Sant Saran Bhojwani
Prem Kumar Dantu

Plant Tissue Culture: An Introductory Text

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ISBN 978-81-322-1025-2 ISBN 978-81-322-1026-9 (eBook)
DOI 10.1007/978-81-322-1026-9
Springer New Delhi Heidelberg New York Dordrecht London

Library of Congress Control Number: 2012954643

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Printed on acid-free paper

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Dedicated to the most Revered
Dr. M. B. Lal Sahab (1907–2002)
D.Sc. (Lucknow), D.Sc. (Edinburgh),
the visionary Founder Director of the
Dayalbagh Educational Institute, for
the inspiration and strength to
undertake and complete the task of
writing this book

Preface

Plant tissue culture (PTC) broadly refers to cultivation of plant cells, tissues, organs, and plantlets on artificial medium under aseptic and controlled environmental conditions. PTC is as much an art as a science. It is the art of growing experimental plants, selecting a suitable plant organ or tissue to initiate cultures, cleaning, sterilization and trimming it to a suitable size, and planting it on a culture medium in right orientation while maintaining complete asepsis. It also requires an experienced and vigilant eye to select healthy and normal tissues for subculture. PTC involves a scientific approach to systematically optimize physical (nature of the substrate, pH, light, temperature and humidity), chemical (composition of the culture medium, particularly nutrients and growth regulators), biological (source, physiological status and size of the explant), and environmental (gaseous environment inside the culture vial) parameters to achieve the desired growth rate, cellular metabolism, and differentiation.

The most important contribution made through PTC is the demonstration of the unique capacity of plant cells to regenerate full plants, via organogenesis or embryogenesis, irrespective of their source (root, leaf, stem, floral parts, pollen, endosperm) and ploidy level (haploid, diploid, triploid). PTC is also the best technique to exploit the cellular totipotency of plant cells for numerous practical applications, and offers technologies for crop improvement (haploid and triploid production, in vitro fertilization, hybrid embryo rescue, variant selection), clonal propagation (Micropropagation), virus elimination (shoot tip culture), germplasm conservation, production of industrial phytochemicals, and regeneration of plants from genetically manipulated cells by recombinant DNA technology (genetic engineering) or cell fusion (somatic hybridization). PTC has been extensively employed for basic studies related to plant physiology (photosynthesis, nutrition of plant cells, and embryos), biochemistry, cellular metabolism, morphogenesis (organogenesis, embryogenesis), phytopathology (plant microbe interaction), histology (cytodifferentiation), cytology (cell cycle), etc. Indeed the discovery of first cytokinin is based on PTC studies.

Thus, PTC is an exciting area of basic and applied sciences with considerable scope for further research. Considerable work is being done to understand the physiology and genetics of embryogenesis and

organogenesis using PTC systems, especially *Arabidopsis* and carrot, which are likely to enhance the efficiency of in vitro regeneration protocols. Therefore, PTC forms a part of most of the courses on plant sciences (Developmental Botany, Embryology, Physiology, Genetics, Plant Breeding, Horticulture, Sylviculture, Phytopathology, etc.) and is an essential component of Plant Biotechnology.

After the first book on "*Plant Tissue Culture*" by Prof. P. R. White in 1943, several volumes describing different aspects of PTC have been published. Most of these are compilations of invited articles by different experts or proceedings of conferences. More recently, a number of books describing the methods and protocols for one or more techniques of PTC have been published which should serve as useful laboratory manuals. The impetus for writing this book was to make available an up-to-date text covering all theoretical and practical aspects of PTC for the students and early career researchers of plant sciences and agricultural biotechnology. The book includes 19 chapters profusely illustrated with half-tone pictures and self-explanatory diagrams. Most of the chapters include relevant media compositions and protocols that should be helpful in conducting laboratory exercises. For those who are interested in further details, Suggested Further Reading are given at the end of each chapter. We hope that the readers will find it useful. Suggestions for further improvement of the book are most welcome.

During the past two decades or so research in the area of plant biotechnology has become a closed door activity because many renowned scientists have moved from public research laboratories in universities and institutions to the private industry. Consequently, detailed information on many recent developments is not readily available.

We would like to thank many scientists who provided illustrations from their works and those who have helped us in completing this mammoth task. The help of Mr. Jai Bhargava and Mr. Atul Haseja in preparing some of the illustrations is gratefully acknowledged.

October 2012

Sant Saran Bhojwani
Prem Kumar Dantu

Contents

1 Historical Sketch 1
1.1 Landmarks/Milestones 8
Suggested Further Reading 10

2 General Requirements and Techniques 11
2.1 Introduction 11
2.2 Requirements 11
2.2.1 Structure and Utilities 11
2.2.2 Washing Room 12
2.2.3 Media Room 13
2.2.4 Glassware/Plasticware 14
2.2.5 Transfer Room 14
2.2.6 Growth Room 15
2.2.7 Cold Storage 16
2.2.8 Greenhouse 16
2.3 Techniques 16
2.3.1 Glassware and Plasticware Washing 17
2.3.2 Sterilization 17
2.4 Appendix I 22
2.5 Appendix II 23
Suggested Further Reading 25

3 Culture Media 27
3.1 Introduction 27
3.2 Media Constituents 27
3.2.1 Inorganic Nutrients 29
3.2.2 Organic Nutrients 29
3.2.3 Plant Growth Regulators 31
3.2.4 Other Supplements 33
3.2.5 Undefined Supplements 33
3.2.6 Gelling Agents 34
3.3 pH of the Medium 34
3.4 Media Preparation 35
3.4.1 Steps in the Preparation of Culture Medium 35
3.4.2 Use of Commercial Pre-Mixes 36
Suggested Further Reading 36

4 Tissue and Cell Culture 39
4.1 Introduction 39
4.2 Callus Cultures 39
4.3 Suspension Cultures 40
4.3.1 Batch Cultures 41
4.3.2 Continuous Cultures 41
4.3.3 Medium for Suspension Cultures 43
4.3.4 Synchronous Cell Suspension Cultures 43
4.3.5 Determination of Growth in Suspension Cultures 43
4.3.6 Tests for Viability of Cultured Cells 44
4.4 Large Scale Cell Culture 45
4.5 Single Cell Culture 46
4.5.1 Isolation of Single Cells. 46
4.5.2 Culture of Single cells 46
4.5.3 Factors Affecting Single Cell Culture 49
4.6 Concluding Remarks 49
4.7 Appendix 49
Suggested Further Reading 50

5 Cytodifferentiation 51
5.1 Introduction 51
5.2 Experimental Systems 52
5.2.1 Tracheary Element Differentiation In Vitro 52
5.2.2 Phloem Differentiation In Vitro 53
5.3 Factors Affecting Vascular Tissue Differentiation 53
5.3.1 Growth Regulators 53
5.3.2 Other Factors 55
5.4 Cell Cycle and Tracheary Element Differentiation . . . 55
5.5 Changes Associated with Tracheary Element Differentiation 56
5.6 Process of TE Differentiation 58
5.7 Concluding Remarks 59
5.8 Appendix 60
Suggested Further Reading 60

6 Cellular Totipotency 63
6.1 Introduction 63
6.2 Factors Affecting Shoot Bud Differentiation 64
6.2.1 Culture Medium 64
6.2.2 Genotype 67
6.2.3 Explant 67
6.2.4 Electrical and Ultrasound Stimulation of Shoot Differentiation 68
6.3 Thin Cell Layer Culture 68
6.4 Totipotency of Crown Gall Tumor Cells 69
6.5 Ontogeny of Shoots 69
6.6 Induction of Organogenic Differentiation 70

6.7 Concluding Remarks . . . 73
Suggested Further Reading . . . 74

7 Somatic Embryogenesis . . . 75
7.1 Introduction . . . 75
7.2 Factors Affecting Somatic Embryogenesis . . . 76
7.2.1 Explant . . . 77
7.2.2 Genotype . . . 77
7.2.3 Medium . . . 78
7.2.4 Growth Regulators . . . 78
7.2.5 Selective Subculture . . . 79
7.2.6 Electrical Stimulation . . . 79
7.2.7 Other Factors . . . 80
7.3 Induction and Development . . . 80
7.3.1 Induction . . . 81
7.3.2 Development . . . 81
7.3.3 Single Cell Origin of Somatic Embryos . . . 82
7.4 Synchronization of Somatic Embryo Development . . . 82
7.5 Physiological and Biochemical Aspects of Somatic Embryogenesis . . . 83
7.6 Molecular Markers and Somatic Embryogenesis . . . 84
7.7 Maturation and Conversion of Somatic Embryos . . . 85
7.8 Somatic Embryos *Versus* Zygotic Embryo . . . 86
7.9 Large Scale Production of Somatic Embryos . . . 86
7.10 Synthetic Seeds . . . 89
7.11 Practical Applications of Somatic Embryogenesis . . . 90
7.12 Concluding Remarks . . . 90
7.13 Appendix . . . 91
Suggested Further Reading . . . 92

8 Androgenesis . . . 93
8.1 Introduction . . . 93
8.2 Androgenesis . . . 93
8.2.1 Techniques . . . 93
8.3 Factors Effecting In Vitro Androgenesis . . . 95
8.3.1 Genetic Potential . . . 95
8.3.2 Physiological Status of the Donor Plants . . . 98
8.3.3 Stage of Pollen Development . . . 98
8.3.4 Pretreatments . . . 98
8.3.5 Culture Medium . . . 100
8.4 Origin of Androgenic Plants . . . 100
8.4.1 Induction . . . 101
8.4.2 Early Segmentation of Microspores . . . 102
8.4.3 Regeneration of Plants . . . 103
8.5 Diploidization . . . 104
8.6 Applications . . . 105
8.7 Concluding Remarks . . . 106
8.8 Appendix . . . 107
Suggested Further Reading . . . 110

9 Gynogenesis 113
9.1 Introduction 113
9.2 Factors Affecting Gynogenesis 113
9.2.1 Genotype 113
9.2.2 Explant 114
9.2.3 Pre-Treatment 115
9.2.4 Culture Medium 115
9.3 Origin of Gynogenic Plants 116
9.4 Endosperm Development 117
9.5 Abnormalities 117
9.6 Ploidy Level 117
9.7 Applications 117
9.8 Concluding Remarks 118
Suggested Further Reading 118

10 Triploid Production 119
10.1 Introduction 119
10.2 Callusing 119
10.2.1 Stage of Endosperm at Culture 119
10.2.2 Culture Medium 121
10.3 Histology and Cytology 121
10.4 Plant Regeneration 121
10.4.1 Culture Medium 122
10.4.2 Cytology 124
10.5 Applications 125
10.6 Concluding Remarks 125
10.7 Appendix 125
Suggested Further Reading 126

11 Zygotic Embryo Culture 127
11.1 Introduction 127
11.2 Technique 127
11.3 Culture Requirements 130
11.3.1 Mineral Nutrients 131
11.3.2 Amino Acids and Vitamins 131
11.3.3 Carbohydrates 131
11.3.4 Growth Regulators 131
11.3.5 Natural Plant Extracts 132
11.4 Culture of Proembryos and Zygote 132
11.5 Changing Growth Requirements of the Embryos 133
11.6 Role of Suspensor in Embryo Development 134
11.7 Precocious Germination 135
11.8 Applications 135
11.8.1 Basic Studies 135
11.8.2 Shortening of Breeding Cycle 137
11.8.3 Rapid Seed Viability 137
11.8.4 Propagation of Rare Plants 137
11.8.5 Haploid Production 137
11.8.6 Transformation 138

11.8.7 Production of Rare Hybrids 138
11.9 Concluding Remarks 140
Suggested Further Reading 140

12 Somaclonal Variation 141
12.1 Introduction 141
12.2 Technique 142
12.3 Methods to Assess Somaclonal Variation 143
12.4 Origin of Somaclonal Variation 144
12.4.1 Pre-Existing Variability 144
12.4.2 In Vitro Induced Variations 145
12.5 Mechanisms Underlying Somaclonal Variation 146
12.5.1 Changes in Chromosome Number and Structure 146
12.5.2 Gene Mutations 147
12.5.3 Amplification of DNA 147
12.5.4 Hypomethylation of DNA 147
12.5.5 Activation of Transposable Elements 148
12.6 Applications 148
12.6.1 Sugarcane 148
12.6.2 Banana 149
12.6.3 Geranium 150
12.6.4 Potato 150
12.6.5 Rice 151
12.6.6 Mustard 151
12.6.7 Tomato 152
12.6.8 Finger Millet 152
12.7 Concluding Remarks 152
Suggested Further Reading 153

13 In Vitro Pollination and Fertilization 155
13.1 Introduction 155
13.2 In Vitro Pollination (IVP) 156
13.2.1 Terminology 156
13.2.2 Technique 156
13.2.3 Preparation of Explant 156
13.2.4 Factors Affecting Seed-Set Following IVP 157
13.3 In Vitro Fertilization (IVF) 158
13.3.1 Isolation of Egg, Central Cell and Sperms 160
13.3.2 Fusion of Gametes 161
13.3.3 Culture of In Vitro Zygotes 162
13.4 Applications 168
13.4.1 Basic Studies on Fertilization and Zygote Development 168
13.4.2 Hybridization 168
13.4.3 Transformation 168

13.5 Appendix 168
Suggested Further Reading 171

14 Parasexual Hybridization 173
14.1 Introduction 173
14.2 Protoplast Isolation 174
14.2.1 Factors Effecting Protoplast Isolation 175
14.2.2 Purification of Protoplasts 175
14.2.3 Viability of the Protoplasts 176
14.3 Protoplast Fusion 176
14.3.1 PEG-Induced Fusion 176
14.3.2 Electrofusion 178
14.4 Protoplast Culture 180
14.4.1 Culture Methods 180
14.4.2 Cell Wall Formation 180
14.4.3 Cell Division and Callus Formation 180
14.4.4 Plant Regeneration 183
14.5 Selection of Somatic Hybrids 184
14.6 Characterization of Somatic Hybrids 185
14.7 Consequences of Protoplast Fusion 185
14.8 Symmetric Hybridization 186
14.9 Asymmetric Hybridization 187
14.10 Cybridization 189
14.11 Applications to Crop Improvement 191
14.12 Concluding Remarks 193
14.13 Landmarks in the History of Somatic Hybridization 193
14.14 Appendix 194
Suggested Further Reading 198

15 Genetic Engineering 199
15.1 Introduction 199
15.2 Gene Transfer 200
15.2.1 Agrobacterium Mediated Transformation . . . 201
15.2.2 Direct Gene Transfer 205
15.3 Selection and Identification of Transformed Cells/Plants 207
15.3.1 Selection 207
15.3.2 Analysis of Putative Transformants 208
15.4 Regeneration of Transformed Plants 209
15.5 Applications 209
15.5.1 Herbicide Resistance 209
15.5.2 Insect Resistance 211
15.5.3 Disease Resistance 212
15.5.4 Virus Resistance 213
15.5.5 Nutritive Quality of Food 214
15.5.6 Abiotic Stress Tolerance 215
15.5.7 Male Fertility Control 215

15.5.8 Parthenocarpy 216
15.5.9 Plants as Bioreactors 217
15.5.10 Biofuel 218
15.5.11 RNA Interference (RNAi) Based Improvement of Plant Products 218
15.6 Biosafety 222
15.7 Concluding Remarks 223
15.8 Appendix 224
Suggested Further Reading 225

16 Production of Virus-Free Plants 227
16.1 Introduction 227
16.2 In Vivo Thermotherapy 228
16.3 In Vitro Therapy 229
16.3.1 Meristem-Tip Culture 229
16.3.2 In Vitro Shoot-Tip Grafting 234
16.3.3 Electrotherapy 235
16.3.4 Virus Elimination Through Other In Vitro Methods 235
16.3.5 Practical Method of Virus Elimination 236
16.4 Maintenance of Virus-Free Stocks 236
16.5 Virus Indexing and Certification 236
16.5.1 Biological Indexing 237
16.5.2 Molecular Assays 238
16.6 Importance of Virus Elimination 239
16.7 Concluding Remarks 240
16.8 Appendix 240
Suggested Further Reading 243

17 Micropropagation 245
17.1 Introduction 245
17.2 Micropropagation of Orchids 246
17.3 General Micropropagation Technique 249
17.3.1 Stage 0: Preparatory Stage 249
17.3.2 Stage 1: Initiation of Cultures 251
17.3.3 Stage 2: Multiplication 251
17.3.4 Stage 3: Shoot Elongation and Rooting 255
17.3.5 Stage 4: Transplantation and Acclimatization 255
17.4 Factors Affecting Micropropagation 258
17.4.1 Initiation of Cultures and Shoot Multiplication 258
17.4.2 Rooting 259
17.5 Problems Inherent with Micropropagation 260
17.5.1 Hyperhydration 260
17.5.2 Contamination 261
17.5.3 Oxidative Browning 261
17.5.4 Recalcitrance of Some Plants 262
17.5.5 Off-Types 262

17.5.6 High Cost 263
17.6 Bioreactors 264
17.7 Photoautotrophic Micropropagation 266
17.8 The Indian Scenario of Micropropagation 267
17.9 Applications of Micropropagation 267
17.10 Concluding Remarks 268
17.11 Appendix 269
Suggested Further Reading 273

18 Production of Industrial Phytochemicals 275
18.1 Introduction 275
18.2 Strategies to Optimize Phytochemical Production in Vitro 276
18.2.1 Culture Conditions 276
18.2.2 Genetic Enhancement 277
18.2.3 Elicitation 278
18.2.4 Biotransformation 279
18.2.5 Immobilization of Cells 280
18.2.6 Permeabilization 280
18.3 Removal of Secreted Products 281
18.4 Hairy Root Cultures 281
18.5 Bioreactors 282
18.6 Commercialization 284
18.7 Concluding Remarks 285
Suggested Further Reading 285

19 Conservation of Phytodiversity 287
19.1 Introduction 287
19.2 In Situ Conservation 287
19.3 Ex Situ Conservation 288
19.4 In Vitro Conservation 288
19.4.1 Medium-Term Storage 289
19.4.2 Long-Term Storage 292
19.5 Concluding Remarks 297
Suggested Further Reading 298

About the Authors 299

Subject and Plant Index 301

About the Book

Plant tissue culture (PTC) is basic to all plant biotechnologies and is an exciting area of basic and applied sciences with considerable scope for further research. PTC is also the best approach to demonstrate the totipotency of plant cells, and to exploit it for numerous practical applications. It offers technologies for crop improvement (haploid and triploid production, in vitro fertilization, hybrid embryo rescue, variant selection), clonal propagation (micropropagation), virus elimination (shoot tip culture), germplasm conservation, production of industrial phytochemicals, and regeneration of plants from genetically manipulated cells by recombinant DNA technology (genetic engineering) or cell fusion (somatic hybridization and cybridization). Considerable work is being done to understand the physiology and genetics of in vitro embryogenesis and organogenesis using model systems, especially *Arabidopsis* and carrot, which is likely to enhance the efficiency of in vitro regeneration protocols. All these aspects are covered extensively in this book.

Historical Sketch

1

Gottlieb Haberlandt, a German botanist, made the first attempts to culture fully differentiated single cells isolated from the leaves of *Lamium purpureum*, petioles of *Eichhornia crassipes*, glandular hairs of *Pulmonaria mollissima*, and stamen hairs of *Tradescantia* in a simple nutrient solution of Knop. The purpose of this experiment was to achieve divisions in these cells and obtain complete plants from them to verify the concept of cellular totipotency inherent in the famous Cell Theory put forward by Schleiden (1838) and Schwann (1839). The cultured cells survived for up to 1 month and also increased in volume but did not divide. Although Haberlandt could not achieve his goals, his genius is apparent in his classic paper presented before the Vienna Academy of Science in Berlin in 1902 wherein he laid down, for the first time, several postulates and principles of plant tissue culture. He had proposed that cells in the plant body stop growing after acquiring the features required by the entire organism without losing their (cell's) inherent potentiality for further growth and are capable of resuming uninterrupted growth on getting suitable stimulus. He also put forward the view that it should be possible to obtain embryos from vegetative cells. With the passage of time, most of the postulates of Haberlandt have been confirmed experimentally, and therefore he is justifiably recognized as the father of plant tissue culture.

GOTTLIEB HABERLANDT
(1854-1945)

A new line of investigation was initiated by Hannig (1904) that later emerged as an important applied area of plant tissue culture. He excised nearly mature embryos of some crucifers and successfully cultured them to maturity on mineral salts and sugar solution. In 1925, Laibach made a very significant contribution when he demonstrated that in the cross *Linum perenne* x *L. austriacum* the hybrid embryos, which normally abort prematurely, could be rescued to obtain full hybrid plants by excising them from the immature seeds and culturing on nutrient medium. Embryo culture has since become a useful tool in the hands of plant breeders to obtain rare hybrids which otherwise fail due to post-zygotic sexual incompatibility (Chap. 11). Van Overbeek et al. (1940) demonstrated for the

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_1, © Springer India 2013

first time, the stimulatory effect of coconut milk on development of young embryos of *Datura*. It was possible only in 1993 that as small as 8-celled embryos of *Brassica juncea* could be cultured successfully using double-layer culture system and a complex nutrient medium (Liu et al. 1993). Almost the same time, Kranz and Lörz (1993) and Holm et al. (1994) succeeded in in vitro cultivation of excised in vitro and in vivo formed zygotes, respectively. However, this required the use of a nurse tissue.

In 1922, Kotte in Germany and Robbins in the USA suggested that the meristematic cells in shoot buds and root tips could possibly be used to initiate in vitro cultures. Their work on root culture, although not very successful, opened up a new approach to tissue culture studies. In 1932, White started his famous work on isolated root culture, and in 1934 he announced the establishment of continuously growing root cultures of tomato. Some of these root cultures were maintained, by periodic subcultures, until shortly before his death in 1968, in India. The medium initially used by White contained inorganic salts, yeast extract, and sugar. Yeast extract was later replaced with the three B vitamins, namely pyridoxine, thiamine, and nicotinic acid. This heralded the first synthetic medium, which was widely used as basal medium for a variety of cell and tissue cultures. During 1939–1950, Street and his students extensively worked on the root culture system to understand the importance of vitamins in plant growth and root-shoot relationship. The other postulate of Kotte and Robbins was realized when Loo (1945) established excellent cultures of *Asparagus* and *Cuscuta* shoot tips. Finally, Ball (1946) succeeded in raising whole plants from shoot tip (apical meristem plus a couple of leaf primordia) cultures of *Lupinus* and *Tropaeolum*.

The discovery of auxin (Kogl et al. 1934) and recognition of the importance of B vitamins in plant growth (White 1937) gave the required impetus for further progress in the field of plant tissue culture. Using indoleacetic acid and B vitamins, Gautheret (1939) obtained continuously growing cultures from carrot root cambium. In the same year, White (1939) and Nobécourt (1939) reported the establishment of callus cultures from tumor tissue of the hybrid *Nicotiana glauca* × *Nicotiana langsdorffii* and carrot, respectively. These three scientists are credited for laying the foundation for further work in the field of plant tissue culture. The methods and media now used are, in principle, modifications of those established by these three pioneers in 1939. The first book on plant tissue culture, authored by White, was published in 1943.

PHILIP R. WHITE
(1901-1968)

ROGER J. GAUTHERET
(1910-1997)

PIERRE NOBÉCOURT
(1895-1961)

During 1950s Skoog and his co-workers, at the University of Wisconsin, USA made several major contributions toward the progress of plant tissue culture. Jablonski and Skoog (1954) tested several plant extracts to induce divisions in mature pith cells of tobacco and found yeast extract to be most suitable in this respect. Miller et al. (1955) isolated the first cell division factor from degraded sample of herring sperm and named it 6-furfurylamino purine, commonly called kinetin. Following this discovery, several natural and synthetic cytokinins were identified, of which benzylamino purine (BAP) is most widely used in plant tissue cultures. The availability of cytokinins made it possible to induce divisions in cells of highly mature and differentiated tissues, such as mesophyll and endosperm from dried seeds. With the discovery of auxins and cytokinins the stage was set for rapid developments in the field of plant tissue culture. The classic experiments of Skoog and Miller (1957) demonstrated chemical regulation of organogenesis in tobacco tissue cultures by manipulating auxin and kinetin ratio in the medium (Chap. 6). Relatively high concentration of auxin promoted rooting whereas higher levels of cytokinin favored shoot bud differentiation. In 1962, Murashige and Skoog formulated the now most extensively used plant tissue culture medium, popularly called MS medium. It contains 25 times higher salt concentration than the Knop's medium, particularly in NO_3^- and NH_4^+ ions (Thorpe 2007).

FOLKE SKOOG
(1908-2001)

TOSHIO MURASHIGE
(Born 1930)

The dream of Haberlandt of cultivating isolated single cells began to be realized with the work of Muir. In 1953, Muir demonstrated that by transferring callus tissues to liquid medium and agitating the cultures on a shaking machine, it was possible to break the tissues into small cell aggregates and single cells. Muir et al. (1954) succeeded in inducing the single cells to divide by placing them individually on separate filter papers, resting on the top of well-established callus cultures that acted as a nurse tissue, and supplied the necessary factors for cell division. Jones et al. (1960) designed a microchamber method for growing single cells in hanging drops of a conditioned medium (medium in which tissue has been grown for some time). This technique allowed continuous observation of the cultured cells. Using this technique, Vasil and Hildebrandt (1965) were able to raise complete plants starting from single cells of tobacco. An important biological technique of cloning large number of single cells was, however, developed in 1960 by Bergmann. It involved mixing single cell suspension with warm, molten agar medium, and plating the cells in a Petri dish where the medium solidified. This cell plating technique is now widely used for cloning cells (Chap. 4) and protoplast culture experiments (Chap. 14). The work of Kohlenbach (1966) came closest to the experiment of Haberlandt. He successfully cultured mature mesophyll cells of *Macleaya cordata* and obtained germinable somatic embryos (Lang and Kohlenbach 1975). Kohlenbach is also credited for providing convincing evidence that an isolated fully differentiated mesophyll cell of *Zinnia elegans* can directly differentiate(transdifferentiation) into a tracheary

element without cell division (Kohlenbach and Schmidt 1975). This provided a model system for detailed cytological, molecular, and genetic studies on the differentiation of tracheary elements by Komamine and his students (Chap. 5).

White (1934) during the course of his work with virus-infected roots observed that some of the subcultures were free of viruses. Limasset and Cornuet (1949) verified that lack of viruses in the meristematic cells is true not only for root tips but also for shoot tips. Taking a cue from this, Morel and Martin (1952) raised virus-free plants of *Dahlia* by meristem culture of infected plants. Shoot tip culture, alone or in combination with chemotherapy or/and thermotherapy, has since become the most popular technique to obtain virus-free plants from infected stocks (Chap. 16).

While applying the technique of shoot tip culture for raising virus-free individuals of an orchid, Morel (1960) realized the potential of this method for rapid clonal propagation. The technique allowed the production of almost 4 million genetically identical plants from a single bud in 1 year. This revolutionized the orchid industry, which was dependent on seeds for multiplication. This method of in vitro clonal propagation, popularly called micropropagation, was soon extended, with modifications, to other angiosperms. Toshio Murashige (USA) was instrumental in popularizing micropropagation for horticultural species. Micropropagation has now become an industrial technology, and several commercial companies round the world, including India, are using it for clonal propagation of horticultural and forest species (Chap. 17).

GEORGES MOREL
(1916-1973)

In 1958, Reinert (Germany) and Steward et al. (USA) demonstrated that plant regeneration in tissue cultures could also occur via embryogenesis. They observed differentiation of somatic embryos in the cultures of root tissue of carrot. These observations fascinated many scientists because in nature embryo formation is restricted to seeds. Backs-Hüsemann and Reinert (1970) achieved embryo formation from an isolated single cell of carrot. Somatic embryogenesis has been projected as the future method of rapid cloning of plants because: (a) the embryos are bipolar with root and shoot primordia, and (b) they can be converted into synthetic seeds by encapsulation in biodegradable substances for direct field planting (Chap. 7).

FREDERICK C. STEWARD
(1904-1993)

HERBERT E. STREET
(1913-1977)

ATSUSHI KOMAMINE
(1929-2011)

By the early 1960s, methods of in vitro culture were reasonably well developed, and the emphasis was shifting toward applied aspects of the technique. Cocking (1960) demonstrated that a large number of protoplasts could be isolated by enzymatic degradation of cell walls. He used culture filtrates of the fungus *Myrothecium verrucaria* to degrade cell walls. Takebe et al. (1968) were the first to use commercially available enzymes, cellulase, and macerozyme, to isolate protoplasts from tobacco mesophyll cells. In 1971, the totipotency of isolated plant protoplasts was demonstrated (Nagata and Takebe 1971; Takebe et al. 1970). At almost the same time, Cocking's group in the UK achieved fusion of isolated protoplasts using $NaNO_3$ (Power et al. 1970). Since then more efficient methods of protoplast fusion, using high pH-high Ca^{2+} (Keller and Melchers 1973), polyethylene glycol (Wallin et al. 1974; Kao et al. 1974), and electrofusion (Zimmermann and Vienka 1982) have been developed. These discoveries gave birth to a new field of somatic hybridization and cybridization (Chap. 14). Carlson et al. (1972) produced the first somatic hybrids between the sexually compatible parents *N. glauca* and *N. langsdorffii*. In 1978, Melchers and co-workers produced intergeneric somatic hybrids between sexually incompatible parents, potato and tomato, but the hybrids were sexually sterile. A unique application of protoplast fusion is in the production of cybrids, with novel nuclear-cytoplasmic combinations. This technique has already been used to transfer male sterility inter- and intraspecifically.

In India, tissue culture started in 1957 at the Department of Botany, University of Delhi under the dynamic leadership of P. Maheshwari. The emphasis was on in vitro culture of reproductive structures (ovary, ovule, nucellus, and embryo) of flowering plants. Some pioneering contributions were made at this school. Incidentally, one of the first International Conferences on plant tissue culture was held at the Department of Botany, University of Delhi in December 1961 (see Maheshwari and Rangaswamy 1963). Prompted by her success with intra-ovarian pollination (Kanta 1960), Kanta et al. (1962) developed the technique of test tube fertilization. It involved culturing excised ovules (attached to a piece of placental tissue) and pollen grains together on the same medium; the pollen germinated and fertilized the ovule. Using this approach, Zenkteler and co-workers (Poland) produced interspecific and intergeneric hybrids unknown in nature (see Bhojwani and Raste 1996; Zenkteler 1999). Kranz et al. (1990) reported a major breakthrough when they electrofused isolated male and female gametes of maize and 3 years later regenerated fertile plants from the in vitro formed zygotes (Kranz and Lörz 1993).

PANCHANAN MAHESHWARI
(1904-1966)

EDWARD C. COCKING
(Born 1931)

ERHARD KRANZ
(Born 1947)

In 1964, the Delhi school made another major discovery when Guha and Maheshwari demonstrated that in anther cultures of *Datura innoxia* the microspores (immature pollen) could be induced to form sporophytes (androgenesis). Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains, and Nitsch and Norreel (1973) succeeded in raising haploid plants from isolated microspore cultures of *Datura innoxia.* Production of androgenic haploids by anther or microspore culture, now reported in several crop plants, has become an important adjunct to plant breeding tools and is being widely used by plant breeders (Chap. 8). Androgenesis also provides a unique opportunity to screen gametophytic variation at the sporophytic level. For some plants, where androgenesis is difficult or not possible, haploids can be obtained by culturing unfertilized ovules or ovaries (Chap. 9). San Noeum (1976) published the first report of gynogenic haploid formation in unfertilized ovary cultures of barley.

SIPRA GUHA-MUKHERJEE
(1938-2007)

SATISH C. MAHESHWARI
(Born 1933)

In 1965, Johri and Bhojwani reported for the first time differentiation of triploid shoots from the cultured mature endosperm of *Exocarpus cupressiformis*. It provides a direct, single step approach to produce triploid plants.

Regeneration of plants from carrot cells frozen at the temperature of liquid nitrogen (−196 °C) was first reported by Nag and Street in 1973. Seibert (1976) demonstrated that even shoot tips of carnation survived exposure to the super-low temperature of liquid nitrogen. This and subsequent successes with freeze preservation of cells, shoot tips and embryos gave birth to a new applied area of plant tissue culture, called in vitro conservation of germplasm. Cultured shoots could also be stored at 4 °C for 1–3 years. These methods are being used at several laboratories to establish in vitro repository of valuable germplasm.

The Pfizer Company made the first attempt for in vitro production of secondary metabolites on industrial scale during 1950–1960 for which Routin and Nickell (1956) obtained the first patent. Tulecke and Nickell (1956) first reported large-scale culture of plant cells in a 134 L bioreactor. Shikonin from cell cultures of *Lithospermum erythrorhizon* was the first in vitro produced phytochemical to be commercialized in 1983 by Mitsui Petrochemical Co., Japan (Curtin 1983). The other industrial compounds under commercial production through tissue culture are taxol and ginseng.

For long the variations observed in ploidy, morphology, pigmentation, and growth rates of cultured cells were ignored as mere abnormalities. Heinz and Mee (1971) published the first report of morphological variation in sugarcane hybrids regenerated from cell cultures. The agronomic importance of such variability was immediately recognized and the regenerants were screened for useful variations. During the next few years, *Saccharum* clones with resistance to various fungal and viral diseases as well as variation in yield, growth habit and sugar content were isolated (Krishnamurthi and Tlaskal 1974; Heinz et al. 1977). Larkin and Scowcroft (1981) reviewed the literature on

spontaneous in vitro occurring variation suitable for crop improvement, and termed the variation in the regenerants from somatic tissue cultures as somaclonal variation. Evans et al. (1984) introduced the term gametoclones for the plants regenerated from gametic cells. Several somaclones (Chap. 12) and gametoclones (Chap. 8) have already been released as new improved cultivars.

Based on his extensive studies on crown gall tissue culture, Braun (1947) suggested that probably during infection the bacterium introduces a tumor-inducing principle into the plant genome. Subsequently, Chilton et al. (1977) demonstrated that the crown galls were actually produced as a result of transfer and integration of genes from the bacteria *Agrobacterium tumefaciens* into the plant genome, which led to the use of this bacterium as a gene transfer system in plants.

ARMIN C. BRAUN
(1911-1986)

MARY-DELL CHILTON
(Born 1939)

The first transgenic tobacco plants expressing engineered foreign genes were produced by Horsch et al. (1984) with the aid of *A. tumefaciens*. Since 1988, biolistic gun, also called particle gun, has become a popular means to deliver purified genes into plant cells (see McCabe and Christou 1993). In 1986, Abel et al. produced the first genetically engineered plants for a useful agronomic trait. The list of genetically engineered varieties with useful traits has considerably enlarged, and since 1993 several transgenic varieties of crop plants, such as canola, cotton, maize, rice, tomato, and soybean, have been released. In 1996, nearly 5 million acres of biotech crops were sown, mainly in the USA and by 2007 these figures rose to 282 million acres in 23 countries (Vasil 2008). Efforts are now being made to genetically modify plants in such a way so as to utilize them as factories for producing desired biomolecules in large quantities (Chap. 15).

These, in brief, are some of the milestones in the history of plant tissue culture. Like any other area of science, plant tissue culture started as an academic exercise to answer some basic questions related to plant growth and development. However, over the years it has emerged as a tool of immense practical value. Plant tissue culture is being extensively used for clonal plant propagation, germplasm storage, production, and maintenance of disease-free plants and as a valuable adjunct to the conventional methods of plant improvement. Plant tissue culture techniques are also being extensively used in basic studies related to plant growth and development, cytodifferentiation, physiology, biochemistry, genetics, and pathology.

Plant tissue culture in India was started way back in 1957 at the Department of Botany, University of Delhi, India. Soon active centers of plant tissue culture were established at the Bose Institute, Kolkata, M.S. University, Vadodra, National Botanical Research Institute, Lucknow, and National Chemical Laboratory, Pune. The creation of the Department of Biotechnology (DBT) by the Government of India in 1986 gave a substantial boost to plant tissue culture research in this country. Many new tissue culture laboratories appeared in several traditional and agricultural universities and institutes across the country. DBT supported the establishment of plant tissue culture pilot plants at

Tata Energy Research Institute, New Delhi and National Chemical Laboratory, Pune in 1989, National Research Centre for Plant Biotechnology at IARI, New Delhi, in 1985, National Facility for Plant Tissue Culture Repository at National Bureau of plant Genetic Resources (NBPGR), New Delhi in 1986 and National Gene Banks of Medicinal and Aromatic Plants at NBPGR, New Delhi, Central Institute of Medicinal and Aromatic Plants, Lucknow, Tropical Botanic Garden and Research Institute, Thiruvananthapuram, and Regional Research Laboratories, Jammu in 1993.

In 1970, International Association of Plant Tissue Culture (IAPTC) was established to promote research and development in this area, and in 1971 it started publishing "IAPTC Newsletter" with one or two feature articles on a current topic, forthcoming events related to PTC, list of recent publications and highlights of major developments in the area. The association organizes international conferences once in 4 years in different parts of the globe. The association was renamed in 1998 as "International Association of Plant Tissue Culture and Biotechnology" and again in 2006 as "International Association of Plant Biotechnology". Similarly, the Newsletter of IAPTC was renamed in 1995 as "Journal of Plant Tissue Culture & Biotechnology". Now it is published as a part of the journal "In Vitro Cellular and Developmental Biology – Plant". For more detailed history of plant tissue culture see White (1943), Krikorian and Berquam (1969), Gautheret (1985), Bhojwani and Razdan (1996), Thorpe (2007) and Vasil (2008).

1.1 Landmarks/Milestones

1. 1902—Haberlandt presented the classic paper describing his pioneering attempt to culture isolated plant cells in a simple nutrient solution at a meeting of the Vienna Academy of Sciences in Germany.
2. 1904—Hannig initiated the work on excised embryo culture of several Crucifers.
3. 1922—Knudson demonstrated asymbiotic in vitro germination of orchid seeds.
4. 1925, 1929—Laibach demonstrated the practical application of embryo culture to produce interspecific hybrids between sexually incompatible parents (*Linum perenne* x *L. austriacum*).
5. 1934—White established continuously growing cultures of tomato root tips.
6. 1937—White formulated the first synthetic plant tissue culture medium (WM).
7. 1939—Gautheret, Nobécourt and White, independently, established continuously growing tissue cultures.
8. 1941—Van Overbeek introduced coconut water as a medium constituent by demonstrating its beneficial effect on in vitro development of immature embryos and callus formation in *Datura*.
9. 1946—Ball succeeded in raising whole plants from excised shoot tips of *Lupinus* and *Tropaeolum.*
10. 1947—Braun proposed the concept of tumor inducing principal (TiP) of *Agrobacterium tumefaciens* responsible for autonomous growth of crown gall tissue.
11. 1950—Braun demonstrated that Ti principal in *Agrobacterium tumefaciens* is transferred to plant genome naturally.
12. 1952—Morel & Martin developed the technique of meristem culture of *Dahlia* to raise virus-free plants from infected individuals.
13. 1954—Muir et al. succeeded in inducing divisions in mechanically isolated single cells cultured in the presence of a nurse tissue.
14. 1955—Miller et al. discovered the first cytokinin (kinetin) from autoclaved herring sperm DNA.
15. 1957—Skoog and Miller put forth the concept of chemical control of organogenesis (root and shoot differentiation) by manipulating the relative concentrations of auxin and kinetin.
16. 1958—Steward (USA) and Reinert (Germany), independently, reported the formation of embryos by the somatic cells of carrot (somatic embryogenesis).

17. 1960—Jones et al. successfully cultured isolated single cells using conditioned medium in microchamber.
18. 1960—Bergmann developed the cell plating technique for the culture of isolated single cells.
19. 1960—Morel described a method for rapid in vitro clonal propagation of orchids (micropropagation).
20. 1960—Cocking isolated plant protoplasts enzymatically.
21. 1962—Kanta et al. developed the technique of in vitro pollination; viable seed formation by in vitro pollination of naked ovules.
22. 1962—Murashige & Skoog formulated the most widely used plant tissue culture medium (MS).
23. 1964—Guha and Maheshwari produced the first androgenic haploid plants of *Datura* by anther culture.
24. 1965—Johri and Bhojwani demonstrated the totipotency of triploid endosperm cells.
25. 1965—Vasil and Hildebrand achieved regeneration of full plants starting from isolated single cells of tobacco.
26. 1966—Kohlenbach succeeded in inducing divisions in isolated mature mesophyll cells of *Macleaya cordata* which later differentiated somatic embryos.
27. 1970—Power et al. published the first report of chemical fusion of plant protoplast.
28. 1970—Establishment of International Association of Plant Tissue Culture (IAPTC).
29. 1971—Heinz and Mee reported somaclonal variation in the regenerants from callus cultures of sugarcane.
30. 1971—Takebe et al. achieved plant regeneration from isolated protoplasts of tobacco.
31. 1971—Newsletter of IAPTC launched.
32. 1972—Carlson et al. produced the first somatic hybrids by the fusion of isolated protoplasts of *Nicotiana glauca* and *N. langsdorffii*.
33. 1973—Nitsch and Norreel succeeded in producing haploid plants from isolated microspore cultures of tobacco.
34. 1973—Nag and Street succeeded in regeneration of plants from carrot cells frozen in liquid nitrogen (−196 °C).
35. 1974—Zaenen et al. identified *Ti* plasmid as the causative factor of *Agrobacterium tumefaciens* for crown gall formation.
36. 1974—Kao et al. and Walin et al. introduced PEG as a versatile chemical for the fusion of plant protoplasts.
37. 1974—Reinhard reported biotransformation by plant tissue cultures.
38. 1976—Seibert reported regeneration of shoots from cryopreserved shoot.
39. 1976—San Noeum reported the development of gynogenic haploids from the cultured unfertilized ovaries of barley.
40. 1977—Chilton et al. demonstrated that only a part of the *Ti* plasmid of *A. tumefaciens* is responsible for crown gall formation.
41. 1984—Horsch et al. produced the first transgenic plants of tobacco by co-culture of leaf discs with *Agrobacterium tumefaciens.*
42. 1986—Abel et al. produced the first transgenic plants with useful agronomic traits.
43. 1987—Sanford et al. invented the biolistic method of direct gene transfer into plant cells.
44. 1987—Fujita and Tabata developed commercial process for the production of shikonin by cell cultures of *Lithospermum erythrorhizon*.
45. 1993—Kranz et al. reported regeneration of full plants from in vitro fertilized eggs of maize (In Vitro Fertilization).
46. 1994—Holm et al. succeeded in raising full plants from excised in situ fertilized eggs (zygotes) of barley.
47. 1995-To date; the existing in vitro techniques were refined to enhance their efficiency and were applied to increasing number of plant species with different objectives.
48. 1995—IAPTC Newsletter developed into Journal of Plant Tissue Culture and Biotechnology.
49. 1998—IAPTC renamed as International Association of Plant Tissue Culture and Biotechnology (IAPTC & B).

50. 2006—IAPTC & B renamed as International Association of Plant Biotechnology (IAPB).

Suggested Further Reading

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White PR (1943) A handbook of plant tissue culture. Jacques Cattell Press, Lancaster

General Requirements and Techniques

2

2.1 Introduction

A plant tissue culture laboratory, whether for research or for commercial purpose, should provide certain basic facilities, such as (i) washing and storage of glassware, plasticware and other labwares, (ii) preparation, sterilization and storage of nutrient media, (iii) aseptic manipulation of plant material, (iv) maintenance of cultures under controlled conditions of temperature, light and humidity, (v) observation of cultures and (vi) hardening of in vitro developed plants. The extent of sophistication in terms of equipment and facilities depends on the need and the funds available. Therefore, establishment of a new tissue culture facility requiring ingenuity and careful planning.

2.2 Requirements

2.2.1 Structure and Utilities

The construction of a laboratory from scratch is a costly affair but there is considerable scope for maneuverability with the design at the conceptional stage and in the selection of construction material. To begin with, a commercial laboratory is best housed in a pre-existing building with suitable modifications. After carefully examining the economic feasibility of the venture an independent facility may be erected. More often than not, for research work the tissue culture laboratory is carved out of the existing infrastructure, and several facilities/equipments are shared with other laboratories. A research facility should have at least four rooms: (i) *Washing Room,* for glassware washing, storage and autoclaving (ii) *Media Room*, for media preparation (iii) *Sterile Area*, for aseptic manipulation and (iv) G*rowth/Culture Room*, to maintain cultures under suitable environmental conditions. The culture room should also have a working table, a stereoscopic microscope and a good light source, preferably cool light (fiber optics), for observing cultures. The sterile transfer cabinets could be placed in the culture room or in a specially designed transfer room. In many research laboratories it is kept in an undisturbed area of a general lab.

In case the facility needs to be constructed, especially for a commercial setup, it would be desirable to locate the unit away from the city to avoid heavy pollution and vehicular vibrations. However, this may require transportation of the personnel. The location of the laboratory should not be near fields to avoid spurts of infection by the combines and threshers during the harvest seasons. The facility needs to be adequately protected from rains and winds as these carry spores, mites and thrips. Thermal insulation of the facility to conserve energy is another aspect requiring proper thought. One way is to have the transfer area and the growth rooms below ground level. In that case care must be taken to protect the lab from seepage and provide adequate ventilation. Alternately, these two rooms could have a double wall or built of hollow

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_2, © Springer India 2013

bricks with air trapped in between, which could be vented during summers.

A tissue culture facility requires large quantities of good quality water. At the designing stage itself adequate attention should be paid to the source of water and waste-water disposal, especially where sewer facilities are not available, keeping in view the local municipal rules for health and environment.

A tissue culture unit must have power backup to save cultures from getting contaminated in the event of power failure or load-shedding from the mains during aseptic manipulations. Valuable cultures may be lost because of temperature shocks in the growth room during electricity breakdowns/shutdown. The generator may be fitted with a self-starting switch.

It is of paramount importance that a tissue culture laboratory is clean and movement of materials from one area to another occurs with minimal backtracking. These aspects should be the guiding principle while designing the layout plan of various rooms, pass-through windows, doors and hallways. It is necessary and desirable to isolate the 'clean area', comprising of transfer room and growth room from rest of the 'unclean area' and it should be treated as 'restricted area', out of bounds for visitors and outsiders. In the passage between these two areas, especially in a commercial set-up, one is required to wash hands and feet and wear sterilized overcoats and headgear before entering the 'restricted area'. Generally, high standards of sanitation need to be maintained and these have to be more stringent where dust, pollen and small insects abound in the environment. It is a good idea to have paved pathways and shrubs around. High levels of cleanliness and freedom from extraneous materials could be achieved by having positive air pressure, at least in the 'clean area'. Depending on the necessity, a Class 1,000 or Class 10,000 standard should be maintained for the clean room. For the movement of material in (sterilized medium, instruments, water, etc.) and out of (glassware, old and infected cultures, tissue culture produced plants for hardening, etc.) the 'clean area' a window with double door hatch should be provided to maintain high asepsis in the 'clean area'.

As far as possible indigenously available construction material, equipment, apparatus, and instruments should be used for cost effectiveness and ease of maintenance. Innovativeness and indigenous fabrication will go a long way in reducing the costs.

2.2.2 Washing Room

Depending on the availability of funds and space the washing and sterilization areas may be in separate rooms or in a common room. In either case, the washing area should have adequate supply of good quality hot and cold running water and an acid and alkali resistant big sink. Adequate steel or plastic buckets and tubs are required for soaking culture vials and other labwares used in medium preparation. Brushes of various sizes and shapes are essential for cleaning glassware, while it is optional to have a dish washing machine. For a commercial set-up an industrial dish washer is desirable. The media room should have a hot air cabinet to dry the washed labware, an oven for dry sterilization, and a dust proof cupboard for storage of plastic and glass-ware. When washing is done in media room a temporary partition can be erected between the two areas to prevent splashing of soap solution and any other interference in the two activities. Alternately, timings of the two activities could be staggered. Where the autoclave is to be housed within the media room an isolated area with provision for ventilation through an exhaust should be chosen.

Even if good quality water is available it cannot be used for final washing of labwares or for medium preparation as it contains impurities such as inorganic and organic compounds, dissolved gases, particulate debris and microorganisms. Water could be purified through distillation, deionization or reverse osmosis. Sometimes a combination of two or more is required. Water purity is measured in terms of resistivity (ohms cm^{-1}) or its reciprocal, i.e.,

conductivity (mhos cm^{-1}). Water for tissue culture should ideally have a conductivity of 5.0 μmhos cm^{-1} although a conductivity level up to 15 μmhos cm^{-1} is acceptable. Deionized water may be used for teaching laboratories or for rinsing labware but for research and commercial purposes, water distillation apparatus, a reverse osmosis unit or a Mili-Q purification system needs to be installed. The choice between the three is one of quality of final water, speed of production and cost. For a research laboratory a glass distillation unit with a handling capacity of 1.5 to 2 L h^{-1} of water should be sufficient. For a commercial set-up or where high purity water is required a Mili-Q purification system that can provide 90 L h^{-1} may be used. Proper storage tanks should be provided for the purified water.

2.2.3 Media Room

The media room is the kitchen of the tissue culture facility. The media room is provided with a working table in the centre and benches along the wall, the tops of which are either covered with granite or laminated board (Fig. 2.1). The tables and benches should be at a height suitable for working while standing and the space below them could be fitted with drawers and cupboards for storage purposes. The benches are required for keeping balances, pH meter, magnetic stirrers, hot plates etc. A top loading electronic balance with tare for weighing large quantities and an analytical balance for small quantities of chemicals must be provided. The balances should be isolated in a small chamber if the media room also houses the autoclave. In a large commercial laboratory it will be of help to have an automatic media dispenser.

For short term storage of certain chemicals, plant materials, and stock solutions a refrigerator and a deep freeze are required. These could be kept in the corridor if sufficient space in the room is not available. A single electrode pH meter that can read conductivity also should be provided. For filter sterilization of medium or solutions of thermolabile compounds an aspirator or vacuum pump may be required. For steam sterilization an autoclave or a domestic pressure cooker, depending on the quantity to be sterilized, is needed.

For emergencies a fire extinguisher and a first aid kit should be kept in this room.

Fig. 2.1 Media preparation

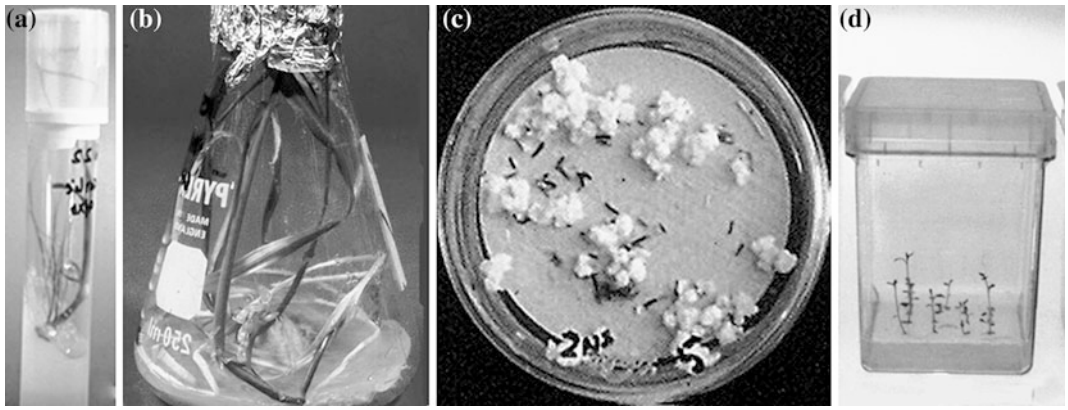

Fig. 2.2 Culture vials. **a** Culture tube with polypropylene cap. **b** Flask. **c** Petri plate. **d** Plastic jar (*Magenta box*)

2.2.4 Glassware/Plasticware

In a tissue culture laboratory culture vessels (Fig. 2.2) are required in bulk. Depending on the type of work, adequate supplies of these should be maintained. For standard tissue culture work rimless test tubes (25 × 150 mm) are widely used (Fig. 2.2a). The culture tubes are important for culture initiation and establishment even in a commercial set-up. For further mass multiplication larger containers such as jam bottles or other wide mouthed bottles are required. Erlenmeyer flasks have also been used as culture vessels (Fig. 2.2b). Only borosilicate or Pyrex glassware should be used.

Plastic culture vials, autoclavable and presterilized, have replaced glass culture vials to a large extent. A wide range of presterilized, disposable culture vials made of clear plastic, especially designed for protoplast, cell, tissue and organ culture, are available in the market under different brand names. The presterilized plastic Petri dishes (Fig. 2.2c), jars (Fig. 2.2d), screw cap bottles, and various cell culture plates come with their closures. For culture tubes and flasks, traditionally, non-absorbent cotton plugs wrapped in a single layer of cheesecloth have been used as a closure. Autoclavable, transparent polypropylene caps with a membrane built into the top are also available (KimKaps, Kimble, Division of Owens, IL). Cotton plugs provide excellent aeration but the medium dehydrates very fast. On the other hand, polypropylene caps reduce the rate of medium desiccation but increase moisture and gaseous accumulation within the container. However, it is important to ensure that the closure allows proper aseptic aeration and does not inhibit the growth of culture materials. In this regard it may be mentioned that Parafilm/Nescofilm, commonly used to seal Petri dishes, releases butylated hydroxytoluene, which is toxic to the cultured plant material (Selby et al. 1996). Alternately, one can use cling film for sealing, as 2-ethyl-1-hexanol released by it is not inhibitory to culture material.

Now it is possible to buy culture vessels made of different synthetic materials. Culture vessels made of polypropylene transmit 65 % light and those made of polycarbonate transmit almost 100 % light. Gas permeable fluorocarbonate vessels are available for use with plant materials that are sensitive to gas build up within the culture vials (Kozai 1991).

Besides culture vials, various other glass-/plasticware such as beakers, measuring cylinders, pipettes, etc. of various sizes are required for media preparation.

2.2.5 Transfer Room

In research laboratories the transfer hoods are placed in the growth/culture room or even in a quite corner of a general laboratory. However, in a commercial facility it is necessary to have separate transfer and growth room(s). There are

no special requirements for a transfer room except that a high degree of cleanliness and a worker friendly environment is to be maintained. Transfer hoods are placed in this room to carry out aseptic manipulations. Often the culture medium to be used is also stored here although, where possible, a separate store room within the 'clean area' is demarcated for this purpose. To transport culture medium or cultures in and out of the transfer room, trolleys with one or more shelves are helpful. These trolleys may also serve as side benches for the operator to hold extra culture medium, stock cultures, and/or freshly raised cultures until transferred to the growth room. Since fire/heat is constantly used in the transfer hoods it is advisable to keep a fire extinguisher in this area.

2.2.6 Growth Room

The inoculated culture vials are transferred for incubation to a growth room with controlled temperature and light conditions. It is of paramount importance to maintain cleanliness in this area. It can be achieved by having positive air pressure in the 'clean area' or an overhead air-curtain at the entry to remove surface dust. This room should have one door, and the windows should be avoided to prevent external light from interfering with the internal light cycle. Desirably, the junction of walls should be rounded rather than angular to prevent cobwebs. The wall paints should be of semi or high-gloss with a linoleum floor to withstand repeated cleaning. Tiller et al. (2002) reported that the polymer Hexyl-PVP when coated on a glass surface killed 99 % of harmful bacteria.

Growth rooms being closed units require devices to control temperature and light. Temperature is controlled by air conditioning. Tower air conditioning is an expensive proposition, but if that is the chosen option then adequate precautions should be taken to filter the cooled air to prevent dust, spores etc. from reaching the 'clean area'. With this type of air conditioning it is easy to maintain positive air pressure. In research laboratories generally window mounted or split air conditioners are used for cooling during summer and hot air blower for heating during winter. The air conditioners and heaters are hooked to temperature controllers to maintain the temperature at 25 ± 2 °C. For higher or lower temperature treatments, special incubators with built in fluorescent lights can be used. These incubators may be kept even outside the growth room with suitable measures to prevent anyone from tampering with the settings.

Generally, cool white fluorescent lamps with electronic ballasts are used for growth rooms because of the uniform light intensity they emit. Cultures are normally maintained in diffuse light (3000–5000 lux). The low light intensity can be achieved in 3.5 feet wide shelves by installing three tubes one foot above the surface of the shelf. A sheet of aluminium foil or coat of aluminium paint provided above the tubes maximizes light intensity below. Some provision should also be made for growing cultures in total darkness or under high light intensities. Automatic timers are used to regulate the photoperiod.

If the relative humidity in the growth room falls below 50 %, humidifiers are required to be used to prevent medium from drying rapidly. Dehumidifiers may be required when the humidity is very high, particularly during the rainy season, to prevent cotton plugs from becoming damp or condensing of water droplets, as both may increase chances of contamination of cultures.

Culture vials in the growth room are maintained on specially designed shelving unit that are either stationary or moveable. Stationary shelves may be fixed on walls of the room, or could be fitted into angular iron frames to form culture racks (Fig. 2.3) that are placed conveniently in the room. Alternately, the racks could be on roller coaster wheels that allows efficient use of space. Open shelves are generally preferred because of better air circulation. The shelves can be made of plywood board or rigid wire mesh. Each shelf is provided with a separate set of fluorescent tubes. In case the cultures are to be maintained under different photoperiod and temperature regimes it is advisable to have more than one growth room.

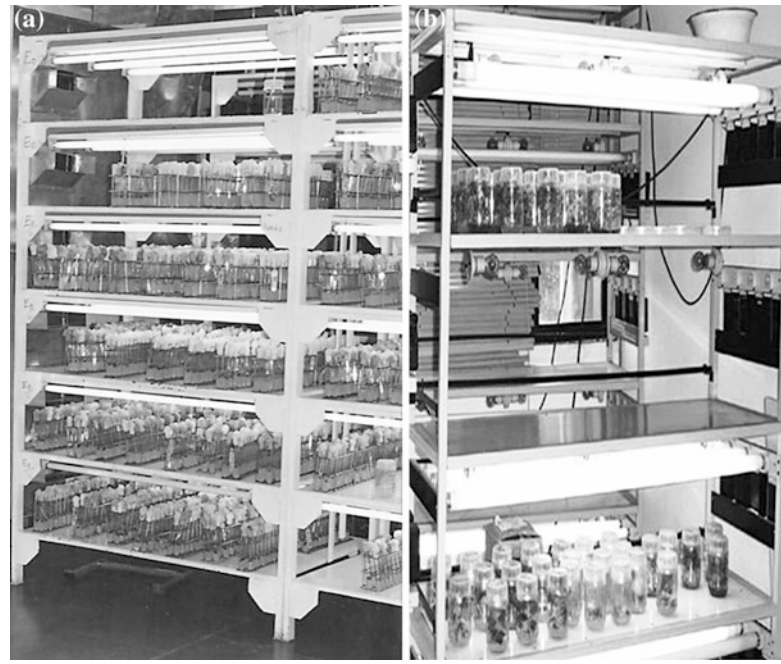

Fig. 2.3 **a** An illuminated culture trolley with six shelves holding culture tubes arranged in metallic racks. **b** An enlarged portion of a trolley as in (**a**), with cultures in screw cap bottles

Culture flasks, jars and Petri dishes can be placed directly on the shelves or in trays of suitable size, while culture tubes require some support such as a metallic wire rack (Fig. 2.3), which can hold 20–24 tubes. In commercial companies, where large quantities of culture vials have to be moved, it is not only convenient but also time saving to use autoclavable plastic or epoxy coated metallic trays for holding the culture vials. The culture vials and the trays holding them should be appropriately labeled to avoid mixing up. For transportation of culture vessels cart trolleys may be used.

The culture room should also have a shaking machine, either of the horizontal type or the rotary type, if cell suspension cultures are to be grown. Shakers with speed, temperature and light controls are also available.

2.2.7 Cold Storage

In a commercial setup it is necessary to have a cold storage maintained at 2–4 °C for temperate plants and 15 °C for tropical plants. These rooms are used to give treatment for breaking dormancy of some plant materials, storing of cultures to schedule workload, maintain 'mother' cultures and to hold harvested plants (Mageau 1991).

2.2.8 Greenhouse

In order to grow the mother plants and to acclimatize in vitro produced plants, a tissue culture laboratory should have a greenhouse made of glass, polythene or polycarbonate depending on the budgetary provisions. This facility should have a provision to maintain high humidity such as fan and pad system. It would be desirable to have a potting room adjacent to this facility. A separate autoclave might be required in this area if one wants to sterilize the potting mixture.

In a commercial laboratory provision for certain other rooms such as, a general storage, and employee's tea room, an administrative office and shipping and receiving centre should be made.

2.3 Techniques

This section deals with the basic techniques of maintaining cleanliness and asepsis in the laboratory and in the cultures.

2.3.1 Glassware and Plasticware Washing

All glassware and plasticware, except pre-sterilized ones, should be thoroughly washed when using for the first time. As a normal practice, the apparatus is soaked overnight in a standard laboratory detergent and scrubbed with a bottle brush manually or by a machine. These are then rinsed under tap water followed by a rinse in distilled water. Dried agar can be removed by heating. The contaminated glass and plastic culture vials should be autoclaved before opening for washing or discarding, respectively, in order to minimize the spreading of bacterial and fungal contaminants in the laboratory. The washed apparatus are placed in wire baskets or trays to allow maximum drainage and dried in a hot air cabinet at about 75 °C and stored in a dust proof cupboard. For transportation of washed labware from washing area suitable trays and mobile carts can be used.

2.3.2 Sterilization

Whether it is labware or culture medium, plant material or environment in the laboratory, instruments used for culture or the operator himself, all are sources of infection. The tissue culture medium being rich in sugar and other organic and inorganic nutrients supports good growth of microorganisms, such as fungi and bacteria. On reaching the medium the microorganisms may grow faster than the plant tissues, finally killing them. The microbes may also secrete toxic wastes into the medium inhibiting growth of cultured tissues. It is, therefore, absolutely essential to maintain a completely aseptic environment inside culture vessels. As a rule, plant tissue culture laboratory facilities should not be shared with microbiologists and pathologists, and contaminated vessels should be removed as soon as detected.

The various sources of contamination and measures to guard cultures against them are discussed in the following pages.

(i) *Glassware and Plasticware*. Culture vials are a major source of contamination, more so, if these have been in long use. The glass culture vials may be dry sterilized before pouring the medium to kill such bacteria, which might withstand autoclaving. Culture vials are generally sterilized together with the culture medium. For pre-sterilized medium the culture vials with proper closure may be sterilized by autoclaving or dry heating in an oven at 160–180 °C for 3 h. For dry heating the oven should have a fan mounted inside for better circulation of hot air. It is important that the oven is not over loaded. The glassware should be allowed to cool down before removing it from the oven. Otherwise, cool air sucked from outside may expose the load to bacterial contamination and also increase the risk of cracking.

Not all types of plastic labware can be heat sterilized. Only polypropylene, polymethylpentene, polyallomer, Tefzel ETFE and Teflon FEP may be repeatedly autoclaved at 121 °C. Sterilization cycle for polycarbonate vials should be limited to only 20 min as it shows loss of mechanical strength on repeated autoclaving. Polystyrene, polyvinyl chlorides, styrene acrylonitrile, are not autoclavable at all.

(ii) *Culture Medium.* The tissue culture medium, as stated earlier, is not only a source of contaminants but also supports their luxuriant growth. Therefore, it must be sterilized properly. Mostly, the culture medium is sterilized by autoclaving. Autoclave (Figs. 2.4, 2.5) is an apparatus in which sterilization is done by steam heating under pressure. The culture vials containing medium and closed with a suitable bacteria-proof closure are autoclaved at 15 psi and 121 °C for 15–40 min from the time the medium reaches the required temperature and pressure. Care should be taken to cover cotton plugs and other things that might get wet with aluminium foil before autoclaving. For sterilization of small quantities of medium a

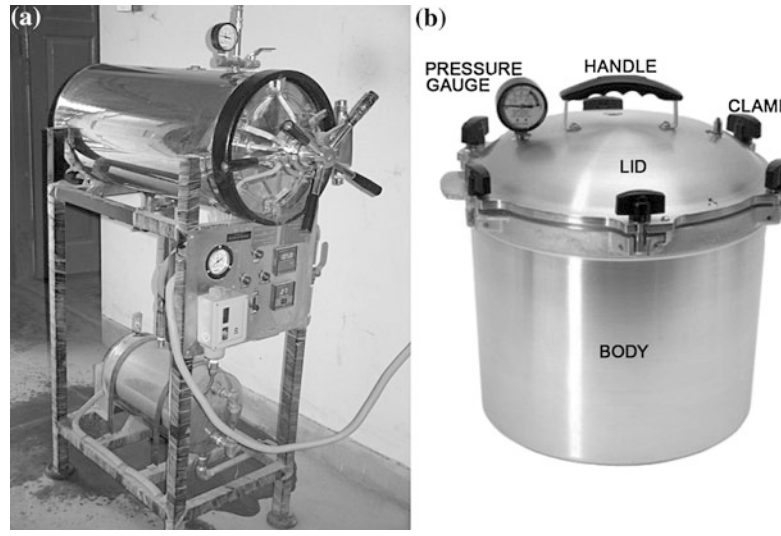

Fig. 2.4 A horizontal autoclave (**a**) and a small vertical autoclave (**b**)

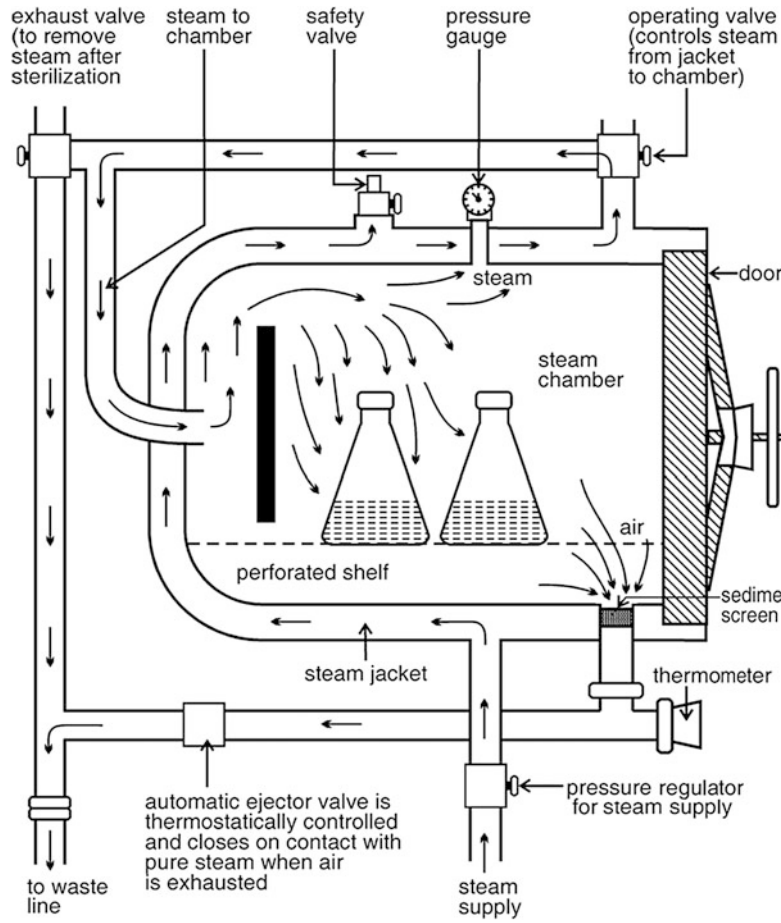

Fig. 2.5 Working diagramme of a horizontal autoclave

Table 2.1 Minimum time required for sterilization by autoclaving

Volume/container (ml)	Minimum sterilization time at 121 °C (min)
20–50	15
75	20
250–500	25
1,000	30
1,500	35
2,000	40

pressure cooker, which works on the same principle as an autoclave, may be used. The exposure time varies with the volume of the liquid to be sterilized (Table 2.1). Prolonged autoclaving may adversely affect gelling of the medium. Care must be taken not to open the pressure valve while autoclave is cooling and loosing pressure, as a rapid loss of pressure, will cause the medium to boil vigorously and overflow, wetting the vial closures. The pressure gauge should be at zero and temperature not more than 50 °C before the autoclave is opened.

It has been observed that 2–5 % of media get contaminated during manual pouring after autoclaving. Moreover, certain *Bacillus* bacteria survive even after autoclaving at 110–120 °C (Leifert et al. 1994). It is, therefore, advisable to incubate the sterilized culture medium at 30–35 °C for 24–48 h before use to ensure that it is free of contaminants.

Autoclaves are either horizontal (Figs. 2.4a, 2.5) or vertical (Fig. 2.4b) and are available in different sizes. The vertical types become cumbersome to use beyond a certain capacity because of the depth to be reached during loading and unloading. Horizontal autoclaves are easy to handle but are costlier. The decision on the type of autoclave to be bought depends on the funds and the objective. Horizontal autoclaves are available with single or double door facility. In the latter case the autoclave is so installed that its one door opens in the media room to load the medium and the other door opens directly into the 'clean area', for unloading the sterilized medium.

Autoclaving has some disadvantages such as change of medium pH and breakdown of some media constituents. The following components will be partly decomposed by autoclaving (Van Bragt et al. 1971):

(1) Sucrose breaks down into glucose, fructose, and some laevulose; the autoclaved medium with sucrose will contain several sugars,
(2) Gibberellic acid looses 90 % of its activity,
(3) Vitamin B_1 disintegrates into pyrimidine and thiazol,
(4) Zeatin, urea, vitamin C, colchicine and antibiotics are thermolabile.
(5) Plant extracts loose some of their effectiveness.

Therefore, thermolabile compounds cannot be autoclaved along with rest of the nutrient medium. These are, instead, filter-sterilized. The whole medium without the heat labile compound/s is autoclaved in a flask and kept in the sterilized hood to cool. The solution of the thermolabile compound is sterilized by membrane filtration and added to the autoclaved medium cooled to 50–40 °C in the case of semisolid medium or to room temperature when using a liquid medium. For filter-sterilization of a solution, bacteria-proof membranes of pore size 0.22–0.45 μm are used. The filter membrane is placed into filter holders (Fig. 2.6) of appropriate size and sterilized by autoclaving after wrapping in aluminium foil. Filters should not be sterilized at temperatures

Fig. 2.6 A filter assembly with syringe

exceeding 121 °C. The thermolabile liquid taken in an unsterilized graduated syringe is gradually pushed through the sterilized filter assembly containing the membrane. The sterilized liquid dripping from the other end is added directly to the autoclaved medium. For large volumes, filter-sterilization can be carried out using a filtering set-up attached to a vacuum pump.

(iii) *Instruments*. The instruments for aseptic manipulation, such as forceps, scalpels, needles, and spatula, should be sterilized before use by wrapping in aluminium foil and autoclaving. Again, during aseptic manipulation the instruments are sterilized several times by dipping in 95 % ethanol and flaming and used after cooling. Alcohol should be regularly changed as some *Bacillus circulans* strains persist in alcohol for more than a week (Leifert and Waites 1990). Heat produced by Bunsen burner can generate eddy currents that could increase incidence of contamination during subculture. Several labs use glass bead sterilizers (Steripot), in which temperature rises to 250 °C within 5–20 min. Embedding the instruments in the heated beads for 5–7 min is adequate to sterilize them. Infrared sterilizers are also available for sterilizing instruments in the hood. The sterilized instruments are rested on a stand inside the laminar airflow cabinet slightly raised from the work table.

(iv) *Plant Material*. Plant surfaces harbour a wide range of microbial contaminants. This source of infection can be avoided by thorough surface sterilization of the plant material before planting it on the nutrient medium. Tissues with systemic fungal and bacterial infection are usually discarded in tissue culture work.

Plant tissues can be surface sterilized using various sterilants. The sterilant type, its concentration and the duration of treatment have to be determined empirically. Table 2.2 gives a guideline to get started.

Hypochlorite solutions (sodium or calcium) have proved to be effective in most cases. For example, 0.3–0.6 % sodium hypochlorite treatment for 15–30 min will decontaminate most tissues. Addition of a few drops of a surfactant (Triton-x or Tween 80) to the sterilant solution or rinsing the tissue for 30 s in ethyl alcohol before surface sterilization can enhance the efficiency of sterilization treatment. It is important to realize that a surface sterilant is also toxic to the plant tissues. Therefore, the concentration of the sterilant and the duration of treatment should be chosen to minimize tissue death. Periodic gentle shaking of the vial during sterilization is recommended. After the sterilization treatment the plant material is washed 2–3 times in sterilized distilled water in an aseptic area (laminar airflow chamber) to remove any traces of the toxic sterilant. To initiate cultures of delicate tissues, such as immature embryos, endosperm, nucellus and shoot tip,

Table 2.2 Effectiveness of some surface sterilizing agents[a]

Sterilizing agent	Concentration (%)	Duration (min)	Effectiveness
Calcium hypochlorite	9–10	5–30	Very good
Sodium hypochlorite	2[b]	5–30	Very good
Hydrogen peroxide	10–12	5–15	Good
Bromine water	1–2	2–10	Very good
Silver nitrate	1	5–30	Good
Mercuric chloride	0.1–1	2–10	Satisfactory
Antibiotics	4–50 mg L^{-1}	30–60	Fairly good

[a] After Yeoman and Macleod (1977)
[b] 20 % (v/v) of a commercial solution

these are sterilized along with the surrounding tissues, and the explant is dissected out under aseptic conditions. Inoculation of the plant material on the medium is done in the laminar airflow cabinet (Fig. 2.7). The sterilized plant material or the plant material to be subcultured is placed on a presterilized ceramic tile, steel tray or Petri plate for cutting to proper size before inoculation.

Ethyl and isopropyl alcohol have also been used to surface sterilize some plant tissues (methanol should never be used). After rinsing in ethanol for a few seconds the plant material is either left exposed in the sterile hood until the alcohol evaporates or, if fairly hardy, flamed (Bhojwani 1980).

Several workers have used antibiotics and antifungal compounds to control explant contamination. Arbitrary use of antibiotics may be counterproductive as majority of the bacteria infecting plants are gram negative, which are less sensitive to commonly used antibiotics. Binomyl has been shown to reduce fungal infection when used with mercuric chloride.

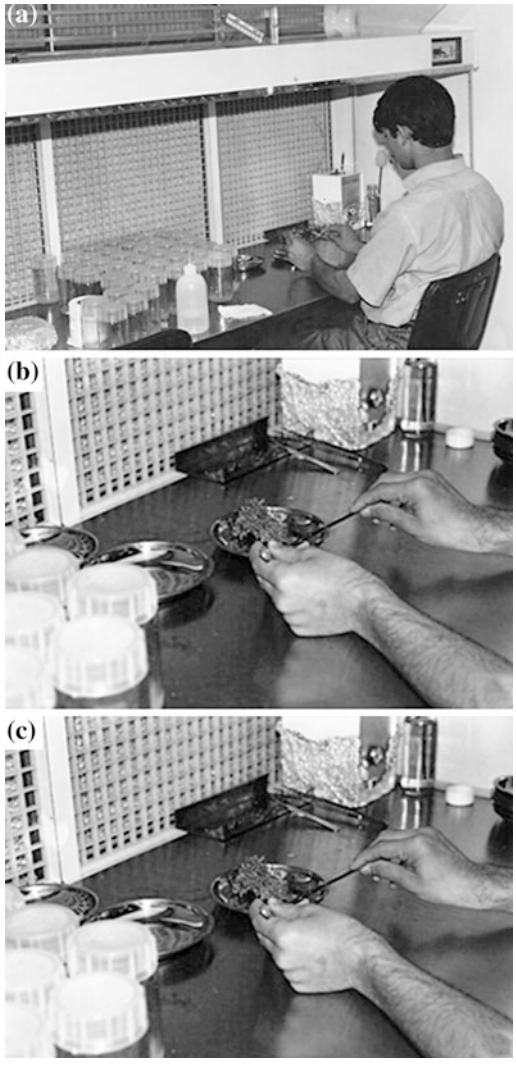

Fig. 2.7 **a–c** Aseptic manipulation in a laminar airflow cabinet

(v) *Transfer Area and Growth room.* The chances of the cultures getting infected exist whenever the culture vials are opened to inoculate the sterilized plant tissue on the medium (inoculation) or for subculturing. To avoid this, all transfer operations are carried out under strictly aseptic conditions. Most laboratories use laminar airflow cabinets to carry out aseptic manipulations (Fig. 2.7). These are very convenient, as work can be stared within 10–15 min of switching on the airflow and can be continued for long hours.

A laminar airflow cabinet basically has drum type fans rotating at high speed to suck air from outside through a coarse filter which removes large particles, and the semi-clean air is thrown in the opposite direction. The dust-free air, which is under pressure, gets pushed through a fine filter, known as the "High Efficiency Particulate Air (HEPA)" filter. The HEPA filter prevents the entry of particles larger than 0.3 μm. The ultra clean air, free of fungal and bacterial contaminants, flows through the working area in the direction of the operator. The velocity of the air coming out of the fine filter is about 27 ± 3 m min^{-1} that is adequate for preventing the contamination of the working area by the worker sitting in front of it. All contaminants such as hair, salts, flakes, etc., get blown away, and a completely aseptic environment is maintained in the

working area as long as the cabinet is on. The flow of air in no way hampers the use of a spirit lamp or a Bunsen burner attached to a LPG cylinder. The working bench is often fitted with germicidal UV lamp and fluorescent tubes for illumination. The airflow cabinet is provided with some power points to facilitate the use of microscope and other minor equipment during inoculation. The laminar airflow cabinet should not be kept facing frequently used doors and windows. It would be ideal, as stated earlier, to maintain these cabinets in the growth room or a separate transfer room in the 'clean area'.

The 'clean area' should, preferably, be under positive air pressure, as stated earlier, to maintain a particle count of 100–1000 ppm (parts per million = particles per m^3) and keep the area reasonably aseptic. Where this is not possible the rooms should be regularly cleaned with germicidal solutions such as ethyl alcohol. Germicidal UV lamps may be used but care should be taken to switch them off before starting the work as UV rays are harmful to eyes.

To check cleanliness in the transfer and growth rooms, a regular spore count should be carried out. This can be done by exposing Petri plates containing sterilized microbial culture medium overnight at different places and incubating them at 30–35 °C for 24–48 h. Such a measure can also be carried out to check for leaks in laminar airflow cabinet. Another measure to maintain cleanliness in the 'clean area' is periodic fumigation, preferably on weekends, with a germicidal gas (such as a mixture of 75 g potassium permanganate and 35 ml of 40 % formalin).

Small arthropods, such as thrips and mites, pose a very serious problem to the cultures. On entering the facility these spread very fast, especially in open shelf systems, thriving on the cultures and contaminating them with microorganisms. These insects may have an easy access with the persons working in the 'clean area', if they frequent lawns and bushes during breaks. So, care should be taken to prevent workers from sitting or strolling on lawns by providing a proper rest/recreation room within the facility. Frequent observation of the cultures and systematic housekeeping will go a long way in keeping contamination at bay.

2.4 Appendix I

A list of apparatus required for plant tissue culture work

1. Flasks (100, 250, 500 ml and 1, 2, 5 L)
2. Volumetric flasks (500 ml and 1, 2, 3 L)
3. Measuring cylinders (25, 50, 100, 500 ml and 1 L)
4. Graduated pipettes (1, 2, 5, 10 ml)/autopipettes of variable volumes
5. Pasteur pipettes and teats for them
6. Culture vials (culture tubes, screw-cap bottles of various sizes, petri dishes, etc.) with suitable closure
7. Plastic or steel buckets, to soak labware for washing
8. Washing machine, for washing labware
9. Hot-air cabinet, to dry washed labware
10. Oven, to dry washed labware and dry heat sterilization of glassware
11. Wire-mesh baskets, to autoclave media in small vials and for drying labware
12. Water distillation unit, demineralization unit, Milli Q unit or reverse osmosis unit, for water purification
13. Plastic carboys (10 and 20 L), to store high quality water
14. Analytical balance, to weigh small quantities and a top pan balance with tare facility, to weigh comparatively larger quantities
15. Hot plate-cum-magnetic stirrer, to dissolve chemicals
16. Plastic bottles of different sizes, to store and deep freeze solutions
17. Refrigerator, to store chemicals, stock solutions of media, plant materials etc.

18. Deep freeze, to store stock solutions of media for long periods, certain enzymes, coconut milk, etc.
19. Steamer or microwave oven, to dissolve agar and melt media
20. pH meter and conductivity, to adjust pH and conductivity of media and solutions
21. Autoclave or domestic pressure cooker, for steam sterilization of media and apparatus
22. Heat-regulated hot plate or gas stove, for steam sterilization in domestic pressure cooker
23. Exhaust pumps, to facilitate filter sterilization
24. Filter membranes and their holders, to filter sterilize solutions
25. Hypodermic syringes, for filter sterilization of solution
26. Medium dispenser, to pour medium
27. Trolley with suitable trays, to transport cultures, media and apparatus
28. Laminar airflow cabinet, for aseptic manipulations
29. Spirit lamp, burner, glass bead sterilizer or infra-red sterilizer, to sterilize instruments
30. LPG cylinder
31. Atomizer, to spray spirit in the inoculation chamber
32. Instrument stand, to keep sterilized instruments during aseptic manipulations
33. Large forceps with blunt ends, for inoculation and subcultures
34. Forceps with fine tips, to peel leaves
35. Fine needles, for dissections
36. Scalpel holder and surgical blades, for chopping of explants
37. Trays or ceramic tile, on which explants is chopped inside the hood
38. Stereoscopic microscope with cool light, for dissection of small explants
39. Digital camera with suitable attachment/s for macro and micro photography
40. Table-top centrifuge, to clean protoplast and isolated microspore preparations, etc.
41. Incubator shaker, for liquid cultures
42. Generator

2.5 Appendix II

A list of suppliers of equipment for setting up a tissue culture laboratory[a]

Equipment/apparatus	Manufacturer(s)
Glassware (culture tubes, flasks, beakers, pipettes, etc.)	*Borosil* Khanna Construction House, 44, Dr. R.G Thandani Marg, Worli, Mumbai 400018
Plasticware (beakers, test tube racks, desiccators, conical flasks, Petri plates)	*Tarsons* 856, Marshal House, 33/1, Netaji Subhash Road Kolkata 700001 *Polylab* AK Scientific Industries, 5531/9, Basti Harphool Singh, Sadar Thana Road, Delhi 110006 *Laxbro* Laxbro Manufacturing Co., W-53, MIDC, Baosari, Pune 411026
pH meter	*Labindia* Labtek Engineers Pvt. Ltd., Vandana House, 4th floor, L.B.S. Marg, Panchpakadi, Thane 400602 *Mettler* Metler Instruments AG, Ch-8606, Griefense, Switzerland

(continued)

(continued)

Equipment/apparatus	Manufacturer(s)
Balances, analytical and top pan	*Anamed* P.O. Box no. 8336, 31, Ujagar Industrial Estate, W.T.P. Road, Deonar, Mumbai 400088 *Mettler* Metler Instruments AG, Ch-8606, Griefense, Switzerland
Filter sterilization membranes	*Millipore (India) Pvt Ltd* 50A, 2nd House, Ring Road, Peewja, Bangalore *Tarsons* 856, Marshal House, 33/1, Netaji Subhash Road Kolkata 700001
Water distillation/purifiers units	*Bhanu units Infusil India* C-251, V Cross, Industrial Estate, Peenaya, Bangalore 560058 *National Physical Laboratory* Pusa Road, New Delhi 110022 *Millipore (India) Pvt Ltd* 50A, 2nd House, Ring Road, Peewja, Bangalore *Ion Exchange India Ltd.* 8, Block B, LSC, Naraina, New Delhi 110028
Laminar airflow cabinet	*Saveer Biotech Ltd.* 1442, Wazir Nagar, Kotla Mubarakpur, New Delhi 110003 *Thermadyne Pvt. Ltd.* 24th K.M. Mathura Road, Faridabad 121003 *Atlantis India Engineering Pvt. Ltd.* 4E/3, Jhandewalan Extension, New Delhi 110055
Autoclaves (horizontal and vertical)	*Nat Steel* Metal Chem Industries, 18, Crescent Industrial Estate, Kanjumarg (E), Mumbai 400042 *Yorco Sales Pvt. Ltd.* 11, Netaji Subhash Marg, Daryaganj, New Delhi 110002 *Hindustan Scientific Instruments Company,* Hindustan House, C-9, Vishal Enclave, New Delhi 110027
Oven, hot plates, magnetic stirrers, vortex	*Associated Scientific and Chemicals* 5531, Basti Harphool Singh, Sadar Thana Road, Delhi 110006 *Hindustan Scientific Instruments Company* Hindustan House, C-9, Vishal Enclave, New Delhi 110027
Shakers	*Hindustan Scientific Instruments Company* Hindustan House, C-9, Vishal Enclave, New Delhi 110027 *New Brunswick Scientific Co. Inc.* Ependorf India Ltd. First Floor, 24 Community Centre, East of Kailash New Delhi 110065
Trolleys for growth room, temperature controller, electronic timers, humidifiers and dehumidifiers	*Saveer Biotech Ltd.* 1442, Wazir Nagar, Kotla Mubarakpur, New Delhi 110003

(continued)

(continued)

Equipment/apparatus	Manufacturer(s)
Stereozoom microscope	*Nikon* Towa Optics (I) Pvt. Ltd., 223, Okhla Industrial Estate, Ph. II, New Delhi 11002
Air conditioners, central cooling system, window/split types	*Frick India Ltd.* Jeevan Vihar, 3 Sansad Marg, P.O. Box 118 New Delhi 110001 *Carrier Aircon Ltd.* Flatted Fact. Complex, Okhla, New Delhi 110021 *Voltas Ltd.* 7/1, Asaf Ali Road, New Delhi 110002
Chemicals	*Qualigen Fine Chemicals* Fisher Scientific Thermo Electron US India Pvt Ltd, A-255, TTC Idustrial Area, Navi Mumbai 400710 *Sigma Chemical Company* P.O. Box 14508, St. Louis, MO 63178, USA *HiMedia* 23, Vadhani Industrial Establishment LBS Marg, Mumbai 400086 *Sigma-Aldrich Chemicals Pvt. Ltd.* 12th Floor, B Block, 148 Statesman House Barakhamba Road, New Delhi 110001 *Merck Specialities Pvt Ltd* Shiv Sagar Estate A, Dr Annie Besant Road Worli, Mumbai 400018

[a] Manufacturers and suppliers mentioned here are for guidance only. Most of the dealers are from India. Every country has their own dealers and representatives of the major foreign companies

Suggested Further Reading

Bhojwani SS, Razdan MK (1996) Plant tissue culture: theory and practice, a revised edition. Elsevier, Amsterdam

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Culture Media

3

3.1 Introduction

The growth, development and morphogenic response of an explant in culture depends on its genetic make-up, surrounding environment and composition of the culture medium. The last of these is the easiest to manipulate. The success of a plant tissue culture experiment largely depends on the selection of right culture medium. The clue for developing a basic culture medium seems to have initially come from the nutritional requirements of plants growing in soil, and later from nutrient solutions used for whole plant culture. Some of the earliest plant tissue culture media, such as callus culture medium of Gautheret (1939) and root culture medium of White (1943), were based on Knop's (1865) salt solution and Uspenski and Uspenskaia's medium (1925) for algae, respectively. The nutrient medium developed for the growth of higher plants by Knop included three salts: calcium nitrate, potassium phosphate and magnesium sulphate. Several media formulations developed in subsequent years are primarily based on White's and Gautheret's media. Compositions of three popular plant tissue culture media are given in Table 3.1. Of these, Murashige and Skoog's medium (MS) has been most widely used. Some other media developed for specific tissues or purposes are listed in respective chapters.

3.2 Media Constituents

The basic requirements of mineral elements such as potassium, calcium, magnesium, nitrogen, phosphorous and sulphur, which are required in large quantities (macronutrients/macroelements/major elements) and others, like iron, manganese, copper, zinc, boron and molybdenum, are required in small quantities (micronutrients/microelements/minor elements) for the growth of plant tissues (Tables 3.1, 3.2) are fulfilled by providing their common salts in the medium. When mineral salts are dissolved in water, they undergo dissociation and ionization. The active factor in the medium is the ions of different types rather than the compounds. One type of ion may be contributed by more than one salt in the medium. For example, in MS medium, NO_3^- ions are contributed by NH_4NO_3, as well as KNO_3, and K^+ ions by KNO_3 and KH_2PO_4. Therefore, a meaningful comparison between two media can be made on the basis of total concentrations of different types of ions in them.

In addition to these inorganic constituents, organic constituents such as vitamins and amino acids are also essential. The inorganic ions and organic compounds have the same function in tissue cultures as in whole plants (Table 3.3). At times, some complex nutritive mixtures of undefined composition, such as coconut milk

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_3, © Springer India 2013

Table 3.1 Composition of some plant tissue culture basal media[a]

Constituents	Amounts (mg L^{-1})		
	White[b]	MS[c]	B_5[d]
Inorganic			
Macronutrients			
NH_4NO_3	–	1,650	–
KNO_3	80	1,900	2,527.5
$CaCl_2.2H_2O$	–	440	150
$MgSO_4.7H_2O$	750	370	246.5
KH_2PO_4	–	170	–
$(NH_4)_2SO_4$	–	–	134
$Ca(NO_3)_2.2H_2O$	300	–	–
Na_2SO_4	200	–	–
$NaH_2PO_4.H_2O$	19	–	150
KCl	65	–	–
Micronutrients			
KI	0.75	0.83	0.75
H_3BO_3	1.5	6.2	3.00
$MnSO_4.4H_2O$	5.0	22.3	–
$MnSO_4.H_2O$	–	–	10.00
$ZnSO_4.7H_2O$	3.0	8.6	2.00
$Na_2MoO_4.2H_2O$	–	0.25	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025
$CoCl_2.6H_2O$	–	0.025	0.025
$Fe_2(SO_4)_3$	2.5	–	–
$FeSO_4.7H_2O$	–	27.8	–
$Na_2EDTA.2H_2O$	–	37.3	–
Sequestrene330Fe	–	–	28.00
Organic Nutrients			
Inositol	–	100	100
Nicotinic acid	0.05	0.5	1.00
Pyridoxine.HCl	0.01	0.5	1.00
Thiamine.HCl	0.01	0.1	10.00
Glycine	3	2	–
Sucrose	2 %	3 %	2 %

[a] Growth regulators and complex nutrient mixtures described by various authors are not included here. Several other media recommended for specific purpose are listed in relevant chapters of the book
[b] White (1963)
[c] Murashige and Skoog (1962)
[d] Gamborg et al. (1968)

(the liquid endosperm from immature coconuts, CM), tomato juice (TJ), casein hydrolysate (CH), yeast extract (YE) and malt extract (ME), have also been used to promote the growth of certain calli and organs. Since the plant parts grown in tissue cultures largely lose their photosynthetic ability, an utilizable source of carbon, such as sucrose, is also included in the culture medium. Successful culture of some systems has been possible only in the presence of actively growing cells, somatic embryos or ovaries (nurse tissue) in the culture medium.

Table 3.2 Stock solutions for Murashige and Skoog's basal medium (MS)[a]

Constituents	Amount (mg L^{-1})
Stock Solution I (*Macronutrients; 20x*)	
NH_4NO_3	33,000
KNO_3	38,000
$CaCl_2.2H_2O$	8,800
$MgSO_4.7H_2O$	7,400
KH_2PO_4	3,400
Stock Solution II (*Micronutrients; 200x*)	
KI	166
H_3BO_3	1,240
$MnSO_4.4H_2O$	4,460
$ZnSO_4.7H_2O$	1,720
$Na_2MoO_4.2H_2O$	50
$CuSO_4.5H_2O$	5
$CoCl_2.6H_2O$	5
Stock Solution III[b] (*Iron; 200x*)	
$FeSO_4.7H_2O$	5,560
$Na_2EDTA.2H_2O$	7,460
Stock Solution IV[c] (*Organic Nutrients; 200x*)	
Inositol	20,000
Nicotinic acid	100
Pyridoxine. HCl	100
Thiamine. HCl	20
Glycine	400

[a] To prepare 1 L of medium take 50 ml of Stock I, and 5 ml each of Stocks II, III and IV

[b] Dissolve $FeSO_4.7H_2O$ and $NA_2EDTA.2H_2O$ separately in 450 ml of distilled water by heating and constant stirring. Mix the two solutions, adjust the pH to 5.5 and add distilled water to make up the final volume to 1 L

[c] Sucrose is added at the time of preparing the medium

3.2.1 Inorganic Nutrients

(i) *Macronutrients*. The macronutrients are required in millimolar (mM) quantities. Calcium (Ca^{2+}), potassium (K^+), magnesium (Mg^{2+}), nitrogen (NO_3^-), sulphur (SO_4^{2-}) and phosphorous (PO_4^{3-}) are the macroelements and the essential ingredients of plant tissue culture media. These are added to the medium as calcium nitrate, potassium dihydrogen phosphate and magnesium sulphate. Alternately, potassium and calcium may be provided as KCl or KNO_3 and $CaCl_2.2H_2O$, respectively. Nitrogen is one of the vital elements required for the growth of plants in cultures as also in nature. Inorganic nitrogen is usually supplied in the form of ammonium (NH_4^+) and/or nitrate (NO_3^-) ions. Sometimes, organic form of nitrogen, such as urea, amino acids (glutamine) and/or casein hydrolysate (a complex mixture of amino acids and ammonium), is also included in the medium.

(ii) *Micronutrients*. Some micronutrients, although required in small quantities, are essential for tissue growth in cultures. They act as cofactors of enzymes. Micronutrients typically include boron (BO_3^{3-}; from H_3BO_3), manganese (Mn^{2+}; from $MnSO_4.H_2O$), iron (Fe^{2+}; from $FeSO_4.7H_2O$), zinc (Zn^{2+}; from $ZnSO_4.7H_2O$), copper (Cu^{2+}; from $CuSO_4.5H_2O$), molybdenum (generally as MoO_4^-; from $Na_2MoO_4.2H_2O$) and cobalt (Co^{2+}; from $CoCl_2.6H_2O$). Some media recipes also include very small amounts of iodine (I^-; from KI). To ensure the availability of iron over a wider pH range, its chelated form (Fe-EDTA) is used or a chelating agent such as Na_2EDTA is added in equimolar concentrations along with the iron salt ($FeSO_4.7H_2O$).

3.2.2 Organic Nutrients

In order to achieve the best growth of plant tissues in cultures, the medium needs to be supplemented with organic compounds, such as vitamins and amino acids. Apparently, the cultured plant cells synthesize them in sub-optimal quantities. Sucrose, a source of carbon or energy, is a very important constituent of plant tissue culture media.

(i) *Vitamins and amino acids*. Vitamins that act as coenzymes are required to be added to the medium for healthy growth of tissue cultures. The most widely used vitamins are those of B group, viz., thiamine (vitamin B_1), nicotinic

Table 3.3 Role of culture media constituents

Constituent	Form of availability	Role
Potassium	K^+	Necessary for normal cell division, and synthesis of proteins and chlorophyll
Magnesium	Mg^{2+}	Component of chlorophyll molecule
Calcium	Ca^{2+}	Constituent of cell wall; involved in the regulation of hormone responses and could have a pre-emptive role in morphogenesis; deficiency may cause shoot tip necrosis
Nitrogen	NO_3^- NH_4^+ Organic Nitrogen (vitamins/ amino acids)	Important constituent of amino acids, vitamins, nucleic acids and proteins; indirectly affects growth by its influence on pH of the medium; NH_4^+ is necessary for somatic embryogenesis in cell and callus cultures
Phosphorus	PO_4^{3-}	Vital for cell division; storage and transfer of energy (part of AMP, ADP and ATP)
Sulphur	SO_4^{2-}	Present in some amino acids (cysteine, cystine and methionine) and proteins
Iron	Fe^{2+}	Part of certain enzymes; functions as respiratory electron carrier through such compounds as cytochrome and oxidative enzymes, peroxidases and catalase
Copper	Cu^{2+}	Part of certain oxidative enzymes such as cytochrome oxidases, tyrosinase and ascorbic oxidase which serve to oxidize phenolic substances
Zinc	Zn^{2+}	Component of the enzyme concerned with the synthesis of the IAA precursor tryptophan; deficiency of zinc may cause rosetting/leaf chlorosis
Molybdenum	MoO_4^-	Component of some plant enzymes, such as nitrate reductase, and therefore, essential for nitrogen metabolism
Boron	BO_3^{2-}	Exact role not known but implicated in enhancing the rate of sugar movement in plants
Thiamine	Vitamin B_1	Involved in biosynthesis of certain amino acids; an essential cofactor in carbohydrate metabolism could have synergistic interaction with cytokinins.
Ascorbic acid	Vitamin C	An antioxidant, prevents blackening during explant isolation
Myo-inositol		Phosphatidyl-inositol is important in signal transduction; inositol phosphate may be acting as a second messenger to the primary action of auxins; probable role as a carrier and in storage of IAA as IAA-*myo*-inositol ester; a crucial precursor in the formation of pectin and hemicelluloses required for cell wall; may have role in the uptake and utilization of ions
Sucrose		Serves as carbon and energy as well as osmotic agent

acid (also known as niacin or vitamin B_3), pyridoxine (vitamin B_6) and *myo*-inositol (sometimes referred to as meso-inositol). *Myo*-inositol, a sugar alcohol, is added in a relatively larger quantity (100 mg L^{-1}). Thiamine, nicotinic acid and pyridoxine are used in the hydrochloride (HCl) form.

Other vitamins included in some media formulations are folic acid (vitamin M), ascorbic acid (vitamin C), riboflavin (lactoflavin, vitamin B_2), pantothenic acid (D-calcium pantothenate, vitamin B_5), biotin (vitamin H) and tocopherol (vitamin E).

Of all the vitamins used in plant tissue culture, only thiamine and *myo*-inositol (considered a B vitamin) are considered essential ingredients of plant tissue culture media. Other vitamins are added due to historical reasons or are found to promote specific in vitro response.

Although the necessity of amino acids in plant tissue cultures is not well demonstrated, they are sometimes added to the culture media. Glycine, the simplest amino acid, is a common constituent of plant tissue culture media. Others include L-glutamine,

asparagine, serine and proline. The amino acids serve as the organic source of reduced nitrogen. However, the presence of inorganic nitrogen in the medium (NH_4^+ and NO_3^-) is generally sufficient to ensure protection against any possible nitrogen deficiency, and supplementation with amino acids may not be required.

(ii) *Carbon source*. Most plant tissue cultures are unable to photosynthesize because of the absence of chlorophyll or poorly developed chloroplasts, limited CO_2 in the culture vessel due to poor gaseous exchange and absence of optimum light intensity. It is, therefore, obligatory to add to the culture medium an utilizable source of carbon necessary for various metabolic activities. The most commonly used carbon source is sucrose at a concentration of 2–5 % (w/v). Generally, sucrose autoclaved along with the medium supports better growth of tissues than filter-sterilized sucrose. Autoclaving causes hydrolysis of sucrose into more efficiently utilizable sugars, such as glucose and fructose. Sucrose not only is an energy source but is also the major osmotic component of the medium. Nutrient salts contribute approximately 20–50 % to the osmotic potential of the medium, and the rest is taken care by sucrose.

Some other forms of carbon that plant tissues are known to utilize include maltose, galactose, mannose and lactose. Maltose has especially been found superior to sucrose in promoting somatic embryogenesis in soybean, alfalfa and rubber. Maltose has also proved to be the best carbon source for androgenesis in wheat and rice (Last and Brettell 1990; Pande and Bhojwani 1999).

3.2.3 Plant Growth Regulators

Addition of growth regulators (Table 3.4) to an otherwise complete medium, containing inorganic and organic nutrients and sucrose called Basal medium (BM), is invariably necessary to trigger various types of growth and differentiation. Although the explants may have certain levels of endogenous growth hormones (naturally synthesized growth regulators), it is often necessary to supplement them exogenously to evoke certain responses. The growth regulators are generally required in very minute quantities (0.001–10 μM). The nature and quantity of growth regulators in the medium is varied according to the variety of plant, nature of the tissue and also the stage of culture (initiation of callus, induction of somatic embryogenesis, shoot differentiation or multiplication, rooting of shoots, etc.).

In order to develop a tissue culture protocol for a new plant species, various types and concentrations of growth regulators in several permutation and combination need to be tested. The concentrations of growth regulators used are very often reported in mg L^{-1}. However, for meaningful comparative studies of the responses induced by various growth regulators in cultures, it is desirable to express them in molar concentrations, which is a reflection of the actual number of molecules of the growth regulator per unit volume of the medium.

(i) *Auxins*. Auxins are involved with many developmental processes in plants, including elongation of stem and internodes, tropisms, apical dominance, abscission and rooting. In tissue cultures, auxins have been used to induce cell division, cytodifferentiation and organogenic and embryogenic differentiation. Generally, auxin at low concentration favours root initiation, whereas at higher concentration induces callus formation. The auxins commonly used in tissue cultures are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2,4-dicholorophenoxyacetic acid (2,4-D) and para-chlorophenoxyacetic acid (p-CPA). Whereas IAA and IBA are naturally occurring auxins, NAA, 2,4-D and p-CPA are synthetic. Some other synthetic auxins that have been used in tissue culture work are naphthoxyacetic acid (NOA), 2,4,5-tricholorophenoxyacetic acid (2,4,5-T), 4-amino-3,

Table 3.4 Some plant growth regulators used in plant tissue culture media, their molecular weights (M.W.) and solvents

Growth Regulator	Abbreviation	M.W.	Solvent
Indole-3-acetic acid	IAA	175.2	Ethanol/1 N NaOH
Indole-3-butyric acid	IBA	203.2	Ethanol/1 N NaOH
2,4-Dichlorophenoxyacetic acid	2,4-D	221.04	Ethanol/1 N NaOH
α-Naphthalene acetic acid	NAA	186.2	Ethanol/1 N NaOH
Naphthoxyacetic acid	NOA	202.2	1 N NaOH
2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	255.5	Ethanol
para-Chlorophenoxyacetic acid	p-CPA	100.59	Ethanol
4-Amino-3,5,6-tricholoro pyridinecarboxylic acid	Picloram	241.5	Acetone
3,6-Dichloro-o-anisic acid	Dicamba	221	Ethanol/Acetone
6-(Furfurylamino)-purine (kinetin)	KIN	215.2	dil HCl/1 N NaOH
6-(Benzylamino)-purine	BAP	225.3	1 N NaOH
2-(Isopentinyl)-adenine	2iP	203.2	1 N NaOH
6-(4-Hydroxy-3-methylbut-2-enylamino)-purine (Zeatin)	ZEA	219.2	dil HCl/1 NaOH
1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thiadiazuron)	TDZ	220.3	DMSO
6-(3-Hydroxybenzylamino)-purine	*m*T	243.26	1 N NaOH
Gibberellic acid	GA_3	346.4	Water
Abscisic acid	ABA	264.31	1 N NaOH
Tri-iodo benzoic acid	TIBA	499.81	1 N NaOH
Phloroglucinol	PG	126.11	Ethanol/water

5,6-tricholoropyridinecarboxylic acid (picloram) and 3,6-dichloro-o-anisic acid (dicamba).

IBA, NAA and IAA are widely used for rooting and, in interaction with a cytokinin, for shoot proliferation. They have also been implicated in tracheidal differentiation in cell and callus cultures. 2,4-D and 2,4,5-T are very effective for the induction and growth of callus. For inducing somatic embryogenesis, 2,4-D is an important factor. Dicamba has been used for monocots whereas picloram has been effective for legumes.

Auxins are usually dissolved in a small amount of ethanol or dilute NaOH.

(ii) *Cytokinins*. This is another major group of plant hormones. Natural cytokinins are N^6-substituted adenine derivatives and occur in plants as nucleosides and nucleotides. Roots are the possible sites of their synthesis. In nature, cytokinins are concerned with cell division, modification of apical dominance and shoot differentiation. Incorporation of these compounds in culture media is mainly to trigger cell division, and to induce differentiation of adventitious shoots from callus and organs, and shoot proliferation by the release of axillary buds from apical dominance. The cytokinins commonly used in tissue cultures are kinetin (6-furfurylamino) purine, BAP [(benzylamino) purine], 2iP [(2-isopentenyl)-adenine or 6-(γ,γ-dimethylallyl amino) purine)] and zeatin [6-(4-hydroxy-3-methyl but-2-enyl amino)-purine)]. Thidiazuron (TDZ), a dipheny1-substituted urea, has also been used as a cytokinin in tissue cultures. However, it is used at very low concentrations (0.05–0.1 μM) as at higher levels it promotes callusing. At times, adenine (2–120 mg L^{-1}) has been added to tissue culture media as a weak cytokinin to promote shoot formation. The most efficient and commonly used cytokinin in plant tissue culture is BAP. It is used in the range of 1–10 μM.

Topolins, are a new class of highly active naturally occurring aromatic cytokinins. These cytokinins, particularly meta-topolin

or *m*T [6-(3-hydroxybenzylamino)-purine], are becoming increasingly popular with plant tissue culturists because of their positive effects on several parameters of tissue culture, such as high rate of shoot multiplication, reduced physiological abnormalities, and better rooting and acclimatization.

Cytokinins are generally dissolved in small amounts of dilute HCl or NaOH. For thidiazuron, DMSO (dimethylsulphoxide) is used as the solvent.

(iii) *Gibberellins*. These are less commonly used in plant tissue culture. There are over 20 known gibberellins, of which GA_3 is used most often. They are reported to stimulate elongation of internodes, meristem growth for some species and more importantly to attain normal development of plantlets from in vitro formed adventive embryos.

GA_3 is readily soluble in cold water (up to 1000 mg L^{-1}). Being heat sensitive (90 % of the biological activity is lost after autoclaving), GA_3 is filter sterilized and added to autoclaved medium after it has cooled.

(iv) *Ethylene and Abscisic acid*. Ethylene (ethane, C_2H_4) is an unusual, gaseous plant hormone. It is produced by ageing and stressed tissues. In plant tissue cultures, ethylene is also produced by the organic constituents of the medium on exposure to heat, oxidation, sunlight or ionizing radiation. Ethylene appears to influence various morphogenic processes, such as embryogenesis and organogenesis, but its effects are not clear. It may be promotory or inhibitory for the same process in different plant systems. For example, ethylene promotes somatic embryogenesis in maize but inhibits it in rubber tree tissue cultures.

Ethrel or ethapon (2-chloroethane phosphonic acid), which releases ethylene upon decomposition, is used to study the effect of this gaseous growth regulator on plant tissue cultures. It generally inhibits growth and differentiation, but in some cases, it promoted somatic embryogenesis.

Abscisic acid, a naturally occurring growth inhibitor, is often required for normal growth, development and maturation of somatic embryos.

3.2.4 Other Supplements

(i) *Polyamines*, derived through decarboxylation of amino acids, have been used to promote organogenesis/somatic embryogenesis (Mengoli and Bagni 1992; Rajam et al. 1998). Of the three polyamines (putrescine, spermidine and spermine), putrescine has proved most effective (Altman 1990; Litz 1993).

(ii) *TIBA* (2,3,5-triiodobenzoic acid) is an inhibitor of auxin polar transport. In carrot cultures, it arrests development of somatic embryos at the globular stage.

(iii) *Phloroglucinol* has been shown to promote rooting in rosaceous fruit trees such as apple.

(iv) *Activated charcoal* is usually added at 0.5 % to culture media to promote rooting and/or to adsorb toxic exudates from cultured tissues.

3.2.5 Undefined Supplements

In order to promote growth of certain calli and evoke a desired morphogenic response in cultures, several complex, undefined organic supplements such as banana homogenate, CH, CM, corn milk, ME, TJ and YE have been used in the medium. These crude natural plant products are generally used when no other combination of defined components yield the desired results. However, the use of such supplements should be avoided, because their active constituents may vary quantitatively and qualitatively with the age of the source tissue and genotype of the plant, affecting the reproducibility of results. If at all necessary to use them in the initial experiments, all efforts should made to effectively replace them by a defined component such

as a single amino acid and arrive at a "synthetic medium" which contains only chemically defined compounds (Straus 1960).

Occasionally, actively growing plant tissues (embryogenic calli, young ovaries) have been used as nurse tissues for successful culture of single-cell systems, such as in vivo and in vitro formed zygotes and isolated microspores of cereals.

3.2.6 Gelling Agents

In static liquid cultures, the tissue would get submerged and die of anaerobic conditions. To circumvent this problem, the medium is solidified with a suitable gelling agent. The most desirable properties of a gelling agent are that it should: (i) be inert (ii) withstand sterilization by autoclaving and (iii) be liquid when hot so that the medium could be dispensed in culture vessels in desired quantities. The semi-solid nature of the medium enables the explants to be placed on its surface so that it remains aerated. Being biological products, the gelling agents do contain organic and inorganic contaminants, the amounts of which vary between different brands depending on the extent of processing and purification. Some of the gelling agents used in plant tissue cultures are agar, agarose and gellan gum (phytagel, Gelrite).

(i) *Agar*. Agar, obtained from red algae, especially *Gelidium amansii*, is the most commonly used gelling agent. Agar is a high molecular weight polysaccharide (built of galactose molecules) that can bind water. Firmness of the gel depends on concentration of agar and pH of the medium during autoclaving. Agar solidifies at 45 °C. It is generally added to the medium at a concentration in the range of 0.8–1 % (w/v).

(ii) *Agarose*. Agarose, comprising β-D(1-3) and 3,6-anhydro-α-L(1-4) galactopyranose molecules, is obtained by further purifying agar to remove agaropectins with its sulphate groups. Consequently, it is costlier than agar. It is used in the range of 0.4–1.0 %. Agarose melts at 30 °C, which makes it suitable for testing medium ingredients that are thermolabile and for protoplast culture.

(iii) *Gellan gum*. A linear polysaccharide obtained from the bacterium *Pseudomonas elodea* and marketed under different trade names, such as gelrite (C.P. Kelco & Co.) and phytagel (Sigma-Aldrich Chemical Co.), has become a popular substitute of agar. Unlike agar, which requires heating, the gellan gum can be prepared in cold solution. To prevent clumping, it should be added to culture medium while rapidly stirring at room temperature. The concentration of divalent cations, such as calcium and magnesium, in the medium must be within restricted range for gelling of the gellan gum.

Gelrite or phytagel is a good alternative to agar not only because of its low cost per litre of medium (0.1–0.2 % is sufficient) but also for many other advantages it offers. It sets as a clear gel, which facilitates easy observation of cultures and detection of any contamination. Unlike agar, their gelling strength is unaffected over a wide range of pH.

Teyssier et al. (2011) reported that raising the concentration of gellan gum in the medium from 0.4 % to 0.8 % improved the maturation of somatic embryos of *Larix eurolepsis*. The embryos had lower osmotic potential but high dry weight.

(iv) *Isubgol*. Indian scientists have shown that the husk of *Plantago ovata* seeds (Isubgol), used at 3 %, could be a cheaper alternative to gel plant tissue culture media (Jain and Babbar 1998).

3.3 pH of the Medium

The pH of medium greatly influences the uptake of ingredients by the tissue in culture, solubility of salts and gelling efficiency of agar. The pH of medium is usually set at 5.8 as at this pH all the salts are maintained in a near buffered form. In

general, a pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar due to its hydrolysis during autoclaving. The pH of culture medium generally drops by 0.3–0.5 units after it is autoclaved and then changes throughout the period of culture both due to oxidation and differential uptake of ions such as NH_4^+ and NO_3^- and secretion of substances by the growing tissue. In some studies, 2-(N-morpholino)ethanesulphonic acid (MES), an organic buffering agent, has been added to the medium, after setting pH to 5.7, to maintain a buffered medium for long periods.

3.4 Media Preparation

Preparation of culture media is a critical step in tissue culture work wherein great precision is required on the part of the investigator. There are two possible ways to prepare medium. One method is to weigh and dissolve the required quantities of the ingredients separately and mix them every time a medium is to be prepared. But this is a cumbersome method and chances of committing error are more.

A more convenient and widely practiced method is to prepare, in advance, a series of concentrated stock solutions and store them in a refrigerator. Whenever a medium is to be prepared, the required amounts are drawn from the stock solutions and mixed. For example, to prepare MS, four stock solutions are prepared (see Table 3.2).

Each salt should be separately dissolved to the last particle and then mixed with the others. In order to avoid any precipitation, the preferred method is to group the compounds by the ions they contain, such as nitrate, sulphate, phosphate and halide, and to first mix the dissolved components of each group and then mixing all the solutions. The stocks of major and minor salts are kept separately.

For making stock solution of iron, the required quantities of $FeSO_4.7H_2O$ and $NA_2EDTA.2H_2O$ are weighed and dissolved separately in 450 ml of distilled water by heating and constant stirring and then the two are mixed. The pH is adjusted to 5.5, and distilled water is added to make up the final volume to one litre. The final stock solution should be deep golden yellow.

Depending on the levels of growth regulators used, their stock solutions may be prepared at a strength of 1 or 10 mM. The growth regulators are dissolved in a minimum quantity of the solvent (Table 3.4), and the final volume made up with distilled water.

For preparing stock solution and media, glass-distilled or purified water, and chemicals of high purity should be used.

All the stock solutions are stored in proper plastic or glass bottles under refrigeration. The stock solution of iron must be stored in amber-coloured bottle. All the bottles containing stock solution should be properly labelled as suggested below, indicating type of medium, type of stock, amount to be used, date of preparation and name of the investigator.

MS STOCK
Major salts
Use 50 ml L^{-1}
25/02/2013
Investigator's Name

For storing coconut milk, the liquid collected from young fruits is boiled to deproteinize it, filtered and stored in a plastic bottle at −20 °C.

Before using the stocks, the bottles must be shaken gently and if any of the solutions shows suspension of a precipitate or a biological contaminant, it should be discarded and fresh stock prepared.

3.4.1 Steps in the Preparation of Culture Medium

(i) Required quantities of agar and sucrose are weighed and dissolved in water, about three-fourth the final volume of the medium, by heating them in an oven or a microwave oven or by autoclaving at low pressure. This step is not necessary for a liquid medium because sucrose would dissolve even in lukewarm water.

(ii) Appropriate quantities of the various stock solutions, including growth regulators and other special supplements, are added. Some investigators prefer addition of vitamins and growth regulators after autoclaving. If there is a special reason to do so, for example, if the substance is thermolabile, it may be filter sterilized (the solution already adjusted to the desired pH) through microfilters of pore size 0.22–0.45 μm and added to the autoclaved medium under aseptic conditions.

(iii) The final volume of the medium is made up with distilled water.

(iv) After mixing well, pH of the medium is adjusted usually to 5.8 using 0.1 N NaOH or 0.1 N HCl.

(v) The medium is poured into desired culture vessels. About 15 ml of the medium is dispensed in 25 × 150 mm glass culture tubes or about 50 ml in 250 ml Erlenmeyer flask. If while pouring the medium starts to gel, it should be reheated in a water bath or microwave oven and poured only when it is in a uniformly liquid state.

(vi) Mouth of the culture vessels are closed with non–absorbent cotton wrapped in cheesecloth (such closures exclude microbial contaminants but allow free gas exchange), or any other suitable closure.

(vii) The culture vessels containing medium are transferred to appropriate baskets, covered with aluminium foil to check wetting of plugs during autoclaving at 1.06 kg cm^{-2} (15 psi) for 15 min (time of sterilization varies with volume). If pre-sterilized, unautoclavable, plastic culture vials (Petri plates or jars) are being used, the medium may be autoclaved in 250 or 500 ml flasks with suitable closure (large flasks are inconvenient for pouring) or narrow-mouthed bottles. The medium is allowed to cool down to 50–40 °C before pouring into the vials under aseptic conditions.

(viii) The medium is allowed to cool to room temperature and stored. If the medium is not to be used within a week, it should be stored at 4 °C. When preparing a semi-solid medium in culture tubes, it is desirable to make slants by keeping the tubes tilted during cooling. Such slants provide a larger surface area for tissue growth. It is also easier to photograph cultures grown on such slants.

To minimize human error, all the steps listed above should be followed very carefully. The ingredients of the medium should be listed on a paper, and after adding a component, it should be cancelled on the sheet. All the tubes, jars, flasks and Petri plates containing the medium should be clearly marked in such a manner that they can be identified even after autoclaving and long storage under light.

3.4.2 Use of Commercial Pre-Mixes

A large variety of basal media and special media formulations are now available commercially in the form of pre-mixed dry powder packed in sachets of various quantities. The powder is dissolved in distilled water (10 % less than the final volume of the medium), and after adding sucrose, agar and other desired supplements, the final volume is made up with distilled water. The pH is adjusted and the medium autoclaved. Such powdered media are more useful in routine commercial micropropagation of specific crops. It avoids storing so many chemicals, preparing their stocks and mixing them and thus saves time.

Suggested Further Reading

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Tissue and Cell Culture 4

4.1 Introduction

The pioneering attempts of G. Haberlandt (1902) to culture plant cells did not succeed but with the refinement of the in vitro techniques and tissue culture media and the discovery of plant growth regulators it has been possible to establish various types of tissue cultures, starting from embryonic, meristematic, and mature tissues. Broadly, there are two types of tissue cultures: (i) unorganized (callus and suspension cultures) and (ii) organized (root cultures, shoot tip cultures, embryo cultures etc.). Organized tissue cultures are dealt with in some of the following chapters. This chapter describes unorganized tissue cultures.

4.2 Callus Cultures

Callus is an irregular mass of parenchymatous tissue with meristematic loci. It lacks any organized structure but often shows cellular differentiation, mostly tracheidal elements. A well-established callus comprised different types of cells. The cellular heterogeneity of the callus is derived from the multicellular explants used to initiate callus cultures and/or induced by the culture conditions. The calli from the same explant may show considerable variation with regard to color, texture, compactness amount of water content, and chemosynthetic and morphogenic potential. The calli may be compact or friable, dry or wet, light, or dark colored (Fig. 4.1a, b). These features may also change with passage of time in cultures due to genetic or epigenetic changes or due to change of culture medium. It has often been possible to isolate different types of calli from the same explants.

The calli can be multiplied as unorganized tissue for unlimited period through periodic subcultures on fresh medium or induced to differentiate organized structures (roots, shoots, embryos) by manipulating the culture medium (Chap. 6 and Chap. 7).

In nature, several plants develop irregular unorganized structures due to disturbance in the endogenous levels of growth regulators caused by infection, by insects (insect galls), microorganisms (crown gall), or specific genetic recombinations (genetic tumours). In vitro normal tissues from different parts of a plant can be induced to divide and undergo irregular cell divisions to form callus by the application of plant growth regulators through the culture medium. In practice, a small piece of plant organ (*explant*) is excised, surface sterilized, and planted on a culture medium containing an auxin and/or a cytokinin. The requirement for exogenous growth regulators for callusing varies with the explants, depending on the endogenous levels of growth regulators. 2,4-D is the most commonly used auxin to induce callus formation. On a favorable medium the explant shows cell divisions, usually starting at the cut ends, and gradually extending over the entire surface of the explant, leading to the formation of an

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_4, © Springer India 2013

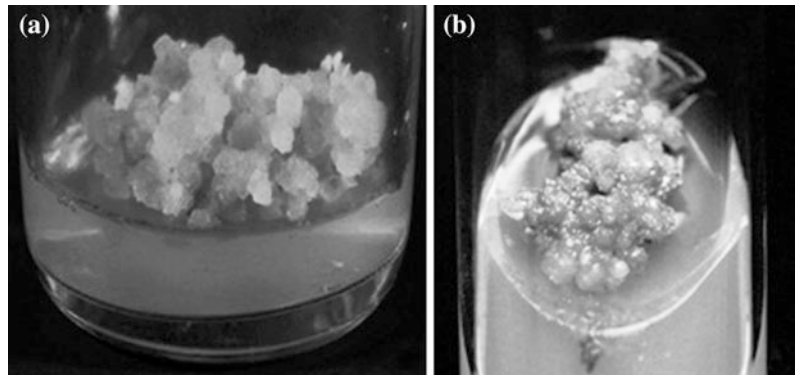

Fig. 4.1 **a** Friable callus of wheat. **b** Compact callus of mulberry

irregular mass of tissue, called callus (Fig. 4.5). After 3–4 weeks, the callus is separated from the parent explant and transferred to fresh medium of the same composition. By periodic subcultures on fresh medium the callus can be multiplied for unlimited period.

Some callus cultures, which initially require an auxin for growth, acquire an irreversible capacity to synthesize excess quantities of auxin, and consequently become autotrophic for auxin. Such callus cultures, also called *habituated*, can grow independent of an exogenous auxin (Gautheret 1946). Similar habituation of cultured tissues with respect to cytokinin requirement has also been observed.

The callus tissue may become progressively friable with repeated subcultures on agar medium. Such a friable callus is highly desirable for raising a fine cell suspension in liquid medium. Friability of callus can also be induced by modifying the growth regulator composition of the medium.

Callus cultures have many applications: (i) serve as the starting material to initiate single cell and suspension cultures, (ii) provide system for various morphogenetic and physiological studies, (iii) produce secondary metabolites, and (iv) generate useful somaclonal variations. This chapter deals with the first aspect. Other applications of cell and callus culture are discussed in other chapters of the book.

4.3 Suspension Cultures

To initiate cell suspension cultures, pieces of established undifferentiated, friable calli are transferred to liquid medium in flasks or some other suitable vials, and the medium is continuously agitated (30–100 rpm) by fixing on a gyratory/orbital shaker (Fig. 4.5e). Agitation of the medium exerts a mild pressure on the tissue, breaking it into smaller cell aggregates and single cells. It also maintains uniform distribution of cells and cell clumps in the medium, and helps gaseous exchange between the culture medium and the air inside the culture vial (aeration of the cells). Ideal suspensions should consist of single cells but this is difficult to

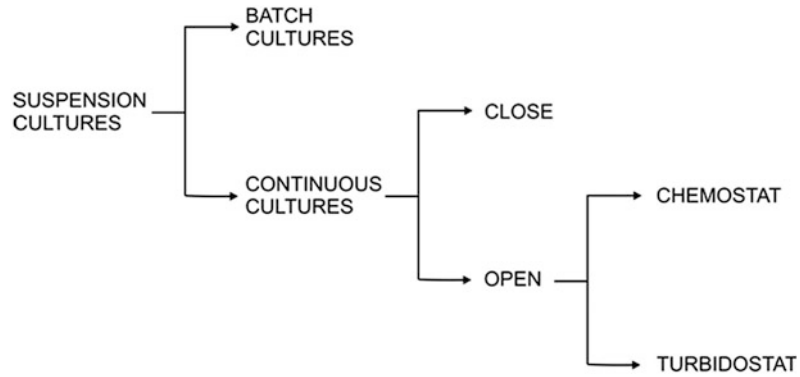

Fig. 4.2 Different types of suspension cultures

achieve. Most suspension cultures that have been established so far consist of both single cells and small cell aggregates. In the suspension cultures of wheat and barley, the presence of multicellular structures in the inoculum at every subculture proved essential to maintain the population of viable single cells (Dong et al. 2010).

A much higher rate of cell division may be exhibited by cells in suspension cultures than in callus cultures on agar medium. However, cell doubling time in suspension cultures varies with the system: *Nicotiana tabacum*, 48 h, *Acer pseudoplatanus*, 40 h, *Rosa* sp., 36 h, and *Haplopappus gracilis*, 22 h.

There are various types of suspension cultures as depicted in Fig. 4.2.

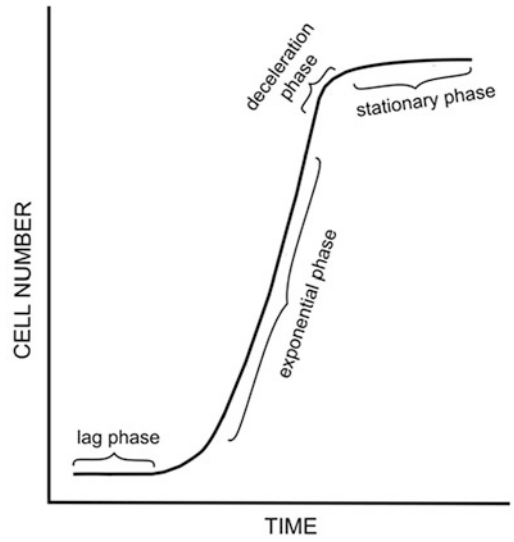

Fig. 4.3 Model curve relating cell number per unit volume of culture to time in a batch-grown cell suspension culture

4.3.1 Batch Cultures

These are used for initiating single cell cultures. In batch cultures cell suspensions are grown in 100–250 ml flasks each containing 20–75 ml of culture medium. The cultures are continuously propagated by routinely taking a small aliquot of the suspension and transferring it to a fresh medium (about five times dilution). A pipette or syringe with an orifice fine enough to allow only single cells and small cell aggregates (2–4 cells) is used for subculture. Batch suspension cultures exhibit a sigmoid pattern of growth with five phases (Fig. 4.3). Initially, the cultures pass through a lag phase where cells prepare to divide. It is followed by a short exponential phase during which the rate of cell divisions is the highest. After a few cell generations cell divisions slow down and cell expansion increases (linear growth phase). Thereafter, the cultures enter the deceleration phase during which cell divisions and expansion decline. Finally, the cultures enter the stationary phase in which the number and size of the cells remain constant. Cultures can be maintained continuously in exponential phase by frequent (every 2–3 days) subcultures. Addition of 'conditioned medium' (in which cell cultures have been grown before) reduces the lag phase dramatically.

A constant change in the pattern of cell growth and metabolism and the composition of the nutrient medium, and consequent absence of steady state of growth are inherent drawbacks of batch cultures. It is, therefore, not a suitable system to study cell growth and metabolism. These problems are overcome, to a certain extent, in continuous cultures.

4.3.2 Continuous Cultures

Periodic addition of fresh medium and draining out the used medium ensures maintenance of suspension cultures under steady state of growth for long periods. Such cultures are called continuous suspension cultures. This system is suitable for mass scale cell culture. A number of culture vessels, also called bioreactors, have been devised to grow continuous cell cultures on a large scale (Fig. 4.4a–d).

There are two types of continuous cultures: close continuous cultures and open continuous cultures.

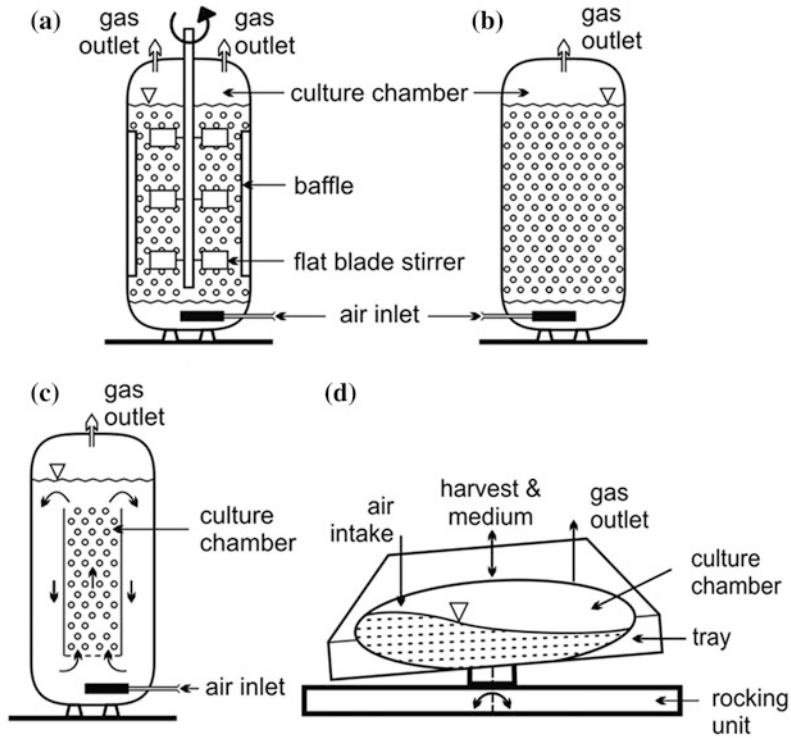

Fig. 4.4 Some bioreactors used for plant cell culture. **a** Stirred Tank Bioreactor; the air is dispersed by mechanical stirring of the medium by low speed rotating impellers (50–150 rpm). **b** Bubble Column Bioreactor; it is the simplest of the gas–liquid reactor in which the medium in the culture chamber is aerated at the bottom by means of nozzle or perforated plates. **c** Air-lift Bioreactor; it has a draft tube as the culture chamber. Air is sparged at the base of the tube lowering the density of the medium which rises up the draft tube pulling fresh medium in the base by which a flow is achieved. **d** BioWave reactor; has a culture chamber made of gas permeable plastic film, which rocks on a platform inducing a wave in the culture bag that contains culture medium and cells resulting in mixing and oxygenation

(i) *Close continuous cultures*. In this system, the addition of fresh medium is balanced by out flow of the old medium. However, the cells from the out flowing medium are mechanically separated and added back to the culture. Thus, cell biomass continues to increase with age of the culture.

(ii) *Open continuous cultures*. The addition of fresh medium is balanced by the harvest of an equal volume of medium, including cells, maintaining indefinitely a constant submaximal growth rate.

Open continuous cultures are also of two types: (a) *chemostat*, and (b) *turbidostat*. In chemostat cultures, a steady state of cell growth is maintained by a constant inflow of fresh medium in which the concentration of chosen nutrient (nitrogen, phosphorus or glucose) is adjusted so as to be growth limiting while other constituents are at a concentration higher than that required for desired rate of cell growth. Any increase or decrease in the growth limiting factor is reflected by an increase or decrease in the growth rate of cells. In turbidostat cultures, the inflow of fresh medium is controlled by an increase in the turbidity of the culture due to cell growth. A desired biomass density is maintained by flushing out cells.

Chemostat cultures offer the possibility of maintaining a steady-state cell growth and metabolism, and to determine the effect of individual growth limiting nutrients on cell growth.

4.3.3 Medium for Suspension Cultures

Generally, a medium on which fast growing friable callus is established is suitable for initiating suspension culture of that species provided, of course, agar is omitted from it. Better cell dispersion can be achieved by manipulating auxin/cytokinin ratio. Inorganic phosphate is rapidly consumed in actively growing suspension cultures. Therefore, phosphate concentration is raised about 2–3 times the prescribed level in the medium. A culture medium (see Table 4.1) formulated for tobacco suspension cultures that could be used for other systems as well is fortified with an auxin, a cytokinin, and casein hydrolysate (Reynolds and Murashige 1979). B_5 medium (see Table 3.1) was primarily developed for suspension cultures of soybean. For the growth of callus and suspension cultures of *Barringtonia racemosa* and lycopene accumulation in these tissues woody plant medium (WPM; Pavingerova and Sediva 1999) was distinctly superior to MS and B_5 media (Behbahani et al. 2010).

Besides medium, cell density is an important factor for raising suspension cultures. For initiating suspension cultures, it is desirable to keep cell density around 5×10^4 cells ml^{-1} or higher.

4.3.4 Synchronous Cell Suspension Cultures

For studying cell cycle or cell metabolism in suspension cultures, it is desirable to use synchronous or partially synchronous cultures. A cell culture in which the majority of cells proceed through each cell cycle phase (G_1, S, G_2 and M) simultaneously is regarded as synchronous. Synchronization in cell suspension cultures could be achieved by *starvation* or *inhibition.* In the former, the cells are starved of a nutrient (phosphate, nitrogen) or hormonal factor required for cell division to arrest them in G_1 or G_2 phase of the cell cycle. When these cells are transferred to complete medium they enter division synchronously.

Synchronization in cell suspension cultures can also be achieved by using the inhibitors of DNA synthesis, such as 5-aminouracil, FudR, hydroxy urea, and thymidine. In the presence of these inhibitors, the cell cycle proceeds only up to the G_1 phase and the cells get collected at G_1/S interphase. Removal of the inhibitor from the culture medium is followed by synchronous division of cells.

4.3.5 Determination of Growth in Suspension Cultures

Growth in plant cell suspension cultures is commonly measured by cell counting, determination of total cell volume (packed cell volume), and fresh and dry-weight increase of cells and cell colonies.

(i) *Cell counting*. For accurate cell counting the cell aggregates should be dispersed into single cells by treating them with chromic acid (5-8 %) or pectinase (0.25 %) before taking sample (Street 1977). For sycamore cell suspensions the following method has been used: one volume of cell culture is mixed with two volumes of 8 % chromic trioxide and heated to 70° C for 2–15 min depending on the growth phase of the culture. The mixture is allowed to cool and agitated vigorously for 10 min before counting the cells in a hemocytometer.

(ii) *Packed cell volume* (PCV). To determine PCV, a known volume of uniformly dispersed suspension is transferred to a 15 ml graduated centrifuge tube and spun at $200 \times g$ for 5 min. PCV is expressed as ml pellet ml^{-1} culture.

(iii) *Cell fresh weight* (FW). This can be determined by collecting cells on a pre-weighed (in wet condition) circular filter of nylon fabric supported in a funnel, washing the cells with water to remove the medium, draining under vacuum, and reweighing.

Table 4.1 Composition of the medium recommended for the culture of isolated mesophyll cells of *Calystegia sepium*

Constituents	Amounts (mg L^{-1})
Macroelements	
KNO_3	950
NH_4NO_3	725
$MgSO_4.7H_2O$	187
$CaCl_2$	169
KH_2PO_4	69
Microelements	
$MnSO_4.4H_2O$	12.5
H_3BO_3	5
$ZnSO_4.4H_2O$	5
$NaMoO_4.2H_2O$	0.125
$CuSO_4.5H_2O$	0.0125
$FeSO_4.7H_2O$	13.9
Na-EDTA	18.6
Vitamins	
Glycine	2
Nicotinic acid	5
Pyridoxine HCl	0.5
Thiamine HCl	0.5
Biotin	0.05
Folic acid	0.5
Myo-inositol	100
Growth regulators	
BAP	0.1
2,4-D	1
Sucrose	10,000
pH	5.5

after Rissini (1972)

(iv) *Cell dry weight* (DW). As in (iii) the cells are collected on a pre-weighed dry nylon filter dried for 12 h at 60 °C and reweighed. Alternately, moisture determination balances can be used to quantify the fresh and dry weight of the callus cells in a very short time. Cell weight is expressed as per culture or per 10^6 cells.

(v) *Noninvasive methods*. All four methods described above require withdrawal of culture samples. Some noninvasive methods to characterize growth in batch cultures have been described. For example, Blom et al. (1992) have described a method in which the culture flask, fitted with a ruler, is tilted at an angle of 30 or 60° (same each time) for 5 min and the height of the sediment recorded. The change in the height of the sediment with age of the culture would represent the change in fresh weight of the cells as there is a direct correlation between the two parameters.

4.3.6 Tests for Viability of Cultured Cells

(i) *Phase contrast microscopy*. Microscopic assessment of cell viability is based on cytoplasmic streaming and the presence of a healthy nucleus. While the phase contrast microscopy gives a better picture of these features, it is often not difficult to observe them under bright field microscopy.

(ii) *Tetrazolium test*. In this test, the respiratory efficiency of cells is measured by reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the red dye formazan which can be extracted and measured spectrophotometrically. Although this method allows quantification of observations, it alone may not always give a reliable picture of the cell viability.

(iii) *Fluorescein diacetate* (FDA) test. This technique offers a quick visual assessment of percentage viability of cells. Stock solution of FDA at a concentration of 0.5 % is prepared in acetone and stored at 0 °C. To test viability, the solution is added to the cell or protoplast suspension (for protoplasts, an appropriate osmotic stabilizer is added to the FDA solution) at a final concentration of 0.01 %. After about 5 min of incubation the cells are examined under an epifluorescent microscope. FDA is nonfluorescent and being nonpolar, freely permeates across the plasma membrane. Inside the living cell it is cleaved by the activity of esterase enzyme, releasing the fluorescent polar portion, fluorescein, which fluoresces green under

UV. Since fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cells, but in dead and broken cells it is lost.

(iv) *Evan's blue staining*. The stain can be used as complementary to FDA. When the cells are treated with a dilute (0.025 %) solution of Evan's blue the damaged cells take up the stain but the intact and viable cells exclude it, and thus remain unstained.

4.4 Large Scale Cell Culture

Plant cell and tissue cultures have been used to produce commercially important low molecular weight secondary metabolites (Chap. 18). Another potential application of plant cell cultures is in the production of foreign recombinant proteins. Indeed, the first in vitro produced plant made protein (PMP) for vaccine against "Newcastle Disease Virus" has been registered (Evans 2006). The feasibility of cell culture technology for commercial production of phytochemicals depends on successful mass culture of plant cells (biomass productivity over 1 g L^{-1} d^{-1}) with moderate metabolite or protein levels, which requires bioreactors well characterized for plant cell culture (Eibl and Eibl 2008). Considerable efforts have been made to design bioreactors for large-scale plant cell culture (Khanna and Srivastava 2005). A bioreactor is a glass or steel vessel fitted with probes to monitor the pH, temperature, and dissolved oxygen in the culture and has provision to allow sampling of cultures, add fresh medium, adjust pH, air supply, mixing of cultures and controlling the temperature, without endangering the aseptic nature of the culture. Thus, a bioreactor allows close control and monitoring of culture conditions.

Most of the bioreactors that have been used for plant cell culture are derived by minor modifications of the microbial bioreactors. Efficient mixing of plant cells is extremely important to provide uniform physiological conditions inside the culture vial. However, plant cells are sensitive to shear stress, and therefore are grown in modified stirred tank bioreactor (Fig. 4.4a) with low agitation speed. Air-lift bioreactors (Fig. 4.4b) are better than stirred tank bioreactors. However, these have a major problem of foaming due to extracellular polysaccharides, fatty acids, and high sugar content in the culture medium. The cells rise with the bubbles, become trapped in foam and eventually die due to lack of nutrients. To some extent, this problem can be offset by the addition of antifoam agents. Another unique feature of the plant cells is the formation of cell aggregates (2–40 cells) in suspension cultures. In older cultures, the cell aggregates and stickiness of the cells cause problem in mixing of the cells. Some of the bioreactors that have been designed and used for large-scale cultivation of plant cells are illustrated in Fig. 4.4.

To overcome the problems associated with the stirred tank, air-lift and bubble column bioreactors (Fig. 4.4c), a variety of low cost disposable bioreactors, possessing a gas permeable cultivation bag of plastic film (Osmotek's Life Reactor, Curtis' Plastic LinedReactor, BioWave reactor, Wave and Undertow Reactor, Slug Bubble Reactor), have been designed and found effective for a number of plant cell systems (Eibl and Eibl 2008). The mechanically driven BioWave bag bioreactor (Fig. 4.4d), with a scale-up capacity of 300 L culture volume and biomass productivity of 40 g fresh weight L^{-1} d^{-1}, could be achieved with doubling time of 2 days. The potential of this bioreactor for tobacco, grape, apple, and yew suspension cells up to 10 L culture volume has been demonstrated (Eibl and Eibl 2008). The Wave and Undertow and Slug Bubble Bioreactors have been successfully used to grow tobacco and soya suspensions expressing isoflavones up to 100 L culture volume (Terrier et al. 2007).

Bioreactors have also been designed for large-scale multiplication of organized structures, such as hairy roots for the production of phytochemicals (Curtis 1993; Shedwick et al. 2004; Kim et al. 2002), somatic embryos (Molle et al. 1993; Nishi et al. 1994; Preil 1991), potato tubers (Akita and Takayama 1993), and shoots (Sreedhar et al. 2008). For hairy root cultures especially designed gas-phase spray (droplet)

and mist reactors have been designed in which the roots, anchored to stainless steel mesh, are exposed to humidified air or gas mixture and nutrients are delivered as droplets by spray nozzles or ultrasonic transducers (Fig. 18.1).

4.5 Single Cell Culture

The significance of single cell culture is well recognized in basic and applied research. Cell metabolism and the effects of various substances on cellular responses could be better studied in single cell cultures than in intact organs and whole plants. The cloning of single cells permits crop improvement through the extension of the techniques of microbial genetics to higher plants such as isolation of spontaneous and induced variants.

4.5.1 Isolation of Single Cells

Single cells could be isolated from intact plant organs (usually leaf) or fine suspension cultures. However, invariably the single cell systems used in basic and applied research are isolated from cultured tissues as it offers several advantages.

Single cells from leaf tissue can be isolated mechanically or by enzymatic degradation of middle lamella.

(i) *Mechanical isolation*. Kohlenbach (1966) reported mechanical isolation of single cells from the leaves of *Macleaya cordata* and their ability to undergo sustained divisions in cultures. Almost at the same time Joshi and Ball (1965 and 1968) demonstrated, with the aid of time lapse photomicrography, sustained divisions of individual mesophyll cells of *Arachis hypogaea* in liquid medium. They isolated the cells first by tearing across the leaf to expose the mesophyll cells, followed by scraping the cells with a fine scalpel.
Photosynthetically active mesophyll cells from mature leaves of several species have been isolated by grinding 10 g of leaves in 40 ml of the grinding medium (20 μM sucrose, 10 μM $MgCl_2$, 20 μM tris–HCl buffer at pH 7.8) in mortar and pestle (Gnanam and Kulandaivelu 1969).
A method for large-scale mechanical isolation of free parenchymatous cells from the leaves of *Calystegia sepium* devised by Rossini (1969, 1972) is described in Appendix. It has been successfully applied to several other plants.

(ii) *Enzymatic isolation*. It involves degrading the middle lamella to release the cells. The enzyme macerozyme is generally used for this purpose. To avoid any adverse effect of macerozyme on cells/cell walls, an osmoprotectant (such as mannitol) is incorporated in the isolation mixture. A protocol for enzymatic isolation of mesophyll cells from tobacco leaves is described in Appendix II. Using this, protocol single mesophyll cells of several plant species, including cereals, have been isolated.

It is relatively much easier and convenient to isolate single cells from cultured tissues. As described earlier, the callus obtained from fresh explants may become friable after several subcultures. Such calli release single cells on continuous agitation in liquid medium. These single cells can be isolated by a micropipette or a fine syringe and subcultured. Several methods have been used to culture single cells (Fig. 4.5).

4.5.2 Culture of Single cells

(i) *Filter paper raft-nurse technique*. This method was developed by Muir et al. (1954) to culture single cells from suspension cultures and friable calli of tobacco and marigold (Fig. 4.5h). Individual cells were isolated from suspension cultures or a friable callus with the aid of a micropipette. Several days before cell isolation, a sterile 8 × 8 mm square of filter paper is placed aseptically on the top of the established callus of the same or a different species. The filter paper is wetted with liquid and nutrients from the nurse

tissue. An isolated single cell is placed on the wet filter paper raft. After a visible callus develops from the cell on the filter paper raft, it is transferred to agar medium for further growth and maintenance of the cell clone.

It is noteworthy that an isolated cell which generally fails to divide when plated directly on a standard, defined culture medium is able to divide under the nursing effect of the callus or on a complex medium such as KM medium (Kao and Michayluk 1980). Apparently, the nurse-callus supplies the cells with not only the nutrients from the culture medium, but also some unknown factor/s, that is/are critical for cell division. The cell division factor/s can diffuse through the filter paper.

(ii) *Microchamber technique*. In this technique, devised by Jones et al. (1960), the nurse tissue is replaced by a conditioned medium, and single cells are grown in a microchamber (Fig. 4.5i). A drop of the medium carrying a single cell is isolated from suspension cultures, placed on a sterile microscope slide and ringed with sterile mineral oil. A drop of oil is placed on either side of the culture drop and a cover glass placed on each drop. A third cover glass is placed on the culture drop bridging the two cover glasses and forming a microchamber to enclose the single cell aseptically within the mineral oil. The oil prevents water loss from the chamber but permits gaseous exchange. The whole microchamber slide is placed in a Petri dish and incubated. When the cell colony becomes sufficiently large, the cover glass is removed and the tissue is transferred to fresh liquid or semi-solid medium. The microchamber technique permits regular observation of the growing and dividing cell.

Using the microchamber technique Vasil and Hildebrandt (1965) demonstrated regeneration of complete flowering plants starting from an isolated single cell of tobacco. Instead of conditioned medium used by Jones et al. (1960), they used a fresh medium containing mineral salts, sucrose, vitamins, Ca-pantothenate and coconut milk.

(iii) *Bergmann's cell plating technique*. This is the most popular technique for single cell culture (Fig. 4.5j) developed by Bergmann, in 1960. Free cells are suspended in a liquid medium at a density twice the finally desired plating cell density. The optimum plating density may vary with the species but cells generally fail to divide below a certain critical cell density. In another vial molten agar (0.6–1 %) containing medium of otherwise the same composition as the liquid medium, is cooled and maintained at 40 °C in a water bath. Equal volumes of the two media are mixed and rapidly spread out in Petri dishes in such a manner that the cells are evenly distributed and fixed in a thin layer (approximately 1 mm thick) of the medium after it has cooled and solidified. The Petri dishes are sealed with Parafilm. Filtration of cell suspension through a sieve should allow exclusion of large cell aggregates and plating of only single cells and small cell aggregates (of 3–4 cells). The plates may be observed on an inverted microscope and single cells marked on outside of the plate by a fine marker to ensure the isolation of pure single cell clones. The culture plates are incubated at 25 °C in the dark.

If the plating density is determined at the time of culture initiation and a known volume of suspension is transferred to each plate, it should be possible to make a quantitative assessment of plating efficiency using the formula:

$$\text{Plating efficiency} = \frac{\text{No. of colonies/plate}}{\text{No. of cell units initially/plate}} \times 100$$

(iv) *Microdrop method*. This method has been especially useful for culturing individual protoplasts (see Chap. 14) but there is no reason why it cannot be equally effective for single cell culture.

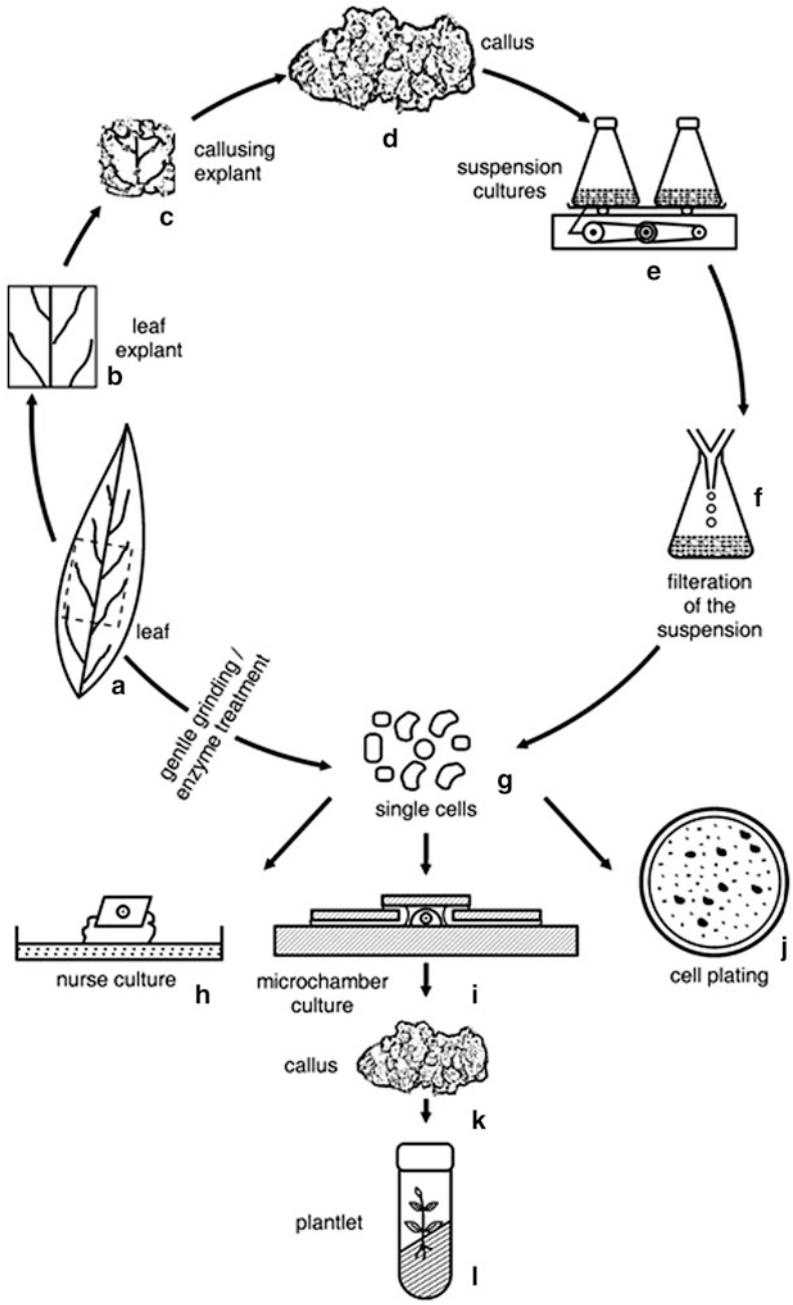

Fig. 4.5 A flow diagram to illustrate the stages in initiation of callus cultures, cell suspension cultures, single cell cultures and regeneration of plants from single cells. Explants (**b**) are excised from surface sterilized leaf (**a**) and planted on a suitable semi-solid medium to form callus (**c**). After 2–3 weeks, callus pieces are excised and transferred to fresh medium of the same composition for further growth **(d)**. By periodic subcultures on the same medium the callus can be multiplied indefinitely, or a part of it transferred to liquid medium in a flask and placed on a shaker to disperse the callus to form cell suspension **(e)**. The suspension cultures can be multiplied by periodic transfer of an aliquot of the suspension to fresh medium. Alternately, the suspension can be filtered to remove large cell clumps **(f),** and single cells (**g**) are picked up mechanically and cultured separately to clone them. The techniques available for single cell culture are Nurse tissue method (**h**), microchamber method (**i**) and cell plating (**j**). Single cells can also be isolated from intact leaves and cultured by the same methods. Under favorable conditions the cells would divide and form mini calli (**k**), which could be multiplied as callus or transferred to another medium to induce plant regeneration (**l**)

4.5.3 Factors Affecting Single Cell Culture

Composition of culture medium and the initial plating cell density are two critical factors for the success of single cell culture. These two factors are intricately linked. For the cells plated at high density a purely synthetic medium with a composition similar to that used for suspension cultures or callus cultures are generally satisfactory. At lower plating density, however, the medium needs to be supplemented with complex nutrient mixtures, such as coconut milk, casein hydrolysate or yeast extract. These undefined factors possibly compensate for the population effect on cell division in a high density cell plating system.

The cell density effect on cell division has been explained on the basis that cells synthesize certain compounds necessary for their division. The endogenous concentration of these compounds should reach a threshold level before a cell can embark on division. The cells continue to lose their metabolites into the medium until equilibrium is reached between the cell and the medium. At high cell density the equilibrium is attained much earlier than at low density, and therefore the lag phase is short. Below a critical cell density the equilibrium is never reached and cells fail to divide. However, a conditioned medium, enriched with essential metabolites leached out of the cells, is able to support divisions at a fairly low cell density. In this regard, a detailed analysis of the conditioned medium to isolate and identify factor(s) responsible for cell division in single cell cultures would be rewarding.

4.6 Concluding Remarks

With the current status of sophistication and developments in plant tissue culture techniques it is possible to establish continuously growing callus cultures of most of the plant species. For a range of plant species long-term suspension cultures have also been established. However, cultivation of plant cells individually or at low plating density still requires the use of complex nutrient mixtures of undefined nature, such as nurse tissue, conditioned medium, CM and so on. Recently, one of the active factors of conditioned medium has been identified as phytosulfokine-α which promoted cell divisions in low density cell cultures of *Asparagus*, *Zinnia* and several other plants (Matsubayashi and Sakagami 1996).

By nature, the plant cells have a tendency to grow as tissue system since the daughter cells after a cell division do not separate and remain adhering to each other by the middle lamella. Therefore, most of the suspension cultures of plants are a mixture of single cells and cell aggregates. Efforts to establish purely single cell suspension cultures have not been successful so far. In wheat and barley, the suspension cultures started with predominantly single cell inoculum exhibited a sharp decline in the population of viable single cells (Dong et al. 2010). Addition of cell aggregates along with the single cells was essential for the production of viable single cells. Possibly, in such cultures viable single cells are released from cell aggregates as a result of shaking or agitation of the medium during culture.

Plant cell cultures have been used for the commercial production of high value phytochemicals (Chap. 18). However, the bioreactors used for the large-scale cultivation of plant cells so far are not entirely satisfactory. Considerable research is being done to design a widely acceptable plant cell bioreactor.

4.7 Appendix

I. Protocol for mechanical isolation of mesophyll cells from the leaves of *Calystegia sepium* (*after* Rossini 1972).
   1. Surface sterilize the leaves by quick immersion in 95 % ethanol followed by rinsing for 15 min in filter-sterilized 7 % solution of calcium hypochlorite. Wash in sterile distilled water.
   2. Cut the leaves into small pieces (less than 1 cm^2).

Table 4.2 Medium for suspension cultures of tobacco

Constituents	Amount (mg L^{-1})
Inorganic salts	As in MS medium
Thiamine.HCl	10
Pyridoxine.HCl	10
Nicotinic acid	5
Myo-Inositol	100
Casein hydrolysate	1000
2,4-D	2
Kinetin	0.1
Sucrose	3 %
pH	5.7

after Reynolds and Murashige (1979)

3. Homogenize 1.5 g of leaf material with 10 ml of culture medium in a glass homogenizer tube. Filter the homogenate through two sterile metal filters, the upper and lower filters with mesh diameters of 61 and 38 μm, respectively.
4. Fine debris can be removed by slow speed centrifugation of the filtrate which would sediment the free cells. Remove the supernatant and resuspend the cells in a volume of the medium sufficient to achieve the required cell density.
5. Plate the cells in a thin layer of solid or liquid medium (Table 4.1).

II. Protocol for enzymatic isolation of mesophyll cells from tobacco leaves (*after* Takebe et al. 1968, as modified by Evans and Cocking 1975).

1. Collect fully expanded leaves from 60 to 80-day-old plants and surface sterilize them by immersion in 70 % ethanol for 30 s followed by rinsing for 30 min in 3 % sodium hypochlorite solution containing 0.05 % Teepol or Cetavlon.
2. Wash the leaves with sterile distilled water and peel-off the lower epidermis with the aid of sterile fine tipped forceps.
3. Excise 4 cm^2 pieces from the peeled areas with a sterile scalpel blade.
4. Transfer 2 g of the leaf pieces to 100 ml Erlenmeyer flasks containing 20 ml of filter-sterilized enzyme solution (0.5 % macerozyme, 0.8 % mannitol, and 1 % potassium dextran sulphate).
5. Infiltrate the enzyme into the leaf tissue by briefly evacuating the flasks with a vacuum pump.
6. Incubate the flasks at 25 °C for 2 h on a reciprocating shaker with a stroke of 4–5 cm at the rate of 120 rpm min^{-1}.
7. Change enzyme solution after the first 30 min. After another 30 min, the enzyme solution should contain largely spongy parenchyma cells and after 90–120 min of incubation the suspension should contain predominantly palisade cells.
8. Wash the cells twice with culture medium (Table 4.2) and culture.

Suggested Further Reading

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Cytodifferentiation 5

5.1 Introduction

The basic event in the development of eukaryotes is the specialization of cells, which is termed as cytodifferentiation. One of the important objectives of developmental biology is to understand the cellular and subcellular events underlying the differentiation of specialized cells. The most studied example of cytodifferentiation is the formation of the vascular system comprised of the tracheary elements and the phloem elements. Vascular elements formed from the meristematic cells of stem apex, root tip, and vascular cambium are the early recognizable products of cytodifferentiation.

Tracheary elements characterized by the formation of a lignified secondary wall with annular, reticulate, spiral, or pitted wall thickenings are characteristic components of the xylem. In primary xylem, the tracheary elements are derived from the procambium of the root and shoot while in the secondary xylem, these are produced from the vascular cambium. In intact plants primary xylem has received much attention because of ease of induction and observation of tracheary element differentiation. Cytodifferentiation of tracheary elements is a striking case of irreversible specialization of cells (such cells cannot be dedifferentiated or redifferentiated), which is accompanied by the loss of nuclei and cell contents at maturity, leaving hollow dead cells that form vessels or tracheids (Fig. 5.1). The final stage of tracheary element differentiation represents a typical example of programmed cell death in higher plants. The morphologically recognizable wall thickenings are accompanied by specific biochemical changes that can be used as marker proteins for studying early stages of this event. Vascular differentiation thus is a typical example of plant morphogenesis exhibiting signal perception, cell lineages, differential gene expression, and programmed cell death.

Tissue cultures have proven useful in understanding some of the fundamental changes at the cellular level associated with vascular element differentiation through in vitro studies on xylogenesis, xylem-like cell differentiation, and phloem differentiation as these involve biosynthesis and modification of the cell wall and cell contents. Tracheary elements can be induced in vitro from various types of cells such as phloem parenchyma, cortex in roots, pith parenchyma in shoots, tuber parenchyma, and the mesophyll and epidermis in leaves (Roberts et al. 1988; Fukuda 1992). In vitro differentiation passes through the four ontogenic stages typical of in situ tracheary element differentiation, which are: (i) acquiring competence by the target cell to commence cytodifferentiation, (ii) cell enlargement, (iii) secondary wall formation, and (iv) lignification, and autolysis of cell contents including the nucleus and selective dissolution of the wall (Fukuda and Komamine 1985).

Tissue and cell cultures are ideal systems for studying cytodifferentiation since simple variations as changing the composition of medium,

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_5, © Springer India 2013

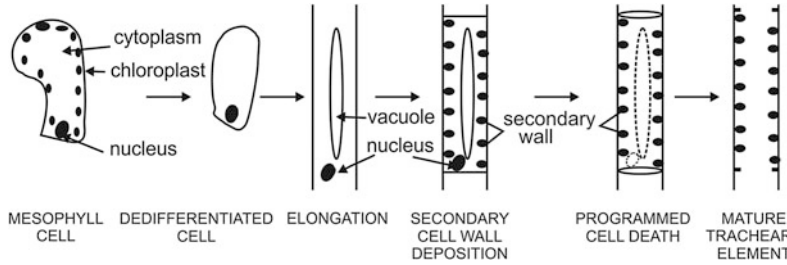

Fig. 5.1 Diagrammatised representation of in vitro transdifferentiation of a mesophyll cell of *Z. elegans* into a tracheary element (*after* Turner et al. 2007)

especially the phytohormones, could regulate in vitro differentiation of tracheary elements. Furthermore, homogenous tissues or cells could be easily obtained to initiate cultures and scale up to produce large quantities of biomass for biochemical studies. Finally, tissue culture systems can be used to understand intracellular signal transduction pathways leading to changes in gene expression (Bolwell and Robertson 1999).

In addition to xylogenesis, induction of phloem sieve elements in tissue cultures has also been used as a model differentiation system and as a source for obtaining molecular probes.

5.2 Experimental Systems

Several cell culture systems have been developed to study aspects of vascular differentiation. In *Zinnia elegans* cell cultures, single mesophyll cells directly differentiate into tracheary elements at a high frequency without cell division (Fig. 5.2) in response to phytohormones (Fukuda and Komamine 1985), and this system has been extensively used to study the sequence of events leading to tracheary element differentiation. Other systems such as the French bean have been useful in studying the biochemical changes associated with secondary wall synthesis in cells that develop to resemble fiber cells. Molecular probes isolated from many such systems have been found to be specific for vascular differentiation in the intact plant using in situ hybridization or immunolocalization.

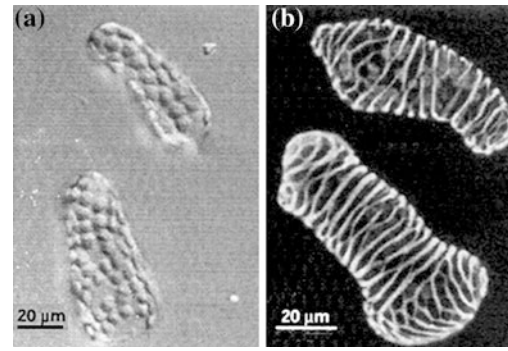

Fig. 5.2 Direct transdifferentiation of a mesophyll cell (**a**) of Z. *elegans* into a tracheary element (**b**), without intervening cell division (*after* Fukuda 2004)

5.2.1 Tracheary Element Differentiation In Vitro

French bean explants have been used to raise callus and suspension cultures that can differentiate tracheary elements (Bevan and Northcote 1979). In response to a high cytokinin to auxin ratio, the French bean suspension cultures have shown differentiation. These cells can be manipulated to produce a wall with characteristic changes in enzyme activity (Church and Galston 1989).

In lettuce, large amounts of undifferentiated parenchyma obtained from the subapical region could be induced to differentiate tracheary elements on appropriate medium (Warren-Wilson et al. 1991). Although tracheids appeared after 3–5 days of culture, vascular differentiation continued up to 14 days. The lettuce pith system

can be advantageously used to mimic intercellular polar transport of signals such as the phytohormones. It has been observed that vessels closest to the auxin source differentiated large tracheary elements while at the far end smaller isodiametric tracheids were formed (Warren-Wilson 1994). Similarly, cells of one type from *Helianthus*, *Sambucus*, *Nicotiana*, and *Coleus* could be manipulated to undergo xylogenesis in cultures (Fukuda and Komamine 1990).

Among the various in vitro systems, mesophyll cell culture of *Z. elegans* has turned out to be a model system to study cellular and subcellular aspects of Tracheidal Element (TE) differentiation (Turner et al. 2007; Fukuda 2004; Motose et al. 2001). Isolated single cell system, especially that of *Z. elegans*, is a striking example of vascular differentiation in cultured cells yet available. Kohlenbach and Schmidt (1975) observed that mechanically isolated mesophyll cells of *Z. elegans* differentiated into TEs within 3 days without cell division when cultured on a suitable medium (for composition see Appendix). This system has been so well refined that it is possible to achieve relatively synchronous differentiation of TEs at high frequencies (50–65 %) within 72 h of culture of the mesophyll cells (Fukuda and Komamine 1980). The high level of synchrony has made it possible to track cytological commitment and determination during differentiation. The merits of this system are: (i) freshly isolated mesophyll cells of *Zinnia* provide a very homogeneous single cell system composed of uniform nonpolyploid cells held in G_1 phase of the cell cycle (Fukuda and Komamine 1981), (ii) controlled differentiation can be achieved by exogenous supply of phytohormones (1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) and sucrose (10 %) in the medium, (iii) the inductive factors effect directly the TE differentiation as cell division is not a prerequisite, (iv) differentiation occurs synchronously and at a high frequency (50 %; up to 80 % under optimal conditions), (v) 10 min exposure of the mesophyll cells to, both, auxin and cytokinin is sufficient to induce TEs differentiation (Milioni et al. 2001), (vi) TE differentiation occurs within 72 h on a synthetic medium, and (vii) mesophyll cells differentiating into tracheary elements can be easily separated from nondifferentiating cells by staining them with fluorescein diacetate and sorting with flow cytometry (Ito et al. 2004).

5.2.2 Phloem Differentiation In Vitro

Phloem sieve element induction in vitro can be used to understand interesting aspects of phloem differentiation, and as these are enucleate new insights into protein synthesis regulation could be had (Bolwell and Robertson 1999). By appropriate manipulation of the medium cultures of *Stephanthus tortuosus* can be successfully induced to differentiate phloem elements (Toth et al. 1994).

5.3 Factors Affecting Vascular Tissue Differentiation

The precise conditions for induction of vascular differentiation are empirically determined and are generally arrived by using combinations of auxin and cytokinin in standard media. The basic design of such experiments involves setting up a matrix of a physiological range of the phytohormones. A carbohydrate source such as sucrose is also essential for differentiation. The cultures are subsequently observed cytologically for differentiated cells. There is evidence to point toward the involvement of ethylene and gibberellin in the process of xylogenesis.

5.3.1 Growth Regulators

(i) *Auxin and Cytokinin*. Early works such as that of Camus (1949), using vegetative buds in graft experiments, clearly demonstrated the involvement of a diffusible substance in differentiation of vascular elements in cultured tissues. It was later confirmed by Wetmore and Sorokin (1955) that shoot buds grafted on undifferentiated callus could induce vascular

differentiation within 20–30 days (Fig. 5.3a). The shoot bud affect could be duplicated with agar containing an auxin and sucrose (Fig. 5.3b; Jeffs and Wetmore 1967). In majority of the species where tracheary element induction has been reported an exogenous auxin was found to be essential, although the optimum concentration varies. Even endogenous levels of auxin seem to affect differentiation. For example, tracheary elements could be induced in habituated cells (Philips 1980) and in crown gall tissue (Basile et al. 1973) without the exogenous auxin. These cells are known to have high levels of endogenous indoleacetic acid.

Physiologically, auxin induces elongation and cell division in plant cells. However, studies show that cytodifferentiation is affected by auxin directly and not through cell division and cell elongation. In *Zinnia* mesophyll cell system, intracellular depletion of auxin caused by polar auxin transport inhibitors prevented tracheary element differentiation (Yoshida et al. 2005).

Cytokinin is the other plant hormone that is important for cyto- differentiation. In the cultures of tobacco (Bergmann 1964) and carrot (Mizuno and Komamine 1978) exogenous cytokinin along with auxin was found to be essential for induction of tracheary elements. Where exogenous supply of cytokinin was not necessary it was found that the endogenous levels were already high enough for such induction. The different level of cytokinin requirements in various species is due to difference in its endogenous levels.

The narrow range of auxin and cytokinin required for maximum differentiation of tracheary elements, specially in *Zinnia*, suggests that the absolute concentrations of the two hormones in the culture medium are more important than auxin/cytokinin ratio. Whereas cytokinin is required only for a brief period in the early stages of differentiation, auxin must be present until the last stage of differentiation (Fukuda and Komamine 1985). In contrast, in tuber disks of *Helianthus* exogenous cytokinin is required for 2 days while auxin is required for only 1 day (Phillips 1987). It has been shown that two differentiation specific proteins are synthesized before morphological changes occur in the presence of cytokinin and auxin (Fukuda and Komamine 1983). Thus, the primary action of hormones in cytodifferentiation is considered to be the expression of some specific genes.

(ii) *Gibberellic acid, Abscisic acid, Ethylene and cAMP*. In Jerusalem artichoke gibberellic acid promoted differentiation of tracheary elements in the presence of auxin and cytokinin (Dalessandro 1973; Philips and Dodds 1977). Contrastingly, Minocha and Halperin (1974) found gibberellic acid to be inhibitory in the same material. This discrepancy may be because of varying endogenous level of gibberellic acid, which is known to change during dormancy in tubers (Philips and Dodds 1977). In bean cultures gibberellic acid delayed auxin/cytokinin-induced tracheary element differentiation but did not affect the frequency of differentiation. Thus,

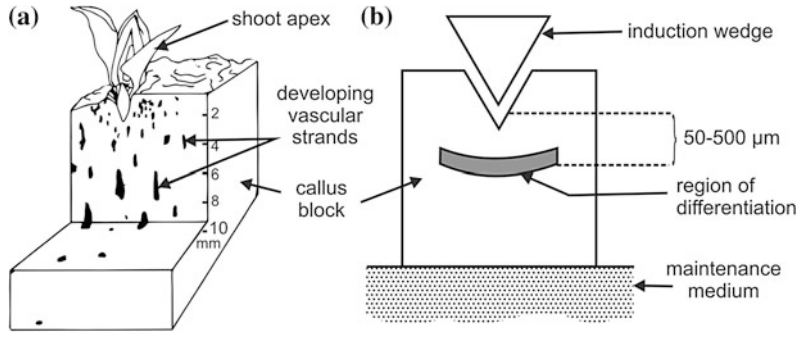

Fig. 5.3 **a** Induction of vascularization in callus tissue of *Syringa* 54 days after grafting of shoot apex bearing 2–3 leaf primordia. **b** Induction of vascularization in a block of *Phaseolus* callus by inserting an agar block wedge containing auxin and sucrose (**a** after Wetmore and Sorokin 1955; **b** after Jeffs and Northcote 1967)

it appears that gibberellic acid does not function directly in the induction of tracheary elements in vitro (Fukuda and Komamine 1985).

Abscisic acid has been found to be inhibitory in the induction of tracheary elements in the artichoke and bean explants. Ethylene is reported to have a positive affect on cytodifferentiation in lettuce pith explants. cAMP also has been found to promote tracheary element formation in the presence of cytokinin and auxin. Probably, cAMP induces cytodifferentiation via production of cytokinin and not as a secondary messenger of cytokinin (Mizuno and Komamine 1978).

5.3.2 Other Factors

(i) *Sucrose*. The effect of auxin on TE differentiation seems to be closely related to the presence of sugars. The relative amounts of xylem and phloem formed in the callus pieces of *Syringa* (Wetmore and Rier 1963) and *Phaseolus vulgaris* (Jeffs and Northcote 1967) could be varied by changing the sucrose concentration in the presence of low amounts of auxin. With 1 % sucrose very few xylem elements were formed. Raising sucrose concentration to 2 %, without altering the auxin concentration, induced better differentiation of xylem with little or no formation of phloem. With 2.5–3 % sucrose, both, xylem and phloem differentiated but with 4 % of sucrose only phloem was formed. Contrastingly, suspension cultures of *Parthenocissus tricuspidata* showed an increase in xylem elements only with increase in sucrose concentration up to 8 % (Rier and Beslow 1967).

Other carbohydrates that could substitute for sucrose are α-glucosyl disaccharides in bean callus (Jeffs and Northcote 1967), glucose, and *myo*-inositol in lettuce explants (Roberts and Baba 1982). Carbohydrates, including sucrose, act as energy source rather than as regulating factors (Fukuda and Komamine 1985).

(ii) *Calcium*. Studies on *Zinnia* system have highlighted the importance of calcium in TE differentiation. Removal of calcium or application of calcium channel blockers or calmodulin antagonists inhibited TE differentiation (Roberts and Haigler 1990). Whereas calmodulin antagonists were effective only when added at the beginning of culture, calcium channel blockers inhibited TE differentiation when applied anytime up to 48 h after culture initiation.

(iii) *Physical and physiological factors*. In *Helianthus* cultures temperature below 17 °C was not favorable for vascular differentiation, and within the range of 17–31 °C xylogenesis increased with the raise in temperature (Gautheret 1961). Wound stress has also been shown to induce TE differentiation in *Zinnia*, probably because of ethylene production (Church and Galston 1989). Light is another factor that influences TE differentiation in *Coleus* (Fosket 1968).

Age of the plant seems to influence in vitro differentiation of vascular elements. Direct differentiation of cells into TE in *Helianthus tuberosus* occurred only in the explants derived from young tubers. This capacity declined with the age of the tuber, and in the explants from mature tubers TE differentiation occurred only after cell proliferation (Philips 1981). Another observation that highlights the importance of the physiological state of the cells in TE differentiation is that the *Zinnia* mesophyll cells isolated mechanically showed higher incidence of differentiation than the cells obtained by enzymatic maceration (Fukuda and Komamine 1985).

5.4 Cell Cycle and Tracheary Element Differentiation

In intact plants the procambium exhibits continued divisions before differentiating xylem and phloem. Similarly, secondary vascular tissues differentiate from the meristematic cells of vascular cambium. In tissue cultures, the chemical factors (auxin, cytokinin, sugar) that favor xylem differentiation are generally the same as those regulating cell division. These observations lead earlier workers to suggest that a cell must divide before it differentiates into a TE (Fosket 1968; Torrey 1971; Torrey et al. 1971). This hypothesis gained further support from the reports that 10^{-5} M BuDR (an inhibitor of DNA

synthesis) completely inhibited TE differentiation in tissue cultures of coleus, pea, lettuce, and so on. (see Bhojwani and Razdan 1996).

However, it has been now well established that cell division is not always necessary for TE differentiation. For example, cells taken from suspension cultures of *Centaurea cyanus* when plated as single cells directly differentiated into TE, although at a low frequency. It could be argued that these cells might have already passed through cell division cycles. More direct evidence was provided by Kohlenbach and Schmidt (1975) when they showed that mature mesophyll cells of *Z. elegans* on culturing could transdifferentiate to TE without cell division. Even mesophyll protoplasts of this plant exhibited direct differentiation into TEs (Kohlenbach and Schopke 1981).

Based on serial observations, microdensitometry and autoradiography, Fukuda and Komamine (1980, 1981) confirmed that in *Zinnia* the majority of TEs (60 %) differentiated directly from cells in G_1 phase of the cell cycle (Fig. 5.4). The differentiation required neither the replication of total genomic DNA nor cell division. However, since various inhibitors of DNA synthesis caused complete inhibition of TE differentiation (Fukuda and Komamine 1981), it has been suggested that some kind of minor repair type DNA synthesis is essential (Sugiyama and Komamine 1990).

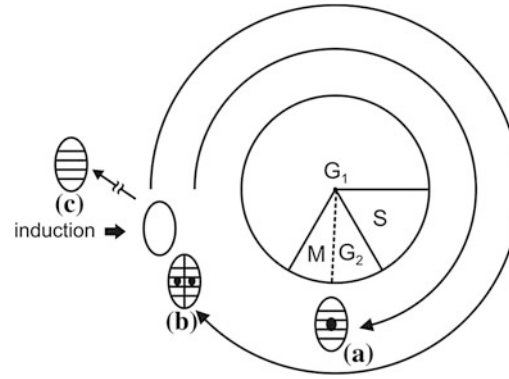

Fig. 5.4 Diagram showing relationship between tracheary element (TE) differentiation and cell cycle in the cultures of isolated mesophyll cells of *Z. elegans*. Hormonal induction of TE differentiation occurs in the G_1 phase, but the cell may get out of the cell cycle to differentiate at different stages in the cell cycle. The induced cell may differentiate into TE without progressing further along the cell cycle (*c*), it may pass through the S phase and differentiate in the G_2 phase (*a*), or it may undergo mitosis and both the daughter cells differentiate into TE (*b*) (*after* Fukuda and Komamine. 1981)

5.5 Changes Associated with Tracheary Element Differentiation

(i) *Cytological change*. Perceptible ultrastructural changes occur during various stages of cytodifferentiation in cultures almost similar to those in intact plants. At the time of culture, most parts of the cytoplasm of the *Zinnia* mesophyll cells are occupied by a line of lens-shaped chloroplasts along the plasma membrane with a large vacuole in the center. Other organelles are hardly visible (Fukuda and Komamine 1985). In a differentiation-inducing medium alteration of intracellular organization indicates some of the earliest events of the cytodifferentiation. There is an increase in the volume of the cytoplasm and numbers of various organelles, excepting chloroplasts. The ordered arrangement of chloroplasts gets disrupted and the space between the plasma membrane and the chloroplasts increases. In cultures, the palisade cells show minimal elongation, if at all, before secondary wall deposition begins. There is also an increase in the dictyosomes and vesicles derived from them.

In the maturing tracheary elements, the breakdown of tonoplast triggers degeneration of intracellular components. The nucleus and the chloroplasts degenerate within 10–20 min of vacuole collapse while chlorophyll degradation takes a couple of hours (Obara et al. 2001). In these cells, a wide range of polysaccharides in the primary cell walls and pectin get degraded into smaller carbohydrate molecules (Ohdaira et al. 2002).

As the differentiation of the cultured cells progresses secondary wall deposition, especially a helical or reticulate type, is observed. Lignification is another event in TE differentiation.

Finally, cell autolysis follows secondary wall thickenings. Within 10 h of secondary wall deposition in the cultured *Zinnia* cells the nucleus is lost (Fukuda and Komamine 1985).

(ii) *Biochemical changes*. The cytological changes during differentiation of tracheary elements are accompanied by changes in various enzymes and their products.

The vacuole which functions as the main lytic organelle accumulates and releases on bursting a variety of programmed cell death-specific hydrolases. The *Zinnia* endonucleases I (*ZEN I*), an SI-type Zn^{2+} dependent nucleases play a major role in nuclear DNA degradation (Ito and Fukuda 2002). Even before comprehensive autolysis is caused by the collapse of the lytic vacuole partial autolysis is initiated in the cytoplasm by serine proteases having neutral pH optima (Bears and Freeman 1997; Ye 2002).

The synthesis of cell wall polysaccharides is a Golgi-based process with the exception of the glucans, cellulose, and callose that are synthesized at the plasmalemma (Bolwell and Robertson 1999). In induction medium, as cytodifferentiation proceeds, there is a loss in the activity of some enzymes such as arabinosyl transferase (French bean cultures, Bolwell and Northcote 1983) and polygalacturonic acid synthase (*Sycamore* cultures, Bolwell and Northcote 1983; *Zinnia* cells, Suzuki et al. 1991) and the enzymes involved in the synthesis of other hemicelluloses such as glucomannan (Dalessandro et al. 1986).

Lignification is another characteristic event in cytodifferentiation to tracheary elements. High activity of enzymes involved in lignification, such as cell wall bound peroxidase and phenylalanine ammonia-lyase, coincide with the active synthesis of lignin at the late stage of tracheary element differentiation (Fukuda and Komamine 1983). In French bean cultures and differentiating *Zinnia* cells specific secondary cell wall proteins have also been localized. During the phase leading up to programmed cell death enzymes specific to autolysis have been recognized in the *Zinnia* system (see Bolwell and Robertson 1999; Fukuda 1997).

(iii) *Molecular changes*. Plants respond to developmental and environmental signals by changes in gene expression (Bolwell and Robertson 1999). A number of molecular markers for tracheary element differentiation have been isolated in the *Zinnia* cell culture system. Based on these markers, the process of tracheary element differentiation in *Zinnia* has been divided into three stages (Fig. 5.5): Stage I, Stage II, and Stage III (Fukuda 1994, 1996, 1997, 2004; Turner et al. 2007). Whereas the Stages I and II last for about 24 h each, the duration of Stage III is 24–48 h.

Stage I. It is characterized by the dedifferentiation of the cells and loss of photosynthetic ability. Two striking features of this stage are change in the organization of the reticulate arrays of actin filaments that anchor the chloroplasts to the plasma membrane, eventually leading to patterned secondary wall thickenings (Kobayashi et al. 1987) and change in the calcium/calmodulin regulation (Roberts and Haigler 1990). Genes activated during this stage are a response to wound than to phytohormones. cDNA clones corresponding to 12 genes have been isolated during this stage. These genes could be grouped as group-I, group-II, and group-III with each group controlling specific events (Fukuda 1997). Nakanomyo et al. (2002)

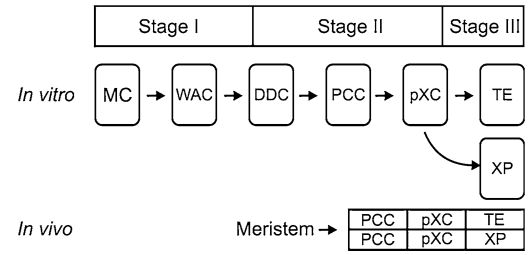

Fig. 5.5 Stages in the process of in vitro differentiation of TEs from a mesophyll cell of *Z. elegans*. *Stage I* the mesophyll cells (MC) loose their ability for photosynthesis and dedifferentiate via wound activated cells (WAC). *Stage II* Dedifferentiated cells (DDC) differentiate into procambial cells (PCC) and then into xylem cell precursors (pXC). *Stage III* Differentiation of TEs and xylem parenchyma (XP) cells occurs from xylem-like precursors, which involve patterned secondary cell wall deposition and programmed cell death (*after* Fukuda 2004)

isolated a Rac *GTPase* gene, *ZeRAC2*, whose product has *GTP*-binding and *GTPase* activity and accumulates preferentially to the site facing developing tracheary elements. During xylem differentiation events such as actin rearrangement, secondary wall thickenings, programmed cell death, and biosynthesis of lignin are putative *Rac* controlled.

Stage II. This stage is characterized by the differentiation of the dedifferentiated cells (DDC) into procambial cells (PCC) and then into xylem cell precursors (pXC). It is accompanied by the expression of three genes, *TED2*, *TED3*, and *TED4* (tracheary element differentiation related 2, 3 and 4; Demura and Fukuda 1993, 1994). At this stage secondary wall deposition does not yet occur. During Stage II, there is continued synthesis of tubulin that brings about an increase in the number of microtubules (Fukuda 1987).

Stage III. The entry from Stage II to Stage III appears to be an irreversible process in the differentiation of TEs. At Stage III, the differentiation of TE and xylem parenchyma cells (XPCs) occurs from the xylem cell precursors (pXC). Stage III involves patterned secondary wall formation and programmed cell death and a corresponding expression of various enzymes and structural proteins involved in these processes. Several genes specific to Stage III have been identified (Fukuda 1997).

Studies involving in vitro xylogenic cultures of *Zinnia* have identified multiple steps of cell–cell interactions that regulate vascular differentiation. *Zinnia* mesophyll cells differentiate in the presence of auxin and cytokinin to become procambium-like cells. Such differentiation occurred only in the conditioned medium (a medium in which cultures have been growing for a long time). It is now known that the medium is conditioned by a sulfated pentapeptide, phytosulfokine-α, released by the fast growing suspension cultures (Matsubayashi and Sakagami 1996). The procambium-like cells may produce more phytosulfokine-α and guide more cells to the procambium-like cell fate. Some of the procambium-like cells differentiate into immature xylem cells and secrete xylogen, an *N*-glycosylated nonclassical AGP (arabinogalactan protein) (Motose et al. 2001). Xylogen influences more neighboring cells into the xylem cell fate (Fig. 5.6). Some of the immature xylem cells may face the xylem parenchyma fate. Transition of tracheary element precursors from Stage II to Stage III requires brassinosteroids that are released by Stage II tracheary element precursors or xylem parenchyma-like cells. TED-4, a proteosome inhibitor protein, is secreted by immature xylem cells as a safety apparatus for the forecasted release of proteosomes into the apoplast (Endo et al. 2001). Finally, the lignification of the maturing tracheary elements is achieved by the monolignols supplied by the adjacent xylem parenchyma-like cells (Motose et al. 2001).

Fate of procambium-like cells into tracheary element precursors or xylem parenchyma-like cells might depend on yet to be understood intercellular communication. Analysis of >8,000 *Zinnia* cDNA clones revealed unique differentiation stage-dependent patterns in gene regulation which might enable to pursue the master gene that controls each stage. Some of these suggest the presence of new steps involved in differentiation, such as an auxin signaling pathway and cell–cell interactions. The discovery of >70 Stage III-specific genes, many of which encode biosynthetic or biodegradation enzymes, implies involvement of many previously unrecognized factors in the morphogenesis of tracheary elements (Demura et al. 2002).

5.6 Process of TE Differentiation

Phytosulfokine-α (PSK-α), a disulfated pentapeptide, was discovered as the conditioned medium factor in the mesophyll cell suspension cultures of asparagus (Matsubayashi et al. 1996). It promoted cell divisions in low density cell cultures of asparagus and some other plants (Matsubayashi et al. 1997; Kobayashi et al. 1997).

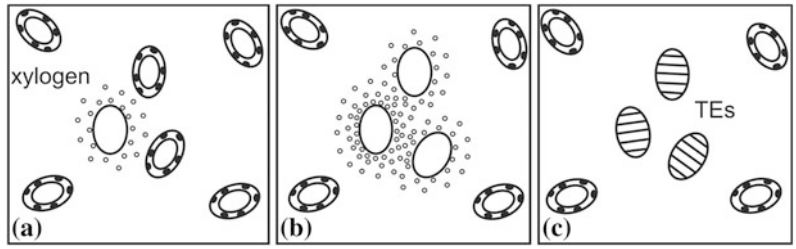

Fig. 5.6 When isolated mesophyll cells of *Z. elegans*, embedded in agarose are cultured as thin sheet on semi-solid medium the differentiation of TEs occurs in groups. It is suggested that a cell which is induced to differentiate into TE synthesizes and secretes xylogen (**a**), which induces the neighboring cells into the pathway of TE differentiation (**b**). The increased number of induced cells results in increased xylogen content, pulling in more neighboring cells into the process of TE differentiation. Thus, the differentiation of TEs is in groups (**c**) rather than at random (*after* Motose et al. 2001)

PSK-α was also observed in considerable amounts in high density mesophyll cell cultures of *Zinnia*. Addition of PSK-α in the medium restored the suppression of TE differentiation in low density mesophyll cell cultures of *Zinnia*. The promotion of TE differentiation by PSK-α did not involve cell multiplication. The work of Motose et al. (2009) strongly suggests the involvement of PSK-α in the attenuation of stress response occurring in the early stages of TE transdifferentiation. It is particularly essential for the progression of Stage II and the entry into Stage III.

In mesophyll cell cultures of *Zinnia* as thin sheet (cells embedded in thin sheets of agarose) on semi-solid medium or as beads (embedded in agarose beads) placed in liquid medium the TE that differentiated was not randomly distributed. They were in aggregates (Fig. 5.6), suggesting the involvement of local intercellular interaction in cellular differentiation (Motose et al. 2001).

In thin sheet cultures, TEs differentiated rarely if the cells were plated at low density ($<1.0 \times 10^5$ cells mL^{-1}). However, differentiation of TEs occurred on low density in thin sheets when they were placed in contact with another plate with high cell density (4×10^5 cells mL^{-1}), suggesting the contribution of a diffusible substance secreted by the cells cultured at high density. This factor has been identified as a proteinaceous molecule larger than 25 kDa with activity similar to arabinogalactan proteins (AGPs) and is termed xylogen. It appeared before the differentiation of TEs and its activity increased as their differentiation progressed.

According to Fukuda (2004), the process of differentiation of *Zinnia* mesophyll cells into TEs can be divided into three Stages or Phases (Fig. 5.5). During Phase I, the mesophyll cells dedifferentiate into procambial-like cells (PCC) with pluricompetence. The PCC cells produce PSK-α which draw more mesophyll cells into the procambial state. The transition from Phase I (24 h) to Phase II (24 h) is mediated by xylogen. In Phase II, a fraction of PC cells differentiate into immature xylem cells. The immature xylem cells secrete xylogen to induce more of PC cells to differentiate into primary xylem cells. Brassinosteroids play an important role in the transition from Phase II to Phase III, leading to the differentiation of TEs (Yamamoto et al. 1997, 2001).

5.7 Concluding Remarks

Z. elegans has turned out to be an elegant single cell system to have insight into the process of tracheary element (TE) differentiation. Several plant growth regulators, such as auxins, cytokinins and brassinosteroids, influence TE differentiation. The information on the cellular, biochemical and molecular basis of TE

differentiation would go a long way in understanding the overall process of vascular differentiation in plants. Transdifferentiation of a photosynthetically active mesophyll cell into a dead functional unit of the vascular system involves processes such as dedifferentiation, apoptosis or programmed cell death, and the formation of lignified secondary walls. An arabinogalactan, xylogen, and a CLE peptide that regulate TE differentiation appear to define new signal pathway. A number of genes associated with TE differentiation have been identified, but their specific role in this transdifferentiation process requires further studies. Involvement of PSK-α in transdifferentiation of TE from mesophyll cell has been highlighted by the recent work of a Japanese group of scientists (Motose et al. 2009).

5.8 Appendix

Induction of xylogenesis in isolated mesophyll cells of *Z. elegans* (Bolwell 1985)

(i) Germinate seeds of *Z. elegans* in moist vermiculite for 1 week in continuous light (4 lux) at 27 °C.
(ii) Transfer the plants under 12 h photoperiod (10 lux) at 27 °C for 1–2 weeks.
(iii) Excise and surface sterilize ~2 g of first or second leaves in a solution of NaOCl (0.05 % w/v) for 10 min.
(iv) Rinse three times in sterile distilled water and cut into pieces (~5 × 5 mm).
(v) Macerate the leaf pieces in 5 ml of 0.2 M mannitol solution using a sterile pestle and mortar.
(vi) Filter through a 50 μm nylon screen and harvest the cells by centrifugation of the filtrate at 150 g for 2 min.
(vii) Resuspend the cells in 150 ml of liquid medium (for composition see Table 5.1).
(viii) Culture the cells in the dark at 27 °C in small flasks on a rotary shaker.
(ix) Observe cultures from second day onwards for the presence of tracheary elements.

Table 5.1 Medium for the culture of isolated mesophyll cells of *Zinnia elegans* (Bolwell 1985)

Constituents	Amount (mg L^{-1})
Macronutrients	
KNO_3	2,020
NH_4Cl	54
$MgSO_4.7H_2O$	247
$CaCl_2.2H_2O$	147
KH_2PO_4	68
Micronutrients	
$MnSO_4.4H_2O$	25
H_3BO_3	10
$ZnSO_4.7H_2O$	10
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$FeSO_4.7H_2O$	28
Na_2EDTA	37
Organic Nutrients	
Glycine	2
Myo-inositol	100
Nicotinic acid	5
Pyridoxine HCl	0.5
ThaimineHCl	0.5
Folic acid	0.05
Biotin	0.05
Growth regulators	
NAA	0.1
BAP	1
Sucrose	10,000
Mannitol	36,000
pH	5.5

Suggested Further Reading

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Motose H, Sugiyama M, Fukuda H (2001) Cell–cell interactions during vascular development. J Plant Res 114:473–481

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Cellular Totipotency

6

6.1 Introduction

All multicellular organisms, including higher plants and animals, are finally derived from a single-celled zygote through successive mitotic divisions and differentiation. In animals most of the cells (other than stem cells), as they differentiate into specific cell types, lose the potentiality to reconstitute a new organism. However, all living plant cells retain the potential to revert back to the meristematic state and form new plants on exposure to favorable conditions, irrespective of their specialization and ploidy level (haploid, diploid or triploid). It has been a routine horticultural practice to use leaf, stem, and root cuttings as source material to regenerate new individuals for vegetative propagation of some plant species. Plant tissue culture has considerably enlarged the scope of regeneration of plants from highly differentiated and structurally and functionally specialized cells of leaves, roots, stem, floral parts, and endosperm. In vitro regeneration of plants is also possible from isolated gametic cells (microspores—Chap. 8; unfertilized egg or synergids—Chap. 9). The potentiality of differentiated and specialized cells to form complete plants like the zygote is referred to as *Cellular Totipotency*. The term was probably coined by T.H. Morgan (1901). However, it was the famous German plant physiologist, Göttlieb Haberlandt, who in his famous address to the German Academy in 1902 introduced the concept of cellular totipotency and suggested that the terminally differentiated plant cells, as long as they contain the entire complement of chromosomes, should be capable of regenerating whole plants. He made a novel approach to prove his hypothesis, in which single cells were isolated from highly differentiated tissues of the plant body and cultured in nutrient medium. Although he did not succeed in his experiments due to technical limitations at that time, his idea attracted the attention of many scientists to pursue this line of investigation, and in 1939 White reported reproducible differentiation of shoot buds in tissue cultures of tobacco. In 1965, Vasil and Hildebrandt, achieved regeneration of full plants from isolated single cells of tobacco, and in 1975 it became possible to regenerate complete plants from mesophyll protoplasts of tobacco (Takebe et al. 1971; also see Chap. 14). The totipotency of plant cells is now well established.

Plant regeneration from differentiated cells is generally preceded by the cells becoming meristematic, followed by divisions to form an unorganized callus. The phenomenon of mature cells reverting back to meristematic state is termed *dedifferentiation*. The callusing phase may be very brief or long, depending on the system. Regeneration of plants from callus or, sometimes, directly from the pre-existing meristematic cells (like stem cells) is termed *re-differentiation*. Differentiation of one organ directly from another organ, such as shoot from root explants of *Arabidopsis,* is referred to as *transdifferentiation* (Sugimoto et al. 2011).

In tissue cultures, cellular totipotency may be expressed via organogenesis (shoot differentiation) or embryogenesis (adventive embryony).

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_6, © Springer India 2013

In more than 75 % of the plant species for which plant regeneration from protoplasts has been achieved, especially those belonging to the most successful families (Solanaceae, Asteraceae, Brassicaceae, and Fabaceae), it occurred via organogenesis (Xu and Xue 1999). Sometimes it is possible to alter the pathway of regeneration by manipulating the culture conditions. The distinctive features of a shoot bud and an adventive embryo are (Fig. 6.1a, b): (i) whereas shoot is monopolar the embryo is bipolar, (ii) the basal end of shoot is open and its provascular strands establish connection with the pre-existing vascular tissue dispersed in the callus or the cultured explant, and therefore if it is removed from the parent tissue the basal end is injured. On the other hand, the basal end (radicular end) of an embryo is closed. It does not have vascular connection with the parent tissue and can be removed from the parent tissue without causing injury.

As the following chapter is fully devoted to somatic embryogenesis, this chapter deals with organogenic differentiation only, especially shoot differentiation (caulogenesis). Detailed studies of de novo shoot organogenesis in vitro, which was first reproducibly demonstrated in tobacco by White (1939), have led to much of the understanding we have about organogenesis. Since then tobacco pith tissue has been the subject of detailed investigations on chemical control of shoot differentiation (Skoog and Miller 1957, 1971), regeneration from Thin Cell Layer (TCL) systems (Tran Thanh Van 1990), and physiological and biochemical aspects of this morphogenic phenomenon (Thorpe 1990, 1994, 1999). During the past 15 years, *Arabidopsis* has emerged as the model system to understand the mechanism and genetic control of adventitious organogenic differentiation (see Sect. 6.6).

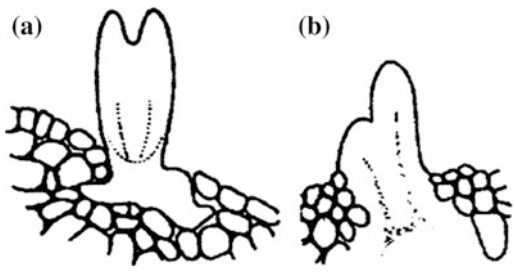

Fig. 6.1 Diagrammes to depict difference of anatomy in the basal ends of an embryo (**a**) and a shoot bud (**b**) differentiated in vitro. The basal end of the embryo is closed, whereas that of the shoot bud is open

By empirically manipulating the composition of culture medium and several other intrinsic and extrinsic factors, it has been possible to induce shoot bud differentiation from explants, cells, protoplasts, callus, pollen, and endosperm of a very large number of angiosperms (monocots and dicots) and gymnosperms.

6.2 Factors Affecting Shoot Bud Differentiation

6.2.1 Culture Medium

Skoog (1944) confirmed the observations of White (1939) on the differentiation of shoot buds from tobacco callus and through systematic studies (Skoog and Tsui 1948; Skoog 1954, 1955; Skoog and Miller 1957), put forth the concept of chemical control of organogenesis in callus cultures of tobacco (Skoog and Miller 1957; Skoog 1971). According to their observations auxin inhibited spontaneous shoot bud formation in tobacco. As low as 5 μM IAA completely suppressed the spontaneous shoot bud formation, and in combination it suppressed the shoot forming activity of kinetin. Two molecules of kinetin were required to offset the inhibitory effect of one molecule of IAA on shoot bud differentiation. Root-shoot differentiation in tobacco tissue cultures is a function of relative concentrations of auxin and cytokinin; relatively higher concentration of auxin favors root formation and relatively higher concentration of cytokinin promotes shoot bud differentiation (Fig. 6.2). This concept of auxin and cytokinin interaction has been successfully applied to several other systems. However, there are exceptions to the rule, which could be due to differences in: (i) the endogenous levels of the two growth regulators, (ii) the level of cell sensitivity to the growth regulators, (iii) uptake of the growth regulators, (iv) degree of glycosylation and hydrolysis of the growth regulators, (v) the type of the growth regulators used, and (vi) the activity of auxin and

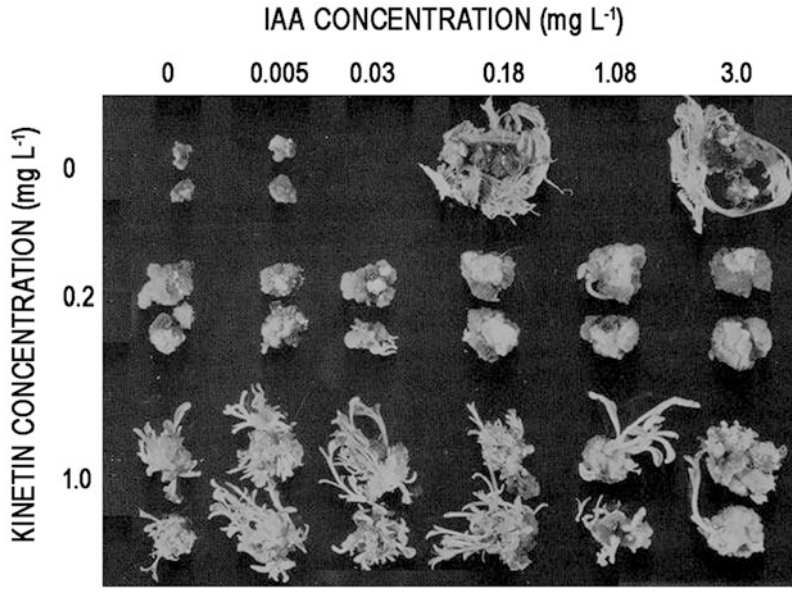

Fig. 6.2 Chemical control of organogenesis in tobacco (Wisconsin No. 38). Effect of increasing concentration of IAA at different levels of kinetin, in the presence of casein hydrolysate (3 mg L^{-1}), on callus growth and differentiation of shoot buds and roots on White's medium. All samples are from 62-days-old cultures. Note root formation in the presence of 0.18–3.0 mg L^{-1} IAA and shoot bud formation in the presence of 1.0 mg L^{-1} kinetin, particularly in combination with IAA in the range of 0.005–0.18 mg L^{-1}. (*after* Skoog and Miller 1957)

cytokinin oxidases (Tran Thanh Van and Trinh 1990).

Like tobacco, spontaneous shoot bud formation in the absence of a growth regulator, also occurs in single cell tissue clones of *Convolvulus*. Addition of auxin or kinetin promotes shoot bud formation. However, maximum shoot bud differentiation occurred in the combined presence of 10^{-7} M IAA and 10^{-5} M kinetin. Some systems do not form buds in the absence of growth regulators (*Scurrula pulverulenta*, *Lactuca sativa* and *Brassica juncea*). In cotyledon cultures of *Brassica juncea*, BAP alone induced shoot bud differentiation from the petiolar cut end. In the absence of BAP or in the presence of an auxin only roots were formed at the same site (Fig. 6.3).

In radiata pine, as also true for many other conifers, exogenous cytokinin alone is sufficient for shoot induction. That the final endogenous concentrations, not exogenous concentrations, are important in determining organogenic differentiation is demonstrated by the lack of necessity for exogenous auxin in conifers, lower cytokinin requirement for shoot differentiation in many graminaceous plants and requirement of high auxin/cytokinin ratio (as against high cytokinin/auxin usually required) for shoot bud formation in some plants, such as alfalfa (Walker et al. 1978, 1979). There are several cytokinins (2iP, BAP, SD8339, thidaizuron and zeatin) that have been used for in vitro induction of shoot buds but BAP has proved most effective and has been used most widely (Joy and Thorpe 1990). In TCL cultures of tobacco CPU (a urea derivative cytokinin related to thidiazuron) induced five times more shoot buds than kinetin (Tran Thanh Van and Trinh 1990).

Transformation of plant cells with modified T-DNA coding for the synthesis of auxin (*iaah* and *iaam*) or cytokinin (*ipt*) enhances endogenous levels of the respective hormones, and allows for appropriate organogenic differentiation in the same manner as exogenous auxins and cytokinins (Schell et al. 1982; Owens and Smigocki 1990). Furthermore, exogenous hormones can reverse the T-DNA induced morphogenesis, suggesting that

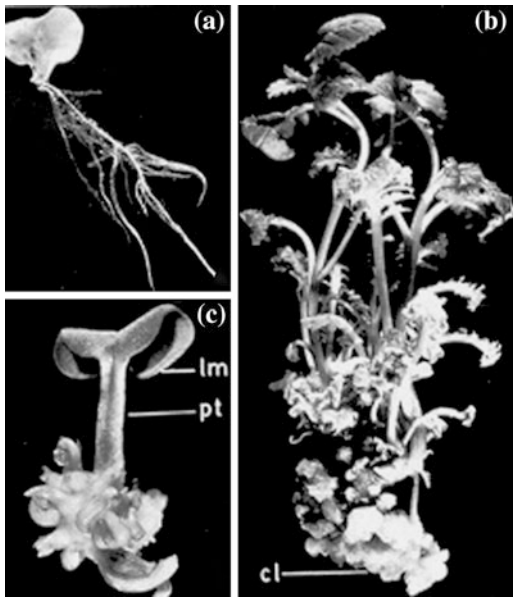

Fig. 6.3 Organogenesis in cotyledon cultures of *Brassica juncea*. **a** Only roots have developed at the cut end of the short petiole, on MS basal medium. **b** Only shoot buds have differentiated at the cut end of the elongated petiole, on MS + $5x10^{-6}$ M BAP. **c** In prolonged culture on medium as in **b**, several shoots have developed. (*lm* lamina, *pt* petiole, *cl* callus) (*after* Sharma and Bhojwani 1989)

the hormones play direct role in organogenesis (Inze et al. 1984).

In most of the cereals, callus is initiated on 2, 4-D-containing medium but organogenesis occurs only when the callus is transferred to 2, 4-D-free medium or containing IAA or NAA. Mostly the callus differentiates roots. Whether the callus would differentiate shoots or roots in 2, 4-D-free medium depends on the innate capacity of the tissue. A two-step process of organogenesis is also reported for alfalfa (Saunders and Bingham 1972; Walker et al. 1978, 1979). Callus is initiated on a medium containing 2, 4-D and kinetin (*Induction Medium*). For organogenesis the callus pieces are transferred to a hormone-free medium (*Regeneration Medium*). The ratio of auxin and cytokinin in the induction medium determines the type of organ formed in the organogenic medium. Higher 2, 4-D to kinetin ratio favors shoot formation and a ratio in favor of cytokinin supports root differentiation. The hormonal ratio is critical during the last 4 days on the induction medium.

GA_3 inhibited shoot bud formation in tobacco (Thorpe and Murashige 1970), *Plumbago indica* (Nitsch and Nitsch 1967), *Begonia* (Heide 1969) and rice (Maeda 1978). A pulse treatment (30–60 s) of GA_3, in dark, is sufficient to inhibit shoot differentiation from tobacco callus. However, GA_3 is effective only during the formation of meristemoids; once shoot bud differentiation is initiated it does not inhibit their further development. In some difficult to regenerate systems unconventional substances such as TIBA (Cassells 1979; Tran Thanh Van and Trinh 1990), ABA (Yamaguchi and Nakajima 1974; Shepard 1980), kanamycin (Owens 1979), and auxin inhibitors (Kochba and Spiegel-Roy 1977) have been effective in inducing shoot differentiation. Raw powder of ginseng, in the presence of auxin and cytokinin, doubled the regeneration frequency in the cultures of cotyledons and hypocotyls of *Brassica oleracea* (Hui and Zee 1980).

Ethylene, a senescence hormone, is known to suppress shoot differentiation in a number of in vitro systems. Therefore, often inhibitors of ethylene synthesis (aminoethoxyvinylglycine; AVG) or action ($AgNO_3$) have been used to induce or promote caulogenesis (shoot differentiation; Pua 1999). An inverse relationship occurs between ethylene production and shoot formation in tobacco (Grady and Bassham 1982). The inhibitory effect of ethylene is further suggested by the studies in which shoot regeneration from the callus of wheat and tobacco (Purnhause et al. 1987) and sunflower (Robinson and Adams 1987) was promoted by AVG or $AgNO_3$. De novo differentiation of shoots in cotyledon cultures of *B. campestris*, a recalcitrant system, was also promoted by ethylene inhibitors (5–10 μM AVG or 10–30 μM $AgNO_3$) (Chi and Chua 1989; Chi et al. 1990; Pua and Chi 1993). In these cultures, the ethylene concentration increased rapidly after 3 days and peaked after 10–14 days (Pua 1993). Implication of ethylene in inhibition of shoot bud differentiation has also been reported in a range of other plant species, including many Brassicaceae members (Pua 1999). Introduction of an antisense ACC oxidase

gene in mustard resulted in highly regenerative mustard plants due to reduction in ACC oxidase enzyme, required for ethylene biosynthesis (Pua and Lee 1995).

However, inhibition of shoot differentiation by ethylene is not universal. There are several examples where ethylene is known to promote shoot differentiation. For example, the capacity to form shoots in lavandin was positively correlated with the level of ethylene (Panizza et al. 1988). The shoot forming cotyledon explants of *Pinus radiata* produced considerable amount of ethylene and carbon dioxide, and the frequency of shoot bud formation could be positively correlated with the concentrations of the two gases inside the culture vials (Kumar et al. 1987). Removal of these gases from the culture vessels completely stopped organogenesis.

The effect of ethylene inhibition on shoot differentiation varies with the explant and the age of the donor plant. In Chinese kale (*B. alboglabra*) stem explants regenerated shoots with high frequency in the absence of ethylene inhibitors but hypocotyl explants, which were otherwise recalcitrant, required ethylene inhibitors, or exogenous putrescine for high frequency shoot differentiation (Pua et al. 1996).

6.2.2 Genotype

Significant genotypic influence on shoot regeneration response has been observed. Indeed, the success in obtaining regeneration in leguminous species, once regarded as a recalcitrant group (Bhojwani et al. 1977), has been mainly due to shift in the emphasis from media selection to genotype selection (Bhojwani and Mukhopadhyay 1986).

Among the three monogenomic species of *Brassica*, *B. oleracea* is most regenerative and *B. campestris* the least, *B. nigra* being intermediate. *B. napus* (amphidiploid of *B. oleracea* and *B. campestris*) is highly regenerative but less than *B. oleracea* because of the presence of *B. campetrsis* genome. Similarly, the regenerative potential of *B. carinata* (an amphidiploid of *B. oleracea* and *B. nigra*) is less than *B. oleracea* because of the presence of genetic component from *B. nigra*. In *Brassica*, both, nuclear and extra-nuclear genomes influence regenerability. The alloplasmic line of *B. oleracea* Ogura R1 male sterile cytoplasm shows an overall lower regeneration than those with normal cytoplasm (Jourdan and Earle 1989). Narasimhulu et al. (1988) noted significant cytoplasmic influence on regenerabilty in *B. carinata* synthesized from reciprocal crosses between *B. oleracea* and *B. nigra*. Several workers have reported that regeneration in wheat is genetically controlled (Rhode et al. 1988; Mathias and Fukui 1986; Galiba et al. 1986). According to Galiba et al. the genes controlling regeneration are present on 7B, 7D, and 1D chromosomes.

The genotypically selected regenerating lines do not show stringent culture requirement and display regeneration ability on a wide range of media (Bhojwani and White 1982; Kurtz and Lineberger 1983).

6.2.3 Explant

The third most important factor, after the composition of the medium and the genotype, is the explant used to initiate cultures. It is not only the source of the explant but the age of the tissue and the plant from which it is derived, preparation, the manner in which it is planted on the medium and inoculum density, all effect the response.

Right choice of explant is of great significance for successful organogenesis and regeneration. In *Crotolaria juncea* and *Glycine* the hypocotyl shows higher potentiality for shoot formation than root segments. Cotyledon was found to be the best explant for plant regeneration in *Lactuca sativa* and *B. juncea*. In *B. carinata*, 86 % of cotyledon explants, 74 % of hypocotyl segments and 26 % of root segments produced shoots (Jaiswal et al. 1987). In cereals and tree species, the regenerative cultures are mostly derived from immature embryos or young seedlings.

Age of the explant donor seedlings is critical in some cases. Five-day-old seedlings of *B. juncea* provided most regenerative cotyledons and those

from older than 10 days did not form shoots at all. The cotyledons of *Pinus radiata* lose the potential to form adventitious shoot buds 3 days after germination. In *P. gerardiana,* the cotyledons derived from ungerminated seeds show high potential to form shoot buds.

The explant is finally trimmed or cut to the requisite size before it is cultured. The final step of preparation of the explant is important as the retention or elimination of adjacent tissues may have a bearing on the kind of response evoked in cultures. In cotyledon cultures of *B. juncea*, shoot buds or roots are formed from the cut end of the petiole but the association of lamina (which lacks potential to form shoot buds) is essential for enabling the petiolar cells to exhibit totipotency. Therefore, the ideal explant to induce regeneration in this example is a short petiole (1 mm) together with the lamina. This is also true for *B. oleracea* (Lazzeri and Dunwell 1986; Horeau et al. 1988).

Orientation of the explant on the medium may be critical for organogenesis in some plants. In cotyledon cultures of *B. juncea* planting the cotyledons with their abaxial side in contact with the medium and the petiolar cut end embedded in the medium gave better response. If the petiole lost contact with the medium due to expansion and curling of the lamina within 3–5 days of cultures there was failure of root or shoot formation. In *Cunninghamia lanceolata* the explants placed horizontally on the medium produced three times more shoots than those planted vertically (Bigot and Engelmann 1987).

6.2.4 Electrical and Ultrasound Stimulation of Shoot Differentiation

Application of very weak (0.1–50 μA) alternating electric current of low frequency (50 Hz) for long periods (22–30 days), in such a way that the callus was made the negative pole and the medium the positive pole, promoted callus growth in tobacco (Goldsworthy and Rathore, 1985), and oil palm (Thavarungkul and Kanchanapoom, 2002) by 70 and 50 %, respectively. Electric stimulation occurred only in the presence of an auxin (IAA or NAA). In tobacco, microampere current to the callus on shoot differentiation medium caused up to 300-fold increase in the rate of shoot bud differentiation (Radu et al. 1994; Carmen 2006).

A brief sonication treatment of the explant is also reported to stimulate shoot bud differentiation (Gaba et al. 2006). Excised cotyledons of a line of squash (*Cucurbita pepo*), which normally produced only one, small fasciated shoot, developed multiple shoots after 30–120 s ultrasonic treatment. The shoots formed by the treated explants also showed good growth (Gaba et al. 2001, 2002). The promotion of shoot differentiation by sonication is ascribed to degradation of the explant surface.

6.3 Thin Cell Layer Culture

The tobacco TCL system developed by Tran Thanh Van (1973) and described in a series of subsequent papers provides a simple system to study the process and control of organogenic differentiation. Single layer epidermal cell layers do not survive in culture. However, TCL, comprising 3–6 layers of epidermal and subepidermal cells, from floral branches could be programmed to differentiate flower buds, shoots, or roots directly from the same cells within 10–18 days by manipulating the hormonal composition of the culture medium (Teixeira da Silva and Tanaka 2010). It is, therefore, regarded as a suitable system to study cytological, physiological, biochemical, and molecular changes associated with each morphogenic expression (Tran Thanh Van 1999).

The TCL from floral branches of tobacco differentiated only flower buds in a medium containing equimolar concentration (10^{-6} M) of kinetin and IAA or NAA. Increasing the concentration of kinetin to 10^{-5} M without changing the auxin concentration resulted in the differentiation of shoot buds from the same cells. With 10^{-5} M IBA and 10^{-7} M kinetin only roots appeared. It is interesting that not all cytokinins had similar affect. With BAP or zeatin or 2iP

both flower buds and shoot buds differentiated. This is probably due to the catabolism of these cytokinins in the system. This is suggested by the fact that the endogenous level of cytokinins with kinetin and dihydrozeatin, which induced 100 % flower formation, was higher than that with the other cytokinins.

The regeneration potential of cells in TCL system has also been demonstrated in many other species, such as *Begonia, Nautilocalyx, Saintpaulia, Brassica*, *Catharanthus, Solanum, Torenia* and *Cichorium*. TCL system has proved to be a very efficient system for clonal propagation of *Cymbidium* hybrids, which are otherwise difficult to propagate (Teixeira da Silva and Tanaka 2010). It is well known that in orchids regeneration from protocorms occurs only from the epidermal cells. Inner cells are not potent for regeneration. A 0.5–1.0 mm thick 3.5 × 3.5 mm TCL from the protocorm like bodies (PLB), developed in vitro from stem segments of *Cymbidium*, produced on an average 14.4 PLB within 20–25 days of culture, which is considerably faster than the conventional method of in vitro propagation of orchids (for more details see Chap. 17).

6.4 Totipotency of Crown Gall Tumor Cells

Crown gall tumor cells, infected with *Agrobacterium tumefaciens*, are characterized by their capacity for unlimited growth independent of exogenous growth regulators (auxin or cytokinin) in vitro and in situ and lack the potential for organogenic differentiation. However, teratoma, a special type of crown-gall tumor cells, shows a pronounced capacity to differentiate shoot buds when they are in the host plant or grown in vitro, but the shoot buds are abnormal morphologically and in their growth form. Braun (1957) and Braun and Wood (1976) could recover completely normal looking shoots from teratoma of tobacco by, (a) isolating single cells from teratoma suspension cultures, (b) raising tissue clones from these single cells by Muir's nurse culture technique, (c) grafting pieces of such a tissue onto the cut ends of stem of healthy tobacco plant, (d) grafting of abnormal shoots developed from grafted tumour tissue onto healthy plants, and (e) repeating the grafting till structurally, histologically, and functionally normal shoots developed. However, such a reversal of teratoma cells to normalcy is not irreversible, as isolated pieces from such 'normal' shoots in cultures reverted to tumor characteristics, such as auxin autonomy, due to the persistence of T-DNA (Yang et al. 1980). Thus, it is a case of suppression of the neoplastic condition of the cells rather than a reversal to normalcy. Nevertheless, somatic tissues of plants raised from seeds formed by the recovered teratoma shoots did not revert back to neoplastic growth (Turgeon et al. 1976). Even the androgenic haploids obtained by culturing anthers from the recovered shoots exhibited permanent and irreversible loss of the neoplastic condition. Stable recovery is attributed to elimination of T-DNA during meiosis (Yang et al. 1980).

6.5 Ontogeny of Shoots

Generally, the origin of roots is endogenous and that of shoots exogenous. In vitro shoot formation is generally preceded by the formation of vascularized or nonvascularized meristematic nests called meristemoids. In suspension cultures of carrot nodules with a central nest of tracheidal elements surrounded by cambium-like cells are formed, which develop roots from the central cells. When such rooted nodules are transferred to a semi-solid medium a shoot bud appears at another locus far removed from the root. Gradually, a vascular connection develops between the root and shoot and a whole plant is formed (Steward et al. 1958).

In tobacco, a case of shoot differentiation from callus, and *P. radiata,* a case of almost direct regeneration of shoots from the epidermal and hypodermal cells of cotyledon explants, the morphogenic response occurs in the region in contact with the medium and shoot bud formation is preceded by the formation of meristemoids with small, compactly packed cells without much intercellular spaces (Joy and Thorpe 1999).

In tobacco, 8 days after culture random cell divisions occur in the region of organogenesis and in another few days distinct meristemoids appear. Shoot primordia are seen after 12 days. In radiata pine, the cotyledons at the time of culture possess meristematic cells and within 3 days of culture cell divisions occur in the epidermal and hypodermal cells. Distinct 6–8-celled meristemoids appear by the fifth day and within the next 5 days the cotyledons become nodular due to the increased size of meristemoids. Leaves appear after 21 days of culture.

Interestingly, in the cultures of root segments of *Arabidopsis thaliana*, both, roots and shoots originate endogenously by the activity of pericycle (Atta et al. 2009). The pericycle cells on callus–induction medium form lateral root-like structures within 5 days which develop shoot primordia if transferred to shoot-induction medium and roots if transferred to root-induction medium. Thus, the pericycle cells are pluripotent.

6.6 Induction of Organogenic Differentiation

Ever since the classic experiments of Skoog and Miller (1957) that put forth the famous concept of chemical control of organogenesis in tobacco by manipulation of the relative concentrations of kinetin and IAA, plant scientists have been intrigued by the ability of the two hormones to control the fate of tissues in cultures. This approach has been extensively applied to achieve shoot/root regeneration from different tissues of a large number of plant species. However, this application has been without the understanding of the mechanism of organogenic differentiation. Consequently, for the same morphogenic expression in different system a wide range of hormonal treatments had to be used and some systems have still remained recalcitrant for *de novo* regeneration of shoots.

The process of organogenesis in response to hormonal treatments is a multistep process. The first phase consists of acquiring competence by the cells to respond to the hormonal treatments. In the second phase, the competent cells are induced to form a specific organ. Once induced to form a specific organ the cells become irreversibly committed to form the organ even if the inductive hormonal treatment is withdrawn. For example, in excised cotyledon cultures of *B. juncea* the cut end of the petiole forms roots (Fig. 6.3a) or shoot buds (Fig. 6.3b) on basal medium and basal medium supplemented with BAP, respectively (Sharma et al. 1990). The cotyledons after 11 days on the BAP containing medium become irreversibly committed to form shoots and continue to form shoots even if transferred to BAP-free medium. Similarly, if the explants are maintained on BAP-free medium for 7 days or more they lose the potential to form shoot buds on BAP-containing medium. In prolonged cultures in the presence of BAP several well-developed shoots were formed (Fig. 6.3c).

Christianson and Warnick (1983, 1984, 1985) described controlled root/shoot differentiation in leaf disc cultures of *Convolvulus arvensis*. These explants formed only shoots on MS + 7 mg L^{-1} 2iP + 0.05 mg L^{-1} IAA (SIM), only roots on MS + 12 mg L^{-1} IBA (RIM) and only callus on MS + 0.3 mg L^{-1} kinetin + 3 mg L^{-1} IAA (CIM). Oraganogenic differentiation is preceded by slight callusing. The leaf cells on SIM for 10–12 days become induced or determined to form shoots and will continue to form shoots even if the leaf pieces are transferred to RIM or CIM (Fig. 6.4). The induction process comprises two major steps. During the first 3–5 days on the induction medium, the cells of the explants acquire competence to respond to the inductive conditions. However, they are still not committed to form one or the other organ. They can form roots or shoots depending on the medium to which they are exposed. On shoot-induction medium they become induced and irreversibly committed to form shoots after 10–12 days. The competence for shoot induction can be achieved even on CIM and RIM for 3–5 days (Fig. 6.4). Some of the genotypes of *C. arvensis,* which exhibit poor or no shoot bud differentiation when directly cultured on SIM, could be induced to form large number of shoots by pre-culture on RIM for 3–5 days, suggesting that these genotypes were blocked to acquire competence in response to SIM. The

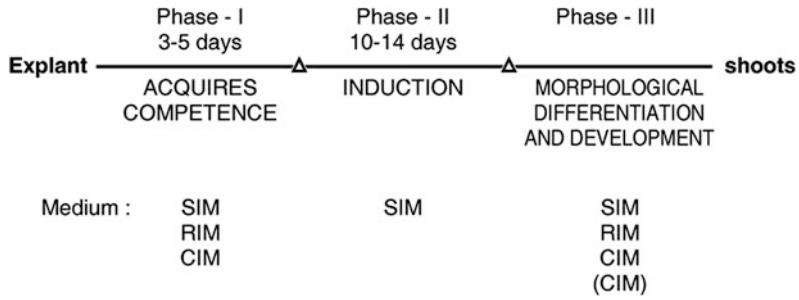

Fig. 6.4 The process of shoot bud differentiation from leaf discs of *Convolvulus arvensis* comprises of three phases: acquiring competence, induction of shoot bud formation, and visible shoot bud formation. The cells acquire competence for shoot bud induction in 3–5 days on shoot induction (SIM), root induction (RIM), or callus induction (CIM) medium. The competent cells become determined to form shoot buds if maintained on SIM for 10–14 days. Thereafter, the cells continue to form shoot buds irrespective of the medium (SIM, RIM or CIM). (adapted from Christianson and Warnick 1984, 1985)

period between the acquisition of competence and determination comprises several substages sensitive to various substances. It includes a stage sensitive to salicylate, followed by a stage sensitive to TIBA and sorbitol. Thus, shoot differentiation in this system is a three-step process: (1) acquisition of competence to respond to the induction conditions, (2) induction or determination to form a specific organ, and (3) differentiation of morphologically visible shoot meristem. Steps 1 and 2 can occur on the same medium.

During the past two decades *A. thaliana* has emerged as a model system to study the process of organogenesis in tissue cultures. Root segments of this plant have been extensively used to understand the ontogeny and molecular basis of shoot differentiation, and a lot of new information has been generated (Atta et al. 2009; Cary et al. 2001, 2002; Che et al. 2006; Gordon et al. 2007; Sugimoto et al. 2010, 2011; Yadav et al. 2009).

A two-step process of shoot differentiation in root explant cultures of *A. thaliana*, as developed by Valveken et al. (1988), has been used by many scientists (Cary et al. 2002; Che et al. 2007). In the first step 5 mm root segments from 7-day-old aseptic seedlings are incubated in an auxin rich callus induction medium (CIM; B_5 + 2.2 μM 2,4-D + 0.2 μM 2iP). On this medium root cells proliferate and form outgrowths that have been referred to as callus. During this phase, the cells are said to acquire competence to form shoots after 2–3 days (Cary et al. 2002; Che et al. 2007). After 4–5 days on CIM, the root explants are transferred to shoot induction medium (SIM; B_5 + 5 μM 2iP + 0.9 μM IAA) or to root induction medium (RIM; B_5 + 0.9 μM IAA). On SIM the so called 'callus' forms shoots (Fig. 6.5).

Gordon et al (2007) traced the spatio-temporal events during callus formation on CIM and shoot regeneration on its transfer to SIM and correlated the pattern formation during these events to the expression of several genes. Atta et al (2009) made a very detailed study of shoot regeneration in root segment cultures of *A. thaliana*. According to them, the outgrowths formed on CIM, which were earlier described as unorganized callus are actually organized Lateral Root Meristem-Like Protuberances (LRM-LP) originating from the pericycle cells opposite the protoxylem poles. Regeneration of Shoot Apical Meristem (SAM) occurs as a result of redetermination of the LRM-LP cells. If cultured directly on SIM, xylem pericycle cells were able to regenerate SAMs directly in places where LRM-LP should have been initiated. It demonstrates the pluripotent nature of the pericycle cells. The pluripotency was accompanied by capacity to retain diploid status even after several rounds of cell divisions, which is a desirable feature for using the system for clonal propagation of plants.

Within 2 days of root segment culture on CIM active cell divisions occurs in the pericycle opposite the protoxylem pole, involving several contiguous pericycle cells. After 3–5 days,

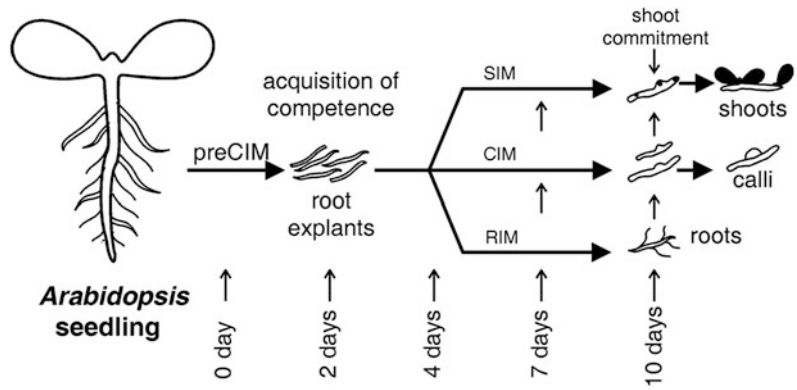

Fig. 6.5 Regeneration of shoots, roots and calli in the cultures of root segments of *A. thaliana*. The illustration shows that explants were preincubated on callus induction medium (CIM; B_5 + 500 mg L^{-1} MES + 2.2 μM 2,4-D + 0.2 μM kinetin + 0.8 % agar) for 4 days and then transferred to cytokinin-rich shoot induction medium (SIM; B_5 + 5 μM 2iP + 0.9 μM IAA), fresh CIM or auxin rich root induction medium (RIM; B_5 + 09 μM IAA). The medium after 4 days determined the nature of morphogenesis. The vertical *arrows* show time during development. (*after* Che et al. 2006)

several periclinal and anticlinal divisions result in multiple LRM-LPs in two longitudinal rows. Each LRM-LP has an outer continuous cell layer and is organized internally into cell files. These LRM-LPs showed many features common to Lateral Root Meristems (LRM), such as their origin from pericycle, occurrence of statolith in the external layer as in root cap, and expression of root specific genes (*QC25, RCH1, PLT1*). Thus, the LRM-LPs initiated on CIM were not true callus but organized structures resembling LRM. If transferred to fresh CIM and maintained for some time the LRM-LP did not elongate and developed root hair-like structures. After 15–20 days on CIM the LRM-LP became disorganized like a callus, the peripheral limiting layer was disrupted and the capacity to form shoots on SIM was lost.

During the 3 days on CIM when competence to respond to shoot-induction medium is acquired (Che et al, 2007) and at the onset of their initiation, the LRM-LP comprised 2–4 layers and expressed the same genes as early LRMs. In addition, it also showed the expression of some genes specific to SAM, such as *CLV3* (*CLAVATA3*) and *LFY* (*LEAFY*), not found in LRM.

Four days after transfer to SIM the LRM-LP increased in size and turned green. The first leafy shoot became visible on the LRM-LP after 5–7 days. Concurrently, there was a dynamic change in gene expression in the LRM-LP. Expression of a number of SAM-specific genes occurred prior to any visible sign of de novo SAM organization (Table 6.1). Correspondingly, after 2–3 days LRM-specific genes (*QC25*, *RCM1* and *PLT1*) and *PIN1* were down regulated. Greening of the explant was accompanied by the expression of WUS (*WUSCHEL*) gene first in the inner pericycle derivatives and later in the rounded domain of the protuberances. It has been observed that a LRM-LP comprises multiple types of cells with difference in gene expression. Only a small proportion of these cells found in small patches initiate development of shoot meristem (Sugimoto et al. 2010).

Atta et al. (2009) have shown that it is possible to induce direct differentiation of SAM from the pericycle cells, bypassing the first step on CIM, by planting fresh explants on SIM or modified SIM. Shoot regeneration occurred in 7 days at various points along the root length. Direct shoot differentiation occurred in situ where LRM would have developed. Such replacement occurred with high concentration of the cytokinin (24.6 or 49.2 μM 2iP or kinetin). At these sites, expression of *CLV3* in the xylem pericycle started within the first day and *WUS* during the second day and the expression of

Table 6.1 Some genes associated with shoot differentiation in tissue cultures of *A. thaliana*. (*after* Phillips 2004)

Gene	Putative Function	Reference
CYCD3	Involved in acquisition of competence for organogenesis	Sugiyama 1999; Fletcher 2002
SRD3	Competence for shoot organogenesis	Sugiyama 1999, 2000
SRD1, SRD2	Competence for redifferentiation of shoots	Sugiyama 1999, 2000
ESR1	Enhances shoot regeneration, vegetative- to-Organogenic transition	Zuo et al. 2002
CRE1	Cytokinin receptor	Zuo et al. 2002
CKII	Cytokinin perception	Zuo et al. 2002
CLV, WUS	Preserve stem cell identity in shoot apical meristem	Fletcher 2002
KNI, STM	Initiate ectopic shoot meristems, shoot apical meristem function	Fletcher 2002
SHO, MGO	Modifiers of the shoot apical meristem involved in leaf founder cell recruitment, lateral organ primordial	Fletcher 2002
CUC1	Shoot meristem differentiation	Daimon et al. 2003

Shoot Meristemless (*STM*) started later after 3–5 days.

Over expression of some genes specific to shoot meristem formation has been shown to promote shoot differentiation from *Arabidopsis* explants. CUP-Shaped Cotyledon1 (*CUC1*) and CUP-Shaped Cotyledon2 (*CUC2*) are essential for shoot meristem differentiation in embryos through *STM* activation. Overexpression of these genes remarkably enhanced the frequency (60–80 % as against 30 % in wild type controls) and rapidity of shoot differentiation in hypocotyl segment cultures (Daimon et al. 2003). However, the promotion required the presence of a cytokinin as neither of them is involved in synthesis or sensitivity of the hormone (Daimon et al. 2003). Enhancer Of Shoot Regeneration (*ESR1*) appears after the cells acquire competence but before the appearance of shoot meristem. It is implicated in initiation of shoot regeneration. The transcripts of this gene increased within 1 h and reached maximum 24 h after transfer of the root explants to SIM (Banno et al. 2003). Overexpression of this gene conferred cytokinin-independent shoot formation in root explants of *Arabidopsis*. It also greatly increased the efficiency of shoot regeneration in the presence of cytokinin, with shifts in the optimum concentration of cytokinin required for the process (Banno et al. 2001). *RAP2.6L* is another gene that is upregulated during early shoot development (Che et al. 2006).

As mentioned earlier, genotype of the plant influences in vitro shoot differentiation capability, which is an inheritable trait, and therefore expected to be located on the chromosomes. Screening for QTLs in inbred lines of two ecotypes of *Arabidopsis*, viz. Columbia and Landsberg erecta, three QTLs related to in vitro shoot regeneration were identified. One major QTL was located on chromosome 5 and 2 minor ones on chromosomes 1 and 4 (Lall et al. 2004).

6.7 Concluding Remarks

In vitro regeneration of plants is critical for the complete success of genetic engineering and micropropagation and many other direct biotechnological applications of plant tissue culture techniques. For long the main approach to induce plant regeneration from cultured tissues has been to manipulate the composition of the culture medium, especially with respect to growth regulators based on the findings of Skoog and Miller (1957). This approach has been fairly

successful with a large number of plant species. However, many other species did not respond to these manipulations or responded with low frequencies. A main reason for the bottleneck has been our poor understanding of the molecular basis of organogenic differentiation.

During the past two decades, tremendous progress has been made in the field of genetics of organogenic differentiation in vitro and in situ (Phillips 2004; Sugiyama 1999, Atta et al 2009) and a number of candidate genes involved in the differentiation of roots and shoots in *A. thaliana* have been identified. Overexpression of these genes transgenically enhanced the regeneration response or induced regeneration in hitherto recalcitrant species (Banno et al. 2001; Daimon et al. 2003, Hibara et al. 2003). This success should help in achieving regeneration in tissue culture systems that have remained recalcitrant so far and developing more efficient, marker-free genetic transformation protocols.

Suggested Further Reading

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Somatic Embryogenesis 7

7.1 Introduction

Embryogenesis is a specialized mode of development by which a fertilized egg through a series of predetermined pattern of cell divisions and differentiation forms an embryos, the precursor of the next generation. In nature, embryogenesis is restricted to ovule and involves the fusion of male (sperm) and female (egg) gametes. Occasionally, as in some varieties of mango and citrus, the sporophytic cells of the nucellus form asexual or adventive embryos independent of fertilization. However, these embryos also mature only inside the embryo sac (female gametophyte). Fertilization-independent embryogenesis is also exhibited by some elements of the embryo sac (egg or synergid) in apomictic plants. Thus, in nature, sexual and asexual embryo formation is restricted to ovular cells and full development of the embryo occurs only inside the embryo sac. This led some scientists to suggest that the embryo formation requires a special environment available only inside the embryo sac. However, tissue culture studies during the past five decades have clearly demolished this myth and demonstrated that most plant cells, irrespective of their specialization and ploidy level, are capable of forming typical embryos that can germinate. The embryos formed by somatic cells are called somatic embryos, and the process by which a somatic cell differentiates into embryo is termed somatic embryogenesis. In vitro somatic embryogenesis has found wide applications in basic and applied areas of plant sciences.

Although Haberlandt conceptualized in the early 1900s that a vegetative cell could form an embryo, the actual formation of somatic embryos in cell cultures of carrot was first demonstrated in 1958 by two scientists working independently, Reinert in Germany and Steward in the USA. Since then somatic embryogenesis has been reported in more than 500 species of dicots and monocots (Thorpe and Stasolla 2001). The list includes many crop plants, such as alfalfa, citrus, coffee, cotton, maize, mustard, rice, sunflower, and wheat. The plants in which somatic embryogenesis has been studied in some details are *Daucus carota*, *Citrus* sp., *Coffea* sp., *Macleaya cordata*, *Medicago sativa*, *Ranunculus sceleratus*, and *Zea mays*. The somatic embryos may arise directly from the explants (Fig. 7.1a) or indirectly after callusing (Fig. 7.1b). Somatic embryogenesis system of *Arabidopsis*, whose complete genome has been sequenced, is being developed as a model system to study the genetics and molecular biology of embryogenesis (Raghavan 2006).

Ever since the first demonstration of somatic embryo formation carrot has become a model system to study various aspects of somatic embryogenesis. Root segments of carrot seedlings cultured in the presence of 2,4-D form callus in about 6–8 weeks. Somatic embryos are produced within 3–4 weeks of the callus being transferred to a medium lacking 2,4-D (Smith and Street 1974).

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_7, © Springer India 2013

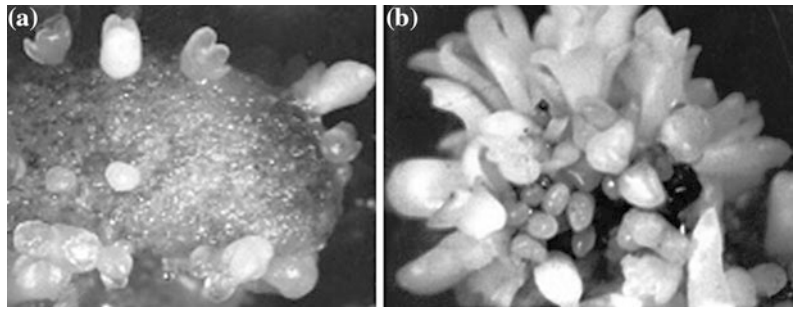

Fig. 7.1 **a** Direct differentiation of somatic embryos from the surface of a cotyledon of alfalfa. **b** A large number of embryos of different stages differentiated from nucellar callus of *Mangifera indica* var. Amrapali. (**a** *after* Merkle et al. 1990; **b** *courtesy* Prof. V.S. Jaiswal, BHU, India)

Fig. 7.2 A large number of somatic embryos directly arising from the stem surface of an in vitro germinated somatic embryo of *Ranunculus sceleratus*. (*after* Konar and Nataraja 1969)

In *Ranunculus sceleratus* both somatic and floral tissues form callus on a medium containing coconut milk with or without IAA. Numerous somatic embryos appear on the callus within 3 weeks on the original medium (Konar and Nataraja 1969). These somatic embryos germinate *in situ* or when transferred, individually, to fresh medium. An extremely interesting feature is the development of several secondary embryos from the epidermal cells of the in vitro germinated somatic embryos (Figs 7.2, 7.3). The nucellar cells of *Citrus* retain their potentiality for differentiating embryos even in tissue cultures. The somatic embryos arise directly from the nucellar cells (Rangan et al. 1968) or from callus in repeated subcultures (Kochba and Spiegel-Roy 1977).

Somatic embryogenesis has been unambiguously demonstrated from even single cells isolated from fully differentiated mesophyll tissue of *Macleaya cordata* (Fig. 7.5; Kohlenbach 1965; Lang and Kohlenbach 1975) and tissue cultures of carrot (Backs-Hüsemann and Reinert 1970).

7.2 Factors Affecting Somatic Embryogenesis

A complete protocol of somatic embryogenesis involves induction of embryogenesis, embryo development, embryo maturation, and their conversion or germination to form complete plants. The general practice for inducing somatic embryogenesis is to culture a suitable plant tissue in an appropriate nutrient medium containing an auxin for callusing and induction of embryogenesis. Post-globular development of embryos requires transfer of callus to an auxin-free medium (Fig. 7.4). However, the requirement for induction of embryogenesis may vary considerably with the species. For successful somatic embryogenesis a judicious selection and manipulation of the explants (plant tissue), nutrient medium, growth regulators, and physical culture environment is required.

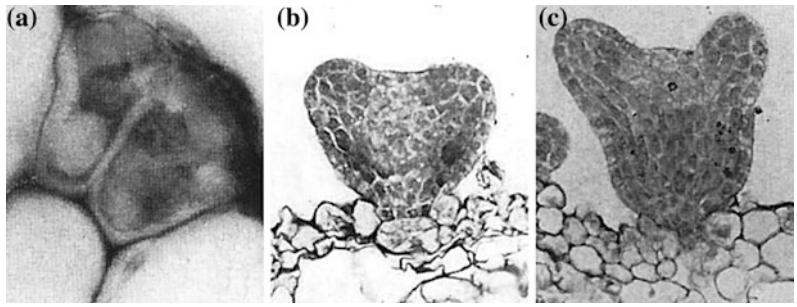

Fig. 7.3 Pictures showing epidermal origin of the somatic embryos formed on the stem of *Ranunculus sceleratus* sapling as shown in Fig 7.2. **a** Epidermal cells entering into division. **b**, **c** Early and late heart shape embryos developed from the epidermal cells

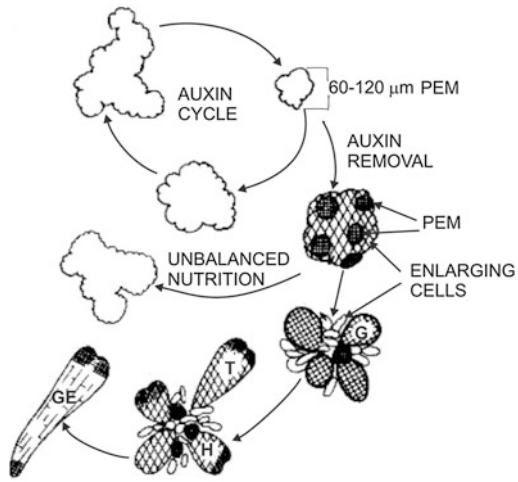

Fig. 7.4 In carrot, somatic embryogenesis occurs in two phases. In the presence of 2,4-D the callus differentiates proembryogenic masses (PEMs) which do not develop beyond the globular stage (auxin cycle). On removal of the auxin the PEMs differentiate further and go through the globular (G), heart (H) and torpedo (T) stages to form dicotyledonous embryos, which may germinate directly (GE) without going through a phase of dormancy. (*after* Wetherell 1978)

7.2.1 Explant

Success in achieving somatic embryogenesis in a large number of plants has been, mainly, due to a shift in emphasis from medium manipulation to explant selection. The choice of explant for the production of somatic embryos is generally limited to immature or less differentiated tissues such as hypocotyl segments, young leaves, embryonic shoot tip, young floral parts, and immature zygotic embryos. The selection of immature zygotic embryos as the explant has been the main factor for successful induction of somatic embryogenesis in monocots (cereals, palms) and many dicots. The zygotic embryos contain cells that already possess embryogenic competence, termed pre-embryogenic determined cells (PEDCs) while, the more differentiated explants have cells that must be inducted to become embryogenic and are termed induced embryogenic determined cells (IEDCs).

In *Arabidopsis thaliana* the best source of cells competent for somatic embryogenesis is immature zygotic embryo. In the cultures of immature embryos of this plant in liquid medium somatic embryogenesis is indirect through a callus phase (Raghavan 2004). However, the older embryos cultured on semi-solid medium exhibit direct somatic embryogenesis from the protoderm cells of the adaxial side of the cotyledons (Gaj 2001; Kurczynska et al. 2007).

7.2.2 Genotype

It is now clear that somatic embryogenesis is a genetically controlled trait. Striking intervarietal differences for somatic embryogenic potential have been reported in rice, alfalfa, groundnut, soybean, and maize. A mutant line (2HA) of *Medicago truncatula* cv. Jemalong exhibited 500-fold greater capacity to form somatic embryos than the parent line (Nolan et al. 2003). Genotypic variations could be due to varying

endogenous levels of growth regulators. In the ovules of highly embryogenic lines of maize the endogenous levels of auxin and cytokinin were considerably lower (16–20 times and 10–15 %, respectively) than in the poorly embryogenic and nonembryogenic lines. In alfalfa, inheritance studies have clearly demonstrated that the capacity to regenerate plants is controlled by two dominant genes (Reisch and Bingham 1980; Kielly and Bowley 1992). It was possible to breed highly regenerating lines, 'Regan-s' (tetraploid) and 'HG2' (diploid), by crossing two poorly responding lines (Du Puits and Sarnac) followed by recurrent selection and chromosome manipulation, respectively (Reisch and Bingham 1980; McCoy and Bingham 1977).

7.2.3 Medium

In 70 % of the successful cases of somatic embryogenesis Murashige and Skoog's (1962) basal medium or its modifications have been used. Very rarely, White's or SH basal medium has been used with suitable supplements. The most commonly used carbon source is sucrose, but for the induction of somatic embryogenesis in scarlet runner bean and citrus nucellus cultures glucose and galactose/lactose, respectively, were superior. Sucrose and auxin concentrations interact with each other, with the optimal concentration of one being dependent on the concentration of the other.

The form of nitrogen in the culture medium significantly affects in vitro somatic embryogenesis. Carrot cultures initiated in White's medium, containing KNO_3 as the sole source of inorganic nitrogen, failed to form somatic embryos when transferred to auxin-free medium. However, addition of a reduced nitrogen source, such as NH_4Cl (5 mM) along with KNO_3 (55 mM) allowed embryo development (Halperin and Wetherell 1965). In general, it appears that 5–12.5 mM NH_4^+ in the medium is required for the induction of somatic embryogenesis.

Other nitrogen sources such as casein hydrolysate (CH) and amino acids have also been found to increase somatic embryo formation. In suspension cultures of orchard grass casein hydrolysate (CH; 3 g L^{-1}) induced development of somatic embryos. Substitution of CH with a combination of proline and serine/threonine was superior in that the somatic embryos had a smoother epidermis, with considerably less incidence of precocious germination, and higher percentage of conversion of the embryos (Trigiano et al. 1992). In alfalfa also, somatic embryo production dramatically increased in the presence of the amino acids, proline, alanine, arginine, and glutamine. The importance of nitrogen requirement during embryogenesis may be correlated to continued synthesis of protein, nucleic acid, and reserve substances and also to maintain an appropriate pH.

7.2.4 Growth Regulators

(i) *Auxin.* The most commonly used auxin for the induction of somatic embryogenesis is the synthetic auxin 2,4-D, also used as an herbicide. Generally, the embryogenic cultures of carrot are initiated and multiplied in a medium containing 2,4-D in the range of 0.5–1.0 mg L^{-1}. On this medium, callus differentiates localized groups of meristematic cells called 'Proembryogenic masses' (PEMs). In repeated subcultures on this proliferation and induction medium, the embryogenic callus continues to multiply without the appearance of fully differentiated embryos. These PEMs develop into dicot embryos (Fig. 7.4) only upon transfer to a medium devoid of an auxin or with very low level of auxin (0.01 mg L^{-1}). This medium is known as Embryo Development medium. The requirement for exogenous auxin for the development of the embryos depends on its endogenous level in the tissue. In long-term callus cultures of *Citrus*, which failed to form somatic embryos in the presence of even a small amount of IAA in the medium turned embryogenic in the presence of auxin synthesis inhibitors.

The other auxins that have been shown to promote somatic embryogenesis, though less frequently, are dicamba (3, 6-dichloro-o-anisic acid) in orchardgrass, 2-(2,4-Dichlorophenoxy) propionic acid in alfalfa, picloram in *Arachis hypogaea*, and IAA and NAA in *Podophyllum hexandrum*.

The embryos of *Arabidopsis* up to 4 days after culture on the embryogenic medium exhibit asymmetric distribution of auxin but thereafter the maximum concentration of auxin occurs in the protoderm cells, which form somatic embryos, suggesting a key role of auxin in somatic embryogenesis (Garbowska et al. 2009). However, all cells with high auxin concentration do not form embryos. It has been suggested that *LEAFY COTYLEDON2* (*LEC2*) gene, which also shows high expression in the embryogenic cells, controls somatic embryogenesis via auxin signaling as this gene regulates *IAA30* gene (Braybrook et al. 2006).

(ii) *Cytokinin.* Cytokinins have been shown to have promontory effects on the induction of somatic embryogenesis. Zeatin, at a concentration of 0.1 μM, promoted the process in carrot, especially when added after 3–4 days of transfer from the proliferation medium. Similarly, for coffee and alfalfa, a cytokinin was found to be necessary. Zygotic embryos of *Podophyllum hexandrum* could be induced to form globular embryos in a medium containing BAP and IAA (Arumugam and Bhojwani 1989).

(iii) *Others.* Sometimes a combination of growth regulators may be needed for somatic embryogenesis. In caraway, a balance among ABA, zeatin, and gibberellic acid was required (Ammirato 1977), while 2iP and GA_3 were needed for grapevine (Mullins and Srinivasan 1976). In carrot, exogenous GA_3 decreased the number of heart and torpedo stage embryos, thus reducing the number of somatic embryos formed (Fujimura and Komamine 1975). Brassinolide proved beneficial for somatic embryogenesis in coconut (Azpeita et al. 2003) and rice (Pullman et al. 2003).

High sucrose concentration and manipulation of pH of the medium have also been reported to induce somatic embryogenesis without the aid of growth regulators.

7.2.5 Selective Subculture

!Multicellular explants are generally a mixture of cells differing in their potentials to form somatic embryos. Only a small proportion of these cells are able to express their cellular totipotency under a set of culture conditions. Therefore, the calli and suspension cultures derived from such explants are also heterogeneous with regard to the embryogenic potential of its constituent cells. Sometimes the embryogenic portions of a callus are distinct from the nonembryogenic tissue on the basis of their morphological appearance and it is essential to make artistic subcultures to establish regenerating tissue cultures. For example, in several Poaceae species embryogenic callus appeared granular and with smooth surface, whereas the nonembryogenic calli were rough, friable, and translucent (Nabors et al. 1983). Similarly, embryogenic calli of *Coffea arabica* are brown (phenolized) and hard while the nonembryogenic calli are pale and friable (Quiroz-Figueroa et al. 2006).

7.2.6 Electrical Stimulation

Mild electric current (0.02 V DC for 20 h) is known to stimulate shoot and embryo differentiation (Dijak et al. 1986). In the alfalfa cv. Rangelander, which shows direct embryogenesis in protoplast cultures, exposure of the protoplasts to low electric field enhanced direct embryogenesis from 40 to 100 % and the number of embryos per plate from 76 to 116. In two other cultivars of this crop, which normally formed embryos via a callus phase, subjecting the protoplasts to electric field induced direct embryogenesis. Electrical stimulation of organized development is ascribed to changes in organization of microtubules which affect the cell polarity (Dijak and Simmonds 1988; de Jong et al. 1993).

7.2.7 Other Factors

Initial cell density is an important factor for the induction of somatic embryogenesis in carrot. For instance, carrot embryogenic suspension cells cultured at high cell density in an auxin-free medium strongly inhibited the formation of somatic embryos, which could be overcome by the addition of activated charcoal. The cells cultured at high density release high amounts of phenolic compounds such as phenylacetic acid, benzoic acid, 4-hydroxybenzoic acid, and p-hydroxybenzyl alcohol (pHBA) that are inhibitory to somatic embryo formation. Of these, the inhibitory effect of pHBA was the strongest. It inhibited somatic embryo formation at a concentration as low as 10^{-6} M (Kobayashi et al. 2001). It specifically inhibited cell divisions at the early globular stage and suppressed transition of globular embryos to heart shape stage.

The gaseous phytohormone ethylene plays an important role in plant morphogenesis. Ehtylene applied exogenously inhibited somatic embryogenesis in carrot. 2,4-D-induced suppression of embryo development in the induction medium may be because of endogenous ethylene production. Ethylene biosynthesis (cobalt, nickel and salicylic acid) and action (silver nitrate) inhibitors were able to increase somatic embryogenesis in carrot and rubber.

Oxygen concentration has been shown to effect embryogenic development in cultures. Reducing dissolved oxygen in wheat cultures improved somatic embryogenesis by almost 6 fold, decreased precocious germination of somatic embryos and reduced abnormal scutellar enlargement.

Interestingly, compounds of prokaryotic origin have been shown to enhance somatic embryogenesis. Extracts from blue green algae such as *Nostoc* and *Anabaena* in carrot cultures (Wake et al. 1991) and co-culture of soybean with *Pseudomonas maltophilia* increased somatic embryo formation (Nowak 1998; Merkle and Parrot 1995). It is not yet clear whether compounds from these organisms act as signal molecules or have growth promoting properties.

Light is a major factor of the culture environment and has definite effect on embryogenesis of some plants. Somatic embryo induction in *Solanum melongena* has an absolute requirement for light, whereas *Populus* has an absolute requirement for darkness. The quality of light also influences somatic embryogenesis. In carrot, green and red light, and darkness were equally effective, while high intensity of white light and blue light proved inhibitory. It has been suggested that in vitro cultured immature embryos of wheat respond to 2,4-D and to light signals by modifying expression of G-proteins, NDP kinase, and arrestin proteins.

7.3 Induction and Development

A somatic cell in a plant, depending on its position and function, is on a pre-set path of morphological, biochemical, and physiological differentiation. Dedifferentiation from this set path is necessary to make the cell receptive to new developmental signals and to acquire embryogenic competence. Cell division is of paramount importance to reset the somatic cell on a new developmental program by general reorganization of chromatin structure, reprogramming of gene expression, and altering cellular metabolism (Feher et al. 2001). The different events leading to embryogenesis though intrinsically coupled to one another are often referred to as dedifferentiation, competence acquisition, induction, and determination.

Depending on the nature of the explant and culture conditions, somatic embryos may arise either directly from cells of the explant or indirectly via a phase of unorganized growth (callus formation) after several cycles of cell divisions. As stated earlier (Sect. 7.2.1.), in the former case explants carry competent cells (PEDCs) while the latter have to acquire competence, and therefore termed IEDCs. Both can be maintained and multiplied in the embryogenic state under appropriate culture conditions.

7.3.1 Induction

To acquire embryogenic competence the prerequisite is dedifferentiation of cells. Plant growth regulators, particularly auxin, are commonly used to induce dedifferentiation, although changes in pH and application of heavy metals also elicit embryogenic response. Auxin is considered to be the main plant hormone required for the activation of cell division in differentiated plant cells both in vivo and in vitro. 2,4-D, the commonly used auxin, is given either continuously in the medium or as pulse treatment of a few minutes to several days to make the cells embryogenically competent.

Polarity. An important phenomenon associated with the induction of somatic embryogenesis is the change of cellular polarity. Plant growth regulators and other treatments employed for the induction of embryogenesis alter the cell polarity and promote asymmetric division. In the single cell suspensions of carrot, the first division was asymmetric and only the smaller daughter cell ultimately developed into somatic embryo (Backs-Hüssemann and Reinert 1970). Since the root pole of the SE is always toward the larger cell the polarity of the entire SE is determined much before the first division of the embryogenic cell. Differential cell fate is determined not by morphological but by physiological asymmetries, with respect to unequal distribution of molecules, such as arabinogalactans, and other constituents (Feher et al. 1999).

In very young spherical SEs of carrot electric gradient exists along the future longitudinal axis (Brawley et al. 1984). Exposure of freshly isolated mesophyll protoplasts to an electric field (0.02 V DC current for 20 h) considerably promoted the embryogenic response in alfalfa (Dijak et al. 1986). The electric stimulus seems to promote the differentiation of organized structures (shoots/embryos) by affecting cell polarity through changes in the organization of microtubules. Another striking effect of electric treatment was the induction of an asymmetric first division in cells coupled with a relatively short period of cell expansion, resulting in spherical structures composed of many small irregularly shaped cells and a few large ones.

7.3.2 Development

In carrot, after re-initiation of cell division and a period of cell proliferation in the presence of the auxin embryogenic cells are released into the medium. The suspension cultures contain, besides the PEMs, single cells of two types: (i) cytoplasmically rich small cells, and (ii) vacuolated long cells. The former type of cells may give rise to proembryogenic masses (PEMs) or globules (Komamine et al. 1990) but most of the PEMs are derived from the pre-existing PEMs (de Vries et al. 1988; Emons et al. 1992). The PEMs comprise embryogenic cells, which are small (400–800 μm^3), angular, connected with the adjacent cells by many plasmodesmatas (2–4 per μm^2), have several small vacuoles (30 % of cell volume), many starch grains (5–25 per cell), high density of ribosomes, numerous profiles of rough endoplasmic reticulum, spherosome-like vesicles, high dehydrogenase activity, many exocytosis configurations on the plasmalemma (0.6 per μm^2), and polylamellate walls at all wall facets. The embryogenic cells are held together by nonembryogenic cells which are larger (1000–3000 μm^3), rounded, have intercellular spaces, fewer plasmodesmata (0.1–1.0 per m^2), larger vacuoles (80 % of cell volume), few starch grains (1–2 per cell), low population of ribosomes, very few endoplasmic reticulum profiles, little or no spherosome-like vesicles, low dehydrogenase activity, and sometimes fewer exocytosis configurations.

The continued presence of auxin even after induction of embryogenesis causes cell elongation and disruption of formerly adhering cells. The enlarged cells permanently lose the competence of developing into somatic embryos. The embryogenic cells secrete certain proteins into the culture medium which not only helps in maintaining the embryogenic potential of the cultures by restricting cell elongation in the presence of 2,4-D but also induce the

appearance of small embryogenic cells in previously nonembryogenic cultures (Kreuger and Van Holst 1993).

When embryogenic cultures of carrot are transferred to auxin-free medium, the disruption of cells from each other stops and the globules develop into globular embryos. In this process, the first differentiation step is the formation of a protoderm outside the globule. The globular embryos then continue further development and form typical embryos (Fig. 7.4). ABA stimulates epidermis development. The developing embryos now synthesize their own auxin and further morphogenesis, beyond globular stage, is dependent on proper polar transport of auxin.

SEs formed on the surface of the callus have intrinsic polarity because these are attached to the callus cells at the radicular end. The future root of such SEs is always oriented toward the callus center. The PEMs develop into complete embryos only when they are anchored. In carrot, the globular embryos exhibit polarity in the form of ionic current; there is influx of potassium ions at the future plumular end and the efflux of hydrogen ions at the redicular end (Brawley et al. 1984). The PEMs of carrot also show polarity in the distribution of calcium-calmodulin complex before morphological polarity is visible.

7.3.3 Single Cell Origin of Somatic Embryos

There are numerous reports of somatic embryogenesis in callus and suspension cultures leading to the formation of embryos resembling the zygotic embryos of the plant. Backs-Hüsse-mann and Reinert (1970) demonstrated differentiation of bipolar somatic embryos resembling zygotic embryos from a single cell isolated from tissue cultures of carrot. The cell divided by unequal division and the smaller derivative continued to divide and formed an embryo.

Kohlenbach (1965) and Lang and Kohlenbach (1975) raised whole plants starting from the mechanically isolated mature mesophyll cells of *Macleaya cordata* (Fig. 7.5). Regeneration of whole plants from isolated protoplasts via organogenesis or somatic embryogenesis has been reported for a large number of species. Some cultivars of alfalfa exhibit direct embryogenesis from isolated protoplasts.

7.4 Synchronization of Somatic Embryo Development

The population of cells undergoing embryogenesis, whether on a semi- solid medium or in a liquid suspension, is highly heterogeneous because of asynchronous development of embryos. It is desirable to have all the differentiating cells and the somatic embryos in a culture at the same developmental stage (synchronous development) for the system to be used effectively for artificial or synthetic seed technology or for studying physiological, biochemical, and molecular changes underlying the process of somatic embryogenesis. In cultures, synchronization is achieved by: (i) physical separation of different embryogenic stages, and/or (ii) physiologically by using growth regulators.

Physical separation of embryos of right stages is achieved by passing the heterogeneous suspension cultures through nylon sieves of varying mesh size or gradient centrifugation. In carrot, 50–100 μm fraction obtained by filtration of embryogenic suspension allowed best synchronization (Molle et al. 1993). The image analysis technique for counting somatic embryos could make way for computer-assisted sorting of embryos of different developmental stages (Harrell and Cantliffe 1991).

In the second method of physiological synchronization of somatic embryogenesis exogenous application of ABA arrests embryo development at a particular stage, generally at the torpedo stage. On withdrawal of ABA the embryos proceed to mature at the same time. Glycerol has also been used to achieve the same result.

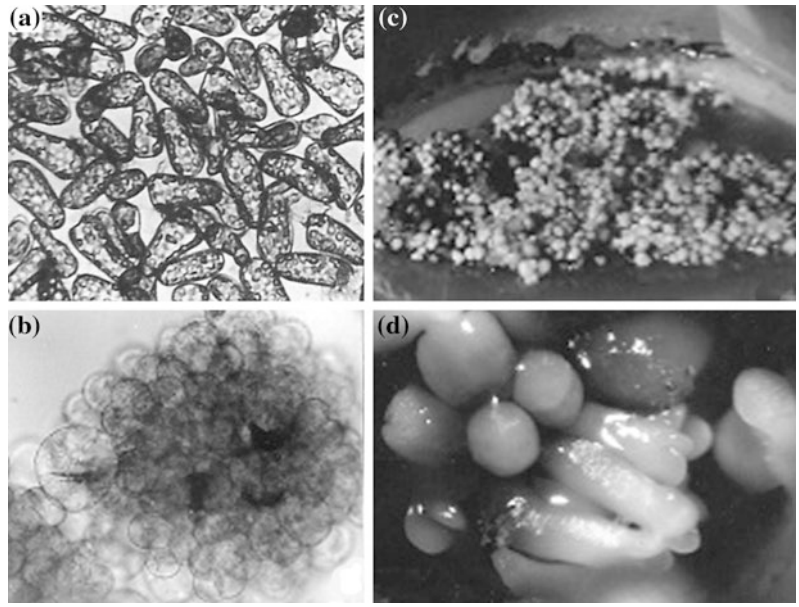

Fig. 7.5 Somatic embryogenesis in the cultures of isolated mesophyll cells of *Macleaya cordata*. **a** Freshly isolated mesophyll cells. **b** A mini callus formed by the mesophyll cells. **c** Embryogenic callus formed by mesophyll cells. **d** A portion from c enlarged to show somatic embryos at different stages of development. (*courtesy* H.W. Kohlenbach, Germany)

7.5 Physiological and Biochemical Aspects of Somatic Embryogenesis

A number of physiological and biochemical changes occur within the cell as it acquires embryogenic competence and much before any morphological differentiation of somatic embryo could be observed.

The most significant change observed is in the endogenous levels of phytohormones and their correlative effects. Once the embryogenic clusters are transferred to 2,4-D-free medium there is a rapid decline of endogenous 2,4-D metabolites and endogenous IAA levels after the globular stage (Michalezuk et al. 1992). In nonembryogenic callus, the levels of polar gibberellic acids were significantly higher than the less polar ones, and as the callus became embryogenic the situation reversed with the levels of less polar gibberellic acid being more than the polar gibberellic acids (Noma et al. 1982). This could probably explain the inhibition of somatic embryogenesis in carrot by exogenously applied GA_3. ABA is another growth regulator that shows changes in endogenous levels depending on the embryo stage (Kamada and Harada 1981).

Studies indicate that the amino acid metabolism of each embryo developmental stage is distinct. The levels of arginine and aliphatic amines were maximum during globular and torpedo stages which subsequently decreased in geminating embryos while glutamate and glutamine increased during embryo development. In nonembryogenic cells and germinating embryos, the glutamine synthase/glutamate synthase (GS/GOGAT) pathway is predominant, and the ornithine cycle is enhanced and predominant during early to mid-embryo development (see Thorpe and Stasolla 2001).

Increase in the endogenous level of polyamines and the enzymes associated with their biosynthesis concomitant with the induction of somatic embryogenesis in carrot and the suppression of somatic embryogenesis by the inhibitors of polyamine biosynthesis suggest the

involvement of polyamines in somatic embryogenesis (Minocha et al. 1990; Altman et al. 1990). Studies on *Solanum melongena* further support the idea of a causative role of polyamines in somatic embryogenesis (Yadav and Rajam 1998) as also in celery (Altman 1990) and mango (Litz 1993). During globular embryo formation there is a substantial increase in the turnover rate of RNA and protein followed by active DNA synthesis (Fujimera et al. 1980).

7.6 Molecular Markers and Somatic Embryogenesis

Changes in gene expression occur as the somatic cell embarks on embryogenic development. Several molecular markers indicating this transition have been recognized, and a number of 'embryo specific' or 'embryo enhanced' genes have been cloned from somatic embryos (Zimmerman 1993; Schmidt et al. 1997; Li et al. 2010; Montero-Cortés et al. 2010).

In *Cichorium* (Dubois et al. 1991) and *Trifolium repens* (Maheswaran and Williams 1985) callose deposition in the cell wall and the presence of vacuolar Ca^{2+} are the first signals that allow the recognition of embryogenic cells. Similarly, JIM8 monoclonal antibody positive cells of carrot suspension cultures formed embryos while the JIM8 negative cells did not (McCabe et al. 1997).

Cell wall associated proteins are known to act as short distance signal molecules in development. Some of these, such as members of the group oligosaccharin participate in the regulation of embryogenesis. In maize, wall of the embryogenic cells contained arabinogalactan proteins (AGPs) while nonembryogenic cells did not (Samaj et al. 1999). Binding the arabinogalactan by addition of the Yariv's reagent (synthetic phenyl-glycoside) to the culture medium blocked the induction of embryogenesis in carrot and *Cichorium* (Tompeon and Knox 1998; Chapman et al. 2000), which could be restored by transfer to fresh medium, confirming a prominent role of AGPs in the induction of somatic embryogenesis. Precipitation of AGPs with an anti-AGP antibody had similar inhibitory effect on somatic embryogenesis (Butwort et al. 1999). Moreover, AGP of tomato promoted somatic embryogenesis in carrot (Kreuger and van Holst 1996).

In proliferating cell cultures of carrot two callus specific proteins (Cl and C2) are synthesized. On transfer to an embryogenic specific medium, the protein profile of callus changes and two new embryo specific proteins (E1 and E2) appear. The callus-specific and embryo-specific proteins are coordinately regulated (Sung and Okimoto 1981, 1983).

To induce somatic embryogenesis in fresh cultures, often a cell-free "conditioned medium", which had been used for the development of somatic embryos, is used. Analysis of the conditioned medium revealed the presence of a number of extracellular proteins (EPs) released by embryogenic cells. So far, three types of extracellular proteins (EPl, EP2, and EP3) have been isolated.

Only nonembryogenic cells secrete EPl (van Engelen et al. 1991). EP2, a secretary lipid-transfer protein, is secreted only by the embryogenic cells and somatic embryos and is expressed in the peripheral cells of PEMs and the protoderm of SEs (Sterk et al. 1991). This protein is probably involved in the transport of cutin monomers to the specific sites of cutin synthesis. The third protein EP3 is a glycosylated acidic endochitinase and is involved in inducing normal protoderm formation (de Jong et al. 1992).

Based on 2D gel electrophoresis, Imin et al. (2005) identified 54 protein spots where significant changes had occurred during somatic embryogenesis in *Medicago truncatula*. Some additional proteins that have been identified at different stages during somatic embryogenesis are germin-like proteins (GLPs), embryogenic cell proteins (ECPs), and the Trx H Protein (Karami et al. 2009; Yang and Zhang 2010).

Schmidt et al. (1997) identified several genes from carrot (*Daucus carota*) cell suspension cultures. One of these genes, termed "Somatic Embryogenesis Receptor Kinase (Dc*SERK*)", is considered as a marker of single competent cells

because of a tight correlation between the ability of single cells to develop into an embryo and Dc*SERK* expression. Expression of this gene could be detected up to the 100 celled early globular stage of embryogenesis. A similar gene (At*SERK*1) has been isolated from *Arabidopsis thaliana*. Overexpression of this gene in the seedlings of *A. thaliana* resulted in 3 to 4-fold increase in the efficiency for initiation of embryogenesis. This gene is expressed in zygotic embryo up to the globular stage and is supposed to effect vegetative to embryogenic transition in carrot suspension culture (Li 2010). In multiple signaling pathway during transition of somatic cell to embryonic cell *SERK*s act as co-receptors via their physical interaction with specific lignad-binding Receptor-like Protein Kinases (RLKs).

A second approach to identify genes that are activated during somatic embryogenesis is the use of loss-of-function mutations as in *Arabidopsis. LEAFY COTYLEDON* (*LEC*) *1* and *2* are two genes identified through this approach. *LEC1* and *LEC2* cause defects in embryo maturation and partially transform cotyledons into leaves. Ectopic expression of both *LEC1* and *LEC2* induce the formation of embryos on vegetative tissues (Lotan et al. 1998; Stone et al. 2008). *LEC 2* induces somatic embryogenesis in vegetative tissues by enhancing auxin activity through its direct action on *YUC4*, an auxin biosynthetic gene. Another pathway depicted for *LEC2* action is through *AGAMOUS–Like 15* (*AGL15*) which induces an enzyme that inactivates GA and thus increases the frequency of somatic embryo formation (Stone et al. 2001, 2008; Wang et al. 2004).

Through another genetic approach of gain-of-function mutations, some important genes that promote somatic embryogenesis from root explants of *Arabidopsis* have been identified. Normally, somatic embryogenesis in *Arabidopsis* has been possible from zygotic embryos. The novel genetic screening method identified the *PLANT GROWTH ACTIVATOR 6* (*PGA6*) gene. Overexpression of this gene promoted somatic embryo formation from various vegetative tissues and zygotic embryo without any exogenous plant hormone. *PGA6* is identical to the homeobox gene *WUSCHEL* (*WUS*), which regulates the fate of stem cells in shoot and floral meristem and plays a critical role in maintaining the identity of embryonic stem cells (Zuo et al. 2002). Two more genes (*MYB118*, *MYB115*) have been identified recently that are capable of inducing vegetative-to-embryonic transition and promote formation of somatic embryos in *Arabidopsis* from vegetative parts. These genes act independently from the *WUS* gene and could be opening up an entirely new regulatory mechanism underlying somatic embryo formation in *Arabidopsis* (Wang et al. 2009).

7.7 Maturation and Conversion of Somatic Embryos

During the final phase of embryogenesis the zygotic embryos undergo maturation, which is characterized by the accumulation of storage substances, acquisition of desiccation tolerance, and synthesis of ABA to induce dormancy. However, somatic embryos generally skip this final stage of embryo maturation and germinate precociously to form weak seedlings. Therefore, morphologically fully developed somatic embryos require special treatment/s to induce maturation. ABA treatment, exposure to high sucrose concentration and gradual desiccation are some of the treatments known to promote maturation of somatic embryos.

Sanaratna et al. (1989, 1990) were able to confer desiccation tolerance upon alfalfa somatic embryos by treating them with ABA at the torpedo to cotyledonary stages. Over 60 % of the ABA treated embryos survived desiccation tolerance up to 10–15 % moisture and converted into plantlets when placed on moist filter paper or sown directly in sterile soil. Moreover, the vigor of these plants was greater than that of the plantlets derived from nondesiccated embryos.

Buccheim et al. (1989) observed that the conversion of soybean somatic embryos increased from 50 to 96 % when matured in the presence of 10 % sucrose. Similarly, somatic

embryos of maize required a maturation phase in a medium with high sucrose concentration to form typical storage organ (scutellum) of this species (Emons and Kieft 1993) which accumulated starch as proglobuline characteristic of zygotic embryos.

Quite often the somatic embryos exhibit structural abnormalities that lead to poor germination. Minimization of such abnormalities should increase the conversion of somatic embryos. The quality of somatic embryos could be improved if culture conditions during somatic embryogenesis simulate the environment inside the embryo sac. Regeneration of somatic embryos is promoted under reduced humidity inside the culture vessel. In liquid medium, somatic embryos exhibit elongation of root and hypocotyls but plant regeneration does not occur. Auxin inhibitors could improve the conversion but they increase the incidence of multiple shoots. Recently, Kim et al. (2011) produced somatic embryos of *Kalopanax septemlobus* in bioreactors in which the embryos were placed on a net and were either temporarily immersed for brief intervals (TIN) or continuously immersed (CIN) in the medium. The quality of embryos, their conversion into plants (85 %), and survival of the plants in vivo were distinctly superior in the case of embryos from TIN bioreactors than those from CIN bioreactors (75 and 29 %, respectively).

7.8 Somatic Embryos *Versus* Zygotic Embryo

The early segmentation pattern of the zygote and its derivatives forms the basis of classification of embryogeny that, in turn, is employed as a taxonomic character (Bhojwani and Bhatnagar 2008). However, according to the available information, the sequence of early development of SEs does not correspond to that followed during zygotic embryogeny. However, irrespective of the early mode of development, the zygotic embryos and SEs share similar gross ontogenesis, with both typically passing through globular, scutellar, and coleoptilar stages in monocots. The SEs also accumulate seed-specific storage reserves and proteins characteristic of that species, although in lesser amounts.

Somatic embryos generally lack a suspensor and even when present it is nonfunctional (Raghavan 1976). Other abnormalities exhibited by somatic embryos are a double or triple vascular systems caused by polar transport of auxin and nondevelopment of shoot in rubber plant. Unlike the zygotic embryos, SEs, often show secondary embryogenesis and pluricotyledony and their development is asynchronous. Some of these abnormalities can be corrected by the application of low concentration of ABA.

7.9 Large Scale Production of Somatic Embryos

Somatic embryogenesis as a system holds great potential for large-scale plant propagation in automated bioreactors. This will not only be cost-effective because of low labor inputs but also amenable to synthetic seed formation.

The major constraint with large-scale somatic embryo production is the lack of proper bioreactor. The first attempt to scale-up somatic embryo production in carrot was reported by Backs-Hüsemann and Reinert (1970). However, in their experiments only a few somatic embryos were formed. Since then, different types of stirred-tank bioreactors, originally designed for microbial culture, have been tested for plant cell multiplication but without much success as the plant cells, and more so the organized structures (such as somatic embryos) suffer sheer damage in it. Airlift or bubble-column bioreactors reduce cell damage but promote undesirable foaming and callus growth.

For the production of poinsettia somatic embryos, Preil (1991) used a round bottom 2 L bioreactor, in which stirring was achieved by vibrating plates and bubble-free oxygen was delivered through stabilized silicon tubing, inserted as a spiral of 140 cm total tube length. The poinsettia plants derived from the bioreactor-raised embryos exhibited high genetic stability.

Another problem associated with scaling-up the production of embryos is the asynchronous development of somatic embryos. To achieve synchrony, Molle et al. (1993) modified the bioreactor by fitting with a stirrer and air sparger. A side tube was provided with nylon filters to collect embryos of specific stage (physical separation method; Sect. 7.4) for immediate transfer to another bioreactor or semi-sold medium for further development.

Ducos et al. (2007), at Nestlé R & D Centre Tours, France, have described a four step protocol for mass propagation of *Coffea canephora* (Robusta coffee) via somatic embryogenesis using an intermittent immersion bioreactor (Figs. 7.6, 7.7), with potential to produce 2.5 million embryos and 1 million plantlets per year (@ 45 % conversion):

Step I. Initiation of embryogenic callus. Fully expanded young leaves were cultured on semi-solid medium (1/4 strength MS mineral salts + B_5 vitamins + 5 μM BAP + 3 % sucrose and gelled with 0.8 % agar; pH 5.8) in Petri plates. The cultures were incubated in dark at 25 °C. After 3–5 months, the callus was subcultured on the same medium. After another 6–8 months yellowish friable callus was selectively subcultured on the same medium except that the agar concentration was raised to 1.5 %. The callus with globular embryos was maintained by 8-weekly subcultures on the same medium; at each subculture yellowish and friable callus tissue was used (0.2–0.4 F.W. of tissue per 40 ml of solid medium). The embryogenic callus could also be multiplied in batch cultures by transferring 0.1 g (F.W.) of friable callus to 250 ml flask containing 25 ml of liquid medium (same as above except agar). The cultures were maintained on a gyratory shaker at 110 rpm. Every 2 weeks 1 g biomass was transferred to 100 ml medium in 250 ml Erlenmeyer flask.

Step II. Production of torpedo stage embryos in liquid medium. About 100 mg of biomass (F.W.), including globular embryos, from batch cultures was transferred to 100 ml of medium (MS salts + B_5 vitamins + 3 % sucrose) in 250 ml flasks. After 2–3 weeks, the contents of 2–4 flasks were pooled in one liter flasks containing 500 ml of the culture medium. Every 2 weeks the medium was renewed. After 8–10 weeks, 95 % of the biomass comprised of torpedo stage embryos (Fig. 7.6a).

Step III. Production of green cotyledonary stage embryos. The contents of 2–5 one liter flasks with torpedo stage embryos were pooled into 500 ml of medium (1/2 strength MS salts + B_5 vitamins + 0.5 μM BAP + 2 % sucrose) and transferred to 10 L Temporary Immersion Bioreactor (TIB; Fig. 7.6d) The embryos were immersed in the medium only for 5 min every 12 h. The cultures were maintained at 24–27 °C under 30–60 μmol m^{-2} s^{-1} irradiance and 16 h photoperiod. After 2–4 weeks, when most of the torpedo stage embryos developed into green dicotyledonous embryos, the medium was replenished with fresh medium of the same composition but devoid of BAP.

Step IV. Storage of dicot embryos. The green cotyledonary embryos (Fig. 7.7b, c) were collected from the TIB after 2–3 months and, after washing with 1 g L^{-1} fertilizer solution for 5 min, spread as thin layer on commercial coconut fiber containing Osmocote fertilizer and minerals. The embryos were transferred to polystyrene boxes covered with a transparent polycarbonate sheet and stored in green house (each box contained 2 L of substrate and 5000 pre-germinated embryos) at 20–32 °C and mid day irradiation of 20–100 μmol m^{-2} s^{-1}. The coconut fibers and the embryos were sprayed with a mixture of fungicides (0.5 mg L^{-1} each of Previcur and Octave), and every 2 weeks 0.5 mg L^{-1} Rovral and Octave were sprayed alternately.

The TIB used in step III above comprised a 10 L glass jar containing the embryos resting on a polyurethane foam disk laid at the bottom of the jar. This culture jar is connected, by a silicon tube, to a 5 L jar containing the culture medium (Fig. 7.7). The medium containing jar is placed below the embryo culture jar and is connected to an air pump. By turning on the pump, twice a day for 5 min each, pressure is applied to push the medium up into the culture jar through the vent filter of the medium jar to immerse the embryos. When the pump is switched off, the

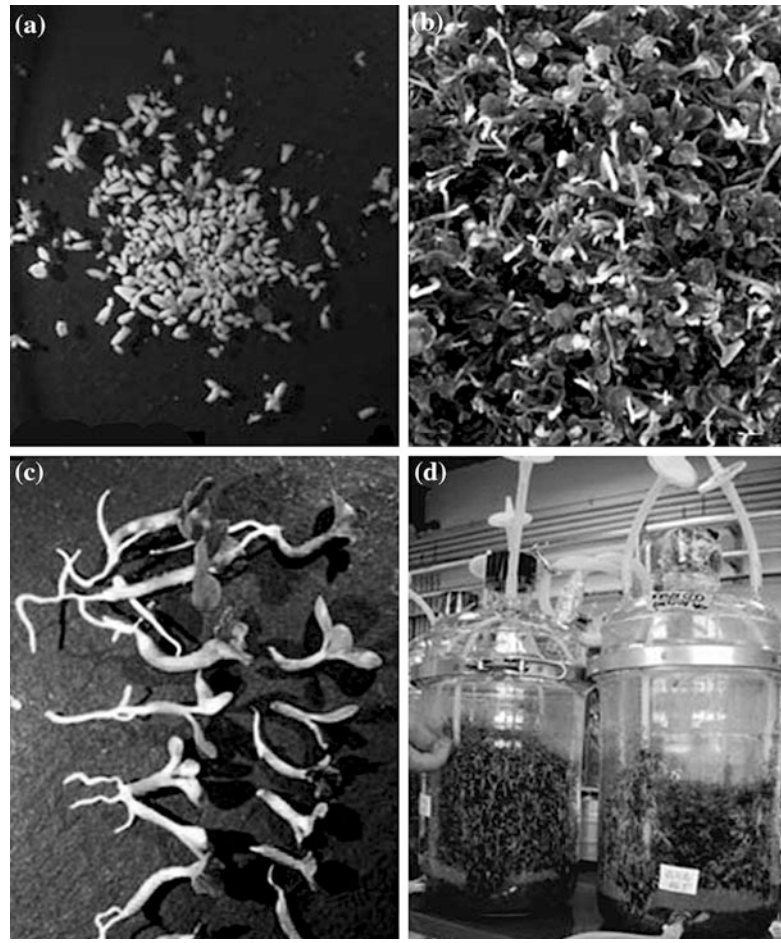

Fig. 7.6 Mass production of somatic embryos of coffee in Temporary Immersion Bioreactors (TIB). **a** Torpedo stage embryos before transfer to TIB. **b** Bulk population of cotyledonary stage embryos harvested from a TIB. **c** An enlarged view of embryos from c; some of the embryos appear germinated with a distinct root. **d** A view of 10 L glass TIB at the end of the pre-germination phase. (*courtesy* Jean-Paul Ducos Nestlé R & D Center–Tours, France)

medium flows down into the medium jar due to gravity. The foam disk isolates the embryos from the thin layer of medium which remains in the vessel. It retains about one liter of medium inside the vessel, and thus maintains sufficient relative humidity (85–90 %). During the immersion the disk acts as an air sparger and facilitates good ventilation of the headspace. The advantages of this reactor as compared to continuous immersion bioreactors are: (i) the hyperhydration of the embryos is limited due to avoidance of continuous immersion in the medium and (ii) limited shear stress due to lack of mechanical agitation or continuous aeration. Kim et al. (2011) used TIB and CIB (continuous immersion bioreactor) for mass multiplication of somatic embryos of *Kalopanax septemlobus* and found that the former was distinctly better in terms of the quality of the embryos formed, per cent conversion of embryos into plants and transplantation survival.

To reduce the cost of production, efforts are being made to develop disposable plastic bioreactors to replace the expensive stainless steel bioreactors traditionally used. The plastic bioreactors (see Fig. 4.4d) are based on the principle of a Wave/Undertow mechanism, providing convenient mixing, and aeration of the plant cell/tissue cultures (WU bioreactors). The WU bioreactors consist of a large flexible plastic container partly filled with the medium and inflated with air. It is made up of biopharmaceutical grade flexible PVC that could be sterilized by autoclaving at 121 °C for 40 min. The system is located on a horizontal table equipped on one side with a platform. Intermittent rising

Fig. 7.7 Temporary immersion bioreactors used for mass multiplication of coffee embryos. The culture vessels on the top shelf are connected with the medium reservoirs on the lower shelf. (*courtesy* Jean-Paul Ducos Nestlé R & D Center–Tours, France)

movement of platform to the rest point and descending movement back to initial position enable continuous mixing and aeration through the Wave/Undertow motion which, in turn, provides liquid culture mixing and bubble-free aeration. Being horizontal the embryos are exposed to light more uniformly. A modified version of plastic reactor, called "Box-in-Bag" bioreactor has been developed by Ducos et al. (2007, 2010) for coffee somatic embryo culture.

7.10 Synthetic Seeds

Somatic embryogenesis is expected to be the only clonal propagation system economically viable for crops currently propagated by seeds. However, like seeds, somatic embryos should also be amenable to mechanical planting. It would be desirable for practical reasons to convert them into synthetic seeds or 'synseeds'

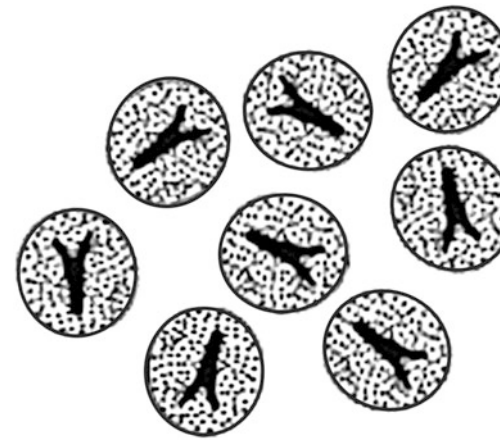

Fig. 7.8 Synthetic seeds. Individual dicotyledonous somatic embryos are encapsulated in Ca-alginate

by encapsulating in a protective covering for field planting (Pinto et al. 2008). The coating of the synseeds need to have the following qualities: (i) it must be nondamaging to the embryo, (ii) the coating material should be sufficiently durable to withstand rough handling during manufacture, storage, transportation and planting, and at the same time be mild and protective to the somatic embryo, (iii) it should be possible to incorporate nutrients, growth regulators, and other components necessary for germination, and (iv) the artificial seeds should be amenable to the existing farm machinery. The success of synthetic seed technology would also depend on the quality of somatic embryos; uniform development stage with reversible arrested growth and high rates of conversion on planting (Pinto et al. 2008).

The most popular method for encapsulation of single, hydrated somatic embryos is coating with calcium-alginate (Fig. 7.8). In practice, somatic embryos are mixed with 2 % solution of sodium alginate and dropped from a plastic pipette into 100 mM solution of calcium nitrate. An ion exchange reaction occurs to form calcium-alginate gel/capsule around the somatic embryo (Redenbaugh et al. 1991). The gel beads can be impregnated with microcapsules containing 3 % sucrose coated with a mixture of 8 % Elvax 4260 and beeswax (for slow release

of sucrose), and 0.1 % Topsin M, a fungicide (Mamiya et al. 2001).

The main advantages of synthetic/somatic/artificial seeds are: (i) easy handling and transportation, (ii) potential long-term storage, (iii) higher scale-up capacity, (iv) uniformity in production, and (v) potential for automation of the entire production process.

7.11 Practical Applications of Somatic Embryogenesis

Improvement of crop plants through manipulation at the cellular level (somatic hybridization, mutation of isolated single cells, and genetic transformation) is possible only if somatic cells are able to regenerate complete plants.

Large-scale multiplication of important plants is one practical application of cellular totipotency. Somatic embryogenesis is the more desirable pathway of regeneration of plants as the embryos are miniature seedlings with root and shoot primordia. It has been possible to induce somatic embryogenesis in majority of crop and forest plants, and considerable success has been achieved in scaling up the process. Somatic embryos can be encapsulated to obtain synthetic seeds. However, the major problem in actual application of this technique on a commercial scale is the genetic instability of cells in long-term cultures.

The study of the process governing the onset and development of the zygotic embryo is limited by its location inside the seed where it is inaccessible to experimental manipulation, particularly during the early stages of embryogenesis. In contrast, somatic embryogenesis is easily observable, its various culture conditions can be controlled and a very large number of somatic embryos can be obtained. In carrot somatic embryos at the same stage of development can be obtained in gram quantities. These characteristic features have made somatic embryogenesis a model system to study physiological, biochemical, and molecular mechanisms underlying the onset of embryogenesis and development of embryo in higher plants (Quiroz-Figueroa et al. 2006).

7.12 Concluding Remarks

Somatic embryogenesis occurs only under the controlled environment of plant tissue cultures. Since 1958, when it was reported for the first time in carrot, the list of species in which this phenomenon has been observed has considerably enlarged. The most potent explant for embryogenic cells is the zygotic embryos, especially at the immature stage. However, by manipulation of culture conditions somatic embryogenesis could be induced from almost all somatic tissues (root, stem, leaf, shoot tip, floral primordial, and pericarp). Some plants that are capable of forming embryos from any part of the plant body are carrot, alfalfa, and buttercup.

Somatic embryogenesis in liquid medium is a powerful alternative to other techniques of clonal propagation of selected varieties of plants. Considerable success has been achieved to scale up the production of somatic embryos using 1–10 L bioreactors. Pilot process for mass propagation of selected clones of *Coffea canephora* (Robusta coffee) via somatic embryogenesis has been developed (Ducos et al. 2007, 2008, 2010).

Somatic embryogenesis offers a model system for understanding the physiological, biochemical, and molecular events occurring during plant embryo development and in transition of a somatic cell into an embryogenic cell. Analysis of proteome and transcriptome has led to the identification and characterization of certain genes involved in somatic embryogenesis. However, most of these genes are up regulated only in the late developmental stages. Better understanding of the key factors that promote vegetative-to-embryogenic transition and identification of genes involved in the induction of competence for embryogenesis in somatic cells and subsequent embryo development should simplify the procedure to induce somatic embryogenesis in different plants (Karmani et al. 2009).

7.13 Appendix

1. Protocol for inducing somatic embryogenesis in *Daucus carota* (*after* Smith and Street 1974).
   (i) Surface sterilize seeds in 10 % calcium hypochlorite for 15 min and, after washing three times in sterile distilled water, germinate them on sterilized moistened filter paper in a Petri dish, in dark at 25 °C.
   (ii) Cut 1 cm long segments of roots from 7-day-old seedlings and culture them individually on a semi-solid medium containing the inorganic salts of Murashige and Skoog's medium, organic constituents of White's medium 100 mg L^{-1} *myo*-inositol, 0.2 mg L^{-1} Kinetin, 0.1 mg L^{-1} 2,4-D, 2 % sucrose, and 1 % agar (Difco bacto agar or of any other brand). Incubate the cultures in dark.
   (iii) After 6–8 weeks, transfer pieces of root calli (0.2 g fresh weight) to fresh medium of the original composition and maintain the cultures in light at 25 °C. The tissue may be multiplied by subculturing every 4 weeks in a similar manner.
   (iv) After the first passage initiate suspension cultures by transferring ca. 0.2 g of callus tissue to a 200 ml Erlenmeyer flask containing 20–25 ml of liquid medium of the same composition as used for callus growth (without agar). Incubate the flasks on a horizontal rotary shaker at 100 rpm in light at 25 °C.
   (v) Subculture the suspension every 4 weeks by transferring 5 ml of it to 65 ml of fresh medium (1:13).
   (vi) To induce embryo development, transfer callus pieces or portions of suspension to 2,4-D-free medium of otherwise the same composition as used before.
   (vii) After 3–4 weeks the cultures contain numerous embryos at different stages of development.
2. Protocol for inducing somatic embryogenesis in *Citrus* sp. (*after* Tisserat and Murashige, 1977).
   (i) Take a 6–8-weeks-old fruitlet of a local cultivar and surface sterilize it with 1 % sodium hypochlorite for 15–20 min. Follow the subsequent steps under aseptic conditions.
   (ii) Bisect the fruit and transfer the ovules to a sterile Petri dish.
   (iii) Excise nucellus tissue from the ovules using a dissecting microscope. Hold the chalazal region of the ovules with pointed forceps. Give a shallow incision longitudinally through the integuments. Cut the ovule transversely into two halves. From the micropylar half, remove the integuments, endosperm, and any embryo (especially zygotic), if present, transfer the nucellar section to the culture vessel in a manner that its cut end is in contact with the medium.
   (iv) Use semi-solid medium containing inorganic salts of Murashige and Skoog's medium, 100 mg L^{-1} *myo*-inositol, 0.2 mg L^{-1} thiamine-HCl, 1 mg L^{-1} pyridoxine–HCl, 1 mg L^{-1} nicotinic acid, 4 mg L^{-1} glycine, 500 mg L^{-1} malt extract, 5 % sucrose, and 1 % Difco bacto agar.
   (v) Incubate the cultures in light (1000 lux) at 27 ± 1 °C.
   (vi) Within 4–6 weeks multiple embryos should develop from the callused nucellar tissue. To stimulate full plant development, transfer the embryos to another medium which differs from the previous medium in having 1 mg L^{-1} GA_3 in place of malt extract.

Suggested Further Reading

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Androgenesis

8

8.1 Introduction

Haploid plants are characterized genetically by the presence of gametic number of chromosomes in their cells (i.e., half the number of chromosomes as in somatic cells). In nature, haploids arise as an abnormality when the egg or a synergid cell develops into an embryo without fertilization (Bhojwani and Bhatnagar 2008). The natural haploids of *Datura stramonium* were first discovered in 1922 by Blakeslee et al. Since then spontaneous occurrence of haploids has been reported in about 100 species (Vasil 1997). Haploids are sexually sterile and require doubling of chromosomes to produce fertile doubled haploids (DH) or homozygous diploids. Haploids and doubled haploids are of considerable importance in genetics and plant breeding programs. However, for long, their full potential could not be exploited because of the lack of a reliable and reproducible method to produce haploids under field conditions. Therefore, the first report of haploid pollen embryo and plant formation in anther cultures of *Datura innoxia*, by Guha and Maheshwari (1964, 1966), generated considerable interest among the geneticists and plant breeders for the production of androgenic haploids (haploids of pollen origin) and their utilization in crop improvement. Since then, the technique of anther culture has been considerably refined and successfully applied to over 200 species, including several major crop plants (Bhojwani and Razdan 1996). The technique is being used routinely in crop improvement programs and has aided the development of several improved varieties (Hu and Guo 1999; Maluszynski et al. 2003; Palmer et al. 2005). Protocols have also been developed for androgenesis in isolated microspore/pollen culture of several species. The other approaches being applied to produce haploids of angiosperms are gynogenesis (formation of sporophytes by haploid cells of the female gametophyte without fertilization) and Distant Hybridization followed by immature Embryo Culture. However, androgenesis is the most popular method for the production of haploids because of the occurrence of a large number of haploid microspores per anther.

This chapter deals with in vitro androgenesis. The next chapter is devoted to gynogenesis, and the technique of haploid production by Distant Hybridization is described in Chap. 11.

8.2 Androgenesis

8.2.1 Techniques

Androgenesis refers to the development of plants (sporophytes) from microspores or immature pollen (male gametophyte). Two techniques are used to produce androgenic haploids, viz. anther culture and isolated pollen culture (Bhojwani and Dantu 2010; Bhojwani et al. 1999). Efficient androgenesis is usually induced by the application of a stress treatment (starvation, temperature shock, osmotic stress, colchicine, n-butanol treatment etc.) to whole plants in vivo, tillers, buds, or isolated anthers or pollen in vitro.

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_8, © Springer India 2013

8.2.1.1 Anther Culture

It is a technically simple and efficient method, requiring minimum facilities. Flower buds, with pollen grains at the most responsive stage are surface sterilized and the anthers, excised under aseptic conditions, cultured on semi-solid or in liquid medium. In some cases, where the flower buds are small, whole buds or inflorescences enclosing the anthers at the appropriate stage of pollen development are cultured. The cultures are exposed to a suitable stress treatment before incubation under normal culture conditions in dark. In some cases, the stress treatment is applied to the donor plants or spikes before excising the anthers and isolation of pollen grains for culture. Depending on the plant species, and to some extent the culture medium, the androgenic pollen either develop directly into embryos (*Nicotiana* sp., *Brassica* sp.; Figs. 8.1a, 8.2a, b) or proliferate to form callus tissue (rice, wheat; Fig. 8.1b). After 2–3 weeks, when pollen embryos or calli become visible, the cultures are transferred to light for their further development and organogenic/embryogenic differentiation. The shoots regenerated from callus often require transfer to another medium for rooting to form complete plants.

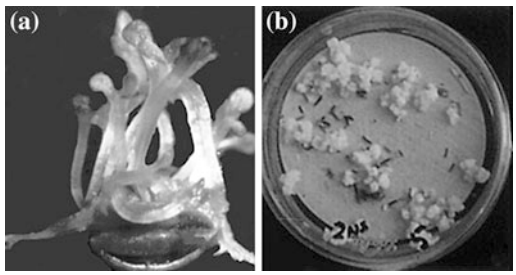

Fig. 8.1 Androgenesis in anther cultures of tobacco (**a**) and rice (**b**), showing direct regeneration of pollen plants and pollen callusing, respectively

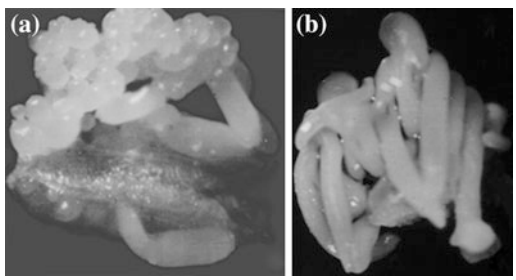

Fig. 8.2 Pollen embryogenesis in anther cultures of *Brassica juncea*. **a** A burst anther has liberated a large number of globular embryos and some older embryos. **b** A cluster of dicotyledonous pollen embryos released from a burst anther

8.2.1.2 Pollen/Microspore Culture

It is now possible to achieve androgenesis in the cultures of mechanically isolated pollen of several plants, including tobacco, *Brassica* species, and some cereals (Fig. 8.3). Besides the culture medium and pretreatment, the plating density (number of pollen grains per unit volume of medium) is a critical factor for the induction of androgenesis in pollen cultures. Generally, 1×10^4 pollen per ml of the medium is satisfactory. In most of the cereals, pollen culture involves pre-culture of the anthers for a few days, or co-culture of pollen with a nurse tissue, such as young ovaries of the same or a related plant (Datta 2001). Treatment of pollen-derived embryos and pollen-derived calli to regenerate complete plants is the same as in anther culture.

The nutritional requirements of isolated pollen in culture are more complex than those of cultured anthers. However, unlike the earlier belief, pollen culture is less tedious and time consuming than anther culture (Fig. 8.4). The additional advantages of pollen culture over anther culture for haploid plant production are as follows:

1. A homogeneous preparation of pollen at the developmental stage most suitable for androgenesis can be obtained by gradient centrifugation.
2. Isolated pollen can be genetically modified by mutagenesis or genetic engineering before culture, and a new genotype can be selected at an early stage of development.
3. Pollen culture considerably improves the efficiency of androgenesis. In rapid cycling *Brassica napus,* the culture of isolated pollen was 60 times more efficient than anther culture in terms of embryo production.

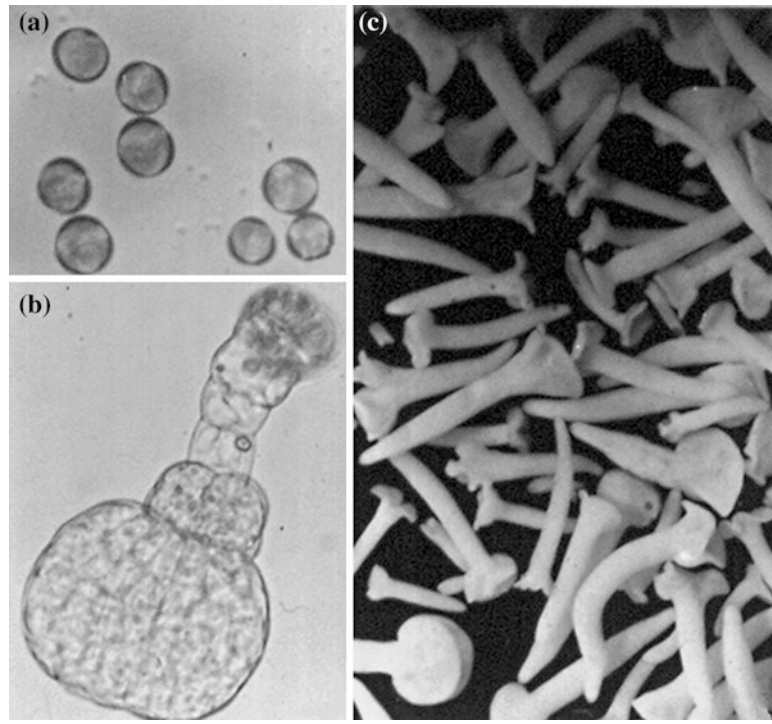

Fig. 8.3 Isolated pollen culture of *Brassica napus*. **a** Isolated pollen at the time of culture. **b** A young pollen embryo with prominent suspensor, from a 7-day-old culture. **c** Mature dicotyledonous embryos, from a 21-day-old culture (*courtesy*, Prof. L. Kott, Canada)

4. The exogenous treatments can be more effectively applied and their precise role in androgenesis studied as the unknown effect of the anther wall is eliminated.
5. The culture of isolated pollen culture provides an excellent system to study cellular and subcellular changes underlying the switch from gametophytic to sporophytic development and the induction of embryogenesis in isolated haploid single cells.

Table 8.1 gives the compositions of some of the media especially developed for anther/pollen culture.

8.3 Factors Effecting In Vitro Androgenesis

There is considerable variation in the requirements for the optimum androgenic response of different species of a genus or even different genotypes of a species. In practice, it has been observed that two batches of cultures of the same genotype often exhibit considerable variation in their response, probably because of change in the physiology and the growth conditions of the donor plants. Therefore, it is advisable to manipulate the published protocols when dealing with a new system to optimize the response. Some of the factors that have a profound effect on the fate of pollen in culture are the genotype and the physiological state of the donor plants, the stage of pollen development at the time of culture, pretreatments, and the culture medium.

8.3.1 Genetic Potential

The androgenic response is influenced considerably by the plant genotype. The observed interspecific and intraspecific variation is often so great that while some lines of a species are highly responsive, others are extremely poor performers or completely nonresponsive.

In general, the indica cultivars exhibit poor response as compared to the japonica cultivars of rice (Miah et al. 1985; Cho and Zapata 1990; Raina 1997). Similarly, among the crop Brassicas, substantial inter- and intraspecific variation has been reported for androgenesis (Duijs et al.

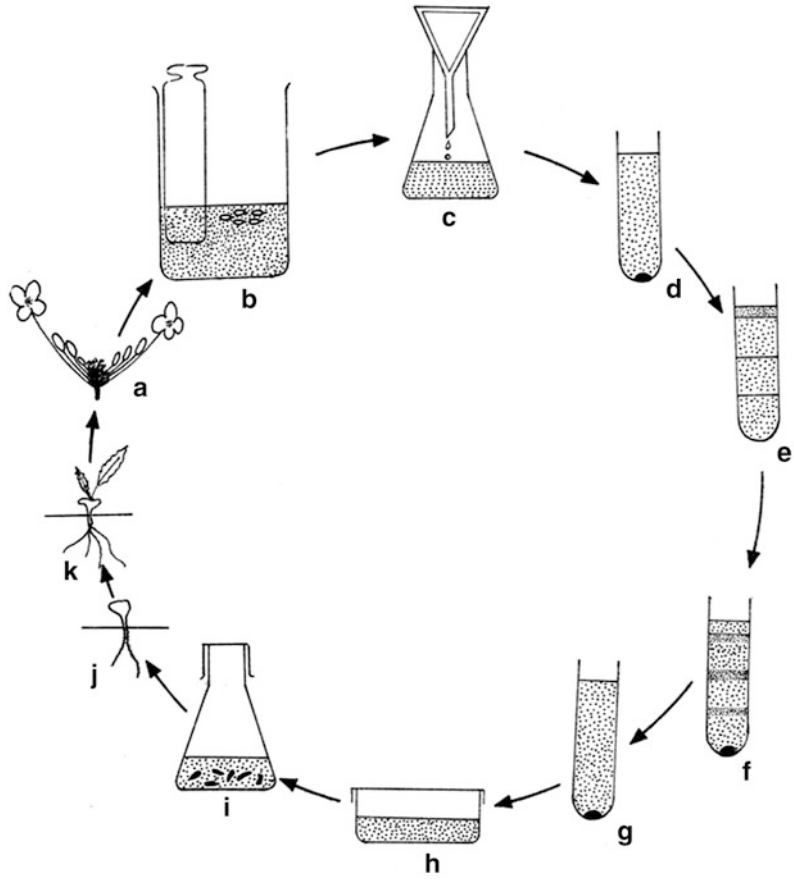

Fig. 8.4 Summary diagram of a protocol for isolated pollen culture of *Brassica napus*. The surface sterilized buds (**a**) of suitable size are crushed to release the pollen grains in B_5 medium containing 13 % sucrose (B_5-13) in a glass homogenizer (**b**) and the medium is filtered through 42 μm nylon mesh to remove the large debris (**c**). The filtrate is centrifuged at 1,000 rpm for 3 min (**d**) and after discarding the supernatant the pellet is resuspended in B_5-13 medium and gently loaded on 24/32/40 % Percoll gradient solution (**e**) and centrifuged at 1,000 rpm for 5 min. The two upper layers (**f**) are pipetted out and mixed with B_5-13 medium. The suspension is again centrifuged at 1,000 rpm for 5 min (**g**) and the supernatant medium is pipetted out and the pollen grains are suspended in NLN medium adjusting the plating density of the pollen grains to 2–5 × 10^4 ml^{-1}. The suspension is plated as thin layer in Petri plates (**h**) and incubated in the dark at 32 °C for 3–5 days and then at 25 °C. The regenerated tissue/embryos are transferred to 18 ml of hormone-free NLN medium in conical flasks (**i**) and maintained on a shaking machine at 60 rpm at 32 °C. Finally, the mature embryos are transferred to solidified B_5 medium containing 2 % sucrose for germination (**j**, **k**). Fertile plants can be obtained by diploidization of pollen plants (*after* Ohkawa 1988)

1992; Chanana et al. 2005). *Brassica napus* is more responsive than *B. juncea*. Optimum culture conditions may also vary with the genotype. For example, the optimum concentration of ammonium nitrogen for indica rice is almost half as that for japonica rice.

In *Melandrium album*, which shows a chromosomal basis of sex determination, only the pollen with X chromosome is competent to form pollen plants. Consequently, the pollen plants are phenotypically and cytologically female (Wu et al. 1990). In tetraploid *Melandrium*, even a single Y chromosome is able to suppress the effect of X chromosomes.

Since plant regeneration from pollen is a heritable trait, it has been possible to improve the androgenic response of poor performers by crossing them with highly androgenic genotypes

Table 8.1 Composition of some of the media used for androgenic haploid production (concentrations in mg L^{-1})

Constituents	MS[a]	B_5[b]	N&N[c]	AT3[d]	KA[e]	NLN-13[f]	N_6[g]	M-019[h]
KNO_3	1,900	2,527.5	950	1,950	2,500	125	2,830	3,101
NH_4NO_3	1,650	–	725	–	–	–	–	–
$NaH_2PO_4.2H_2O$	–	150	–	–	150	–	–	–
KH_2PO_4	170	–	68	400	–	125	400	540
$(NH_4)_2SO_4$	–	134	–	277	134	–	463	264
$MgSO_4.7H_2O$	370	246.5	185	185	250	125	185	370
$CaCl_2.2H_2O$	440	150	166	166	750	–	166	440
$Ca(NO_3)_2.4H_2O$	–	–	–	–	–	500	–	–
$FeSO_4.7H_2O$	27.8	–	27.8	–	–	27.8	27.8	27.85
$Na_2.EDTA.2H_2O$	37.3	–	37.3	–	–	37.3	37.3	37.25
Sequestrene 330Fe	–	28	–	–	40	–	–	–
Fe-EDTA	–	–	–	37.6	–	–	–	–
KI	0.83	0.75	–	0.83	0.75	–	0.8	0.83
H_3BO_3	6.2	3	10	6.2	3	10	1.6	6.2
$MnSO_4.H_2O$	–	10	–	–	–	25	4.4	–
$MnSO_4.4H_2O$	22.3	–	25	22.3	10	–	–	22.3
$ZnSO_4.7H_2O$	8.6	2	10	8.6	2	10	1.5	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	–	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.25	0.025	–	0.025
$CoCl_2.6H_2O$	0.025	0.025		0.025	0.025	0.025		
Myo-inositol	100	100	100	100	100	100		100
Thiamine HCl	0.1	10	0.5	10	10	0.5	1	0.5
Pyridoxine HCl	0.5	1	0.5	1	1	0.5	0.5	2.5
Nicotinic acid	0.5	1	5	1	1	0.5	0.5	2.5
Glycine	2	–	2	–	–	2	2	2
L-Glutamine	–	–	–	1,256	800	800	–	–
Glutathione	–	–	–	–	–	30	–	–
L-Serine	–	–	–	–	100	100	–	–
Folic acid	–	–	5	–	–	0.5	–	–
Biotin	–	–	0.5	–	–	0.5	–	–
NAA	–	–	–	–	0.1	0.5	–	2.5
2,4-D	–	–	–	–	0.1	–	2	0.5
BAP	–	–	–	–	–	0.05	–	–
Kinetin	–	–	–	–	–	–	0.5	0.5
MES[i]	–	–	–	1,950	–	–	–	–
Sucrose	30,000	20,000	20,000	–	10,0000	1,30,000	50,000–1,20,000	–
Maltose	–	–	–	90,000	–	–	–	90,000
Agar	8,000	8,000	8,000	–	8,000	–	8,000	–

[a] MS medium; [b] B_5 medium; [c] for anther culture of tobacco; [d] for isolated microspore culture of tobacco (the medium is filter sterilized); [e] for anther culture of *Brassica*; [f] for isolated microspore culture of *Brassica*; [g] for rice anther culture; [h] for microspore culture of indica rice (for japonica rice NAA is omitted and the concentration of 2,4-D is raised to 2 mg L^{-1}); [i] 2-(N-morpholino) ethanesulfonic acid

(Rudolf et al. 1999; Cloutier and Landry 1995; Foroughi-Wehr et al. 1982; Petolino et al. 1988; Barloy et al. 1989). Efforts are also being made to identify the genes associated with androgenesis (Agasche et al. 1989; Cowen et al. 1992; Wu et al. 1992; Devaux and Zivy 1994).

In *Brassica napus*, most of the work has been on cv. Topas line DH4079 in which >10 % of the microspores subjected to high temperature shock form embryos. With the same protocol *B. napus* cv. Westar is completely nonandrogenic. However, it formed a few embryos when 24-epibrassinolide (1×10^{-6} M) was added to the culture medium. The dihaploid plants developed from these embryos proved heritable, stable androgenic lines (DH1–DH4). One of these lines (DH2) produced up to 30 % the number of embryos produced by the cv. Topas in the absence of brassinosteroid (Malik et al. 2008).

8.3.2 Physiological Status of the Donor Plants

The environmental conditions and the age of the plant, which affect the physiology of the plants, also affect their androgenic response. Generally, the first flush of flowers yields more responsive anthers than those borne later. However, in *B. napus* (Takahata et al. 1991) and *B. rapa* (Burnett et al. 1992) pollen from older, sickly looking plants yielded a greater number of embryos than those from young and healthy plants. Similarly, the late sown plants of *B. juncea* yielded more androgenic anthers than the plants sown at the normal time (Agarwal and Bhojwani 1993). Application of ethrel, a feminizing hormone (Wang et al. 1974; Agarwal and Bhojwani 1993), or fernidazon potassium, a gametocidal agent (Picard et al. 1987), to the donor plants enhanced the androgenic response.

8.3.3 Stage of Pollen Development

The competence of pollen to respond to various external treatments depends on the stage of their development at the time of culture. Generally, the labile stage of pollen for androgenesis is just before (late uninucleate stage), at, and immediately after the first pollen mitosis (early binucleate). During this phase, the fate of the pollen is uncommitted because the cytoplasm is cleaned of the sporophyte-specific information during meiosis, and the gametophyte-specific information has not been transcribed by this time. Once the vegetative cell starts accumulating starch grains the pollen loses the capability to undergo androgenesis (Maraschin et al. 2005a). However, it is important to appreciate that the most vulnerable stage of pollen for responding to exogenous treatments may vary with the system. Late uninucleate pollen is most suitable for androgenesis in rice (Raghavan 1990), and for most crop Brassicas (Dunwell et al. 1985; Sharma and Bhojwani 1985). The pollen of *Atropa belladonna* and *Nicotiana sylvestris* are most responsive at the early binucleate stage.

Binarova et al. (1997) reported that pollen of *B. napus* isolated at the late 2-celled stage can also be induced to form embryo by the application of an additional short but more severe heat shock (1–2 h at 41 °C) before incubating at 31 °C.

The stage of pollen at culture may also affect the ploidy level of the pollen plants. In *Datura innoxia* (Engvild et al. 1972) and *Petunia* sp. (Engvild 1973; Raquin and Pilet 1972), whereas the unicellular pollen produced mainly haploid plants, the bicellular pollen formed plants with higher ploidy.

8.3.4 Pretreatments

Application of a variety of stresses, such as temperature shock, osmotic stress, and sugar starvation, at the initial stage of anther or pollen culture has proved promontory or essential for the induction of androgenesis. However, the type, duration and the time of application of these pretreatments may vary with the species or even the variety (Datta 2001).

Of the various treatments, the application of a temperature shock has been most common.

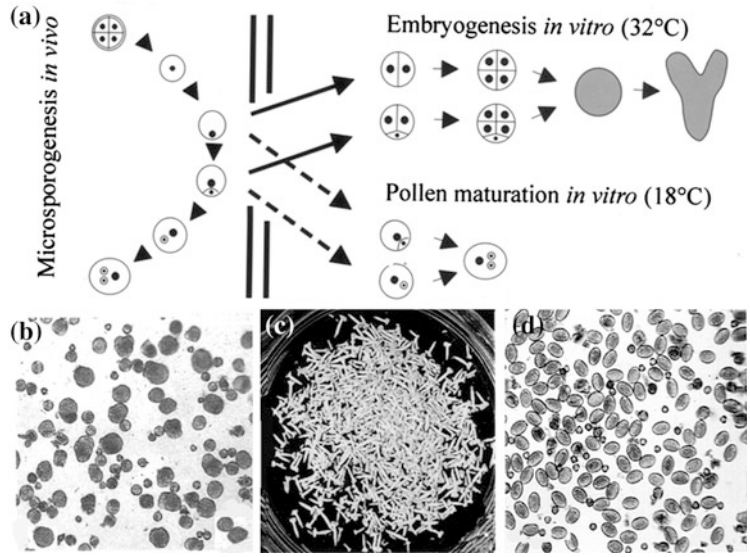

Fig. 8.5 Embryogenesis in microspore culture of *Brassica napus*. **a** Diagram, showing in vivo microgametogenesis from tetrad to mature 3-celled pollen. The pollen at late unicellular and early bicellular stages is competent to form embryos. At 18 °C the pollen continues normal gametogenesis, whereas at 32 °C they exhibit embryogenesis. **b**, **c** Preglobular and torpedo-shaped embryos after 2 days of culture at 32 °C followed by 4 and 14 days at 25 °C, respectively. **d** Normal pollen after 6 days of culture at 18 °C (*after* Custers et al. 2001)

In many species, incubation of anther/pollen cultures at low temperature (4–13 °C) for varying periods before incubation at 25 °C enhanced the androgenic response. In rice, excised panicles of rice are cold treated (10 °C) for 10 days before excising the anthers for culture (Datta 2001; Ogawa et al. 1992; Raina and Irfan 1998; Pande and Bhojwani 1999). The duration of cold treatment is critical to obtain high frequency green plants of pollen origin. For indica rice, cold treatment at 10 °C is essential for the induction of androgenesis, but the treatment for longer than 11 days, although increased the androgenic response, adversely affected the frequency of production of green plants (Pande 1997; Gupta and Borthakur 1987).

In some other plants, such as *Capsicum* (Dumas de et al. 1982; Rines 1983) and some genotypes of wheat (Li et al. 1988), an initial high temperature shock has proved essential or beneficial for androgenesis. A heat shock of 30–35 °C for 2 h to 4 days is a prerequisite for inducing pollen embryogenesis in most of the *Brassica* species (Custers et al. 2001; Fig. 8.5). However, the optimum requirement of high temperature pretreatment varies with different species. The time lapsed between isolation of pollen and high temperature treatment can radically affect embryo induction. For example, embryogenesis was completely inhibited when the pollen of *B. napus* was held for 24 h at 25 °C before the application of heat shock application (Pechan et al. 1991).

To induce androgenesis in ab initio pollen cultures of barley (Wei et al. 1986) and indica rice (Raina and Irfan 1998) an application of 0.3 or 0.4 M mannitol stress for 4–7 days was found to be essential. Initial starvation of developing pollen of important nutrients, such as sucrose (Aruga et al. 1985; Wei et al. 1986) and glutamine (Kyo and Harada 1985, 1986) favored androgenesis in tobacco and barley, respectively.

The other types of stresses applied within the responsive developmental window of pollen grains to induce or promote androgenesis are γ-irradiation (Wang and Yu 1984; McDonald et al. 1988; Ling et al. 1991), centrifugation (Sangwan-Norreel 1977; Aslam et al. 1990), and chemicals such as colchicine (Zaki and

Dickinson 1990,1995; Obert and Barnabás Obert and Barnabás 2004; Soriano et al. 2007) brassinosteroids (Malik et al. 2008) and n-butanol (Soriano et al. 2008).

8.3.5 Culture Medium

Most of the species require a complete plant tissue culture medium with some growth regulators for in vitro androgenesis. The most widely used media for this purpose are MS (Murashige and Skoog 1962), N&N (Nitsch and Nitsch 1969) and their modifications. In general, the requirements of isolated pollen cultures are more elaborate than that of anther cultures. Some of the media developed for anther and pollen culture of tobacco, *Brassica* and rice are listed in Table 8.1. Apparently, all the media specifically developed for androgenic haploid production are low salt media as compared to the MS medium, which is most commonly used in plant tissue culture studies.

Anther cultures of many cereals are very sensitive to inorganic nitrogen, particularly in the form of NH_4^+. Based on this observation, Chu et al. (1975) developed N_6 medium, which is extensively used for cereals. Anther and pollen cultures of indica cultivars of rice are even more sensitive to the concentration of NH_4^+ in the culture medium. Lower ammonium concentration in the medium also improved the yield of green plants in *Lolium perenne* (Olesen et al. 1988).

Sucrose is an essential constituent of media for androgenesis and it is mostly used at 2–4 % (w/v). However, some plants require higher concentration of sucrose to exhibit an optimum response. For potato (Sopory et al. 1978) and some cultivars of wheat (Ouyang et al. 1973) sucrose at 6 % (w/v) was distinctly superior than at 2 % (w/v). However, anther and pollen cultures of all crop *Brassica* species require 12–13 % sucrose for androgenesis.

For several cereals, maltose has proved superior to sucrose as the carbon source (Raina 1997; Pande 1997; Datta and Schmid 1996; Xie et al. 1995; Letini et al. 1995; Last and Brettell 1990). Substitution of sucrose by maltose in the medium in ab initio pollen cultures of wheat allowed genotype-independent plant regeneration (Mejza et al. 1993) and promoted direct pollen embryogenesis (Navarro-Alvarez et al. 1994).

Regeneration of androgenic plants may occur directly via pollen embryogenesis or via callus production followed by organogenesis or embryogenesis. In the latter case, androgenesis is a two-step process, each requiring different media and culture conditions.

8.4 Origin of Androgenic Plants

In the normal course of pollen development, the uninucleate microspores undergo asymmetric division, cutting a small generative cell and a large vegetative cell. The former is initially attached to the inner pollen wall (intine) but eventually comes to lie freely in the cytoplasm of the vegetative cell. Whereas the generative cell divides further, forming two sperms, the vegetative nucleus remains arrested in the G_1 phase of the cell cycle (Zarsky et al. 1992; Bhojwani et al. 2008). Thus, in situ development of pollen is programmed for terminal differentiation into highly specialized gametophytic cells. Manipulation at an early stage of pollen development can alter this highly controlled developmental program. Induction of androgenesis, thus, involves masking of the existing gametophytic program and acquisition of competence for sporophytic development via direct embryogenesis or through a callus phase.

The process of androgenesis can be divided into three overlapping phases (Maraschin et al. 2005a; Fig. 8.6):

(i) *Induction.* Repression of gametophytic developmental program and dedifferentiation of the cell to acquire potential to form sporophyte by a stress treatment.

(ii) *Early segmentation of microspores*. Formation of multicellular structure (MCS) within the pollen wall.

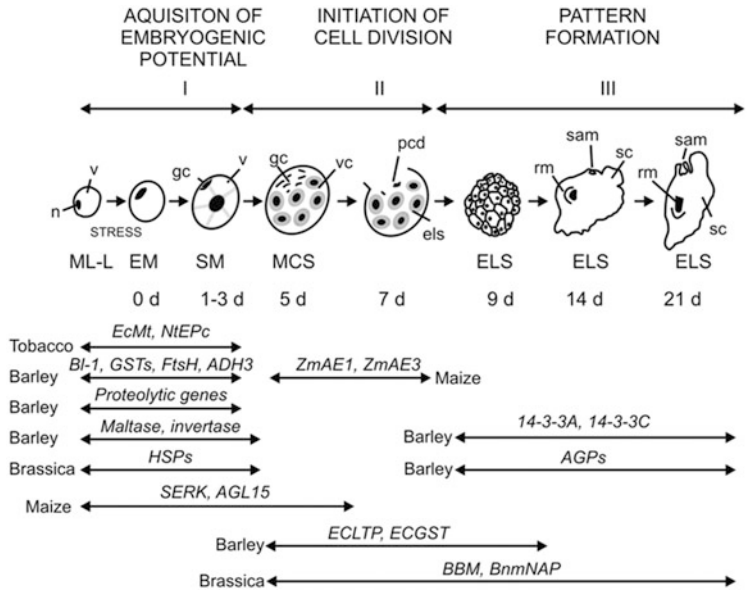

Fig. 8.6 Cellular and molecular aspects of androgenesis. Time line of in vitro androgenic development in the model species of barley illustrating the three different phases of embryogenic development is seen at the top, and the gene expression programs associated with each phase, based on gene expression data from barley and other androgenic model species, are displayed underneath the time-line (*ELS* Embryo-like structure, *EM* enlarged microspore, *gc* generative cell, *MCS* multicellular structure, *ML-L* mid-late to late uninucleate microspore, *n* nucleus, *pcd* programmed cell death, *rm* root meristem, *sam* shoot apical meristem, *sc* scutellum, *SM* star-like microspore, *v* vacuole, *vc* vegetative cell) (*after* Maraschin et al. 2005a)

(iii) *Regeneration of plants*. Release of the MCS by the bursting of the pollen wall and its development directly into an embryo or to form a callus, which later differentiates plants via organogenesis or embryogenesis.

8.4.1 Induction

Due to high regeneration efficiency in microspore cultures, rapeseed (*Brassica napus*), tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) are regarded as model systems to identify the genes involved in the induction of androgenesis and for the development of molecular markers (Maraschin et al. 2005a).

Generally, the androgenic plants arise from the mid to late-unicellular microspores to young bicellular pollen. During this phase, the pollen is noncommittal in their developmental potential. Most of the sporophyte-specific gene products are eliminated from the cytoplasm during meiosis (Porter et al. 1984) and the gametophyte-specific gene products are generally transcribed after pollen mitosis (Scott et al. 1991). During this labile developmental period a variety of stress treatments, which may vary with the plant, can mask the gametophytic program and induce the expression of sporophyte-specific genes, and thus induce the grains to switchover from gametophytic mode of development to sporophytic mode of development.

Starvation of freshly cultured anthers of tobacco for 6 days caused a loss of the ability of the generative cell to synthesize DNA and triggered the vegetative cell nucleus to synthesize DNA (Aruga et al. 1985). In this case, androgenic plants arise from the vegetative cell, which normally remains suspended in G_1 phase of the cell cycle.

The induction of androgenesis by stress involves swelling of the microspores, fragmentation of the large vacuole, and structural

reorganization of the cytoplasm, which result in the appearance of organelles-free region. The existing transcriptional and translational profiles are erased to block the gametophytic development. In barley, the induction of androgenesis is accompanied by the appearance of a thin intine, in addition to the above changes (Maraschin et al. 2005a).

Another important cytological change induced by the stress treatments before the sporophytic division is the rearrangement of the cytoskeleton, particularly microtubules and actin filaments, which is responsible for displacing the asymmetrically placed nucleus to the center of the cell. Disruption and rearrangement of microtubules and actin filaments by treatment with colchicine (Soriano et al. 2007; Herrera et al. 2002; Zhou et al. 2002) and n-butanol (Soriano et al. 2008) is known to enhance pollen embryogenesis in many plants. Centralization of the nucleus is accompanied by fragmentation of the large vacuole into small vacuoles interspersed by radially oriented cytoplasmic strands giving the cell a star-like appearance (Fig. 8.6). The star-like morphology in many plants (barley, rapeseed, tobacco, and wheat) is correlated with the acquisition of androgenic competence (Maraschin et al. 2005a). This stage is followed by sporophytic division. However, in barley, under inductive conditions 47 % of the microspores enlarged, acquired star-shaped morphology but only 7 % formed embryos capable of germination. A large majority of the multicellular pollen did not burst open to liberate the tissue mass for further development into embryo, suggesting a dual control of androgenesis; one responsible for the formation of multicellular structure and the other responsible for pattern formation (Maraschin et al. 2005b).

Induction of pollen embryogenesis in *B. napus* (Cordewener et al. 1994, 1995) and tobacco (Zarski et al. 1995) by high temperature and starvation, respectively, was accompanied by the appearance of new heat shock proteins or enhancement of their transcriptional levels. However, Zhao et al. (2003) observed that induction of pollen embryogenesis in *B. napus* by colchicine was without the appearance of HSP19 or the elevation of HSP70/HSC70. According to these authors, reorganization of the cytoskeleton during colchicine and high temperature shock treatment is more critical during induction of microspores to form embryos in *B. napus* cv. Topas. Cytoskeleton reorganization in plants is also disrupted by low temperature and n-butanol treatments, which stimulated androgenesis in many plants.

Molecular and biochemical analyses of changes during the stress treatment to induce androgenesis in barley, tobacco, and rapeseed have identified a number of genes that can be associated with the acquisition of embryogenic competence (Fig. 8.6). They are related to sucrose-starch metabolism, stress response, proteolysis, and cytoprotection but their precise role in the induction process remains to be determined.

8.4.2 Early Segmentation of Microspores

By the end of the induction period and before the first androgenic division, the pollen acquires a thick somatic wall around the plasma membrane, giving it a sporophytic characteristic (Sangwan-Norreel 1978; Rashid et al. 1982; Zaki and Dickinson 1990). Based on the first few divisions in the microspores, five modes of in vitro androgenesis have been identified (Fig. 8.7):

Pathway I. As commonly observed in *Brassica napus* (Zaki and Dickinson 1992), the microspores divide by an equal division, and both the daughter cells contribute to sporophyte development. This pathway also occurs in wheat (Indrianto et al. 2001).

Pathway II. The uninucleate microspores divide by the characteristic unequal division, and the sporophytes arise by further divisions in the vegetative cells. This mode of development is commonly encountered in *Capsicum annuum* (Kim et al. 2004), *Nicotiana tabacum* (Touraev et al. 1996), *Hordeum vulgare, Triticum aestivum* (Rybczynski et al. 1991), and *Zea mays* (Pescitelli and Petolino 1988).

Pathway III. In *Hyoscyamus niger*, the pollen embryos are predominantly formed from the

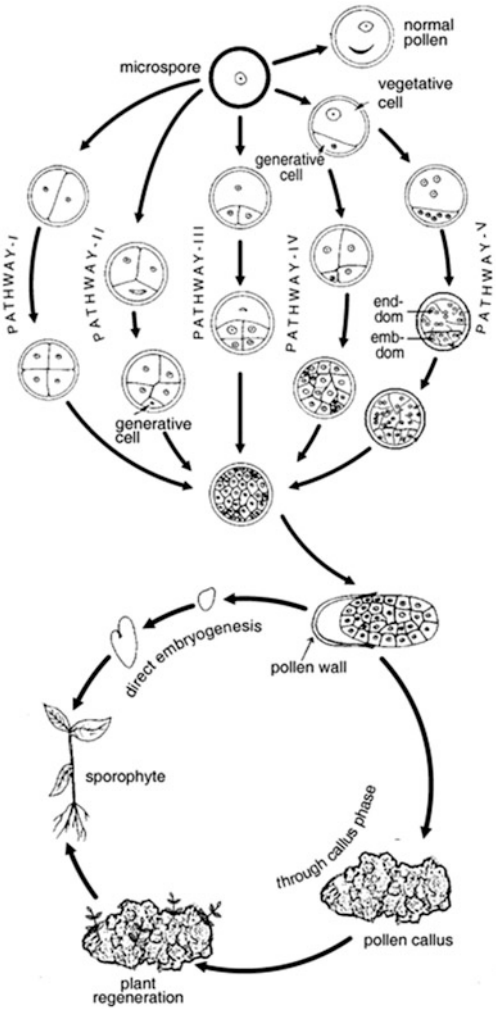

Fig. 8.7 Diagrammatized summary of the origin of sporophyte from pollen grains. A microspore may follow anyone of the five pathways to form a multicellular tissue within the confines of its original wall. Upon bursting of the pollen the multicellular tissue is released, which may directly form an embryo or produce sporophytes via the callus phase

generative cell alone; the vegetative cell either does not divide at all or does so only to a limited extent (Raghavan 1976, 1978).

Pathway IV. As in pathways (II) and (III), vegetative and generative cells are formed normally and both the cells participate in the formation of sporophytes (e.g., *Datura innoxia*).

Pathway V. The two nuclei formed after symmetrical or asymmetrical division of the microspores may fuse to form 2n nucleus, which continues to divide and the microspores becomes multinucleate and eventually multicellular by the formation of walls resulting in diploid plants via embryogenesis. This is common in cereals such as barley, maize, and wheat (Shim et al. 2006). In these plants, two sectors of multinuclear or multicellular cells are formed. The sector comprised of cytoplasmically rich cells with straight walls and diploid nuclei formed by nuclear fusion at an early stage, called embryo domain (emb-dom), forms an embryo. The other sector, comprised of multinuclear or polyploidy cells with irregular walls, called endosperm domain (end-dom), eventually degenerates.

A system may show more than one of the above pathways. Moreover, the type and time of application of the stress can change the developmental pathway in a plant. For example, in barley, with low temperature stress, the first division of the microspore is mostly (94 %) asymmetrical but with 0.3 M mannitol it was predominantly (60 %) symmetrical (Kasha et al. 2001).

8.4.3 Regeneration of Plants

Irrespective of the early pattern of divisions, the androgenic grains finally become multicellular and burst open to release the tissue, which has an irregular outline. In several cases (*Brassica, Hyoscyamus, Nicotiana*), the cellular mass gradually assumes the shape of a globular embryo and continues normal post-globular embryogenic differentiation (Fig. 8.2). In monocots, like wheat and barley, the multicellular mass becomes polarized while still within the pollen wall. The bursting of the pollen wall is not a passive process. It is pre-determined and occurs opposite to the germ pore (Maraschin et al. 2005b; Indrianto et al. 2001). It is interesting that the pollen embryos of *B. napus* and *B. juncea* formed multicellular, uniseriate suspensor at the radicular end like the zygotic embryos (Chanana et al. 2005; Fig. 8.3). By this method a

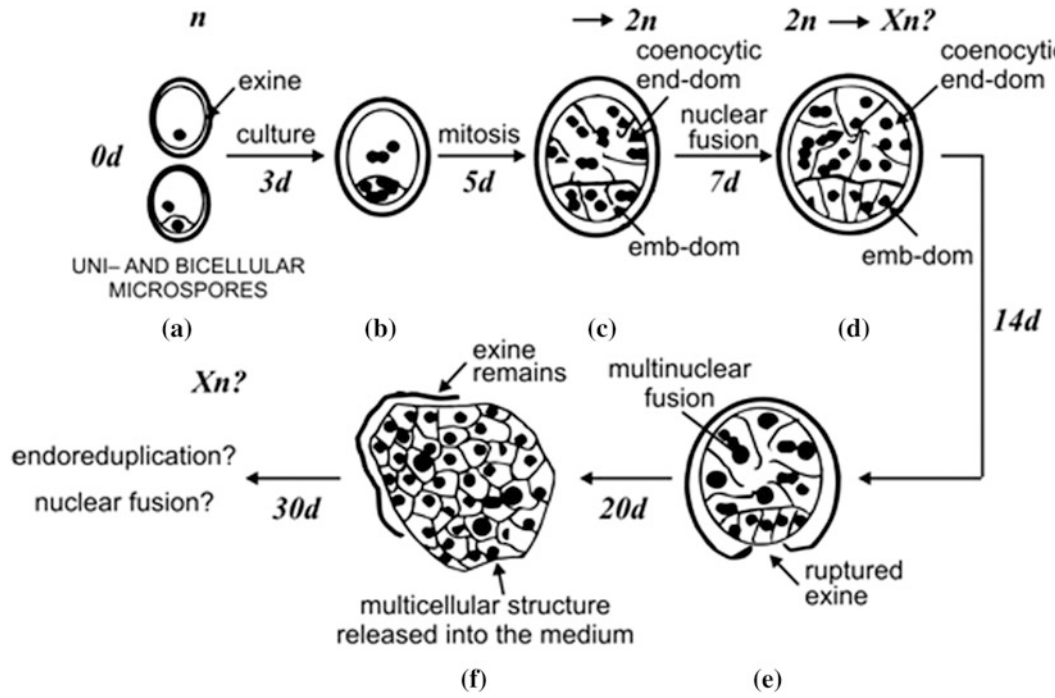

Fig. 8.8 Nuclear fusion during pollen embryogenesis in maize. The cultured microspores (unicellular or bicellular) (**a**) exhibit nuclear divisions (**b**), and by the 5th–7th day of culture two domains are formed within the exine (coenocytic endosperm domain (end-dom) with incomplete wavy walls and the embryo domain (emb-dom) with uninucleate or binucleate cells (**c**, **d**). After 14 days multiple nuclear fusions can be detected in the end-dom (**e**). When the exine bursts the pollen releases the multicellular mass (**f**) derived largely from the emb-dom; the end-dom degenerates (*courtesy* Prof. Elisabeth Matthys-Rochon, France)

pollen grain forms a single plant. However, in several cases (*Arabidopsis, Asparagus, Oryza*), the tissue liberated from the pollen grain undergoes further proliferation to form a callus (Fig. 8.1b), which may later differentiate plants on the same or a modified medium.

8.5 Diploidization

Haploid plants are sexually sterile. In the absence of homologous chromosomes meiosis is abnormal, and as a result viable gametes are not formed. In order to obtain fertile homozygous diploids, the chromosome complement of the haploids must be duplicated. In many of the cereals, like barley (Kasha et al. 2001; Shim et al. 2006; Gonzalez-Melendi et al. 2005), maize (Testillano et al. 2004), and wheat (Hu and Kasha 1999), spontaneous doubling of chromosome number, mainly by nuclear fusion after the first androgenic division, occurs with high frequencies (40–85 %). In these plants, the multicellular androgenic grains show two tissue domains with different cellular organization. In maize, the two domains appear 5–7 days after culture (Fig. 8.8). One of the domains, with small polygonal, regular cells with straight walls, dense cytoplasm, and large rounded nucleus containing one large nucleolus each, is called the embryo-domain (emb-dom). The cells of the emb-dom have either one diploid nucleus or two haploid nuclei which later fuse. The other domain, called endosperm-domain (end-dom), possesses partially coenocytic organization with incomplete walls (Testillano et al. 2004). The diploidized cells of the emb-dom form an embryo, whereas the end-dom degenerates. In *Brassica napus* induction of androgenesis by high temperature stress is also accompanied by

spontaneous chromosome doubling but at low rate (5–10 %). However, if colchicine or trifluralin was used as the induction treatment, up to 90 % of the regenerants were dihaploids (Zhao et al. 1996; Zhou et al. 2002). In such cases, there is no requirement for a special treatment to obtain fertile homozygous diploids.

In other cases, the pollen plants should be treated with 0.1–0.4 % colchicine solution to diploidize them. Different methods have been followed to colchidiploidize the haploid plants. Generally, the pollen-derived plants, with 3–4 leaves, are soaked in a 0.5 % aqueous solution of colchicine for 24–48 h and, after washing with distilled water, transferred to a potting mixture for hardening and further growth. In *Brassica* species, the roots of the pollen-derived plants, in a bunch of 25–30 plants, are immersed in 0.25 % (w/v) colchicine solution for 5 h in light. The treated roots are rinsed with distilled water, and the plants are transferred to a potting mix for hardening and further growth.

8.6 Applications

1. Haploids and DHs are of considerable importance in genetics and plant breeding programs (Hu and Guo 1999; Maluszynski et al. 2003). The main advantage of haploids in plant breeding programmes is to achieve complete homozygosity in a single step by doubling the chromosome number. The best known application of haploids is in the F_1 hybrid system for the fixation of recombinations to produce homozygous hybrids, allowing easy selection of phenotypes for qualitative and quantitative characters. The doubled haploid method reduces the time needed to develop a new hybrid cultivar by 2–4 years, in comparison to the conventional method, involving time consuming, labor intensive, and recurrent back crossing. Pollen-derived doubled haploids are being routinely used in crop improvement programs, which has aided the development of several improved varieties (Maluszynski et al. 2003; Palmer et al. 2005).

   Combining the conventional breeding methods and anther culture, Chinese scientists have developed and released several cultivars of rice (38 varieties), wheat (10 varieties), and maize (22 varieties), which have good agronomic characteristics, such as high yield, well adaptation, and resistance (Hu and Guo 1999; Datta 2001).
2. Mutations are difficult to detect in higher organisms because they are usually recessive and do not express themselves in the presence of their dominant alleles on the homologous chromosomes. Repeated selfing of the plants carrying the mutations is essential to bring out the recessive traits, which is not possible in self-incompatible plants, and where it is possible, only one out of four plants will bear the recessive character, and where more recessive mutations are present the possibility of getting individuals showing all the mutations is extremely low. On the other hand, mutations induced in haploids can be easily detected because they express the full complement of genome, including the recessive characters, at the phenotypic level and the lethal mutations and gene defects are eliminated. Haploids with desirable mutations can be selected and their chromosomes duplicated by colchicine treatment to get fertile diploids with all desirable mutations in a single generation. Following this approach, Heszky et al. (1992) produced an improved variety of rice (DAMA; Fig. 12.3). Liu et al. (2005) used haploid androgenic saplings of *Brassica napus* to raise callus and made in vitro selection of lines more resistant to *Sclerotinia sclerotorum* than the parent plant.
3. Androgenesis is also a good source of genetic variation (Gametoclonal variation). It enables analysis of gametic variation, generated through recombination, and segregation during meiosis, at plant level (Schaeffer et al. 1984; Matzinger and Burk 1984; Siebel and Paul 1989).
4. The commercially desirable features of *Asparagus officinalis* are uniform male plants with inflorescences (spears) having low fiber content. In this dioecious crop plant inbred

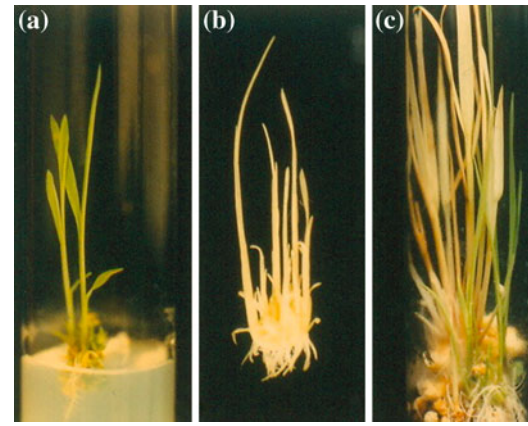

Fig. 8.9 **a–c** Plants regenerated from pollen-derived calli of rice. A callus may differentiate to give only green plants (**a**), only albino plants (**b**) or both, green and albino plants (**c**)

population is produced through sib crosses between pistillate (XX) and staminate (XY) plants. Consequently, 50 % plants are male and 50 % female. Thevinin (1974) and Tsay et al. (1982) cultured anthers from male plants and raised homozygous supermales (YY). When such supermales are crossed with the female plants (XX) the entire progeny consists of males. Corriolis et al. (1990) announced the release of the first homogeneous all male F_1 hybrid of *A. officinalis*, called "Andreas", using homozygous supermales derived from pollen embryos and a homozygous female parent obtained by diploidizing of a spontaneous parthenogenetic haploid. Andrea is a very regular, high yielding variety with large spear diameter and very tight head.

5. Androgenesis provides an excellent model system to study the developmental aspects of induction of embryogenesis and embryo development from single haploid microspores. Freshly isolated uninucleate microspores of *B. napus* can be induced, by 8 h high temperature shock (32 °C), to develop directly into an embryo within 14 days of culture initiation with a frequency as high as 70 % (Custers et al. 2001; Pecham and Keller 1988; Pecham et al. 1991). Similarly, the existing gametophytic developmental program of unicellular microspores of barley and wheat can be inhibited and the competence for sporophyte development, via direct embryogenesis, with high frequency, can be induced by a single stress treatment, such as osmotic shock or starvation (Maraschin et al. 2005a).
6. The doubled haploids of self-pollinating crops (barley, tomato, wheat) have been used to construct linkage maps of molecular markers and to estimate the number of genes controlling complex traits and even to directly release cultivars (Veilleux 2004). In outbreeding crops (maize, potato, cucumber), DHs have been applied to study the effect of inbreeding and to derive inbred lines. Such lines can be used for hybrid cultivar production or for studying the inheritance of useful traits.

8.7 Concluding Remarks

Androgenic haploids have been produced for a very large number of angiosperms, and the technique of anther/pollen culture for haploid production has become an integral part of plant breeding programs. However, many agronomically important crop plants, such as onion, cucumber, sunflower, sugar beet, legumes, and cucurbits have so far proved recalcitrant to androgenesis. Moreover, androgenesis is not applicable to male sterile or female clones of the dioecious plants (e.g., Mulberry).

The main factors that hinder the application of anther and pollen culture to cereals are low rates of androgenesis and the high frequency of albinism (up to 80 %) among the regenerants

(Fig. 8.9). The albino plants cannot survive in the field, and therefore are of no agronomic value (Babbar et al. 2000; Serazetdinova and Lörz 2004; Soriano et al. 2008). Nuclear fusion and endomitosis during early stages of androgenesis, especially in cereals, results in non-haploids (Testillano et al. 2004; Gonzalez-Melendi et al. 2005; Shim et al. 2006).

These are some of the limitations of in vitro androgenesis that need to be addressed by the scientists.

8.8 Appendix

1. Anther culture to produce androgenic haploids of *Nicotiana tabacum.* (*after* Touraev et al. 2003).
   (i) Grow the plants of *Nicotiana tabacum* in a glasshouse at 20–25 °C under 16 h photoperiod with a light intensity of 219–270 μmol m^{-2} s^{-1} provided by sodium lamps.
   (ii) Harvest the flower buds from the first flush of flowers and bring them to the laboratory in a nonsterile Petri dish.
   (iii) Classify the buds according to their corolla length. Excise anthers from one of the buds of each category and crush them in acetocarmine to determine the stage of pollen development. Identify and select the buds (ca 10 mm) with pollen just before, at, and immediately after first pollen mitosis.
   (iv) Incubate the buds at 7–8 °C for 12 days in a pre-sterilized Petri plate sealed with parafilm.
   (v) Surface sterilize the chilled buds with a suitable sterilant (0.1 % mercuric chloride for 10 min or 5 % sodium hypochlorite for 10 min).
   (vi) Rinse the buds 3–4 times in sterile, double distilled water in a laminar air flow cabinet.
   (vii) Using forceps and a needle, flame-sterilized and cooled, tease out the buds, and excise the anthers in a pre-sterilized Petri dish. Carefully detach the filament and place the anthers on MS medium (Table 3.1), supplemented with 2 % (w/v) sucrose and 1 % activated charcoal in Petri dishes (five anthers from a bud per 50 × 18 mm dish containing 5 ml of medium). Seal the Petri dishes with Parafilm and incubate the cultures at 25 °C in dim light (10–15 μmol m^{-2} s^{-1}).
   (viii) After 3–4 weeks, when the anthers have burst to release the pollen-derived embryos, transfer the culture to 16 h photoperiod and light intensity of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. At this stage, if the responding anthers are crushed in acetocarmine (0.5–1.0 %) and observed under the microscope, different stages of pollen embryogenesis can be seen which is asynchronous.
   (ix) Complete green plants will develop after 4–5 weeks of culture.
   (x) Isolate the plantlets emerging from the anthers and transfer them to MS basal medium with 1 % (w/v) sucrose and 1 % (w/v) activated charcoal to allow root development. During this period, incubate the cultures under continuous light (3.6 μmol m^{-2} s^{-1}) from white fluorescent tubes.
   (xi) When the plants attain a height of about 5 cm, transfer them to potting mix in small pots or polythene bags and maintain under high humidity. Gradually reduce the humidity and transfer the plants to field.
2. Pollen culture to produce androgenic haploids of *Nicotiana tabacum* (*after* Touraev et al. 2003)
   (i) Follow steps 1–6 as in Protocol 8.8.1.
   (ii) Squeeze the anthers from 10 buds in a glass vial (17 ml) with about 3 ml of medium B (Kyo and Harada 1986), containing (in mg L^{-1}) KCl (149), $CaCl_2.2H_2O$ (147), $MgSO_4.7H_2O$ (250), KH_2PO_4 (136) and mannitol (54,700).

(iii) Place a magnetic bar in the vial and stir for 2–3 min at maximum speed until the medium becomes milky.
(iv) Collect the suspension of pollen and debris using a Pasteur pipette and filter it through a 40–60 μm pore size metallic or nylon sieve.
(v) Centrifuge the filtrate for 2–3 min at 250 g. Discard the supernatant along with the upper green pellet using a 200 or 1,000 μl pipette.
(vi) Suspend the lower whitish pellet in 2–10 ml of medium B and centrifuge again. Repeat the fifth step 2–3 times until there is no green layer above the white pellet.
(vii) Suspend the white pellet, comprised of purified pollen, in the B-medium and plate the suspension in a pre-sterilized Petri dish. Seal the Petri dish with Parafilm and incubate in dark at 33 °C for 5–6 days. The induction of androgenesis occurs during this starvation stress treatment.
(viii) After the pretreatment, transfer the suspension to a screw capped centrifuge tube and pellet by centrifugation at 250 g for 5 min.
(ix) Discard the supernatant and suspend the pellet in AT-3 medium (for composition see Table 8.1) and plate back in the original dishes (1 ml per dish). Seal the dishes with Parafilm and incubate the cultures in dark at 25 °C for 6–8 weeks.
(x) After 4–5 weeks, when fully differentiated pollen embryos have developed, transfer the culture dishes to 16 h photoperiod and light intensity of 50 μE m^{-2} s^{-1}.
(xi) After another week, transfer individual embryos to culture tubes or jars containing MS basal medium with 1 % sucrose and 1 % activated charcoal for germination and full plant development. During this period incubate the cultures in light as above.
(xii) After the plants attain a height of about 5 cm transfer them to potting mix in small pots or polythene bags and maintain them under high humidity. Gradually reduce the humidity, and finally transfer the plants to field.

3. Pollen culture to produce androgenic haploids of *Brassica juncea.* (*after* Channa et al. 2005)
(i) Sow the seeds in 20 cm pots containing an artificial potting mixture, such as Agropeat PV, and maintain them at 25 °C under natural light.
(ii) At the bolting stage, move the plants to a growth chamber at 10 °C/5 °C day/night temperatures with 16 h photoperiod and 150–200 μE m^{-2} s^{-1} light intensity from cool white fluorescent tubes.
(iii) After 2 weeks, when 2–3 flowers have opened, collect the young green inflorescences and transfer them to the laboratory in nonsterile Petri dishes.
(vi) Classify the buds into 2–4 categories on the basis of their length (2.7–2.9 mm, 3.0–3.1 mm, 3.2–3.3 mm and 3.4–3.5 mm) with the help of a Vernier Caliper.
(v) Determine the stage of pollen development in the buds of the different categories by staining with DAPI (4, 6-diamino-2-phenylindole) and observing under UV light using a fluorescence microscope. Select the buds at the late uninucleate stage, when the nucleus has migrated to one side, for culture. Hereafter, all operations must be performed under aseptic conditions in a sterile air flow cabinet.
(vi) Transfer the selected buds to a tea egg and immerse in 0.1 % (w/v) mercuric chloride or 2 % (w/v) sodium hypochlorite solution, with a drop of Tween or Teepol for 10–12 min with continual shaking.
(vii) After three rinses each of 5 min in cold sterile distilled water, transfer the buds (maximum 20) to an autoclaved 25 ml

beaker containing 7 ml of cold liquid B_5 medium with the salts reduced to half strength and 13 % (w/v) sucrose (½ B_5-13; Table 8.1).

(viii) Homogenize the buds with the aid of an injection piston, applying turning pressure movement to release the pollen. Wash the piston with ½ B_5-13 medium.

(xi) Filter the pollen suspension through a double layer of nylon sieve (Nytex 63 μm pore size top and 44 μm bottom) in a 15 ml sterilized, screw cap centrifuge tube. Rinse the nylon sieve with 2 ml of ½ B_5-13 medium and adjust the volume to 10 ml with medium.

(x) Wash the pollen twice with ½B_5-13 medium by pelleting at 100 g for 3 min in a refrigerated centrifuge precooled to 4 °C.

(xi) Wash the pollen in NLN-13 medium containing 0.83 mg L^{-1} KI (NLN-13-KI; Table 8.1).

(xii) Suspend the pellet in 1 ml of NLN-13-KI medium and determine the density of pollen using a hemocytometer. Adjust the density to 1×10^4 pollen grains ml^{-1} using NLN-13-KI medium.

(xiii) Dispense the suspension into sterilized Petri dishes (3 ml per 60 mm dishes) as thin layers. Seal the dishes with Parafilm and incubate at 32 or 35 °C in the dark.

(xiv) After 3 days, transfer the culture dishes to 25 °C in dark.

(xv) After another 3 weeks of culture, transfer individual embryos to B_5 medium with 2 % (w/v) sucrose for germination. Place the dishes in a culture room with 16 h photoperiod (50–100 μE m^{-2} s^{-1} provided by cool fluorescent tubes) at 25 °C. If necessary, after 4–5 days reorientate the embryos in a vertical plane to facilitate their germination.

(xvi) After 2 weeks, transfer the plants to culture tubes with their roots immersed in 1–2 ml colchicine solution (0.1–0.2 %) and leave overnight. Wash the roots with sterile distilled water and transplant them to a 1:1(v/v) mixture of Agropeat and soil in Hycotrays (Sigma) and maintain in a glasshouse under high humidity. Gradually move the plants to the areas of decreasing humidity. The plants should be ready after another 3 weeks for transfer to the field.

4. Anther culture to produce androgenic haploids of *Oryza sativa* (*after* Zapata-Arias 2003).

(i) Collect, at 8–9 a.m., the tillers from glasshouse-grown plants with the central florets at the middle to late uninucleate stage of the pollen.

(ii) Wipe dry and wrap the spikes in aluminum foil and store at 8–10 °C for 8 days.

(iii) Rinse the spikes in 70 % (v/v) ethanol for 30 s before surface sterilizing them with 2 % (v/v) Chlorax (a commercial bleach with 5.2 % $NaOCl_2$) containing a drop of Teepol for 20 min. Carry out all further steps in a sterile air flow cabinet.

(iv) Rinse the spikes three times in sterile distilled water.

(v) To excise and culture the anthers, cut the base of the florets just below the anthers with sharp scissors. Pick the floret at the tip with forceps and tap on the rim of the Petri dish, so that the anthers fall on N_6 medium (Table 8.1) supplemented with 5 % (w/v) sucrose, 0.5–2.0 mg L^{-1} 2,4-D (callus induction medium). About 60 anthers may be cultured in a 55 mm diameter Petri dish containing 6 ml of callus induction medium.

(vi) Incubate the cultures at 25 °C in the dark.

(vii) After 4–5 weeks, transfer the pollen-derived calli (each 2–3 mm in diameter) to MS-based regeneration medium with 0.5–4.0 mg L^{-1} kinetin and

incubate the cultures in light (12 h photoperiod with 50–100 μE m^{-2} s^{-1} of illumination) at 25 °C.

(viii) Transfer the regenerated shoots to MS-based rooting medium lacking growth regulators.

5. Pollen culture to produce androgenic haploids of rice (*after* Raina and Irfan 1998).
   (i) Follow steps (i), (iii), and (iv) as in Protocol 8.8.4.
   (ii) Collect 150 aseptic anthers in a 6 cm diameter Petri dish containing 0.4 M mannitol solution, following the procedure described in step (v) of protocol 4. Incubate them in dark at 33 °C.
   (iii) Simultaneously, isolate the unfertilized ovaries from the same batch of florets and culture in Petri dishes containing 3 ml of M-019 medium (Table 8.1) to condition the medium for pollen culture. Culture 30 ovaries per plate and incubate the plates in dark at 25 °C.
   (iv) After 4 days of osmotic stress in the mannitol solution, some of the pollen grains will be liberated from the anthers into the pretreatment medium. Transfer the pollen suspension with the pretreated anthers to a small beaker and stir at slow speed for 2–3 min using a Teflon-coated magnetic bar to release the remaining pollen.
   (v) Filter the above suspension through a nylon/metallic sieve (40–60 μm pore size), pipette out the filtrate, transfer it to a screw cap centrifuge tube and centrifuge at 500 rpm for 2–3 min. Discard the supernatant and suspend the pellet in fresh mannitol solution and wash again by centrifugation. Give the final wash in M-019 medium. Finally, suspend the pollen in M-019 medium conditioned by the cultivation of unfertilized ovaries (1 ml suspension per 3.5 cm dish) for 4 days. Transfer 10 ovaries into each dish. Seal the Petri dish with Parafilm and incubate the cultures in dark, at 25 °C.
   (vi) After 4 weeks of initiation of the pollen cultures, transfer the embryo-like structures (ELS) or calli, each measuring 2–3 mm in size, to semi-solid MS-based regeneration medium (Table 8.1) supplemented with BAP (2.0 mg L^{-1}), Kinetin (1.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) and gelled with 6 % (w/v) agarose (Sigma). Incubate the cultures under 12 h photoperiod and 50–100 μE m^{-2} s^{-1} illumination. After 7–10 days, more ELS/calli from the induction medium may be transferred to regeneration medium.
   (vii) Transfer the regenerated plants to hormone-free ¼ strength MS-based medium containing 2 % (w/v) sucrose and gelled with 0.25 % (w/v) Phytogel in culture tubes.
   (viii) When the plantlets attain a height of about 15 cm, transfer them to liquid 1/10 strength MS-based medium without sucrose, vitamins or hormones for hardening before transfer to pots.

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Gynogenesis

9

9.1 Introduction

Normally, the egg cell requires the fertilization stimulus to form an embryo. However, it has the necessary genetic information to initiate embryogenesis independent of fertilization (Parthenogenesis). In some apomictic plants, the egg cell divides in total absence of a stimulus from the male gametes. In several other plants, it is possible to induce the egg or some other cell of the embryo sac (Apogamy) by external treatments, to divide and form an embryo (Bhojwani and Thomas 2001).

Since the first report of parthenogenic haploid formation following cold treatment of *Datura stramonium*, by Blakeslee et al. (1922), various treatments, such as low temperature shock, application of growth regulators or other chemicals, delayed or early pollination, and pollination with pollen in which the normal development of male gametes has been disturbed by irradiation or chemical treatment, have been shown to induce parthenogenesis. The idea behind the use of irradiated pollen is that while normal fertilization would be prevented but the growth substances diffused from the pollen might induce parthenogenesis. Indeed, this technique has yielded haploids of some important crop plants such as cabbage, cucumber, muskmelon, onion, rose, sunflower and wheat (Todorova et al. 1997).

In vitro culture of unfertilized ovules, ovaries and flower buds is another approach to trigger parthenogenesis and/or apogamy. This method of haploid production, called "In Vitro Gynogenesis", provides an attractive alternative to produce haploids of plants where androgenesis is either not applicable or unsuccessful or is fraught with problems such as formation of non-haploids and albinos with high frequencies. San Noeum (1976) was the first to report gynogenic haploid plant production in the unfertilized ovary cultures of *Hordeum vulgare.* To-date in vitro gynogenesis has been achieved in about 25 species, including some important crop plants (Table 9.1).

9.2 Factors Affecting Gynogenesis

9.2.1 Genotype

The genetic make-up of the donor plant is a major factor in determining the success of work on in vitro gynogenesis. Significant interspecific and intraspecific variation for gynogenesis has been reported in the genus *Allium* (Juðkevieiènë et al. 2005). Keller (1990) observed a clear-cut and stable genotypic effect on gynogenic response of 273 donor plants, representing different onion cultivars, grown under identical glasshouse conditions. Bohanec and Jakše (1999) observed inter-accession variation for gynogenic response in onion. The variation ranged from 0 to 22.6 %. Similarly, Javornik et al. (1998) identified an highly gynogenic gametoclone of onion (G126; 118 % response in terms of number of embryos per 100 buds) among the first cycle gynogenic plants of a

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_9, © Springer India 2013

Table 9.1 Some crop plants for which gynogenic haploids have been produced by unfertilized ovule, ovary or flower bud culture.*

Species	Reference
Allium cepa (Onion)	Muren (1989), Bohanec and Jakše (1999), Javornik et al. (1998), Juðkevieiènë et al. (2005)
A. porum (Leek)	Juðkevieiènë et al. (2005)
A. fistulosum (Japanese Leek)	Juðkevieiènë et al. (2005)
Beta vulgaris (Sugar beet)	Hosemans and Bossoutrot (1983)
Brassica oleracea (Cabbage)	Doré (1989)
Cucumis melo (Melon)	Katoh et al. (1993)
Cucurbita pepo (Summer squash)	Metwally et al. (1998)
Helianthus annuus (Sunflower)	Cai and Zhou (1984)
Hevea brasiliensis (Rubber tree)	Chen (1990)
Hordeum vulgare (Barley)	San Noeum (1976, 1986)
Morus alba (Mulberry)	Thomas et al. (1999)
Oryza sativa (Rice)	Asselin de Beauville (1980)
Solanum tuberosum (Potato)	Tao et al. (1985)
Triticum aestivum (Wheat)	Zhu and Wu (1979)
T. durum (Wheat)	Mdarhri-Alaoui et al. (1998)
Zea mays (Maize)	Truong-Andre and Demarly (1984)

*For a detailed list *see* Bhojwani and Thomas (2001)

poorly responding cultivar (3.5–5.1 % response). This response was 20 times higher than the best previously known response in onion. Notable genotypic variation for gynogenic response has also been observed in *Beta vulgaris* (Znamenskaya et al. 1994), *Helianthus annuus* (Yan et al. 1985), and *Zea mays* (Truong-Andre and Demarly 1984). Genotypic effect has been observed for not only the frequency of gynogenesis but also the degree of haploid formation. Schum et al. (1993) observed that one of the three genotypes of leek yielded 89 % haploids but the other two did not produce any haploid regenerant.

Bohanec et al. (2004) reported that at least in the inbred line B2923B of *Allium cepa* the high gynogenic haploid regeneration trait is inherited quantitatively with dominance towards low regeneration.

9.2.2 Explant

Excised ovules, ovaries and floral buds have been cultured to produce gynogenic haploids. However, to minimize complications due to callusing of sporophytic tissues, excised ovule is regarded as the best explant for gynogenic haploid production. In future, it may be possible to culture excised embryo sac that would facilitate determining its stage at the time of culture and follow the cytological changes during gynogenesis.

Gynogenic plants of *Beta vulgaris* and *Gerbera jamesonii* have been produced almost exclusively by ovule culture (Höffer and Lespinasse 1996). For *Helianthus annuus* ovule culture proved superior to ovary or floret culture (Yan 1988). Aselin de Beauville (1980), Zhou and Yang (1980) and Kuo (1982) developed a "young floret float culture" method for the production of gynogenic haploids of rice, which was applicable to all the 19 cultivars tested by these authors. Gynogenic plants of onion could be produced by ovule, ovary or flower bud culture but flower bud culture is regarded as the most practical method for large-scale production of gynogenic plants of this crop plant. In many cases where floral buds or ovaries have been used to initiate cultures, it required ovary or ovule excision, respectively, after some time and their culture in isolation (Thomas et al. 1999; Fig. 9.1).

Besides the nature of the explant the stage of the female gametophyte (embryo sac) at the time of culture is also very important. Since it is difficult to assess the stage of embryo sac development every time the cultures are raised, some external marker, such as the stage of

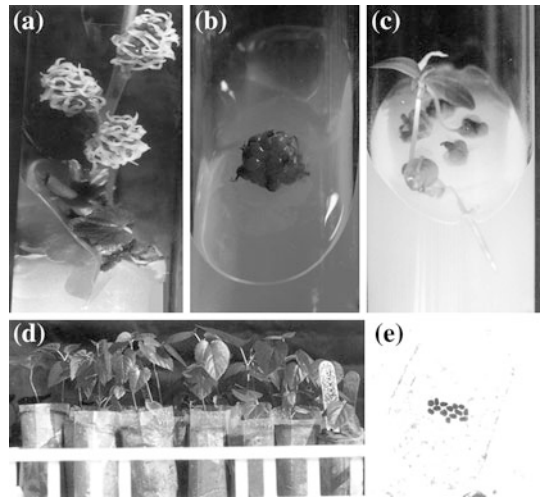

Fig. 9.1 In vitro gynogenesis in mulberry. **a** 3-week-old culture of a nodal segment, showing the development of three female inflorescence. **b** 3-week-old culture of an inflorescence segment derived from in vitro developed inflorescence. The ovaries have enlarged. **c** A culture of excised ovaries from **b,** showing the emergence of gynogenic plants. **d** Hardened gynogenic plants in polybags. **e** A root tip cell of a gynogenic plant, showing haploid chromosome complement ($2x = n = 14$). (*after* Thomas et al. 1999)

pollen, size of the bud and/or days before anthesis have been used to select the buds of the desired stage.

The mature embryo sac stage seems to be suitable for the induction of gynogenesis. However, for rice (Zhou et al. 1983) and *Melandrium album* (Mól 1992; Ferrant and Bouharmount 1994) 1–4 nucleate stage of the embryo sac was most responsive. Even in these two systems the female gametophyte continues its development until all its constituent cells have differentiated. Gynogenesis commences only after the embryo sac has fully organized.

In sugar beet, the ovules planted with their funiculus in contact with the medium showed better response than those planted randomly (Pedersen and Keimer 1996).

9.2.3 Pre-Treatment

Cold treatment of the donor plant or the explants soon after culture strongly influences the gynogenic response. Treatment of the donor plants (Svirshchevskaya and Bormotov 1994) or the flower buds (Lux et al. 1990) of sugar beet at 4 °C for 4–5 days before excising the ovules increased embryo yield. Similarly, cold treatment of *Salvia sclarea* inflorescence prior to ovule isolation promoted haploid callus formation by a factor of 1.5–2 (Bugara and Rusina 1989). Treating the rice panicles at 7 °C for one day promoted the gynogenic response (Cai et al. 1988). The umbels of onion stored at 6 °C for 15 days gave best results (Geoffriau et al. 1997).

The onion plants maintained at 15 °C provided flower buds 10 times more responsive than those grown at 10 °C or, in a glasshouse, at 18 °C (Puddephat et al. 1999).

9.2.4 Culture Medium

It is not possible to suggest any one medium suitable for all species. Even for the same species, different authors have used different media, particularly with regard to the growth regulators. In many cases, the protocol involved two steps, viz. induction and plant regeneration, each requiring different media. In some studies, more than two media were used. Generally MS (Murashige and Skoog 1962), B_5 (Gamborg et al.

1968) and N_6 (Chu et al. 1975) basal media, gelled with agar, have been used.

In plants such as *Gerbera*, sucrose was optimum at a fairly low level (1 %; Ahmim and Vieth 1986; Cappadocia and Vieth 1990). However, sucrose concentration higher than the usual level of 2–3 % gave best gynogenic response in maize (12 %), onion (10 %) and sunflower (12 %).

Addition of one or more growth regulators to the basal medium seems to be essential to induce gynogenesis. Alatorceva and Tyrnov (1994) observed gynogenic embryo development in unfertilized ovary cultures of maize in the absence of a growth regulator but its addition to the medium considerably enhanced the response. It is important that the concentration of the growth regulator is selected judiciously to avoid callusing of the sporophytic tissue of the explant.

An auxin, in combination with a cytokinin, is generally essential to induce gynogenesis. Yang and Zhou (1982, 1990) routinely used the auxin MCPA (2-methyl-4-chlorophenoxyacetic acid) in the induction and regeneration media for rice. For onion (Muren 1989; Martínez et al. 1997) and most other species 2,4-D (1–2 mg L^{-1}) has been widely used. For *Beta vulgaris*, *Gerbera jamesonii* and *Helianthus annuus*, IAA, NAA or IBA in combination with BAP or kinetin (1–2 mg L^{-1}) has been commonly used. Cagnet-Sitbon (1981) tested 12 media and found MS + BAP (2 mg L^{-1}) + kinetin (2 mg L^{-1}) + IAA (0.5 mg L^{-1}) to be the best for gynogenesis in *Gerbera*. However, Cappadocia and Vieth (1986) found MS + BAP (1 mg L^{-1}) + IAA (0.1 mg L^{-1}) to be the best for induction and MS + kinetin (1 mg L^{-1}) + NAA (0.1 mg L^{-1}) for plant regeneration in this taxon. The optimal hormonal combination may vary with the season of culture. Martínez et al. (2000) observed that maximum gynogenesis in *Allium cepa* occurred in the combined presence of two polyamines, viz. putrescine (2 mM) and spermidine (0.1 mM).

9.3 Origin of Gynogenic Plants

Since several layers of ovular tissues enclose the embryo sac, it involves a lengthy and tedious process of sectioning the material to study the ontogeny of gynogenic plants. Therefore, this aspect is less investigated.

Generally, ovules are cultured at the mature embryo sac stage. In the cultures of younger ovules, the female gametophyte seems to continue further development, and gynogenesis commences only after all the cells of the embryo sac are organized. Mostly, the gynogenic plants are derived from the egg cell (parthenogenesis), as reported in *Beta vulgaris, Helianthus annuus, Hevea brasiliensis, Hordeum vulgare, Melandrium album, Morus alba* (Fig. 9.2) and *Nicotiana tabacum*. However, in rice the gynogenic calli and embryos developed from the synergid.

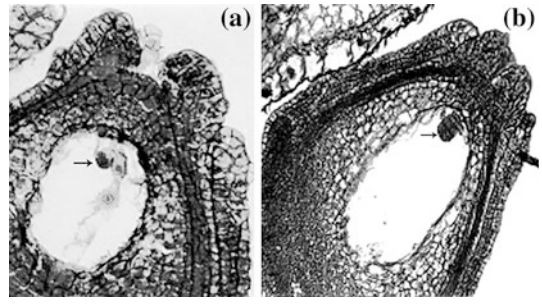

Fig. 9.2 Gynogenesis in mulberry. **a** Longitudinal section of an ovule just before culture, showing the enlarged egg (*arrow marked*) with a prominent nucleus at the micropylar end of the embryo sac. **b** An ovule from three-week-old inflorescence segment culture, showing a parthenogenic proembryo (*arrow marked*). (*after* Thomas et al. 1999)

Irrespective of the source of gynogenic plants, their mode of regeneration may be by direct embryogenesis (*Allium cepa, Beta vulgaris, Melandrium album*, *Morus alba*) or by regeneration from the callused gametic cells (rice). In rice, the proembryos usually grew into big protocorm-like structures, which later regenerated roots and shoots. The other species where gynogenesis generally occurs via callusing are *Gerbera, Malus x domestica* and *Lilium davidii*. Sometimes both pathways of gynogenesis occur in the same plant. Direct embryogenesis is desirable, as it minimizes the chances of somaclonal variations.

9.4 Endosperm Development

The development of endosperm is not essential for the induction and in vitro development of gynogenic embryos. In sugar beet and *Melandrium album* parthenogenic embryos developed in the complete absence of endosperm (Mól 1992; Ferrant and Bouharmont 1994). Autonomous development of endosperm up to 3–40 nucleate stage occurred in ovule/ovary cultures of *Beta vulgaris* (Ferrant and Bouharmont 1994), *Melandrium album* (Mól 1992), *Oryza sativa* (Zhou and Yang 1981) and *Viola odorata* (Wijowska et al. 1999). Cellular endosperm was never formed.

9.5 Abnormalities

A large proportion of the gynogenic embryos degenerate at different stages of development, probably due to the expression of lethal genes, which remain suppressed in the heterozygous state. In *Allium porrum,* only 24 % of the embryos produced plantlets; others failed to regenerate root/shoot (Smith et al. 1991). Similarly, in onion cv. Stuttgarten Riesen, 33 % of the regenerants degenerated due to abnormalities, such as irregular swelling of the hypocotyl, poor vigour, and hyperhydration (Bohanec et al. 1995; Keller and Korzun 1996). Almost half of the gynogenic embryos of *Melandrium album* possessed deformed cotyledons and primary leaves, and the plants died early. Out of 23 embryos only 9 regenerated green plants, of which two reached maturity; one of the plants was albino.

9.6 Ploidy Level

The gynogenic plants of sugar beet (Speckmann et al. 1986), barley (San 1976; Castillo and Cistué 1993) and durum wheat (Mdarhri-Alaoui et al. 1998) were mostly haploids (up to 100 %). However, in onion the frequency of haploids varied from 40 to 88 %. Similar variation also occurred in *Gerbera* (Cappadocia et al. 1988; Meynet and Sibi 1984) and rice (Zhou et al. 1986).

9.7 Applications

In vitro gynogenesis has been reported in at least 25 species. Considering that the number of haploid cells per ovule is considerably less than the number of pollen per anther, the potential of anther/pollen culture for the production of haploids is much more than that of unpollinated ovule/ovary/flower bud culture. However, there are families, such as Asteraceae, Chenopodiaceae and Liliaceae, where androgenic response has been very poor or nil but gynogenesis has been successful. Moreover, gynogenesis is the only approach to produce haploids of male sterile or female clones of dioecious plants, such as mulberry.

Another problem associated with the production of androgenic haploids is the occurrence of very large numbers of albinos among the pollen plants of all the major cereals. In contrast, most of the gynogenic plants of barley, rice, wheat were haploid and green. It is in such situations that gynogenesis may be useful for haploid plant production. Gynogenic haploids are being routinely used in breeding programmes of sugar beet, onion and rice (Mól 2003).

The gynogenic plants are also distinct from androgenic plants as they contain cytoplasmic traits of the maternal origin.

The dihaploid plants of *Nicotiana tabacum* produced by in vivo gynogenesis following pollination with *N. africana* were agronomically superior to the dihaploids produced via androgenesis and more closely resembled the parent plant. The yields of the two categories of plants were 91 and 80 %, respectively (Wernsman et al. 1989).

9.8 Concluding Remarks

Haploid production through gynogenesis is of considerable importance for species where androgenesis is either not applicable or unsuccessful or is fraught with other problems. However, further efforts are required to enhance the gynogenic response by manipulation of intrinsic and extrinsic factors. In this regard the reports of Bohanec and Jacśe (1999) and Javornik et al. (1998) are noteworthy. They identified a plant of onion showing gynogenic response as high as 52 %, which is more than double the average response of the parent genotype. They also isolated a highly gynogenic gametoclone ('Gynoclone') of a poor gynogenic plant (3.5–5.1 %) of onion, which showed 118 % response, in terms of number of embryos per 100 buds.

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Triploid Production

10

10.1 Introduction

Double fertilization is unique to the flowering plants. The male gametophyte (pollen) produces two sperms, which fertilize two different constituent cells of the female gametophyte (embryo sac). One of them fuses with the egg nucleus (syngamy) forming the diploid zygote and the other one fuses with the two nuclei in the central cell (triple fusion) resulting in a triploid primary endosperm cell. Whereas the zygote develops into a well-organized diploid embryo, the progenitor of the next generation, the primary endosperm cell forms an unorganized, short-lived triploid endosperm tissue, the main source of nutrition for the developing and, in some cases, also for the germinating embryo. Endosperm is also a dynamic center of developmental influences on the embryo (Hong et al. 1996). Depending on the plant, the endosperm may be consumed during seed development (cucurbits, legumes, and mulberry) or persist in the seeds as a massive tissue (cereals, castor bean, and palms).

As a rule, the endosperm does not form any organized structure. Therefore, the early embryologists regarded endosperm as the second embryo whose development is maimed from the very beginning by the introduction of third nucleus to ensure the survival of the first embryo without competition (Sargant 1900). *Brachiaria setigera*, an apomictic grass, is the only example where endosperm has been reported to produce triploid embryos and seedlings (Muniyamma 1978). However, in vitro studies have clearly demonstrated that the endosperm tissue has the potential for unlimited growth and its cells are totipotent, capable of forming full triploid plants which hold great potential in genetics and crop improvement programs (Hoshino et al. 2011).

10.2 Callusing

10.2.1 Stage of Endosperm at Culture

The work on endosperm culture was initiated in 1933 by Lampe and Mills, but the establishment of continuously growing tissue cultures of maize endosperm was first achieved in 1949 by LaRue. Although maize endosperm persists in mature grains, but callus cultures could be established only from immature endosperm. The best stage to initiate maize endosperm cultures was 8–11 days after pollination (DAP). This is also true of other cereals, where the bulk of mature endosperm is dead. Thus, the age of the endosperm could be a critical factor in raising tissue cultures of some species.

In 1963, Mohan Ram and Satsangi demonstrated that divisions could be induced in endosperm cells from mature, desiccated seeds of castor bean by treating it, before germination, with 2,4-D. In the same year, establishment of

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DOI: 10.1007/978-81-322-1026-9_10, © Springer India 2013

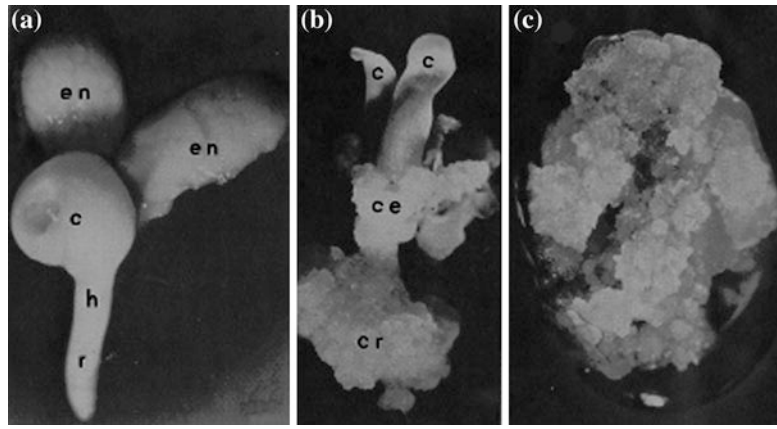

Fig. 10.1 In vitro proliferation of mature endosperm in the cultures of de-coated seeds of *C. bonplandianum*. (*ce* callused endosperm, *c* cotyledon, *cr* callused radicle, *en* endosperm, *h* hypocotyl, *r* radicle). **a** Ten days old culture of decoated seed, showing germination of embryo with enlarged cotyledons and elongated hypocotyl; also note proliferation on the inner side of the two halves of the endosperm. **b** Four weeks old culture, showing enlarged cotyledons. The radicle as well as the endosperm have callused. **c** Profusely growing endosperm callus (*after* Bhojwani and Johri 1971)

continuously growing callus cultures from the mature endosperm of sandalwood was reported by Rangaswamy and Rao (1963). Since then it has been possible to initiate tissue cultures from mature endosperm of several plants, such as *Croton, Jatropha, Osyris, Putranjiva* and *Ricinus*. A critical factor in the induction of cell divisions in mature endosperm is its initial association with the embryo. If only endosperm pieces are cultured they fail to proliferate. In seed cultures once the endosperm starts callusing the embryo can be removed without adversely affecting its growth (Fig. 10.1). There is ample evidence to suggest that the germinating embryo contributes some factors which are essential to trigger cell divisions in the mature endosperm. Brown et al. (1970) observed that endosperm pieces from dried seeds of castor bean did not grow in cultures. However, if endosperm was excised from germinating seeds they exhibited callusing, and there was direct relationship between the number of days for which the seed had germinated and the number of endosperm pieces showing callusing (Fig. 10.2). For tomato, soaking the seeds for 3 days was optimum in terms of the number of cultures showing endosperm callusing (Kang-zur et al. 1990). Therefore, to initiate callus cultures from mature endosperm, it is generally cultured along with the embryo, and once endosperm callusing has initiated (7–8 days) the embryo is discarded to ensure that it does not callus and contaminate the

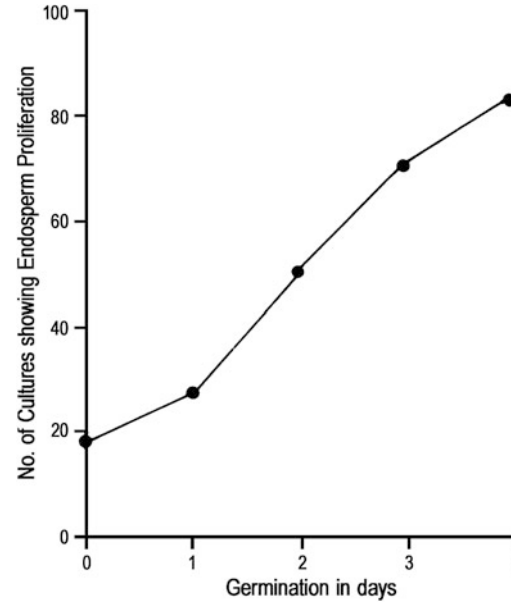

Fig. 10.2 *Ricinus communis*. Effect of germination period on the proliferation of excised endosperm; 0 days refers to seeds soaked for 22 h (based on the data of Brown et al. 1970)

endosperm callus. In some plants, the embryo factor could be substituted by gibberellic acid (Bhojwani 1968). Although it is now possible to induce divisions in mature endosperm of several species, immature endosperm continues to be the explant of choice in cereals and those species where endosperm is consumed before seed maturation (acacia, citrus, and mulberry).

10.2.2 Culture Medium

Lampe and Mills (1933) grew young maize endosperm on a medium containing an extract of potato or young maize grains and observed slight proliferation of endosperm layers adjacent to the embryo. Many years later, LaRue (1949) was able to raise, for the first time, continuously growing tissue cultures from the immature endosperm of maize on a medium containing, besides its basic constituents, tomato juice, green corn juice, yeast extract, or cow's milk. Of these, tomato juice supported maximum callus growth but on this medium the results were inconsistent. Yeast extract could fairly substitute tomato juice in supporting callus growth, and the results were also reproducible. Straus (1960) reported that asparagine (1.5×10^{-2} M) could suitably replace yeast extract. Nakajima (1962) carried out a series of experiments and concluded that for satisfactory growth of immature endosperm of *Cucumis* a nutrient medium supplemented with an auxin, a cytokinin and a rich source of organic nitrogen (yeast extract, casein hydrolysate) was necessary. Nutrient media with similar composition have proved satisfactory for the culture of mature endosperm of several other plant species.

10.3 Histology and Cytology

The decoated seeds of *Croton bonplandianum* on a medium containing 2,4-D, kinetin and yeast extract burst open 10–12 days after culture, exposing the callusing inner surface of the endosperm (Fig. 10.1a, b). At this stage, the embryo showing the initial stages of germination is discarded and the endosperm is transferred to fresh medium of the original composition for its continued proliferation (Fig. 10.1c). Irrespective of the culture medium, the endosperm calli of cereals remain purely parenchymatous. However, in Euphorbiaceous, Santalaceous, and Loranthaceous species tracheidal elements differentiate in the endosperm callus either scattered or in nests. Organogenic differentiation from endosperm tissue is generally associated with tracheidal differentiation.

As a rule, the endosperm tissue exhibits a high degree of polyploidization and cytological abnormalities during in vivo development (Bhojwani and Bhatnagar 2008). In established cultures of maize endosperm, the polyploid, hypoploid, and aneuploid cells were as common as those showing normal chromosome number (Straus 1954). About 30 % of the cells in anaphase exhibited chromosome bridges and laggards. Cells of ploidy higher than 3*n* have also been reported in endosperm cultures of *Croton* (Bhojwani and Johri 1971), *Jatropha* (Srivastava 1971), and *Lolium* (Norstog 1956; Norstog et al. 1969). It is, however, interesting that up to 10 years the majority of cells in rye-grass endosperm callus remained triploid (Norstog et al. 1969). A remarkable stability of chromosome number of endosperm cells in vivo and in vitro also occurs *in* the mistletoes, *Dendrophthoe falcata, Taxillus vestitus*, and *T. cuneatus* (Johri and Nag 1974). Neither the culture medium nor the culture duration altered the chromosome number of endosperm cells in *D. falcata*.

10.4 Plant Regeneration

By 1964, the cellular totipotency of plant cells was well established, but endosperm cells were still regarded as recalcitrant for plant regeneration. In 1965, Johri and Bhojwani, for the first time, reported the differentiation of well-organized shoot buds from the mature endosperm of *Exocarpus cupressiformis*, a root parasite of the family Santalaceae (Fig. 10.3). Histological studies confirmed the origin of the shoots from the peripheral cells of the endosperm. As expected, the shoots were triploid. Since then

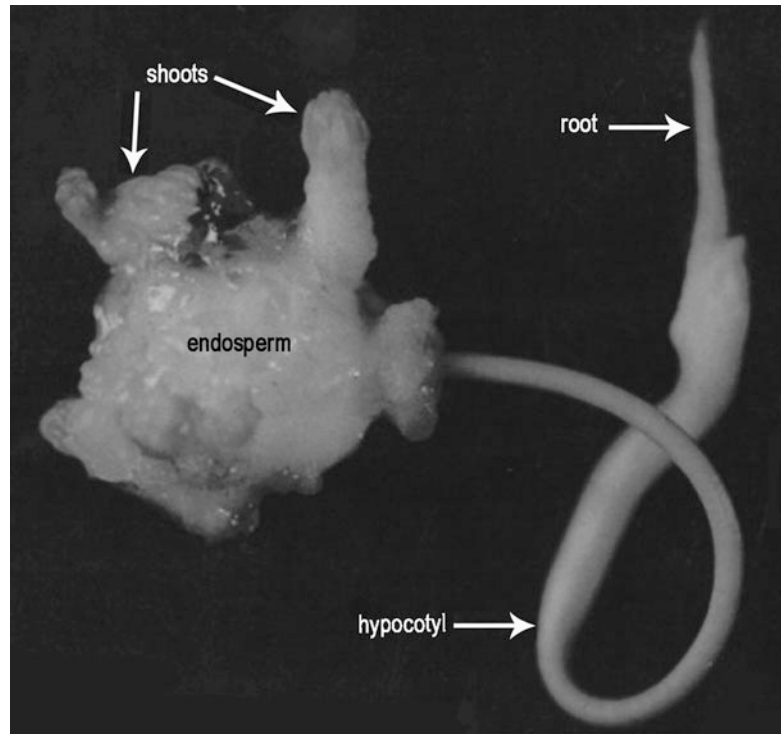

Fig. 10.3 A 5-week-old culture of decoated seed (endosperm + embryo) of *Exocarpus cupressiformis*, showing germination, and several shoots arising from the surface of the endosperm (*after* Johri and Bhojwani 1965)

shoot/embryo/plantlet regeneration from endosperm has been reported in at least 23 species (Table 10.1). In the cultures of mature endosperm, shoot bud differentiation may occur directly from the endosperm (Fig. 10.3) or after callusing (Fig. 10.4). However, in immature endosperm cultures it is always preceded by a callus phase. Plant regeneration from the callused endosperm of *Acacia nilotica, Citrus* sp. *Santalum album,* and *Juglans regia* occurred via embryogenesis.

10.4.1 Culture Medium

For shoot bud differentiation from endosperm tissue, a cytokinin is generally necessary. Whereas in *Scurrula pulverulenta* and *T. vestitus* shoot bud differentiation from the endosperm tissue occurred in the presence of a cytokinin alone, in *D. falcata* and *Leptomeria acida* an auxin was required in addition to a cytokinin.

The immature endosperm (Fig. 10.4a) of mulberry (17–20 DAP) exhibited good callusing on a medium supplemented with 5 μM BAP and 1 μM NAA (Thomas et al. 2000). Further addition of coconut milk and yeast extract improved the callusing response. Continuously growing callus was established on MS + 5 μM 2,4-D (Fig. 10.4b). Maximum shoot regeneration occurred on MS + 5 μM BAP and 1 μM NAA (Fig. 10.4c). In *Actinidia* sp. the callus which developed on MS medium fortified with 2,4-D (6 μM) and kinetin (23 μM), differentiated shoots and roots upon transfer to MS medium containing 2 μM IAA and 25 μM 2iP (Machno and Przywara 1997). In *Actinidia deliciosa*, endosperm callusing occurred with 80 % efficiency on MS + 2,4-D (9 μM) + Kn (23 μM). However, shoot regeneration occurred only in the presence of 2 μM TDZ (Goralski et al. 2005).

The immature endosperm of *A. nilotica* produced nodular callus on MS + 2,4-D (10 μM) + BAP (25 μM) + CH (1000 mg L^{-1}). In the third passage of the callus on the same medium, in dark, several somatic embryos differentiated (Garg et al. 1996). Germination of the embryos occurred on a modified MS basal medium supplemented with glutamine

Table 10.1 A list of species that are known to regenerate shoots, embryos and/or plants from endosperm tissue in vitro

Species (common name)	Reference
Actinidiaceae	
Actinidia chinensis (Kiwifruit)	Gul et al. (1982)
Actinidia deliciosa (Kiwifruit)	Machno and Przywars (1997), Goralski et al. (2005)
Apiaceae	
Petroselinum hortense (Parseley)	Masuda et al. (1977)
Caricaceae	
Carica papaya (Papaya)	Sun et al. (2011)
Euphorbiaceae	
Codiaeum variegatum (Croton)	Chijjannaiah and Gayatri (1974)
Jatropha panduraefolia (Jatropha)	Srivastava (1971)
Putranjiva roxburghii (Putranjiva)	Srivastava (1973)
Juglandaceae	
Juglans regia (Walnut)	Tulecke et al. (1988)
Loranthaceae	
Dendrophthoe falcata (Leafy mistletoe)	Nag and Johri (1971, 1974)
Scurrula pulverulenta (Leafy mistletoe)	Bhojwani and Johri (1970)
Taxillus vestitus (Leafy mistletoe)	Nag and Johri (1971)
Taxillus cuneatus (Leafy mistletoe)	Nag and Johri (1971)
Meliaceae	
Azadirachta indica (Neem)	Chaturvedi et al. (2003)
Mimosaceae	
Acacia nilotica	Garg et al. (1996)
Moraceae	
Morus alba (Mulberry)	Thomas et al. (1999)
Poaceae	
Oryza sativa (Rice)	Nakano et al. (1975)
	Bajaj et al. (1980)
Rosaceae	
Prunus persica (Peach)	Shu-quiong and Jia-qu (1980)
Pyrus malus (Apple)	Mu et al. (1971)
Rutaceae	
Citrus grandis (Orange)	Wang and Chang (1978)
Citrus sinensis (Orange)	Gmitter et al. (1990)
Santalaceae	
Exocarpus cupressiformis (Cherry Ballart)	Johri and Bhojwani (1965)
Leptomeria acida (Native Current)	Nag and Johri (1971)
Santalum album (Sandalwood)	Lakshmi Sita et al. (1980)

(500 mg L^{-1}), CH (200 mg L^{-1}), and coconut milk (10 %). The endosperm callus of *Citrus sinensis* and *C. grandis* initiated on Murashige and Tucker (1969) medium containing 2,4-D (10 μM), BAP (22 μM) and CH (1000 mg L^{-1}), differentiated globular embryos upon transfer to 2MT medium (salt concentration in MT medium doubled) fortified with 6 μM GA_3 (Gmitter et al. 1990). However, the optimum medium for embryogenesis was 2MT + GA_3 (6 μM) + BAP (1 μM) + Adenine

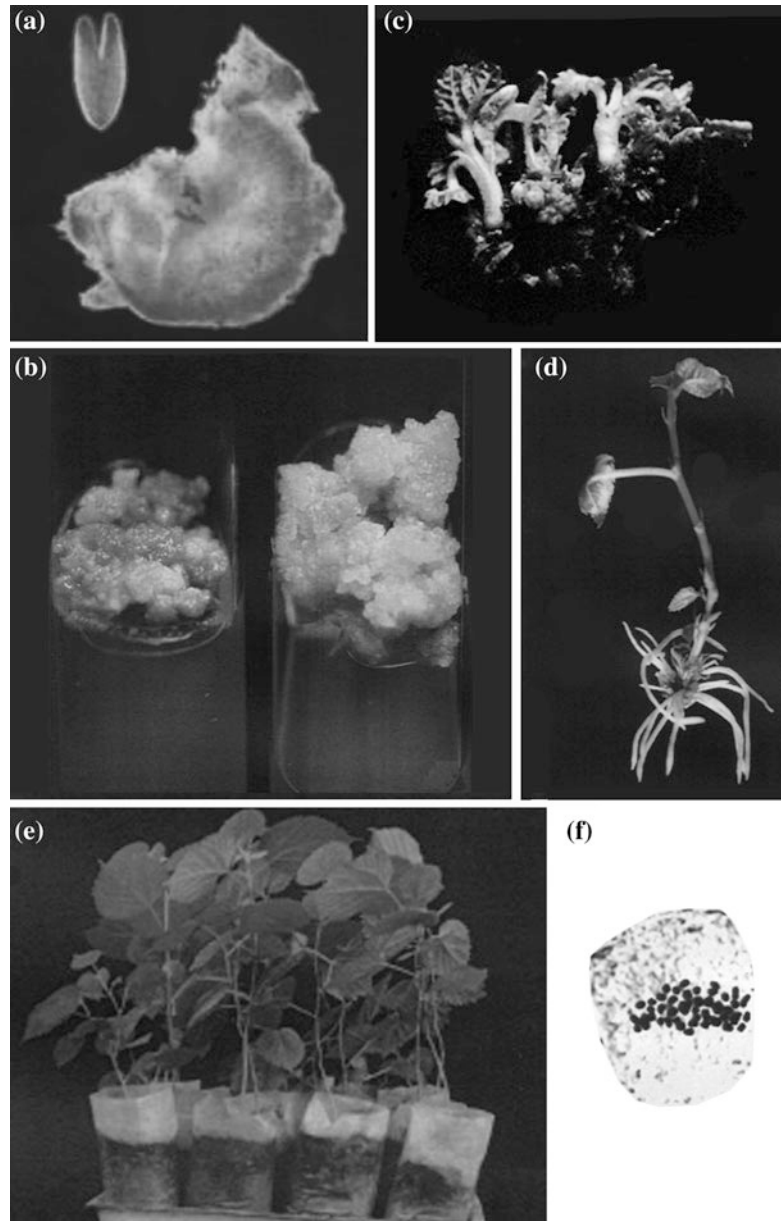

Fig. 10.4 Regeneration of triploid plants from immature endosperm of mulberry. **a** Embryo and endosperm at the time of culture. **b** Well-established endosperm calli. **c** The endosperm callus has differentiated multiple shoots. **d** A plantlet of endosperm origin. **e** Hardened plants of endosperm origin in polythene bags. **f** A root tip cell of a plant regenerated from endosperm callus, with triploid number of chromosomes (42) (*after* Thomas et al. 2000)

(10.5 μM) + CH (500 mg L^{-1}). On this medium dicotyledonous embryos developed only in the case of *C. sinensis*. These embryos germinated on 2MT + GA_3 (14 μM) but root development did not occur. Plantlets were recovered by micrografting the shoots on to the diploid rootstock seedlings. Eventually, triploid plants of endosperm origin were established in soil (Gmitter et al. 1990).

10.4.2 Cytology

Most of the organs and plants regenerated from endosperm cells were triploid. Both the plants of *J. regia* derived from endosperm callus were triploid ($3n = 3x = 48$). In *Citrus*, the stability of ploidy level was maintained throughout the regeneration period. The triploid nature of the

shoots regenerated from endosperm callus of *A. nilotica* was established through Feulgen cytophotometry (Garg et al. 1996). All the 10 field grown mulberry plants of endosperm origin were triploid (Fig. 10.4d–f; Thomas et al. 2000). About 66 % plants of *Azadirachta indica* (Chaturvedi et al. 2003) and 75 % plants of *Carica papaya* (Sun et al. 2011) regenerated from endosperm callus were triploid.

Full triploid plants of endosperm origin have been developed for apple, kiwifruit, mulberry, papaya, and sandalwood. The triploid plants of mulberry (Fig. 10.4e), sandalwood, and citrus have been established in soil.

10.5 Applications

Triploid plants are usually seed sterile, and therefore undesirable where seed is of commercial importance. However, there are examples where seedlessness caused by triploidy is of no serious concern or is a desirable feature. It is with reference to these plants that triploidy can be exploited for plant improvement. The triploids of several varieties of apple, banana, mulberry, sugarbeat, tea, and watermelon are already commercially important (Elliot 1958). The triploid plants of *Petunia axillaris,* raised from microspores, were more vigorous and much more ornamental than their anther donor diploids (Gupta 1982). Natural triploids of tomato produced larger and tastier fruits than their diploid counterparts (Kagan-Zur 1990).

Traditionally, triploid production involves chromosome doubling to raise tetraploids, followed by crossing of the tetraploids with the diploids. This approach is not only laborious and time consuming but in many cases not possible due to high sterility of the autotetraploids (Gupta 1982; Esen and Soost 1973; Hoshino et al. 2011). Despite these problems, some triploid varieties of crop plants with superior traits than their diploids have been developed. The triploid cassava variety, Sree Harsha, produced by the traditional method has many desirable characters, such as higher yield and harvest index, increased dry matter, and starch content in the roots, rapid bulking, early harvesting, shade tolerance, and tolerance to cassava mosaic virus as compared to its diploid counterpart (Sreekumari et al. 1996). Similarly, a triploid variety of *Coccinia grandis* (little gourd), developed by Suresh Babu and Rajan (2001), produces larger, tastier, and less stringent fruits than the diploids.

Regeneration of plants from endosperm has opened up a direct approach to raise triploids. However, so far this technique has not been exploited commercially.

10.6 Concluding Remarks

There is enough experimental evidence to suggest that endosperm cells are totipotent, and plants can be regenerated from mature and immature endosperm tissues. Therefore, the technique of endosperm culture holds great potential in raising triploids of crop plants with endospermous or nonendospermous seeds.

10.7 Appendix

Protocol for the production of mulberry triploids (*after* Thomas et al. 2000)

(i) Collect immature seeds (20 DAP) from the donor plants and wash them thoroughly in 1 % Savlon solution. After rinsing in 70 % ethanol for 15 s surface sterilize them in 0.1 % mercuric chloride solution for 7 min.

(ii) Excise endosperm with intact embryo from the surface sterilized seeds and culture them on MS + 5 μM BAP + 1 μM NAA. Incubate the cultures in light.

(iii) After a week, discard the germinated embryo and incubate the cultures in dark.

(iv) After 5 weeks, transfer the callused endosperm to MS + 5 μM 2,4-D for the maintenance of endosperm callus cultures. Maintain the cultures in dark.

(v) To regenerate shoots from the callus, transfer it to MS + 5 μM BAP + 1 μM NAA.

(vi) After 4 weeks, excise 3–4 cm long shoots and transfer them, individually, to 1/2 MS + 7.5 μM IBA for rooting.

(vii) After about 4 weeks, wash the roots of the plants and transfer them to small pots or polythene bag and maintain them under high humidity chambers for hardening.

Suggested Further Reading

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Zygotic Embryo Culture 11

11.1 Introduction

The zygote (fertilized egg), through a series of well-defined developmental stages, forms an embryo (Fig. 11.1) the progenitor of the next generation. Several physical and chemical factors regulate the growth and development of the embryo. The surrounding tissues, especially the endosperm, also control the predetermined pattern of embryo development. Any disturbance in these factors causes abnormalities and, in extreme cases, abortion of the embryo. In vitro culture of excised zygotic embryos at different stages of development has provided useful information on the developmental and physiological aspects of embryogenesis. Embryo culture is one of the in vitro techniques, which found a practical application even before it was well established. It is now widely used to produce rare hybrids which are normally not possible due to premature abortion of the embryo. This chapter describes the technique of embryo culture, its importance in understanding the physiological and developmental aspects of embryogenesis and, finally, its applied aspects.

The first systematic attempt to grow the embryos of angiosperms in vitro under aseptic conditions was made by Hannig (1904). He cultured excised mature embryos of Brassicaceae members, *Cochlearia danica, Raphanus caudatus, R. landra* and *R. sativus* on a mineral salt medium supplemented with sucrose and obtained transplantable seedlings. Dieterich (1924) observed that on a semi-solid medium containing minerals and 2.5–5 % sucrose, mature embryos of several plants showed normal growth, but the embryos excised from immature seeds failed to achieve the organization of a mature embryo. These, instead, grew directly into seedlings, skipping the intermediary stages of embryogenesis. Dieterich described this phenomenon of precocious germination as 'kunstliche Fruhgeburt'. Stimulus for further work on embryo culture came from the work of Laibach (1925, 1929). In interspecific crosses between *Linum perenne* and *L. austriacum*, Laibach noted that the seeds were greatly shrivelled, very light and incapable of germination. By excising the embryos from such seeds and growing them on a moist filter paper or cotton wadding soaked in sucrose or glucose solution, he was able to raise full hybrid plants. This technique of hybrid embryo culture has since been widely used to raise numerous such hybrids which normally fail due to post-zygotic sexual incompatibility. The technique of growing embryos outside the ovule (ex ovulo embryo culture) provides an excellent opportunity to study the physical and chemical regulation of growth and differentiation during embryogenesis. Raghavan (2003) has published an excellent historic account of embryo culture in the centenary year of this technique. More recently, Thorpe and Yeung (2011) have reviewed the subject.

11.2 Technique

There are two important aspects of the embryo culture technique: (i) composition of the culture medium and (ii) excision of the embryo.

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DOI: 10.1007/978-81-322-1026-9_11, © Springer India 2013

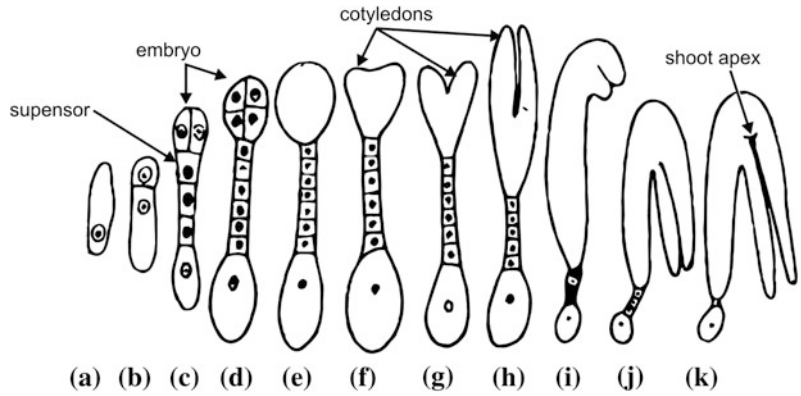

Fig. 11.1 Stages in the normal development of embryo in *Capsella bursa-pastoris*. **a** Zygote, **b** 2-celled proembryo, **c–e** Globular, **f** Early heart-shaped, **g** Late heart-shaped, **h** Torpedo-shaped, **i** Walking stick–shaped, **j** Inverted U-shaped and **k** Mature embryos (*after* Raghavan 1966)

The composition of the culture medium varies with the material and the age of the embryo to be cultured. Some of the media used for embryo culture are given in Tables 11.1, 11.2 and 11.4. For the culture of isolated zygotes and very young proembryos, the media formulations developed so far have proved inadequate. They require co-cultivation with feeder cells/nurse tissue, such as actively growing suspension cell aggregates or embryogenic microspores.

For the introduction of the technique to the students, a plant that has large seeds and whose embryos can be easily dissected out is ideal, such as some legumes and crucifers. For researchers, it would depend on the experimental objective. *Capsella bursa-pastoris* (Shepard's purse) has been a favourable material for many basic studies on embryo culture. It has racemose inflorescence, and each raceme bears flowers at various stages of development, with younger flowers at the top and older below. Each capsule bears 20–25 ovules, which are more or less at the same stage of development (Fig. 11.2a, b). When the objective is to rescue a hybrid embryo which otherwise aborts in situ, it is essential to determine the stage at which the degeneration of the embryo sets in. In such cases, the embryo is allowed to grow inside the ovule as long as possible and excised well before the abortion starts. Younger the embryo, more difficult it is to culture.

Table 11.1 Improved nutrient medium for the culture of *Capsella bursa-pastoris* embryo culture (*after* Monnier 1976, 1978)

Constituents	Amount (mg L^{-1})
KNO_3	1,900
$CaCl_2.2H_2O$	880
NH_4NO_3	825
$MgSO_4.7H_2O$	370
KCl	350
KH_2PO_4	170
Na_2.EDTA	14.9[a]
$FeSO_4.7H_2O$	11.1[a]
H_3BO_3	12.4
$MnSO_4.H_2O$	33.6
$ZnSO_4.7H_2O$	21
KI	1.66
$Na_2MoO_4.2H_2O$	0.5
$CuSO_4.5H_2O$	0.05
$CoCl_2.6H_2O$	0.05
Glutamine	400
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Sucrose	120,000
Agar	7,000

[a] 2 ml of a stock solution containing 5.57 g $FeSO_4.7H_2O$ and 7.45 g Na_2.EDTA L^{-1}

The ovules enclosing the embryo are well protected inside the ovary wall and are sterile. Therefore, the ovaries are suitably surface

Table 11.2 Composition of Kao90 medium for the culture of barley zygote (*after* Holm et al. 1994)

Constituents	Amount (mg L^{-1})	Constituents	Amount (mg L^{-1})
Inorganic nutrients			
NH_4NO_3	165	$MnSO_4.4H_2O$	22.3
KNO_3	1,900	$ZnSO_4.4H_2O$	8.6
$CaCl_2.2H_2O$	440	$Na_2MoO_4.2H_2O$	0.25
$MgSO_4.7H_2O$	370	$CuSO_4.5H_2O$	0.025
KH_2PO_4	170	$CoCl_2.6H_2O$	0.025
KI	0.83	$FeSO_4.7H_2O$	27.8
H_3BO_3	6.2	$Na_2.EDTA.2H_2O$	37.3
Organic nutrients			
Inositol	100	Biotin	0.005
Nicotinamide	1	Choline chloride	0.5
Pyridoxine HCl	1	Riboflavin	0.1
Thiamine HCl	10	Ascorbic acid	1
D-Calcium pantothenate	0.5	Vitamin A	0.005
Folic acid	0.2	Vitamin D_3	0.005
p-Aminobenzoic acid	0.01	Vitamin B_{12}	0.01
Organic acids			
Sodium pyruvate	5	Malic acid	10
Citric acid	10	Fumaric acid	10
Hormone			
2,4-D	1		
Others			
Vitamin-free casamino acid	250	Coconut water	20 ml L^{-1}
Maltose	9 %	–	–

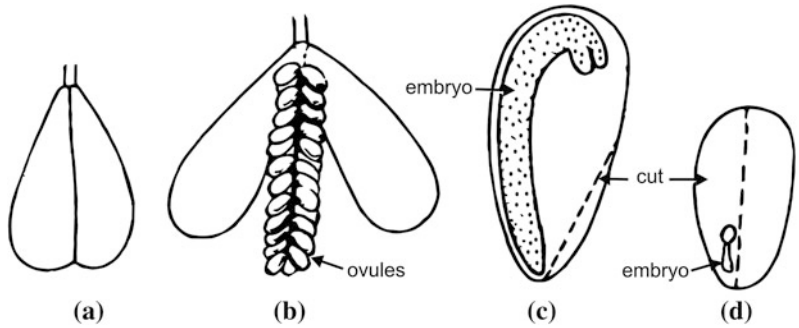

Fig. 11.2 Isolation of the embryos of *Capsella bursa-pastoris*. **a** A capsule. **b** The capsule has been opened to expose the ovules. **c** An ovule with walking stick–shaped embryo inside; the *dotted line* shows the region of incision of the ovule to release the embryo. **d** An ovule with globular embryo. A cut along the *dotted line* exposes the embryo (*after* Raghavan 1966)

sterilized, and the ovules and embryos are dissected out inside a sterile chamber. The dissections are carried out in a drop of suitable medium such as sucrose/glucose solutions or a culture medium to prevent desiccation and provide osmotic protection to the embryo.

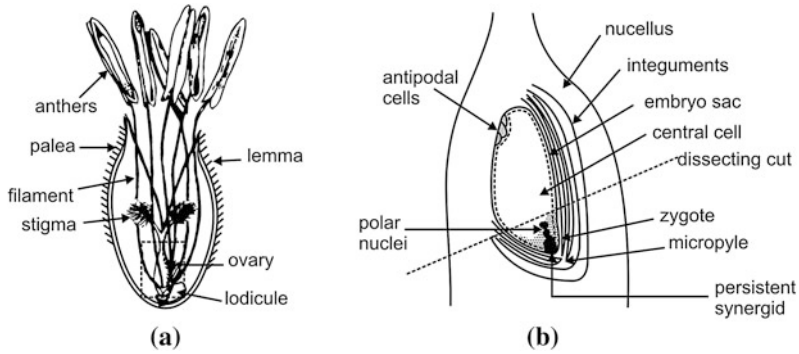

Fig. 11.3 Structure of a rice spikelet during anthesis (**a**) and dissection of zygote (**b**). The ovary emblocked in (**a**) is excised and cut open one-third its length from the micropylar end which houses the zygote (**b**). Slight pressure at the micropylar end pushes out the zygote from the cut end (*after* Zhang et al. 1999)

The procedure used for the isolation of *Capsella* embryos by Raghavan and Torrey (1963) is depicted in Fig. 11.2. The surface sterilized capsules are placed in a few drops of sterile culture medium, the outer wall is removed by an incision in the region of the placenta, and the two halves are pulled apart to expose the ovules (Fig. 11.2b) Torpedo-shaped and younger embryos are confined to longitudinal one-half of the ovule (Fig. 11.2c, d) and are clearly visible through the chalaza, either because of their green colour (older embryos) or because of transparent vesicle of their suspensor. To excise immature embryo, an ovule is placed in a drop of medium on a microscope slide and split longitudinally with the help of a sharp mounted blade (Fig. 11.2d). By carefully teasing apart the ovular tissues, the entire embryo, along with the suspensor, is carefully removed. For excising older embryos, a small incision is made in the ovule on the side lacking the embryo (Fig. 11.2c), and the embryo is pushed out by a slight pressure with a blunt needle.

To isolate zygote, time taken from pollination to fertilization or from anthesis to syngamy is determined, preferably under controlled light and temperature regimes. In cereals, the spikelets at the appropriate stage are removed, surface sterilized and washed thoroughly before mechanically isolating the zygote in a suitable medium. Whereas Holm et al. (1994), working with barley, used a modified MS medium supplemented with 9 % maltose, Zhang et al. (1999) used Kao and Michayluk (1975) medium with 9 % maltose for rice. For the isolation of rice zygote, the ovaries excised from surface-sterilized spikes were placed in a drop of the isolation medium and decapitated at approximately one-third of their length from the micropyle to open the embryo sac (Fig. 11.3a, b). The sudden pressure change in the embryo sac releases the zygote. The section of the ovary containing the zygote is then transferred to 35 mm plastic Petri dish containing 3 ml of the isolation medium. The zygote is gently pushed out of the embryo sac through the cut end of the ovary by carefully pressing from the micropylar end towards the cut end. The isolated zygotes are picked up with micropipette and transferred to culture dish. In maize, softening of nucellar tissue by a brief treatment with a mixture of cell wall–degrading enzymes prior to microdissection of zygote proved useful (Kranz and Kumlehn 1999).

11.3 Culture Requirements

Brown (1906) applied the technique of embryo culture to study the relative efficiency of various organic nitrogenous compounds on the growth of excised barley embryos and demonstrated that the amino acids, aspartate, glutamate, amide and asparagine, are superior nitrogen sources, causing increased dry weight and nitrogen content of the cultured embryos. Further studies have contributed significantly to our knowledge about

the importance of mineral nutrients, source and concentration of carbohydrate, nitrogenous compounds and growth regulators for the development of isolated zygotic embryos.

11.3.1 Mineral Nutrients

Many different mineral formulations have been used for embryo culture without much critical evaluation of the role of individual elements. Monnier (1976) studied the effect of various mineral solutions for the culture of zygotic embryos of *Capsella bursa-pastoris* and concluded that there was no correlation between the growth and survival of embryos on a particular medium. MS medium supported maximum growth, but the survival frequency of the embryos was very low. On the other hand, the Knop's medium was least toxic, but growth of the embryo was very poor. After a detailed study, Monnier developed a medium (for composition see Table 11.1) that supported as good growth of the embryo as MS and the survival was high. As compared to the MS medium, this medium has higher concentrations of K^+ and Ca^{2+} and lower level of NH_4^+ ions.

For immature embryos of many plants, such as barley (Umbeck and Norstog 1979), *Datura stramonium* (Paris et al. 1953) and *D. tatula* (Matsubara 1964), NH_4^+ was either essential or a preferred source of inorganic nitrogen. This has been ascribed to the lack of necessary enzyme to reduce NO_3^- to NH_4^+ at the early stage of embryo development.

11.3.2 Amino Acids and Vitamins

Hannig (1904) reported that asparagine was very effective in enhancing the embryo growth. Generally, glutamine has proved to be the most effective amino acid for the growth of excised embryos (Rijven 1955; Matsubara 1964). Casein hydrolysate, a complex mixture of amino acids, has been widely used as an additive to embryo culture media, especially for young embryos (Sanders and Burkholder 1948; Rangaswamy 1961).

Vitamins have been used in embryo culture media, but their presence is not always necessary.

11.3.3 Carbohydrates

Sucrose is by far the best form of carbohydrate and has been most commonly used for embryo culture. Sucrose is added to the medium not only as the source of energy but also to maintain a suitable osmolarity which is extremely important for immature embryos (Liu et al. 1993). The optimum concentration of sucrose, therefore, varies with the stage of embryo development. Mature embryos grow well with 2 % sucrose, but younger embryos require higher levels of the carbohydrate. This is in harmony with the observation that in situ, the proembryos are surrounded by a fluid of high osmolarity that gradually drops as the embryo grows (Ryczkowski 1960). Sucrose at 8–12 % is generally adequate for the culture of proembryos of *Datura* (Rietsema et al. 1953), *Hordeum* (Ziebur and Brink 1951) and *Capsella* (Monnier 1978). Artificially increasing the osmotic pressure of the culture medium by the addition of enough mannitol, in the presence of a moderate level (2 %) of sucrose, has enabled the culture of proembryos of wheat (Fischer and Neuhaus 1995) and *Capsella bursa-pastoris* (Rijven 1952).

11.3.4 Growth Regulators

Growth regulators are not always necessary for the normal development of embryo. Addition of hormones may cause structural abnormalities (Monnier 1978).

The requirement for high osmolarity of the culture medium for globular embryos (55 μm long) of *Capsella bursa-pastoris* could be substituted by a balanced combination of IAA, kinetin and adenine sulphate (Raghavan and Torrey 1963, 1964). Some other examples of growth promotion of proembryos by growth regulators are as follows: *Linum usitatissimum* by kinetin (Pretová 1986), heart-shaped embryos

of *Medicago scutellata* and *M. sativa* by IAA and BAP (Bauchan 1987) and proembryos of maize by zeatin and BAP (Matthys-Rochon 1998). Substitution of suspensor by GA_3 (see Sect. 11.5) is another example of the involvement of a growth regulator in embryo development. ABA is a well-known hormone to support morphologically and biosynthetically normal embryogenesis in cultured embryos (Fischer-Iglesias and Neuhaus 2001).

Involvement of polar auxin transport has been implied in modulating cell divisions in a localized region of the globular embryo where the cotyledons arise. Treatment of cultured globular embryos with auxin transport inhibitors (TIBA, N-1-naphthylphthalamic acid) leads to the formation of a ring-like structure around the shoot apex akin to fused cotyledons, instead of two separate cotyledons in *Brassica juncea* (Liu et al. 1993) and differentiation of additional shoots in wheat (Fischer et al. 1997).

11.3.5 Natural Plant Extracts

The use of liquid endosperm of coconut (coconut water or coconut milk) enabled van Overbeek to culture 200–500-μm embryos of *Datura stramonium,* which otherwise could not be cultured on a defined medium. This led to the suggestion that CM contains an 'Embryo Factor'. This was followed by several reports on in vitro culture of proembryos of other plants by the use of plant extracts of endospermic or non-endospermic origin or by using endosperm as the nurse tissue. Matsubara (1962) found alcohol diffusates from young seeds of *Lupine* to be as effective as CM for the culture of heart-shaped embryos (150 μm) of *Datura tatula*.

11.4 Culture of Proembryos and Zygote

The nutritional requirements of very young embryos and zygote remain undefined. Liu et al. (1993) successfully cultured 35 μm long early globular embryos (8-celled) of *Brassica juncea* using double-layer culture system of Monnier (1976, 1978), involving two complex semi-solid culture media differing only in their osmolarity. In vitro culture of excised zygotes has been possible so far only by co-cultivation with actively growing feeder suspension cells or embryogenic microspores. Holm et al. (1994) cultured zygotes of barley on a complex Kao90 medium (Table 11.2), which is a modified MS medium with NH_4NO_3 reduced to 165 mg L^{-1} and supplemented with vitamins and organic acids of the Kao and Michayluk (1975) medium, 20 ml L^{-1} liquid endosperm of coconut (CM), 250 mg L^{-1} vitamin-free casamino acids, 1 mg L^{-1} 2,4-D and 9 % maltose as the source of carbohydrate. On this medium, the isolated zygotes of barley, excised 6 days after pollination, divided a few times and formed spherical microcalli, after which the cells degenerated. However, co-cultivation with embryogenic microspores of barley (younger than 19 days old) in the liquid Kao90 medium for 23–30 days resulted in 75 % of the zygotes developing beyond the microcalli stage, and most of the cultures formed plantlets. Kumlehn et al. (1998) reported regeneration of fertile plants from isolated wheat zygotes through direct embryo differentiation with 90 % success. In contrast, only 36 % of the zygotes of a japonica cultivar (Taipei 309) of rice grew into fertile plants, which was similar to the frequency of zygote regeneration in maize (Leduc et al. 1996). In rice, the zygotes divided 24–48 h after culture and formed calli, which differentiated fertile plants only after transfer to MS medium (with 3 % maltose) supplemented with 0.4 mg L^{-1} BAP (Zhang et al. 1999).

Kumlehn et al. (1999) developed a method in which the wheat zygotes were immobilized in 100 μl droplets of 0.75 % agarose placed on a microscope slide and co-cultured with an appropriate population of embryogenic microspores in liquid medium (Fig. 11.4). The system facilitated direct observation and photographic documentation of the developmental changes during 5–40 days after pollination. The first division of

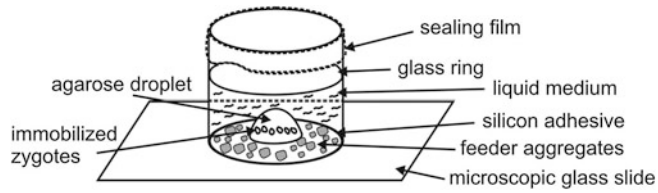

Fig. 11.4 Representation of the culture technique employed to monitor individual development of isolated wheat zygote (*after* Kumlehn et al. 1999)

the zygote was symmetrical, and it developed into a germinable normal embryo which formed fertile plants.

So far, the success with excised zygote culture is restricted to monocots. In *Arabidopsis thaliana*, as in many other dicots, in ovulo culture of zygote has been successful, but excised embryos could be cultured only after the heart-shaped stage (Sauer and Friml 2008). For the culture of in vitro formed zygotes, see Chap. 13.

11.5 Changing Growth Requirements of the Embryos

With respect to its nutrition, Raghavan (1966) recognized two phases of embryo development: (i) *Heterotrophic Phase*—the early phase of development that may last up to the globular or even a slightly later stage, during which the embryo draws upon the endosperm and suspensor for its nutrition and (b) *Autotrophic Phase*—this phase starts at the late heart-shaped stage when the embryo becomes metabolically capable of synthesizing substances necessary for its growth and morphogenesis from basic mineral salts and sugar and draws upon its own metabolites. The critical stage at which the embryo enters the autotrophic phase varies with the plant species (Rhagavan 1976). Even within the two phases, the exogenous requirements of the cultured embryo become simpler as it ages. This can be illustrated by two well-known examples, viz. *Datura* (van Overbeek et al. 1941, 1942) and *Capsella bursa-pastoris* (Raghavan 1966).

In *Datura*, excised mature embryos develop into normal seedlings on mineral salts and dextrose. Young torpedo-shaped embryos (up to 0.5 mm) require a medium containing mineral nutrients, dextrose, glycine, thiamine, ascorbic acid, nicotinic acid, vitamin B_{12}, adenine sulphate, succinic acid and pantothenic acid (basal medium). Embryos smaller than 0.5 mm in length could be cultured only with the addition of coconut milk to the basal medium. A similar increasing autonomy of *Capsella* embryos with age is evident from the data presented in Table 11.3.

As mentioned before, the osmotic value of the nutrient medium seems to be an important factor for the proper growth of the embryo. Ryczkowski (1960–1972) had shown that in

Table 11.3 Progressive nutritional autonomy during embryogenesis in *Capsella bursa-pastoris* (*after* Raghavan 1966)

Developmental stage	Length of embryo (μm)	Nutritional requirement
Early globular	21–60	Could not be cultured
Late globular	61–80	Basal medium (macronutrients + trace elements + vitamins + 2 % sucrose) + kinetin (0.002 mg L^{-1}) + IAA (0.1 mg L^{-1}) + adenine sulphate (0.001 mg L^{-1})
Heart-shaped	81–450	Basal medium alone
Torpedo-shaped	451–700	Macronutrients + vitamins + 2 % sucrose
Walking stick–shaped and mature	>700	Macronutrients + 2 % sucrose

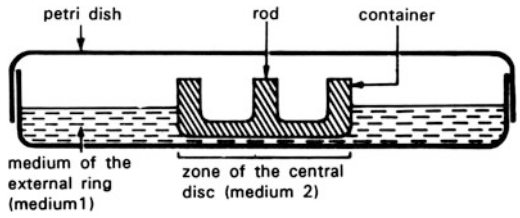

Fig. 11.5 Device allowing the juxtaposition of two media with different compositions (see Table 11.4). The first agar medium is liquified by heating and then poured around the central glass container. The medium gives the external ring. After cooling and solidification of the medium, the container is removed. In the central ring, thus formed, a second medium of a different composition is poured. The embryos are cultivated on the second medium in the central part of the Petri dish. As a result of diffusion, the embryos are subjected to the action of variable medium with time (*after* Monnier 1976, 1978)

monocots as well as in dicots, the values of osmotic pressure, viscosity, specific gravity and concentration of sugar and amino acids of the ovular sap that surrounds the developing embryo decrease with the age of the ovule. Accordingly, isolated mature embryos grow fairly well with 2 % sucrose in the culture medium, but younger embryos require higher levels of the carbohydrate for normal embryogenic development. With the lower level of sucrose, proembryos directly develop into weak seedlings displaying only those structures already present at the time of embryo excision (see Sect. 11.7).

Changing growth requirements of developing embryos necessitates their transfer from one medium to another in order to achieve their optimal in vitro growth. Monnier (1976, 1978, 1990) described a culture method which allowed complete development of 50 µm long embryos (early globular stage) of *Capsella* into embryos that germinated in the same culture plate without moving the embryos from its original position (for details see Fig. 11.5). The compositions of the two media used in the culture dish are given in Table 11.4. Following a similar procedure, Fischer and Neuhause (1995) obtained normal direct embryogenesis in the cultures of globular embryos (100–160 µm) of wheat. Liu et al. (1993) used a similar technique for ex ovulo culture of 8-celled proembryos of *Brassica juncea*.

Table 11.4 Composition of the two media used in different parts of the same Petri dish to obtain uninterrupted growth of *Capsella* embryos from the globular stage (ca. 50 µm) to maturity (*after* Monnier 1976)

Constituents	Amounts (mg L^{-1})	
	Medium 1 (external ring)	Medium 2 (central ring)
KNO_3	1,900	1,900
$CaCl_2.2H_2O$	484	1,320
NH_4NO_3	990	825
$MgSO_4.7H_2O$	407	370
KCl	420	350
KH_2PO_4	187	170
Na_2.EDTA	37.3	–
$FeSO_4.7H_2O$	27.8	–
H_3BO_3	12.4	12.4
$MnSO_4.H_2O$	33.6	33.6
$ZnSO_4.7H_2O$	21	21
KI	1.66	1.66
$Na_2MoO_4.2H_2O$	0.5	0.5
$CuSO_4.5H_2O$	0.05	0.05
$CoCl_2.6H_2O$	0.05	0.05
Glutamine	–	600
Thiamine HCl	0.1	0.1
Pyridoxine HCl	0.1	0.1
Sucrose	–	18 %
Agar (%)	0.7	0.7

11.6 Role of Suspensor in Embryo Development

Suspensor is an ephemeral structure found at the radicular end of the embryo. It attains maximum size by the globular or early heart-shaped stage. Thereafter, it starts degenerating, and in mature embryos, only remnants of suspensor may be seen. Based on detailed structural, cytological, physiological and biochemical studies, an active involvement of suspensor in early embryo development has been suggested (Bhojwani and Bhatnagar 2008). Embryo culture provides direct experimental evidence to support the nutritive role of suspensor in embryo development. Continued in vitro growth of proembryos of *Eruca sativa* (Corsi 1972), *Phaseolus coccineus* (Yeung and Sussex 1979) and *Capsella bursa-pastoris*

(Monnier 1984, 1990) is enhanced by the presence of suspensor.

Cionini et al. (1976) observed that while the older embryos (5 mm or larger) of *Phaseolus coccineus* grew equally well when cultured with or without the suspensor, in the cultures of heart-shaped or early cotyledonary stage embryos the removal of suspensor drastically reduced the frequency of plant formation. Confirming the importance of suspensor for the growth of young embryos of *P. coccineus*, Yeung and Sussex (1979) showed that intact suspensor when detached but placed in the proximity of the embryo on the culture medium greatly stimulated further development of the embryo as compared to the embryo cultured in the absence of the suspensor (Table 11.5). The growth promoting activity of the suspensor was maximal at the early heart-shaped stage of the embryo. Of the various growth regulators tested, gibberellin at a concentration of 5 mg L^{-1} most effectively substituted the suspensor. This is in accordance with the observation of Alpi et al. (1975) that gibberellin activity in the suspensor of *P. coccineus* is maximal at the heart-shaped stage.

11.7 Precocious Germination

From the viewpoint of plant physiologists and biochemists, embryo development is a continuous process starting from zygote to germination. Walbot (1978) identified five stages in embryo development (Table 11.6). Initially, the growth of the embryo is essentially by cell divisions, resulting in the formation of a proembryo with small meristematic cells. This is followed by tissue differentiation, during which the meristematic activities become localized. After its full development, the embryo accumulates reserve food and gets dehydrated, leading to a phase of metabolic quiescence and developmental arrest (dormancy) during which the embryo is normally incapable of germination.

Embryos of mangroves and some other plants germinate while still attached to the parent plant (vivipary). Excised immature embryos of several other plants when cultured on nutrient medium not only bypass the stage of dormancy but also cease to undergo further embryogenic mode of development and develop into weak seedlings displaying only those structures already present at the time of embryo excision. This phenomenon of seedling formation without completing normal embryogenic development is called precocious germination. It can be prevented and normal embryogenic development promoted in the cultures of immature embryos by increasing the osmotic value of the culture medium having moderate level of sucrose (2 %) by raising the concentration of sucrose or adding a suitable concentration of mannitol.

It has been suggested that the beneficial influence of the high osmotic pressure may be mediated through its effect on endogenous pool of growth regulators. Raghavan and Torrey (1969) noted that for continued cell divisions and growth of the globular embryos (80 μm) of *Capsella bursa-pastoris* in vitro, a combination of IAA, kinetin and adenine sulphate added to the medium with only 2 % sucrose was better than high concentration of sucrose (12–18 %). Similarly, abscisic acid has been shown to replace high level of osmoticum in preventing precocious germination of excised immature embryos of cotton (Dure 1975).

11.8 Applications

Embryo culture is one of the few techniques that found an important application, while it was still in its infancy. Over the years, it has found many other applications in basic and applied areas of plant sciences.

11.8.1 Basic Studies

Embryo culture offers a useful system to study very fundamental problems of embryogenesis, which are normally difficult to address due to difficult approach to embryo under the in situ conditions. Embryo culture has unravelled many

Table 11.5 The effect of suspensor on in vitro growth and development of *Phaseolus coccineus* embryos (*after* Yeung and Sussex 1979)

Initial stage (fresh weight in mg) ±SE	Treatment	Fresh weight[a] 10 days after culture (mg) ±SE (N)	% embryos[c] forming plants (No. of embryos cultured)
Early heart-shaped (0.87 ± 0.02)	Embryo proper only	3.9 ± 0.52 (10)	41.5 (89)
	Embryo proper with suspensor attached	8.91 ± 1.16[b] (10)	88.4 (95)
	Embryo proper with detached suspensor in direct contact	6.22 ± 0.78[b] (10)	72.5 (51)
	Embryo proper with heat-killed detached suspensor in direct contact	4.10 ± 0.43 (5)	37.0 (43)
	Embryo proper with suspensor 1 cm away	–	33.3 (30)
Late heart-shaped (1.07 ± 0.07)	Embryo proper only	17.2 ± 2.84 (5)	94.4 (18)
	Embryo proper with suspensor attached	15.4 ± 1.41 (6)	94.4 (18)
Early cotyledon (3.92 ± 0.19)	Embryo proper only	20.3 ± 2.5 (7)	100 (18)
	Embryo proper with suspensor attached	24.4 ± 2.75 (11)	100 (19)

[a] Figures in parenthesis represent the sample size
[b] significant at 1 % level
[c] Assessed 8 weeks after culture

Table 11.6 Major stages in the development of *Phaseolus* embryos (*after* Walbot 1978)

Stage	Characteristics
1. Cleavage and differentiation	Cell division with little growth; differentiation of all major tissues
2. Growth	Rapid cell expansion and division
3. Maturation	Little or no cell division or expansion; synthesis and storage of reserve material
4. Dormancy	Developmental arrest
5. Germination	Renewed cell expansion and division; embryo growth

intricacies related to embryogenesis, such as the nutrition of embryo, the physical and chemical control of orderly development of embryo, role of various parts of the embryo in its development, maturation and induction of dormancy. Embryo culture has provided experimental support to the view that suspensor plays an important role in the nutrition of proembryos. Nutritional requirements of zygote and very young proembryo still remain a challenge.

In *Arabidopsis thaliana*, ex ovulo embryo culture has been possible only after the heart-shaped stage. However, embryos as young as the 4-celled stage could be cultured in ovulo (Sauer and Friml 2004, 2008). Embryo culture is also being used to study the physiology and genetics of embryogenesis (Fischer-Iglesias and Neuhaus 2001; Schrick and Laux 2001).

Embryo culture has been applied to study morphogenesis in the cultured seeds with partially differentiated embryos and those of parasitic plants. For several crop plants, including legumes, cereals, cotton and a number of tree species, highly regenerable callus and cell cultures could be established only from immature embryos. This enabled extending the application of biotechnological methods of crop improvement to these economically important species.

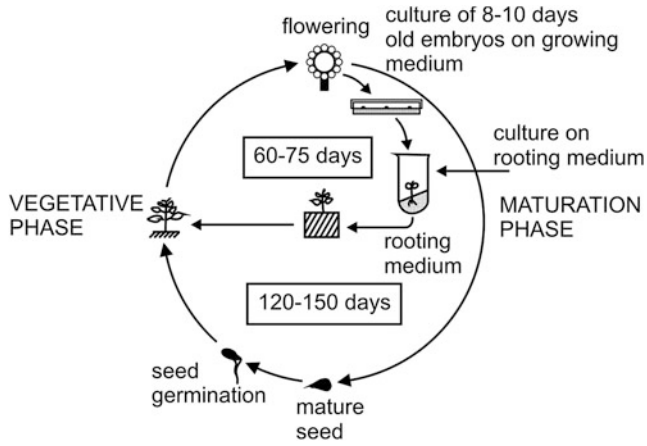

Fig. 11.6 Diagrammatized summary of immature embryo (8–10 DAP) culture to shorten the life cycle duration of sunflower from 120–150 days to 60–65 days (*after* Serieys 1992)

11.8.2 Shortening of Breeding Cycle

Breeding work on horticultural plants can be delayed due to long dormancy periods of their seeds. Excised embryo culture can circumvent the various causes of dormancy and thus reduce the period from seed to flowering. Randolph and Cox (1943) could reduce the life cycle of *Iris* from 2–3 years to less than a year by embryo culture. In cultures, the excised embryos of weeping crab apple *(Malus* sp.) start germination within 48 h, and in 4 weeks, transplantable seedlings are formed (Nickell 1951).

Seed maturation in soya bean and sunflower takes 50–60 % of the life cycle duration, which is 120–150 days (Serieys 1992). By in vitro culture of 10-day-old immature embryos of sunflower, Plotnikov (1983) could reduce the duration of the life cycle by half. Similarly, Alissa et al. (1986) and Aspiroz et al. (1988) could raise four generations of sunflower in a year by culturing 7-day-old and 10- to 18-day-old embryos, respectively (Fig. 11.6).

11.8.3 Rapid Seed Viability

The possibility of breaking seed dormancy by embryo culture also allows the use of this technique for rapid testing of the viability of a particular batch of seeds. A good correlation occurs between the growth of excised embryos of non-after-ripened seeds (freshly harvested dormant seeds) and germination of after-ripened seeds (which have completed the dormancy period) of peach (Tukey 1944). Germination of excised embryo is considered as more exact and reliable test than the commonly used staining methods for seed viability (Barton 1961).

11.8.4 Propagation of Rare Plants

As an abnormality, some coconuts develop soft, fatty tissue in place of liquid endosperm. Such nuts are called macapuno. Being rare, macapunos are expensive. Plants propagated from macapuno nuts are largely macapuno. Using the technique of excised embryo culture, De Guzman (1969) and De Guzman and Del Rosario (1974) raised plants from macapuno nuts, 85 % of which bore macapuno nuts (De Guzman et al. 1976)

11.8.5 Haploid Production

A unique application of embryo culture has been in the production of haploids through selective elimination of chromosomes, following distant

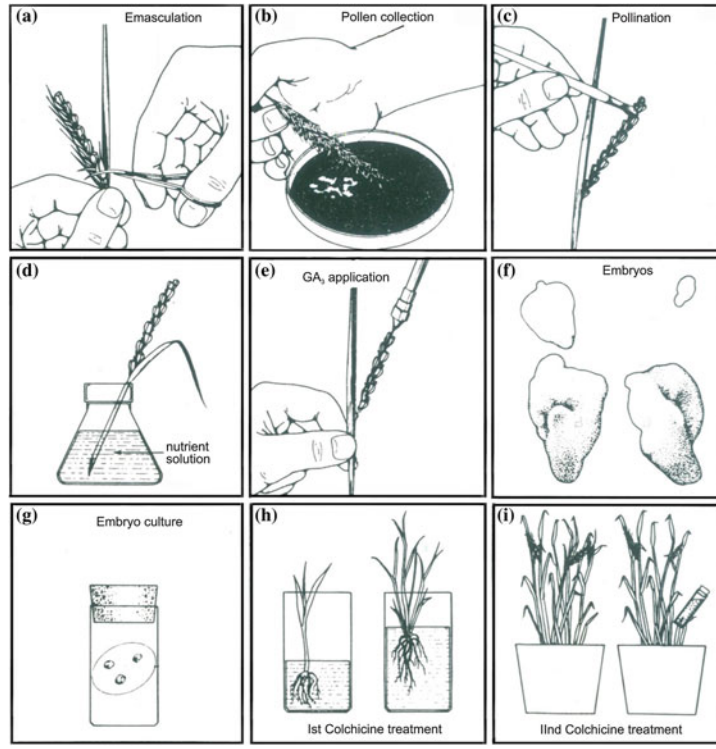

Fig. 11.7 General procedure for haploid production in barley following interspecific hybridization (*courtesy* N.C. Subrahmanyam, India)

hybridization. In the cross *Hordeum vulgare* × *H. bulbosum*, double fertilization proceeds readily, but the chromosomes of *H. bulbosum* are preferentially lost during the first few divisions of embryogenesis. As a result, haploid embryos, with single set of chromosomes of *H. vulgare*, are formed. However, the haploid embryos abort due to disintegration of endosperm 2–5 days after fertilization. In this cross, full haploid plants of barley can be raised by in vitro culture of excised immature embryo (Fig. 11.7). Wheat haploids are routinely produced by crossing it with maize, followed by immature embryo culture (Laurie and Reymondie 1991). Lei et al. (2004) reported development of haploid plants of *Cucumis sativus* by pollination with irradiated pollen, followed by embryo culture.

11.8.6 Transformation

Protoplasts of zygotes, capable of regenerating whole plants, are ideal targets for marker-free transformation by microinjection. This is particularly attractive for the agronomic crops because it avoids the toxic marker genes that have raised concern about the safety of the edible transgenic crops (Wang et al. 2006).

11.8.7 Production of Rare Hybrids

Embryo abortion is a common problem in breeding programmes. Distant crosses are often unsuccessful even after normal fertilization because the hybrid embryo aborts on the mother plant due to the failure of normal endosperm

development or embryo–ovular tissue incompatibility. In several such crosses, in vitro culture of excised embryo (ex ovulo) has been very successful in rescuing the embryo and raising full hybrid plants.

The first successful interspecific hybrid produced with the aid of embryo culture was in the genus *Linum* by Laibach (1925). In these crosses, the embryo was unable to grow to maturity in situ, but when it was removed from the seed and cultured on nutrient medium, it developed into a full plant. This work of Laibach laid the foundation of a method to surmount post-zygotic barriers to crossability where fertilization occurs normally. The technique has become a routine tool in the hands of plant breeders to enhance the scope of hybridization to raise new genotypes.

For instance, hybrids between *Triticum* and *Aegilops* were attempted to introduce resistance to leaf rust in wheat. However, pollination of wheat with *Aegilops* pollen yielded only very few and mostly abortive seeds. A large number of hybrid plants could, however, be raised by means of embryo culture (Chueca et al. 1977). Utilizing the same technique, Ben Rajeb and Bendalis (1989) could make a cross between *Phaseolus coccineus* and *P. acutifolium*, providing useful drought-resistant hybrids. Similarly, the interspecific cross *P. vulgaris* x *P. lunatus*, which normally fails due to abortion of embryo after the heart-shaped stage, could be successfully made with the aid of embryo culture (Kuboyama et al. 1991).

All attempts to cross *Lilium henryi* and *L. regale* were unsuccessful until the embryo culture technique was adopted (Skirm 1942). The seeds obtained on crossing *L. speciosum album* and *L. auratum* enclosed a large embryo and endosperm. However, during the storage of the seeds or soaking them in water, the embryo degenerated due to embryo–endosperm incompatibility. By growing excised immature embryos, Emsweller and Uhring (1962) could raise full hybrid plants from this cross.

The cross *Lycopersicon esculentum* × *L. peruvianum* is highly desirable with the view to transfer pest- and disease-resistant traits from *L. peruvianum* to *L. esculentum*. However, in this cross, fertilization occurs normally, but the embryo aborts, and no viable seeds are formed. Smith (1944), Chowdhury (1955) and Alexander (1956) obtained hybrid plants from this cross through embryo culture. Thomas and Pratt (1981) also raised hybrids from this cross but found that the poorly developed embryo excised 35 days after cross-pollination callused, followed by differentiation of plants. This embryo-callus approach has also yielded hybrids from the crosses *L. esculentum* × *L. chilense* and *L. esculentum* × *Solanum lycopersicoides*, in which embryos capable of direct plant formation do not develop (Scott and Stevens; cited in Thomas and Pratt 1981).

Where embryo abortion occurs at an early stage when it is either difficult to excise and/or culture the embryo in isolation, in ovulo embryo culture (ovule culture) has been used to rescue the hybrid embryo. In the cross *Gossypium arboreum* × *G. hirsutum*, the hybrid embryo develops only up to 8–10 days after pollination. Efforts to raise full plants by culturing excised embryo were unsuccessful (Beasley 1940; Weaver 1958). Pundir (1967) excised the ovules 3 days after cross-pollination, when they contained zygote or a 3-celled proembryo and cultured them on MS medium containing 50 mg L^{-1} inositol. By the 5th week, in culture, fully differentiated embryos were formed, and by the 7th week, 70–80 % ovules developed hybrid seedlings. Following a similar technique, Steward and Hsu (1978) raised four different interspecifc hybrids in the genus *Gossypium*, which were otherwise unknown.

The cross *Trifolium repens* × *T. hybridum* has not been successful by the conventional or by embryo rescue method. However, excising the ovules 12–14 days after pollination and culturing them on a medium containing 15 % cucumber juice for 5–6 days provide culturable embryos

(Przywara et al. 1989). Similarly, Espinasse et al. (1991) succeeded in obtaining some hybrid plants from the crosses *Helianthus annuus* × *H. maximiliani* and *H. annuus* × *H. tuberosum* with a combination of in ovulo embryo culture for 1 week, followed by excised embryo culture.

11.9 Concluding Remarks

Zygotic embryo culture is a well-established in vitro technique being routinely used for the production of rare hybrids that could not be raised by the conventional method of hybridization due to post-zygotic sexual incompatibility between the parents. This application of embryo culture was demonstrated as early as in 1925 when the technique of tissue culture in general and embryo culture in particular was not well developed. During 1960s and 1970s, considerable work on embryo culture was done to understand the nutritional and hormonal requirements of zygotic embryos of different developmental stages. It is now possible to rescue hybrid embryos which abort even at the globular stage of development, and the technique of hybrid embryo culture has become an integral part of the techniques of plant breeding.

It has been possible to successfully culture isolated in vivo and in vitro formed zygotes, but not without supplementing the medium with a suitable nurse tissue. The role of nurse tissue, however, remains to be understood.

Over the years, zygotic embryo culture has found many other applications, such as haploid production by distant hybridization, shortening of breeding cycle, propagation of rare plants, in vitro fertilization (Chap. 13) and understanding the physiology and developmental genetics of embryogenesis (Schrick and Laux 2001; Bhojwani and Bhatnagar 2008).

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Somaclonal Variation 12

12.1 Introduction

The terminally differentiated cells in plants, such as pith cells and cortical cells, often exhibit cytological and genetic changes (D'Amato 1990) that go unnoticed and do not cause abnormalities in the plant because they do not form part of the germ line cells. However, in cultures these cells may be induced to undergo redifferentiation and express the inherent variability at the whole plant level. Additional variations may be induced by the culture conditions. While some of these variations are transient physiological and developmental changes (e.g., rejuvenation), others are a result of epigenetic changes which can be relatively stable but are not transmitted to the seed progeny. Some other in vitro induced variations are caused by specific genetic changes or mutations and are transmitted to the progeny. The genetic and epigenetic changes observed in plants regenerated from cultured somatic cells are referred to as *somaclonal variation* (*soma* = vegetative, *clone* = identical copy), a term coined by Larkin and Scowcroft (1981). For distinction, the variation observed in the cultures of gametic cells is termed *gametoclonal variation* (Evans et al. 1984). The other terms used in this context are *calliclones* (somaclones from callus cultures) and *protoclones* (somaclones from protoplast cultures). In broader sense, somaclonal variation also encompasses the changes observed at the level of cell cultures that have been utilized to select cell lines tolerant/resistant to salt (Ochatt et al. 1999), heavy metals (Chakravarty and Srivastava 1997), diseases (Jones 1990), and herbicides (Smith and Chaledd 1990) or for higher productivity of industrial phytochemicals (Fujita et al. 1985).

Variations in plants regenerated from tissue cultures have been observed for a very long time, but until the beginning of 1970s these off-types were ignored as abnormalities. However, since the first formal report of morphological variations in the plants regenerated in tissue cultures of sugarcane (Heinz and Mee 1971) this phenomenon has proved to be of wide occurrence.

The occurrence of somaclonal variation is, both, a boon and a bane. When the objective is clonal multiplication of valuable elite clones, this variation can result in unwanted off-types that reduces the commercial value of the propagules. Similarly, genetically engineered plants are carefully screened to avoid unwanted and unintended somaclonal variation so that the commercially released clones have only beneficial effects from the transgene. On the other hand, the somaclonal variation may yield desirable genotypes as novel cell lines or plants of agronomic and commercial advantages.

Sugarcane is one of the plants in which the somaclonal variants were first observed (Heinz and Mee 1971). Since then several useful variants of sugarcane have been isolated in Fiji, Hawaii, India, and Taiwan. Larkin and Scowcroft (1981) reviewed the scattered literature on somaclonal variation and emphasized that the

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_12, © Springer India 2013

Table 12.1 Some examples of somaclonal variants released as new cultivars[a]

Crop	Somaclonal trait	Cultivar name	Reference
Barley	Improved yield and Downey Mildew resistance	AC Malone	Larkin (2004)
Wheat	Improved yield and agronomy	HeZu 8	Gao et al. (1991)
Potato	Reduced tuber browning	White Baron	Arihara et al. (1995)
Blackberry	Thornless	Lincoln Logan	Hall et al. (1986)
Flax	Salt and heat tolerance	Andro	O'Conn et al. (1991)
Celery	*Fusarium* resistance	UC-TC	Heath-Pagliuso and Rappaport (1990)
	Fusarium yellows resistance	MSU-SHK5	Lacy et al. (1996)
Tomato	*Fusarium* resistance	DNAP-17	Evans (1989)
Rice	*Picularia* resistance and Improved cooking quality	DAMA	Heszky et al. (1989), Heszky and Kiss (1992)
Sugarcane	Disease resistance and higher yield	Ono	Daub (1986)
	Early, high sucrose and high yielding	Co 94012	Jalaja et al. (2006)
Geranium	Vigorous plants with attractive flowers	Velvet Rose	Skirvin and Janick (1976)
Brown mustard	Yield	Pusa Jai Kisan	Katiyar and Chopra (1995)
Sweet potato	Color, shape, and baking quality of roots	Scarlet	Moyer and Collins (1983)
Banana	Fusarium wilt resistant and high yielding	Tai-Chiao No. 1	Hwang and Ko (2004)
	Fusarium wilt resistant, higher yield	Formosana	Hwang and Ko (2004)
	Resistant to Yellow Sigatoka disease	CIEN BTA-03	Trujillo and Garcia (1996)
Amaryllus	Red and White double color flowers	Suryakiran (IARI)	IARI Newlett.

[a] Based on Larkin (2004)

variation in tissue culture derived plants could serve as a novel source of rich variability suitable for development of new plant varieties. The first International Symposium devoted to somaclonal variation and their potential role in crop improvement was held in Belgium (Semel 1986). During the past 25 years, somaclonal variations for karyotype, isozyme pattern, precocity for bearing, ploidy level, growth, yield, quality, pigmentation, disease resistance, and resistance to adverse soil and climatic conditions have all been reported in different plant species (Patil and Navale 2000). A limited number of new cultivars of important crop plants have been released through somaclonal variant selection (Table 12. 1).

This chapter describes the causes and cytological and molecular basis of somaclonal variants and some examples of improved varieties, lines, and genotypes of crop plants selected utilizing the tissue culture derived variability.

12.2 Technique

Somaclonal variation provides a novel and exciting option for obtaining increased genetic variability relatively rapidly and without sophisticated technology. It may be especially valuable for limited improvement of an existing crop plant, because new traits can appear without disturbing the array of desirable traits that

are already present, unlike the traditional plant breeding, which shuffles all the traits of the plants that are bred.

In practice, to create somaclonal variation, the explants (root, leaf or stem segments) are placed on a suitable culture medium that supports cell proliferation. The tissue soon looses its identity due to dedifferentiation of its cells and grows as an unorganized callus mass. After a period of proliferation, the callus differentiates plants on the same medium or after transfer to a different medium. If necessary, the callus is multiplied on the proliferation medium for several cycles to enhance the degree of variation, before subjecting it to plant regeneration treatment. The regenerated plants are hardened and transferred to the field as R_0 plants. The R_0 plants are screened for various agronomic traits, and the individuals with minor changes of interest in the presence of the original desirable traits of the cultivar are selected. The selected plants are multiplied and their seed progeny is screened for the stability of somaclonal traits. Many of the recessive mutations induced in vitro may not be visible in the R_0 generation. In finger millet, the R_0 somaclones exhibited developmental and growth lag compared to the parent cultivar but none of the plants of R_1 generation exhibited pronounced developmental aberrations, although they differed in height, number of spikelets, aerial biomass, and in terms of maturity as compared to the parent form (Baer et al. 2007). It is, therefore, recommended that selection is made at the R_1 or R_2 generation.

For a number of traits, such as resistance to fungal toxins, herbicides, a pollutant, salt concentration and extreme of temperatures, as well as for increased production of secondary metabolites enrichment of somaclonal mutants can be made during cell/tissue cultures. The advantage of this approach is that millions of cells can be challenged with the desired stress and the surviving lines selected in a very small place under controlled conditions with small inputs of efforts and resources. However, the stability of the traits for which a cell line has been selected must be checked at the level of whole plants regenerated from them, because not all phenotypes selected at the cell level would express at the whole plant level. Moreover, not all genes of interest may be active at the single cell level.

12.3 Methods to Assess Somaclonal Variation

Earlier, evaluation of somaclonal variation was based on cytological and phenotypic analyses. However, during the past two decades a number of molecular techniques (RFLP, RAPD, and AFLP), based on the use of polymerase chain reaction (PCR), have become available for quick detection and characterization of somaclonal variation at the DNA level and are being used widely (Gostimsky et al. 2005). RFLP (Restriction Fragment Length polymorphism) was the first molecular technique applied to DNA sequence somaclonal variants. It requires the use of probes that hybridize to known sequences. RAPD (Randomly Amplified Polymorphic DNA) is based on the premise that, because of its complexity, eukaryotic nuclear DNA may contain paired random segments that are complementary to single decanucleotides and these segments have the correct orientation and are located close to each other. RAPD–PCR uses single primers of arbitrary oligonucleotide sequence to initiate DNA synthesis. Muhammad and Othman (2005) concluded that characterization of somaclonal variants requires more than one DNA marker system to detect variation in diverse components of the genome. They used RAPD and IRAP (Inter-Retrotransposon Amplified Polymorphism) to characterize somaclonal variants of banana.

Another PCR-based technique, applied to study somaclonal variation is AFLP (Amplified Fragment Length Polymorphism; Polanco and Ruiz 2002). It is a DNA-finger printing procedure based on a selective PCR amplification of fragments from restriction digestion of genomic DNA.

12.4 Origin of Somaclonal Variation

The variability observed in tissue culture derived plants is a combined effect of the genetic heterogeneity of the cells of the initial explant and the genetic and epigenetic variations occurring during the course of culture. In general, the in vitro conditions could be very stressful on plant cells and may set in motion highly mutagenic processes during explant establishment, callus induction, and plant regeneration (Lörz et al. 1988). The in vitro factors that induce somaclonal variation are the age of the culture, mode and rate of in vitro multiplication etc. The genotype of the donor plant also affects the degree of somaclonal variation induced by culture conditions.

12.4.1 Pre-Existing Variability

The extent of variation originating from the explant is dependent on, among other things, the age (Brossard 1975; Castorena Sanchez et al. 1988) and type of tissue and organ (Sree Ramulu and Dijkhuis 1986), genotype of the donor plant (Krikorian et al. 1993), and even the mode of cultivation of the donor plant (Pijnacker et al. 1989). Polyploid plants with higher chromosome number exhibit greater variability in plants regenerated in vitro (Watson et al. 1992; Skirvin et al. 1994).

In most of the angiosperms (90 %), normal development of plant is accompanied by a range of direct changes in nuclear DNA (D'Amato 1990). In the apical meristems (root tip and shoot tip), where cells divide by normal mitotic divisions, the cells are maintained at a uniform diploid level. However, their derivatives during the process of differentiation into specialized tissues may undergo duplication and endoreduplication to various degrees, resulting in somatic cells with 4C, 8C, or even higher levels of DNA. This phenomenon of polyploidization of body cells is termed *polysomaty*. In cucumber, most of the organs exhibit polysomaty, which is dependent on the (Gilissen et al. 1993). *Crepis capillaris, Dendrophthoe falcata, Helianthus annuus, Helianthus tuberosus,* and *Lilium longiflorum* are some of the nonpolysomatic species. Torrey (1965) demonstrated, by autoradiographic studies that in root cultures of pea the first set of tetraploid mitoses were derived from the explant.

Another type of pre-existing chromosomal variability, viz. aneusomaty, occurs only rarely in hybrids and polyploids of recent origin. Every individual of sugarcane clone H50-7209 showed chromosomal number mosaicism ($2n = 108$–128). In this plant the apical meristems, and consequently the mature tissues comprise mosaic of cells with varying proportion of different aneuploid chromosome numbers. The aneusomaty is frequently enhanced in callus cultures derived from such tissues.

Breakdown of genetic chimera during callusing or direct differentiation of adventitious organs may be another source of somaclonal variation, particularly in vegetatively propagated species. Highly differentiated tissues such as roots, leaves, and stems generally produce more variation than explants with the pre-existing meristems, such as axillary buds and shoot tips. However, in banana somaclonal variation occurred with higher frequencies in the plants derived from shoot tip cultures of cv. Grand Naine (5.3 %) than those derived through somatic embryogenesis (0.5–3.6 %) (Shchukin et al. 1998), which could be due to the chimeric nature of the shoot tip, and dissociation of the chimera in cultures resulting in the recovery of variants (Israeli et al. 1996).

The extent of variation contributed by the explant is expected to vary with the source of the explant. Accordingly, meristematic and embryonic tissues are likely to yield more stable cells and regenerated plants than explants comprising mature and differentiated cells. In pineapple, all the plants regenerated from syncarp (compound ovary) or slip (small shoot just below the fruit) calli were variants while only 7 % of the plants obtained from the crown (shoot at the top of the fruit) exhibited variations (Wakasa 1979). Similarly, many of the plants regenerated from seed callus of *Cymbopogon* sp. were atypical but

those obtained from inflorescence callus closely resembled the parents, with only a negligible variation (Chandra and Sreenath 1982). The plants obtained directly from spadix explants of *Anthurium scherzerianum* were less variant than those produced from leaf explants (Geier 1987). Similarly, in geranium the plants from in vivo stem cuttings were uniform but those from in vivo root and petiole cuttings were quite variable (Skirvin and Janick 1976). In potato, cotyledon-derived protoplasts produce more tetraploids than leaf-derived protoplasts (Osifo et al. 1989). In cucumber, the plants micropropagated from meristem lacked variation, and very low variation occurred in direct regeneration from leaf explants (Plader et al. 1998). However, a high frequency of changes was recorded in plants directly regenerated from protoplasts.

12.4.2 In Vitro Induced Variations

Excision of tissue from the stable environment of the plant body and its transfer to culture medium under artificial conditions inside the culture vial could be a traumatic shock, causing a range of abnormalities, such as numerical and structural changes in the chromosomes, organization of DNA, mutations and so on. A direct evidence of tissue culture induced variation is the occurrence of chromosomal changes in the cultured tissues of nonpolysomatic species, such as *C. capillaris* and in cultures derived from single cells or protoplasts. The representational difference analysis of DNA fragment sequence studies have revealed that while some portions of the genome are more susceptible to stress and show higher rearrangement and mutation rates than other portions of the genome (Thomas et al. 2002). The occurrence of hot spots of instability in the genome is also suggested by Olmos et al. (2002). Therefore, somaclonal variation may not be a random process. Some of the factors known to induce somaclonal variation under in vitro conditions are: (1) age of the culture, (2) culture medium, especially growth regulators, (3) growth habit of the tissue, and (4) genotype of the donor plant.

In vitro culture conditions and rapid multiplication of a tissue affect its genetic stability and lead to somaclonal variation. The frequency of variation increases with increase in the number of multiplication cycles, probably due to increased mutation rate with each cell cycles and/or accumulation of mutations over a period of time. In shoot tip cultures of banana cv. Nainco somaclonal variation appeared after the fifth subculture, its frequency increased thereafter (Rodrigues et al. 1998). Shepherd et al. (1996) reported similar observations with two other cultivars of banana.

The culture medium, especially the growth regulators, is one of the various factors suspected to be the cause of somaclonal variation. In tissue cultures 2,4-D has proved highly mutagenic. It either induces polyploidy or selectively favors division of polyploid cells. In the presence of 2,4-D, suspension cultures of *Haplopappus* changed from wholly diploid state to wholly tetraploid state within 6 months (Sunderland 1977). Substitution of 2,4-D by NAA or IAA reduced chromosomal aberrations (Chand and Roy 1980). Interestingly, in pea 2,4-D at hormonal concentration (0.25 mg L^{-1}) increased polyploid mitoses but at herbicidal concentration (20 mg L^{-1}) favored divisions in diploid cells. High concentrations of BAP and kinetin in cultures are also shown to induce genetic variability (Trujillo and Garcia 1996). Embryogenic suspension cultures of cucumber in auxin (2,4-D)-containing medium showed greater variation than those in cytokinin-containing medium (Ładyżyński et al. 2002).

In vitro clonal propagation of oil palm (*Elaeis guineensis*), a monoecious tree crop, was developed in 1970s but several forms of somaclonal variation, particularly in the form of floral structures regularly occurred in the micropropagated plants. A common floral abnormality observed is "mantled phenotype" (Fig. 12.1), which refers to feminization of male parts in flowers of both sexes (Corley et al. 1986), resulting in the reduction of productivity or total

loss of harvest due to abortion of abnormal fruits (Duval et al. 1995; Eeuwens et al. 2002). The intensity of abnormality varies with the nature of the embryogenic calli from which the plants are recovered. Whereas slow growing, nodular and compact calli produce, on an average, 5 % variant palms, the plants derived from fast growing calli exhibited up to 100 % variations, thus demonstrating the importance of the rate of callus growth and the nature of the callus in the determination of trueness to type of the regenerants.

12.5 Mechanisms Underlying Somaclonal Variation

Among the mechanisms causing somaclonal variation are numerical and morphological changes of chromosomes, gene mutations, gene amplification, changes in extranuclear genes, activation of transposable elements and DNA hypomethylation. The latter two, are responsible for epigenetic changes.

12.5.1 Changes in Chromosome Number and Structure

Ploidy changes, including polyploidy, aneuploidy, or mixoploidy, are one of the most commonly observed changes associated with the plants regenerated from cultured cells (D'Amato 1985; Geier 1991). In the polysomatic species, the polyploid population of cells may arise from the explant or induced by the culture conditions. With the exception of plants of hybrid origin (*Saccharum officinarum*; Heinz et al. 1969) and polyploids of recent origin, aneuploidy does not occur in nature but is not a rare feature in cultured cells. Selection of certain types of cells in culture plays a significant role in establishing a dominant karyotype or modal chromosomal number. In mixed cultures of diploid and tetraploid cell lines of carrot (both lines show identical initial growth rates in monoculture) the frequency of tetraploid cells gradually increased, and finally the cultures attained tetraploid mode (Smith and Street 1974).

The banana somaclone variant CIEN BTA-03, selected for its resistance to Yellow Sigatoka (YS) disease from tissue cultures of the susceptible cv. Williams (Trujillo and Garcia 1996), did not exhibit much difference from the parent cultivar in terms of DNA sequence polymorphism as analyzed by RAPD. However, the number of chromosomes and DNA content of the somaclones was higher than the parent cultivar (Giménez et al. 2001). Both, the parent cultivar and the somaclone were mosaic of binucleate, aneuploid, and polyploidy cells and cells with laggard chromosomes. However, the somaclone exhibited 65 % cells with more and 35 % cells with less number of chromosomes than the normal chromosome number ($2n = 3x = 33$) as compared to 22 and 78 % cells, respectively, for the parent cultivar.

The regeneration process itself acts as a screen to eliminate a portion of varying karyotypes. Generally, a strong selection acts in favor of diploids or at least euploids during plant regeneration from callus and suspension cultures. Selection may occur at different stages in the regeneration process. Some of the cells with chromosomal variation may be totally impaired in their regeneration process. The shoots regenerated from potato protoplasts showed significant difference in their rooting ability depending on whether they were euploid (good rooting) or aneuploid (poor rooting) (Fish and Karp 1986). The plants regenerated from mesocotyl segments of Durum wheat, within 10–15 days of culture, showed chromosome number mosaicism in their root tips and shoot tips, comprising hypohaploids, haploids, and hypodiploid cells besides the diploid cells (Bennici and D'Amato 1978). Aneusomaty persisted until advanced stage of spike development but was completely eliminated before meiosis in micro- and megaspore mother cells (Lupi et al. 1981).

Structural changes in chromosomes due to deletion (loss of genes), inversion (alteration of gene order), duplication (duplication of genes), and translocation (movement of chromosome

segments to new location) could also bring about somaclonal variation without altering the chromosome number.

12.5.2 Gene Mutations

Several somaclonal variants due to recessive or dominant single or multiple gene mutations have been reported. Recessive mutations may not express in R_0 generation of somaclones but can be detected in their selfed progeny.

Through conventional genetic complementation tests of several tomato plants regenerated from leaf callus, 13 distinct gene mutations have been well characterized and mapped to specific loci on the chromosome (Evans and Sharp 1983, 1986; Evans and Bravo 1986). The yellow fruit (chromosome 31), orange fruit (chromosome 10), and jointless pedicel (chromosome 11) were recessive single gene mutations while the *Fusarium* Race 2 resistance (chromosome 11) was a single dominant gene mutation. Somaclones showing single gene recessive mutations are also reported for rice (Fukui 1983; Sun et al. 1983), tobacco (Prat 1983), and maize (Edallo et al. 1981). In red clover, variation was due to additive effects of mutated genes (Keyes et al. 1980).

It has been suggested that somaclonal variation may not be a random phenomenon as is generally perceived. There is specific labile portion of the genome that is especially susceptible to stress and prone to higher mutation rates than others during in vitro culture (Thomas et al. 2002).

12.5.3 Amplification of DNA

Enhancement of herbicide resistance by a factor of 20 to 100 folds in alfalfa cell lines was associated with 4 to 11-fold amplification of glutamine synthase (GS) gene and consequential 3 to 7-fold increase in GS enzyme (Donn et al. 1984). Similarly, selection of tobacco cell lines tolerant to glyphosate herbicide was accompanied by amplification of the gene for 5-enolpyrovylshikimate-3-phosphate synthase (EPSPS). The elevated level of EPSPS mRNA was maintained even in the absence of the herbicide, suggesting that the change was a stable genetic modification (Goldsbrough et al. 1990).

12.5.4 Hypomethylation of DNA

In oil palm, the calli or embryos forming abnormal plants do not show any change in DNA content or organization. Moreover, the abnormal phenotypes revert to normal types up to 100 % in the case of slightly mantled individuals and up to 50 % in the case of severely mantled ones (Fig. 12.1) after 9 years in the field (Rival et al. 1998). The high frequency with which this abnormality occurs in independent lines and the high reversal rate suggests that the abnormal phenotype is due to epigenetic changes. It gains support from the observations that a correlation occurs between DNA hypomethylation and the mantled somaclonal variation (Jaligot et al. 2000).

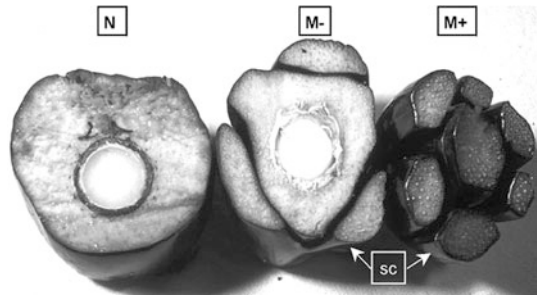

Fig. 12.1 Cross-sections of fruits from normal (*N*), slightly mantled (*M*−) and severely mantled (*M*+) somatic embryo-derived oil palm. (*sc* supernumerary carpels) (*after* Jaligot et al. 2000)

Alwee et al. (2006) reported that transcription activity of B-type palm oil MADS box genes, such as EgMADS16, specifying the stamen and carpel structure may be causal or at least instrumental in the formation of the mantled flowers.

12.5.5 Activation of Transposable Elements

Transposons or transposable elements (also called jumping genes) are mobile DNA segments that can move around in the genome and get inserted into coding regions and modify gene expression. It has been suggested that tissue culture stress can activate the previously silent transposable elements and cause somaclonal variation (Hirochika et al. 1996).

Activation of maize transposable elements in vitro has been reported more than once. The maize plants regenerated from tissue cultures were found to contain an active *Ac* element, whereas none had been detected in the initial explants (Evola et al. 1985; Peschke et al. 1987; Phillips et al. 1990). Evola et al. (1984) had earlier observed activation of Spn (*En*) element in half of the regenerated plants of maize. More than 20 % of the alfalfa plants regenerated from tissue cultures of a white flowered somaclone exhibited the wild-type purple flowered phenotype (Groose and Bingham 1986). Genetic analysis indicated that while the wild type and mutated alleles were stable and sexually transmitted the culture process appeared to trigger reversion, suggesting the involvement of transposable elements.

12.6 Applications

Somaclonal variability can be regarded as an efficient tool for breeders to create new plant varieties. Somaclonal variation can provide genotypes that can be directly used as new improved cultivars or as additional genetic variability suitable for combining with the conventional breeding methods to accelerate the process of creating new superior varieties. Somaclonal variation occurs for traits of both nuclear and cytoplasmic origin.

Tissue culture-induced variation does not have the socioethical hurdle like GM crops. Moreover, it does not require sophisticated technologies nor has significant technology ownership issue that has become problematic with genetic engineering. Further, genetic engineering has been largely successful with herbaceous species and has still not been commercialized in perennial woody species. Some examples of useful somaclones selected to develop new improved varieties or genotypes of crop plants are described in this section.

12.6.1 Sugarcane

The potential use of somaclonal variation in crop improvement was first demonstrated in sugarcane (Heinz et al. 1977). Plants regenerated from calli of sugarcane exhibit marked phenotypic and genotypic variation, such as cane yield, tillering, fiber content and numerous fine morphological features. Nickell and his co-workers in Hawaii and Krishnamurthi and his associates in Fiji isolated, through tissue culture, several subclones of well-established local cultivars which showed resistance to eyespot disease (caused by *Helminthosporium sacchari*) and, Fiji disease (caused by an aphid transmitted virus) and downy mildew (caused by *Sclerospora sacchari*).

Krishnamurthi (1974) and Krishnamurthi and Tlaskal (1974) isolated several somaclones of the sugarcane cultivar Pindar resistant to Fiji disease. While most of the resistant clones gave poor yield, the clone *Pindar 70-31*, which was also resistant to downy mildew, performed as well or even better than the parent cultivar which was highly susceptible to Fiji disease. The disease resistance of the somaclone was maintained through several cane generations in the field. The somaclone *Pindar 70-31* was released as a new cultivar (see Daub 1986).

Doule (2006) identified several somaclones of sugarcane for higher cane yield, sugar content and millable quality. From the plants regenerated from four-subculture-old leaf callus of the Indian cv. *CoC671*, Jalata et al. (2006) released an improved variety of sugarcane (*Co 94012*). It is an early, high sugar and high yielding variety with high CCS yield.

12.6.2 Banana

Banana and plantain (*Musa* sp.) are the fourth most important crop of the world. *Fusarium oxysporum* is a devastating fungal pathogen affecting banana plantations. Sexual breeding of banana for resistance to *Fusarium* wilt is hampered because of its triploid and sterile nature and the production of parthenocarpic fruits, besides being a vegetatively propagated crop. Although in nature, the vegetatively propagated population is genetically stable the micropropagated plants from shoot tips exhibit 3 % variation. Somaclonal variations have been observed for height, variegated leaves, reduced lamina, early flowering, disease resistance and so on. Taiwan Banana Institute obtained many useful somaclones from the major local cultivar Giant Cavendish (Hwang and Ko 2004), which showed resistance to *Fusarium* wilt and was also high yielding. It has been claimed that release of the somaclone "*Tai Chiao No.1*" for commercial planting in 1990 saved Taiwan banana industry from destruction by *Fusarium* wilt.

Hwang and Ko (2004) planted about 20,000 in vitro propagated plants of Giant Cavendish in pathogen infested nursery with a titer of 300–1,000 propagules of *F. oxysporum* per gram of the soil. After 1 year 28 clones survived. Cleaned suckers from all the surviving clones were replanted in the disease nursery. Six of these clones survived and remained healthy after 1 year. Plants from second- and third-generation suckers from the six resistant clones maintained high resistance to the pathogenic fungus as compared to the parent cultivar. Large number of plants raised in vitro from the six resistant clones were inferior with regard to the horticultural traits such as excessive height, week petiole, lengthened growth period, and inferior fruit quality. In subsequent generations, some of the vegetatively multiplied plants of the resistant clones showed improved fruit quality but none of them were suitable for commercial planting. However, through a similar experiment with the suckers of in vitro raised Giant Cavendish plants from different locations in Taiwan a clone (*GCT CV-215-1*) with high resistance to *Fusarium* wilt was isolated and released as cv. *Tai Chiao No. 1* in 1990. This somaclonal cultivar was not only resistant to *Fusarium* but also produced fruit bunches with same number of hands and fingers as Giant Cavendish, although its fruit bunches were slightly lighter because of short and slender fingers as compared to the parent cultivar. However, its fruit was acceptable not only locally but also in Japan. A serious drawback of this cultivar was that the mature plants were taller and slender than the Giant Cavendish, and therefore susceptible to breakage by wind during typhoon season. It also has a longer crop life. Therefore, search for a better clone continued. Another somaclone (*GCT CV-218*) was selected from in vitro raised plants, which, besides being equally resistant to *Fusarium* wilt, surpassed in many horticultural traits such as robust pseudostem, stronger petiole, thicker leaves, better hand formation, and more uniform hand size. Above all, the fruit bunches of this somaclone were 50 % heavier than the Giant Cavendish (Fig. 12.2). This somaclone became very popular and rapidly replaced the cv. *Tai Chiao No. 1* for commercial planting. It was released as cv. *Formosana*, in 2002.

Trujillo and García (1996) reported the selection of a tetraploid somaclone (*CIEN BTA-03*) of banana resistant to YS disease through adventitious bud regeneration from sucker shoot tip cultures of the triploid cv. Williams susceptible to the YS disease. The resistance was maintained over 5 years of asexual reproduction.

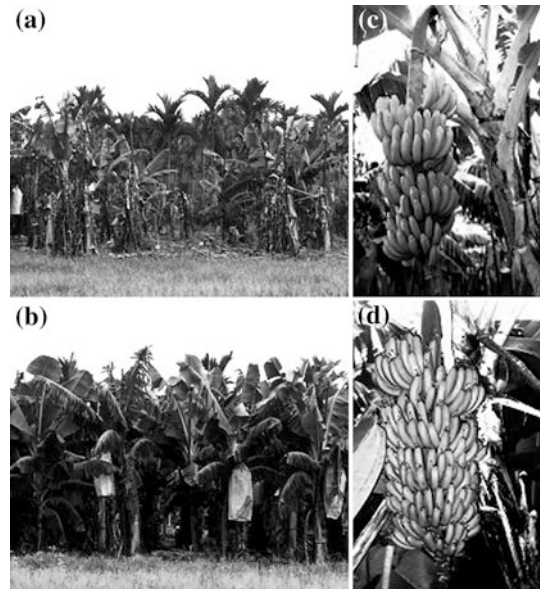

Fig. 12.2 Comparison of parental cultivar of banana, Giant Cavendish (**a**) and its somaclone, *Formosana* (**b**) planted in a *Fusarium oxysporum* infested orchard, along with their fruit bunches; **c** Giant Cavendish, **d** *Formosana* (*after* Hwang and Ko 2004)

12.6.3 Geranium

This ornamental plant is traditionally propagated from leaf cuttings. Skirvin and Janick (1976) compared the plant populations raised from root, stem, and petiole cuttings in vivo and those differentiated from their calli. Whereas the plants from in vivo stem cuttings were uniform, those from in vivo root and petiole cuttings and from callus were quite variable. They released a new scented variety '*Velvet Rose*' from a calliclone obtained from the cv. '*Rober's Lemon Rose*' (Skirvin and Janick 1976). The new cultivar had double the number of chromosomes of the parent and was selected for its general attractiveness and vigor.

12.6.4 Potato

Being highly heterozygous and sexually sterile, most of the popular cultivars of potato are not readily amenable to conventional breeding. In this regard, somaclonal variation is of special significance for this important crop plant.

Somaclones of potato have been selected for improved foliar characters, tuber number and shape, starch content, earliness, and disease resistance. Many of the traits were stable through potato generation in the field. The degree of variation depends not only on the procedure used to regenerate plants but also on the genotype and specific characters of the donor plants (Thieme and Griess 2005).

Shepard et al. (1980) screened 2,500 plants regenerated from the protoplasts of potato cv. Russet Burbank and identified about 60 protoclones with stable improved agronomic traits, including resistance to late blight (caused by *Phytophthera infestans*) and early blight (caused by *Alternaria solani*). Gunn and Day (1986) reported protoclones of potato which had tuber yield equal to or better than the parent cultivar and were resistant to the common scab. Somaclones of potato resistant to Potato Virus Y (PVY) and Potato Leaf Roll Virus have also been isolated (Thompson et al. 1986; Semal and Lepoivre 1990).

Due to difficulties in performing sexual crosses and because of the tetraploid nature of

most of the potato cultivars no genetic analysis of potato somaclones have been made.

12.6.5 Rice

Several reports of the selection of somaclonal variants from somatic and gametic tissue cultures of rice have been published (Oono 1978; Chaleff 1980; Chen et al. 1980; Fukui 1983). Oono (1988) reported that in 72 % of the 762 regenerated lines that were screened variation were noted for such characters as seed viability, plant height, tillering and chlorophyll deficiency, and the somaclonal variants were stable through, at least, one seed generation. From a single callus formed in seed cultures of rice, Fukui (1983) raised 12 plants with 90 % fertility. The progeny of these plants showed variation for leaf color, early heading, albino, and short culm. The author has concluded that the mutation for the four traits occurred independently and successively.

Anther and pollen culture are good sources of variation. The pollen plants being haploid stable variants can be obtained in one generation. Schaeffer et al. (1984) obtained useful variants by selfing anther-derived plants of rice. Chinese scientists have produced new varieties of many cereals, including rice, through anther culture. Through a combination of anther culture and somatic tissue culture, Heszky and Kiss (1992) evolved an improved variety of rice 'DAMA'. The Pollen Haploid Somaclone (PHS) method is summarized in Fig. 12.3.

12.6.6 Mustard

Several species and types of Brassicas are important oilseed crops, vegetables, forage crops, and are used in the production of condiments. The oilseed Brassicas are found within *Brassica juncea*, *Brassica carinata*, *Brassica rapa* (syn. *Brassica campestris*), and *Brassica napus* and are collectively referred to as oilseed

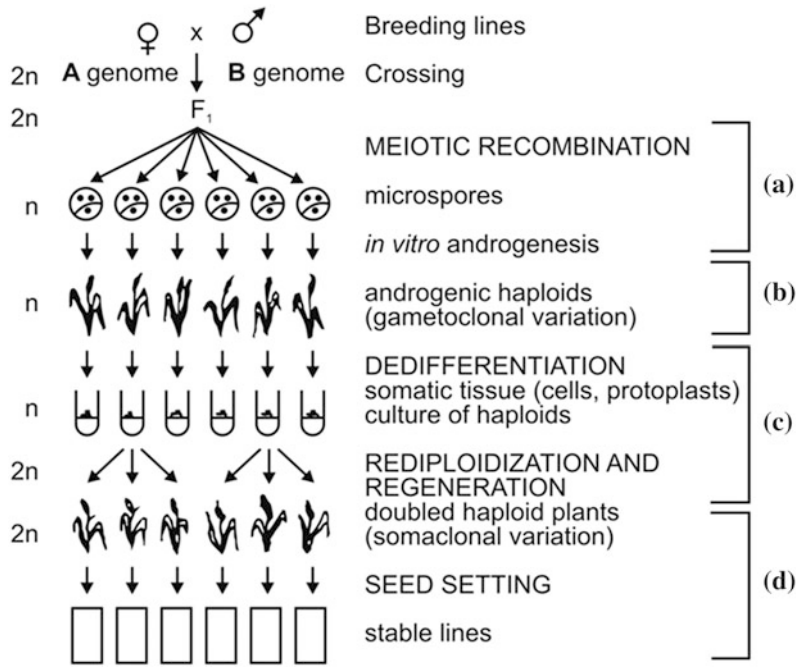

Fig. 12.3 Diagrammatized summary of the Pollen Haploid Somaclonal method (PHS method) for increasing genetic variability in breeding material (*after* Heszky et al. 1989)

rape. George et al. (1987) reported yellow-seeded variants in the progeny of the plants regenerated from cotyledonary explants of *B. juncea* cv. *TM-4*. A large variation for all the characters evaluated were also observed in the R_1 progeny of plants regenerated from cotyledon callus of *B. juncea* cv. Prakash by Jain et al. (1989). Some of the plants also showed significantly higher yield and other improved agriculturally important characteristics compared with the control. Somaclonal variation led to the selection of a dwarf mutant and true breeding lines in the R_2 generation.

Nehnevajova et al. (2007) regenerated plants from in vitro selected metal (Cd, Cu, Pb) tolerant callus lines of *B. juncea* and found that 7 out of the 30 individuals extracted significantly higher amounts of metals than the control plants. Such lines could be useful for phytoremediation of toxic metals.

A somaclone of *B. juncea* cv. Varuna, called *Pusa Jai Kisan* (*BIO 902*), was released for commercial cultivation in 1994 (Katiyar and Chopra 1995). It was regenerated from seedling callus. The new cultivar is an improvement over the earlier ones in terms of higher yield (20 %), early maturity (6–8 days) and seed boldness. It has become immensely popular among farmers even in the zones for which it was not recommended.

12.6.7 Tomato

Somaclonal variants of tomato for several characters, such as fruit color, plant architecture, and characters for mechanical harvesting, have been isolated by Evans and Sharp (1983; see also Evans et al. 1984; Evans and Bravo 1986). Detailed genetic analysis of these variants revealed that stable genetic changes were caused by single gene mutations, fruit color being recessive and *Fusarium* resistance a dominant trait. One of the somaclones of tomato, with very high (20 %) dry matter content and enhanced taste and better texture and color was registered as a new variety by DNA Plant Technology Corp., USA (Evans 1989).

12.6.8 Finger Millet

Baer et al. (2007) screened 30 lines of regenerants from callus cultures of Finger millet (*Eleusine caracana*) of which the somaclone *SE-7* was most promising due to the high yield of biomass and seeds, rapid and good seed germination at low temperatures (15 and 20 °C) and early maturing (by 10–20 days) as compared to the parent plant. These changes were not associated with any detectable alteration in the number and morphology of chromosomes. It is regarded as a promising somaclone to select a new cultivar of finger millet.

12.7 Concluding Remarks

Plant tissue cultures exhibit considerable variations in their morphology, growth rate and regeneration potential, which increase with the age of the culture. It is the combined effect of the pre-existing aberrant cells within the explant used to initiate the cultures and the changes induced by the culture environment. Many tissues which exhibit good regeneration of plants in the initial stages of culture lose this morphogenetic potential at a later stage. In cereals, irrespective of the nature of the explant, two types of calli are formed: (1) white, off-white, or pale yellow, compact and often nodular, and (2) soft, granular and translucent. Of these, only the first type of calli exhibit somatic embryogenesis. In successive cultures, the embryogenic calli continue to produce some amount of nonembryogenic calli. The loss of morphogenic potential in long-term cultures, a phenomenon of wide occurrence, could be due to altered hormonal balance of the cells or their sensitivity to exogenous growth regulators. The cultured plant cells undergo varying degrees of cytological and genetic changes.

Table 12.2 Advantages and disadvantages of somaclonal variation

S.No.	Disadvantages	Advantages
1.	May not occur for complex agronomic traits	Changes can occur in agronomically important traits
2.	Many characters change in the opposite or negative direction	Changes occur at a high frequency
3.	Variations are unpredictable in nature	Some changes can be novel and may not be achieved by conventional breeding
4.	In vitro selected somaclones may not be genetically stable	In vitro selection helps in isolation of lines tolerant to biotic and abiotic stresses
5.	Selected somaclones require extensive field testing	In vitro selection shorten the time in somaclone isolation with desirable trait
6.	Somaclones could be unstable due to DNA methylation and transposon elements	Large population of cells can be used for in vitro selection

Appearance of off-types during in vitro multiplication of horticultural and forest species, regeneration of plants from genetically engineered cells, or during industrial production of phytochemicals by cell culture is a serious limitation in the practical applications of plant tissue culture technique. Understanding the causes and the mechanisms underlying the occurrence of somaclonal variation has helped minimizing/eliminating the appearance of aberrant types during micropropagation.

On the positive side, induced variation continues to be the best option for the improvement of perennial crops, especially those propagated asexually. Somaclonal variation has proved to be an additional source of variation useful for modifying one or a few characters of a cultivar or genotype while preserving its genetically determined traits. Indeed, several superior cultivars of crop plants have been developed from the variability generated in tissue cultures. An added advantage of tissue culture variation is the selection of cell lines resistant to certain biotic and abiotic stresses with the economy of space and time. Some of the advantages and disadvantages of somaclonal variation are listed in Table 12.2.

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In Vitro Pollination and Fertilization 13

13.1 Introduction

In angiosperms, the female gamete (egg) is formed and remains fixed at the micropylar end of the embryo sac deeply embedded in the sporophytic tissues of the ovule, which is enclosed in the ovary well removed from the stigma. The male gametes (sperms) are enclosed in the pollen grain. To effect fertilization, the pollen germinate on the stigma to form a pollen tube that transports the two non-motile sperms to the embryo sac and delivers them in the vicinity of the egg. Whereas one of the sperms fertilizes the egg (syngamy), the other fertilizes the central cell (triple fusion). The fertilized egg (zygote) develops into an embryo, the progenitor of the next generation, and the fertilized central cell forms the endosperm tissue, the main source of nutrition for the developing and germinating embryo. Thus, in the angiosperms, the gametes, the process of double fertilization, zygote, early stages of embryo and endosperm development are not readily accessible to study the cellular and molecular aspects of fertilization and embryogenesis. Therefore, for almost 100 years since the discovery of double fertilization in angiosperms, by Nawaschin (1898), not much progress could be made in this area. Whatever little information is known is based mainly on mutant analysis in *Arabidopsis*.

Sexual incompatibility is a serious handicap in developing desirable hybrids. In this, the pollen either fail to germinate on the stigma or growth of the pollen tube gets arrested before it reaches the ovary. To overcome these problems, several in vivo and in vitro techniques have been developed. One of the methods to bypass prezygotic barriers to crossability, developed in the early 1960s, is In Vitro Pollination (IVP). With further sophistication of equipment and refinement of microtechniques, in 1990s, it became possible to perform In Vitro Fertilization (IVF) in higher plants (Bhojwani and Raste 1996; Kranz 2001; Kranz et al. 2004, 2008; Wang et al. 2006). IVF technique is proving to be an invaluable approach to directly observe and analyse fertilization and post-fertilization processes in flowering plants (Wang et al. 2006). This chapter describes the technique of IVF after a brief introduction to IVP.

13.2 In Vitro Pollination (IVP)

As early as 1926, Dahlgren reported fertilization and seed development in *Codonopsis ovata* following in vitro pollination of the stump after cutting off the style at its base. That was perhaps the beginning of what almost 40 years later developed into the technique of IVP (Kanta et al. 1962).

IVP of excised pistils (stigmatic pollination) resulting in the production of viable seeds has been achieved following self (*Antirrhinum majus*) as well as cross-pollination (*Nicotiana rustica, Petunia violacea*). The presence of intact calyx lobes and pedicel was found to be essential for viable seed formation following in vitro stigmatic pollination in *Trifolium*

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_13, © Springer India 2013

species. Considerable success has been achieved with IVP and IVF in maize (Bhojwani and Raste 1996; Kranz and Kumlehn 1999; Okamoto and Kranz 2005; Kranz and Scholten 2008). Dhaliwal and King (1978) and Raman et al. (1980) reported seed-set following both stigmatic and ovular IVP in maize.

13.2.1 Terminology

Kanta et al. (1962) and, following them, several other authors (Zenkteler 1965, 1967, 1970; Balatkova and Tupy 1968; Rangaswamy and Shivanna 1969) have described the technique of seed development through IVP of ovules as "Test Tube Fertilization". Seed development following stigmatic pollination of cultured pistils has been referred to as "In Vitro Pollination". However, in either case, fertilization of egg occurs inside the ovule by sperms delivered by the pollen tube almost in a natural fashion. Strictly speaking, the term "Test Tube Fertilization" or "In Vitro Fertilization" should, as in animals, refer to plant regeneration through in vitro fusion of isolated single egg and sperm cells as reported, first time, by Kranz et al. (1991; see also Kranz and Lörz 1993). Therefore, in this chapter, in vitro application of pollen to excised ovules is referred to as "In Vitro Ovular Pollination", to the ovules attached to a piece of placenta as "In Vitro Placental Pollination" and to the stigma of intact pistil as "In Vitro Stigmatic Pollination" under the general term of in vitro pollination (Fig. 13.1).

13.2.2 Technique

The success with IVP depends, to a great extent, on a thorough knowledge of the floral biology of the experimental materials, such as time of anthesis, the time of anther dehiscence, viability of pollen grains, viability of the female gametophyte, receptivity of stigma to pollen grains, pollen germination and pollen tube growth, fertilization and embryo and endosperm development. If the

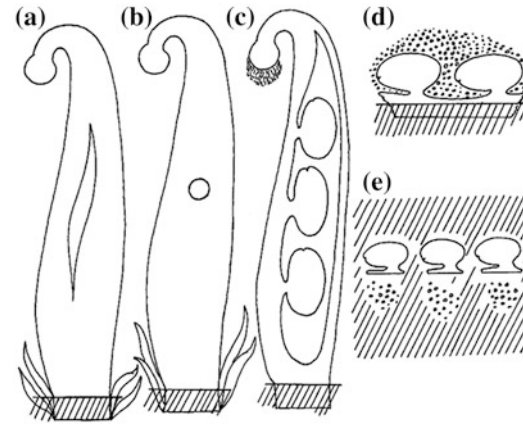

Fig. 13.1 Diagrams depicting various types of in vitro pollination. **a**, **b** Intra-ovarian pollination; pollen suspension is injected into the ovary through a hole or a slit. **c** Stigmatic pollination; pollen are applied to the stigma of a cultured pistil. **d** Placental pollination; pollen are dusted on the ovules cultured along with a piece of placental tissue. **e** Ovular pollination; individual ovules are cultured and pollinated

flowering seasons of the desired parents are different or they are growing in geographically different locations, suitable methods for pollen storage and transport need to be adopted. The choice of nutrient media to support optimal pollen germination and pollen tube growth as well as development of in vitro fertilized ovules to form viable seeds is most critical. In some instances, full development of seeds and embryos may require a transfer of ovules, 7–10 days after IVP, to fresh medium of a different formulation (Marubashi and Nakajima 1985; Bino et al. 1992).

13.2.3 Preparation of Explant

In order to prevent chance pollination, flower buds of the female parent are emasculated and bagged. The buds are excised 1–4 days after anthesis and prepared for aseptic culture. Both, sepals and petals are removed, and the pistil with pedicel rinsed quickly in 70 % ethanol, surface sterilized with suitable sterilizing agent and, finally, washed thoroughly with sterile distilled water before planting them on the culture medium under aseptic conditions. For in vitro

stigmatic pollination, the excised pistils are sterilized without wetting the stigma and wiped dry with a sterile filter paper before implanting them on the culture medium.

For placental and ovular pollination, the stigma and style are removed and the ovary wall is peeled off to expose the ovules. The whole placenta, bearing the ovules on a short pedicel, is generally used for placental pollination. The placenta may also be divided into two or more pieces, each carrying a certain number of ovules and cultured individually. In species such as *Trifolium*, where removing the ovary wall or cutting a slit along the length of the ovary wall causes browning of the ovules, the best method to achieve IVP is to introduce pollen grains into the ovary through a pore in the ovary wall (in vitro intra-ovarian pollination).

For collecting pollen, undehisced anthers are aseptically removed from surface-sterilized flower buds and kept in sterile Petri plates until dehiscence. When anthers are taken from already opened flowers, they need to be surface sterilized and kept on a filter paper inside a sterilized Petri plate. The pollen grains are aseptically collected and deposited on the ovules or the stigma as the case may be. Pollen deposited on the ovules or placenta have performed better than those placed on the medium around the ovules.

The procedure described above may require modification according to the type of experimental material. In maize, where ovaries are well protected by several layers of husks, surface sterilization is not necessary. Ears are bagged prior to the emergence of silks (styles) to prevent uncontrolled pollination. Such bagged ears are collected 2–6 days after the emergence of silks. The protruding silks are excised and all but the innermost husks are removed. The removal of the innermost husk and all subsequent operations are performed aseptically. Pieces of cob, each carrying a total of 4–10 ovaries in two rows are placed on the medium so that their silks hang out of the Petri plate after replacing the lid. It is also possible to keep this plate inside a larger Petri plate so that the silks are contained within it. The silks are pollinated, not necessarily aseptically. Twenty-four hours after pollination, the silks are clipped off and the Petri plate sealed with Parafilm. Several reports have described single-pollen pollination in maize using this technique.

For in vitro pollination of ovules, pollen are deposited directly on the ovules either attached to the placenta or detached from it. However, mostly the ovules are left attached to the placenta as the pollination of detached ovules has not been successful except in *Brassica oleracea* (Kameya et al. 1966; Kameya and Hinata 1970). In vitro self-pollination has been attempted in 47 species belonging to 34 genera, and viable seeds have been obtained in 28 species (Bhojwani and Raste 1996). Interspecific, intergeneric and interfamily in vitro cross-pollination of ovules has been attempted in 37 crosses, but success has been achieved in only 16 combinations.

13.2.4 Factors Affecting Seed-set Following IVP

The number of viable seeds produced is the measure of success in IVP. Some of the factors that affect the success of the technique are the nature of the explant, culture medium and the culture conditions.

In *Petunia axillaris,* excised ovules or pieces of placenta each bearing a group of ovules did not set viable seeds following IVP. However, pollination of the ovules on intact placenta resulted in normal germination of pollen and development of viable seeds. In *P. hybrida*, even the removal of stigma and style proved deleterious. In maize, the number of kernels formed on the pieces of cob after IVP was directly related to the number of ovaries attached to the explant and the amount of accompanying cob tissue (Gengenbach 1977). The ovular and stigmatic tissues in the explant should be dry. Traces of water at the site of pollination lead to pollen bursting. The ovules for IVP should be excised 1–2 days after anthesis instead of on the day of anthesis; for maize, the optimal stage of spike for IVP is 3–4 days after silking. The

physiological state of pistil is affected by pollination and pollen tube growth. In many cases, unfertilized ovules excised from pollinated pistils responded better than those from unpollinated pistils.

The efficacy of IVP, to a large extent, depends on the composition of the culture medium, which should support pollen germination and pollen tube growth to cause fertilization and, secondly, development of the fertilized ovules into mature seeds with a viable embryo. At times, it may be necessary to transfer the fertilized ovules, 7–10 days after IVP, to a different medium to support normal seed development. It is, therefore, imperative to optimize, before proceeding with IVP, the nutritional and hormonal requirements of fertilized ovules (containing zygote or 2-celled proembryo) of the plant to be used as the female parent.

The genotype of the parents also influences the response. The mineral salts commonly used in the medium for IVP are those prescribed by Nitsch (1951) for ovary culture. To this, sucrose and vitamins are added as recommended by White (1943). The composition of the modified Nitsch's medium widely employed for IVP is given in Table 13.1. The addition of growth regulators and supplements to this medium needs to be optimized for every system.

The in vitro pollinated ovule cultures are generally kept in dark at normal culture room temperature (25 °C).

Table 13.1 Composition of the modified Nitsch's medium widely used for the culture of in vitro pollinated ovules

Constituents	Amount (mg L^{-1})
$Ca(NO_3)_2.4H_2O$	500
KNO_3	125
KH_2PO_4	125
$MgSO_4.7H_2O$	125
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.025
$ZnSO_4.7H_2O$	0.5
$MnSO_4.4H_2O$	3.0
H_3BO_3	0.5
$FeC_6O_5H_7.5H_2O$	10.00
Glycine	7.5
Ca-Pantothenate	0.25
Pyridoxine.HCl	0.25
Thiamine.HCl	0.25
Niacin	1.25
Sucrose	50,000.00
Agar	7,000.00

after Kanta and Maheshwari (1963)

13.3 In Vitro Fertilization (IVF)

Unlike higher plants, gametes of animals and lower plants can be easily obtained in large numbers because they are either released from the body for fertilization in the surrounding water (Fucoid algae, sea urchin, frogs, fishes) or fertilization occurs in the lumen of the oviduct (mammals). Therefore, IVF in these organisms has made considerable progress and is being used routinely for animals and human beings but it remained elusive in higher plants because in the latter, the gametes remain enclosed in the gametophyte. The female gametophyte, enclosing the egg cell, is deeply embedded in the sporophytic tissues, which makes its examination and manipulation difficult. IVF in these plants requires isolation of live eggs and sperms and their in vitro fusion followed by the culture of in vitro zygotes to regenerate full plants via embryogenesis or organogenesis. Difficulties in isolating gametes impeded our understanding of gamete physiology, cellular and molecular aspects of fertilization, activation of zygote and early embryogenesis in flowering plants.

Kranz et al. (1991) reported the first successful in vitro fertilization experiments with an angiosperm. The zygotes generated in vitro by electrofusion of isolated egg and sperm cells of maize formed multicellular colonies in cultures. Two years later, Kranz and Lörz (1993) succeeded in regenerating fertile plants from the in vitro zygotes of maize (Fig. 13.6). With this, the first complete IVF system was established. The only other angiosperm for which complete IVF system has been developed is rice (Uchiumi

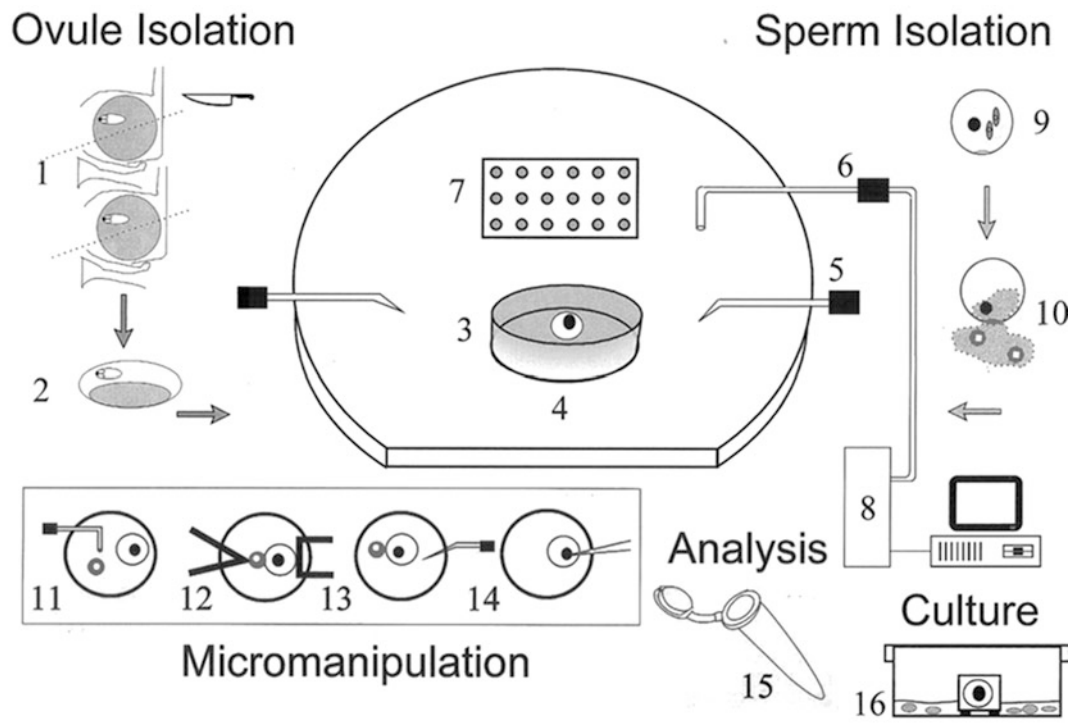

Fig. 13.2 Diagrammatic summary of the procedure and set-up for isolation, manipulation, analysis and culture of single maize gametes and zygote. Nucellar tissue pieces containing the embryo sac are dissected (*1*, *2*). The tissue pieces, collected in a Petri dish (*3*), are transferred into an insert of a sliding stage (*4*). Individual embryo sacs and cells of the embryo sac are isolated manually with two needles (*5*). Isolated cells are transferred with a capillary (*6*) into microdroplets on a cover slip (*7*) using a computerized pump (*8*). Manual transfer of cells using a microcapillary connected with a small tube is also possible. Subsequently, sperm cells are isolated from pollen grains (*9*) by osmotic shock (*10*) in a plastic dish, selected under microscopic observation and transferred to microdroplet on the cover slip (*7*). Manipulations are performed in microdroplets: staining (*11*), electrofusion (*12*), calcium or PEG-mediated fusion (*13*) or microinjection (*14*). Individual cells are transferred by a microcapillary into tubes (*15*), for further biochemical studies, or into an insert which is previously placed into larger plastic dish for growth (*16*). The larger plastic dish is filled with feeder cell suspension (*courtesy* Prof. Erhard Kranz, Germany)

et al. 2006, 2007). Unlike the rice system, in maize, endosperm development from in vitro fertilized central cell has also been successful (Fig. 13.6; Kranz and Lörz 1993). The only other system in which in vitro double fertilization has been accomplished is tobacco (Tian and Russell 1997; Sun et al. 2000). In tobacco, polyethylene glycol solution stimulated the fusion of sperm cell with egg cell and central cell, but the fusion products did not continue their further development (Tian and Russell 1997; Sun et al. 2000). Kovács et al. (1995) were able to fuse pairs of isolated male and female gametes of wheat using the same IVF technique. The in vitro formed zygotes divided to form multicellular colonies which failed to develop further.

In vitro fertilization involves three basic microtechniques (Fig. 13.2): (1) isolation, handling and selection of egg cell, sperm cell and central cell, (2) in vitro fusion of isolated sperm cell with egg cell or central cell and (3) culture of the in vitro formed zygotes and primary endosperm cells (both single cells).

13.3.1 Isolation of Egg, Central Cell and Sperms

Methods have been developed for the isolation of sperm and egg cells for a wide range of plants, including maize, wheat, rice, rye grass, barley (all monocots), *Brassica napus*, *Plumbago zeylanica*, *Nicotiana tabacum* and *Alstromeria* (Dicots; Uchiumi et al. 2007). Healthy and well-grown gamete donor plants are used.

(i) *Sperm cells.* The first attempt to isolate male gametes of *Hordeum vulgare* was made by Cass in 1973, but mass isolation of live sperms in *Plumbago zeylanica* was achieved 13 years later by Russell (1986). Since then, the technique has been considerably refined and the isolation of viable sperm cells has been reported for several angiosperms (Theunis et al. 1991; Southworth 2001).

The osmotic potential of the vegetative cell is lower than that of the sperm cells enclosed in it (Southworth 2001). Taking advantage of this feature, sperms are isolated by placing the pollen (for 3-celled pollen) or germinated pollen (for 2-celled pollen) in a solution with osmotic potential lower than the vegetative cell cytoplasm but isotonic or hypertonic to sperm cells. In practice, the pollen or pollen tubes are burst by osmotic shock, and the sperms are selected under microscopic observation (Fig. 13.3a, b).

For the isolation of rice sperms, the anthers were harvested from flowers before anthesis and transferred to 0.3 M mannitol in plastic dishes, and the tissue was then torn with a forceps to free the pollen grains. After 3–5 min, the pollen grains burst and their contents, including the two sperms, were released. For the bursting of maize pollen, mannitol solution of the osmotic potential 540 mosmol kg^{-1} H_2O (Kranz and Lörz 1993) or 650 mosmol kg^{-1} H_2O (Hoshino et al. 2004) was used. Sperms can also be isolated by gentle grinding of the pollen grains in a tissue homogeniser (Southworth 2001). Since the sperms are protoplasts, they become spherical on release. The size of the sperms is around 7 μm.

(ii) *Egg cell.* The female gametes can be isolated mechanically (Holm et al. 1994; Kovács et al. 1994; Kranz et al. 1995; Katoh et al. 1997; Kumlehn et al. 1997) but softening the ovular tissues by treatment with cell wall-degrading enzymes (cellulase, hemicellulase, pectinase and pectolyase) before mechanical isolation facilitates the process and avoids damage to the gametic protoplasts (Kranz 2001). The enzyme treatment should be short to avoid spontaneous fusion of egg cell with other protoplasts of the embryo sac or nucellar protoplasts. The nucellar tissue softened by enzyme treatment is dissected with fine-tipped glass needles. The mechanical step is essential because the enzymes do not

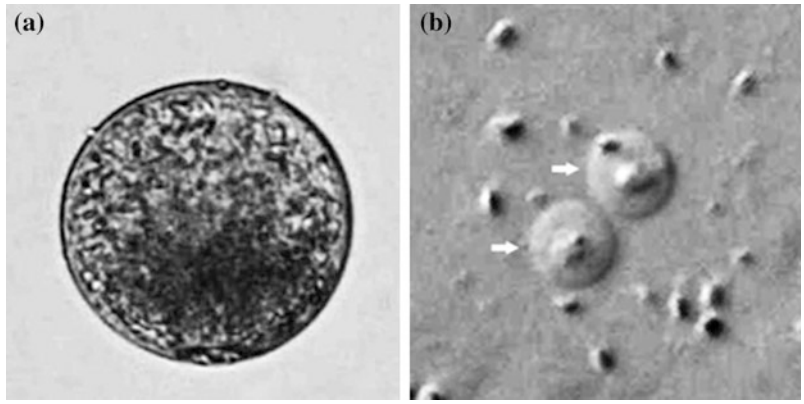

Fig. 13.3 Isolation of sperm cells of rice. **a** A pollen grain before releasing its contents. **b** A pollen grain, burst by immersion in 0.3 M mannitol solution has released its contents, including the two sperms (*arrow marked*). The two sperm cells generally remain side-by-side (*courtesy*, Prof. Takashi Okamoto, Japan)

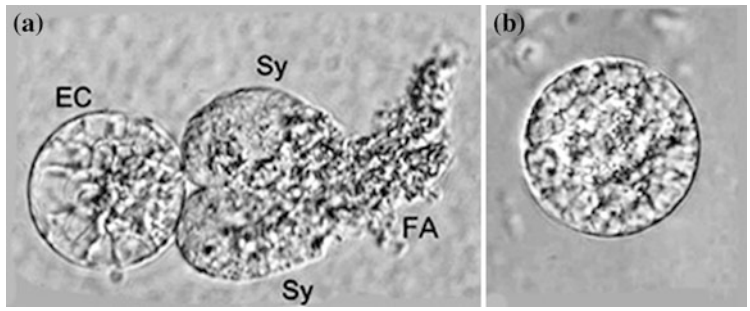

Fig. 13.4 Isolation of egg cell of rice. **a** An isolated rice egg apparatus released by dissecting an ovule treated with hydrolytic enzymes. Two synergids (*Sy*) with putative filiform apparatus (*FA*) are associated with an egg cell (*EC*). **b** An isolated rice egg cell, with granular cytoplasm around the nucleus (*courtesy*, Prof. Takashi Okamoto, Japan)

digest the embryo sac wall. Isolation of adequate number of egg cells for in vitro fertilization has been achieved in maize (Kranz et al. 1991; Faure et al. 1999), barley, wheat and tobacco. In maize, routinely 20–40 egg cells and zygotes can be isolated by an experienced person per day. Under optimal conditions, up to 60 of these cells can be obtained. In rice, an experienced person can isolate 5–10 egg cells by enzymatic method and 30–40 egg cells mechanically per day. The isolated egg cells are protoplasts and, therefore, become spherical (Fig. 13.4). The egg cell of rice (40–50 μm) is smaller than that of maize (65–77 μm), wheat (50–70 μm) and ryegrass (50–60 μm).

(iii) *Central cell.* Compared with egg cell isolation, some modification is generally necessary to isolate central cell, which is fairly larger than the egg cell. Firstly, the enzymatic treatment is longer (45 min). Enzyme solution of slightly higher osmolarity is used to plasmolyse the central cell so that it separates from the wall of the embryo sac. It facilitates the mechanical isolation step. Starting near the antipodals, the central cell is pushed by a microneedle towards the micropylar end of the embryo sac where it will be liberated (Fig. 13.5). In maize, 3–8 central cells can be isolated from 160 nucellar tissue pieces within 2–3 h. The central cell of rice is also smaller (100–120 μm) than that of maize (200 μm). Isolated central cell is also a protoplast and, therefore, becomes spherical (Fig. 13.5).

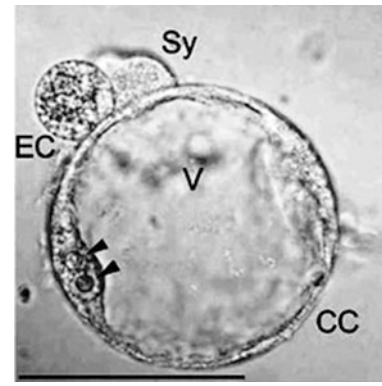

Fig. 13.5 Isolation of central cell from an unpollinated rice ovule. A central cell (*CC*), with a large vacuole *(V)*, apparently adhering to an egg cell (*EC*) and a synergid (*Sy*). The cell was isolated from an ovule treated with hydrolytic enzyme. *Arrowheads* indicate putative nucleoli (*courtesy* Prof. Takashi Okamoto, Japan)

13.3.2 Fusion of Gametes

Because the gametes are protoplasts, they can be fused chemically or electrically as in the case of somatic cell protoplast fusion. The electrofusion method is well established, and it efficiently produces sufficient zygotes for IVF, growth

studies and molecular analysis. The various parameters that need to be optimized in electrofusion include dielectrophoresis for alignment of egg and sperm cells, strength of DC pulse for fusion and distance between the two electrodes. Generally, the final distance between the electrodes should be approximately twice the sum of the diameters of the two gametes.

Isolated sperms are short-lived and, therefore, should be used for fusion soon after isolation. Whereas the maize sperms can be used for fusion within 30 min of isolation, those of wheat are useful only for few minutes (Kranz et al. 2008). Oval or spindle-shaped sperms fuse more efficiently than the rounded ones.

In practice, for maize, a pair of isolated sperm and egg cells are transferred to a 0.5–1.0 µl fusion droplet of mannitol solution (370 mosmol kg^{-1} H_2O) overlaid with mineral oil on a cover slip. The gametes are aligned on one of the electrodes under an AC field (1 MHz, 70 V cm^{-1}), and 0.5–1.0 µl of the mannitol solution (520 mosmol kg^{-1} H_2O) is gently added to the fusion drop. Fusion is induced by a single negative DC pulse (0.9–1.0 kV cm^{-1}) of 50 µs. The alignment in a manner that the egg is in contact with the electrode favours better fusion. The fusion product is washed by transferring it to fresh mannitol solution (520 mosmol kg^{-1} H_2O) twice and then transferred to the culture medium. In maize (Kranz 2001) and rice (Uchiumi et al. 2007), electrofusion frequencies of sperm and egg cells are about 85 %.

Despite the large difference in the size, the isolated central cell can also be fertilized in vitro by a careful adjustment of the electrodes which leads to a mean cell fusion frequency of 44 % (Kranz and Kumlehn 1999). Fusion is better when the cells are turgid. Therefore, osmolality of the fusion mixture is slightly reduced as compared with the isolation medium. It also helps to prevent floating of unfertilized and fertilized egg and central cells (Kranz 2001).

After fusion of the male and female gametes, the sperm nucleus migrates towards the egg nucleus, aligns and fuses with it within 1 h as demonstrated by ultrastructural observations. In electrofusion of rice and maize gametes, fusion is completed in less than a second, and the zygote recovers the spherical shape within 10 s of fusion.

13.3.3 Culture of In Vitro Zygotes

The compositions of the media used for the culture of in vitro zygotes of maize and rice are given in Table 13.2. Generally, co-cultivation with feeder cells is essential for sustained growth of in vitro formed zygotes and primary endosperm cells as in the case of isolated in vivo formed zygotes. The feeder cells comprise of actively growing non-embryogenic cell suspensions or microspore-derived cell aggregates. The in vitro zygotes are planted on the semipermeable membrane of the 12-mm-diameter Millicell-CM dishes, which is placed in the centre of a larger plate (35 mm) filled with 3 ml of culture medium containing the feeder cells (Fig. 13.2). The medium generally contains glucose and hormones, such as 2,4-D, and the cultures are maintained on a rotary shaker (40 rpm) at 25 °C under 16-h photoperiod and a light intensity of 50 µ$Em^{-2}s^{-1}$. The zygotes can also be immobilized in low–melting point agarose droplets surrounded by feeder cell suspension in liquid medium. The culture medium should fulfil the requirements of the cultured cell as well as the feeder cells. Once mini calli have developed they are transferred to another medium for further growth and plant regeneration.

Isolated egg cells of barley, wheat and maize and the fusion products of two maize egg cells did not divide under standard culture conditions. However, a brief treatment with high concentration of 2,4-D triggered division of unfertilized egg (Kranz et al. 1995). Fusion with the sperm cell activates the inert egg cell. It involves cell wall formation as early as 30 s after fusion, a triggering of Ca^{2+} influx for sperm incorporation followed by elevation of cytosolic Ca^{2+}, changes in membrane-bound Ca^{2+} and calmodulin levels, expressional abundance of gene for calreticulin (a Ca^{2+} binding protein, strongly correlated with

Table 13.2 Composition of media for the culture of in vitro zygotes of maize (ZMS) and rice (N_6Z)[a]

Constituents	Amount (mg L^{-1})	
	ZMS[b]	N_6Z[c]
NH_4NO_3	165.00	–
$(NH_4)_2SO_4$	–	231.00
KNO_3	1,900.00	1,415.00
KH_2PO_4	170.00	200.00
$CaCl_2.2H_2O$	600.00	83.00
$MgSO_4.7H_2O$	370.00	93.00
$Na_2FeEDTA$	40.00	25.00
$MnSO_4.4H_2O$	22.30	4.00
H_3BO_3	6.2	0.50
$ZnSO_4.7H_2O$	7.0	0.50
$Na_2MoO_4.2H_2O$	0.25	0.025
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	–	0.025
KI	0.83	–
Retinol	–	0.01
Thiamine HCl	10.00	1.00
Pyridoxine HCl	1.00	1.00
Nicotinic acid	1.00	1.00
Ca-Pantothenate	–	1.00
L-glutamine	750.00	1,000.00
Proline	150.00	–
Asparagine	100.00	–
Riboflavin	–	0.20
Folic acid	–	0.40
Cobalamine	–	0.02
Ascorbic acid	–	2.00
Calciferol	–	0.01
Biotin	–	0.01
Cholin chloride	–	1.00
p-Aminobenzoic acid	–	0.02
Malic acid	–	40.00
Citric acid	–	40.00
Fumaric acid	–	40.00
Na-Pyruvate	–	20.00
Casein hydrolysate	–	250.00
Myo-inositol	100.00	100.00
Xylose	150.00	150.00
Glucose	90,000.00	85,000.00
2,4-D	2.00	0.20
pH	5.5	5.7
Osmolality (adjusted with glucose)	600 mosmol kg^{-1} H_2O	450 mosmol kg^{-1} H_2O

[a] Media are filter sterilized
[b] *after* Kranz and Lörz (1993) and Kranz (1999)
[c] *after* Kumlehn et al. (1998) and Uchiumi et al. (2007)

cell division) and several novel ribosomal proteins at about 18 h, alteration in ER dynamics and change in microtubule organization.

Development of in vitro zygotes has been achieved so far only in monocots (maize, rice and wheat). Whereas the in vitro zygotes of wheat formed only mini cell colonies, those of maize (Fig. 13.6a–e) and rice (Figs. 13.7, 13.8) exhibited sustained divisions leading to the formation of fertile plants. In addition, in maize, even the in vitro fertilized central cell developed into unorganized endosperm tissue (Fig. 13.6f–j). Thus, both the embryo and endosperm are able to organize themselves without the mother tissue in a manner similar to in vivo development and are able to develop independent of each other.

A significant finding is the burst of free Ca^{2+} during egg–sperm fusion. Ca^{2+} signalling, involved in a wide range of processes in signal transduction in higher plant cells, also plays a key role in activation and development of zygote and early embryogenesis in higher plants. In maize, influx of extracellular Ca^{2+} occurs immediately after the fusion of sperm with the egg cell (1.8 s). It starts from the site of fusion and spreads through the whole egg cell plasma membrane as a wave front. Calcium influx is a necessary condition for cell wall deposition. The Ca^{2+} influx precedes a transient elevation of free cytosolic Ca^{2+} which occurs 40–120 s after IVF and peaks around 10 min after IVF (Antonie et al. 2001; Digonnet et al. 1997). This free Ca^{2+} may be sufficient for egg activation. Rapid formation of cell wall by maize egg after fusion with sperm suggests that cell wall material is stored in the egg cells which are secreted by exocytosis.

Through indirect immunocytochemistry, using anti α-tubulin antibody, it has been shown that the maize egg cell has only few cortical microtubules. Fertilization induces the appearance of distinct cortical microtubules, which remain visible up to 7 h after IVF. Following this phase, strands of cytoplasmic microtubules radiating from the nucleus into the periphery of the cell are formed and persist throughout the remainder of zygote development (Hoshino et al. 2004). It may be associated with deposition of cellulose microfibrils for wall formation that occurs soon after gametic fusion. A transient change in the dynamics of endoplasmic reticulum in wheat egg occurs within 3 min of in vitro fusion involving temporary loss of ER connections after sperm–egg fusion. ER seems to be the main Ca^{2+} store in the cells and might have a putative role in egg activation (Pónya et al. 2004).

The zygote forms a wall very rapidly, probably to check polyspermy. In rice, nuclear fusion is complete within 4 h of gametic fusion. Granular appearance of the cytoplasm, probably because of starch appearance, is observed after 12 h (Fig. 13.7a). The first division of the in vitro zygote occurs 15–24 h after fusion (Fig. 13.7b), and 4-8-celled embryos are formed after 30 h (Fig. 13.7c). A high percentage (83.5 %) of in vitro zygotes formed globular embryos after 40–50 h (Fig. 13.7d). Irregular mass of tissue, observed 5 days after fusion (Fig. 13.8a), attained a size of 1 mm after another 2 weeks (Fig. 13.8b). After 22 days of fusion or after 4 days of transfer of mini calli to the regeneration medium green loci appeared (Fig. 13.8c) and after 8 days multiple shoots differentiated (Fig. 13.8d). The shoots rooted in 11–13 days after transfer to a hormone–free medium (Fig. 13.8e). A similar calendar of events following in vitro fertilization in maize is shown in Fig. 13.9.

The technique used to fuse the gametes may affect the developmental potential of the in vitro zygotes. In maize, the zygote produced by chemical fusion formed only unorganised tissue, whereas the electrically fused gametes resulted in zygotes capable of forming complete plant via embryogenesis (Kranz et al. 1993). However, the in vitro zygotes of wheat, formed through electrofusion of the gametes, developed only up to the unorganized callus stage (Kovács et al. 1995).

IVF has demonstrated that in heterologous in vitro fertilization, the pattern of zygote division is under the maternal control. The zygote derived by in vitro fertilization of maize egg with the sperm of barley, coix or sorghum

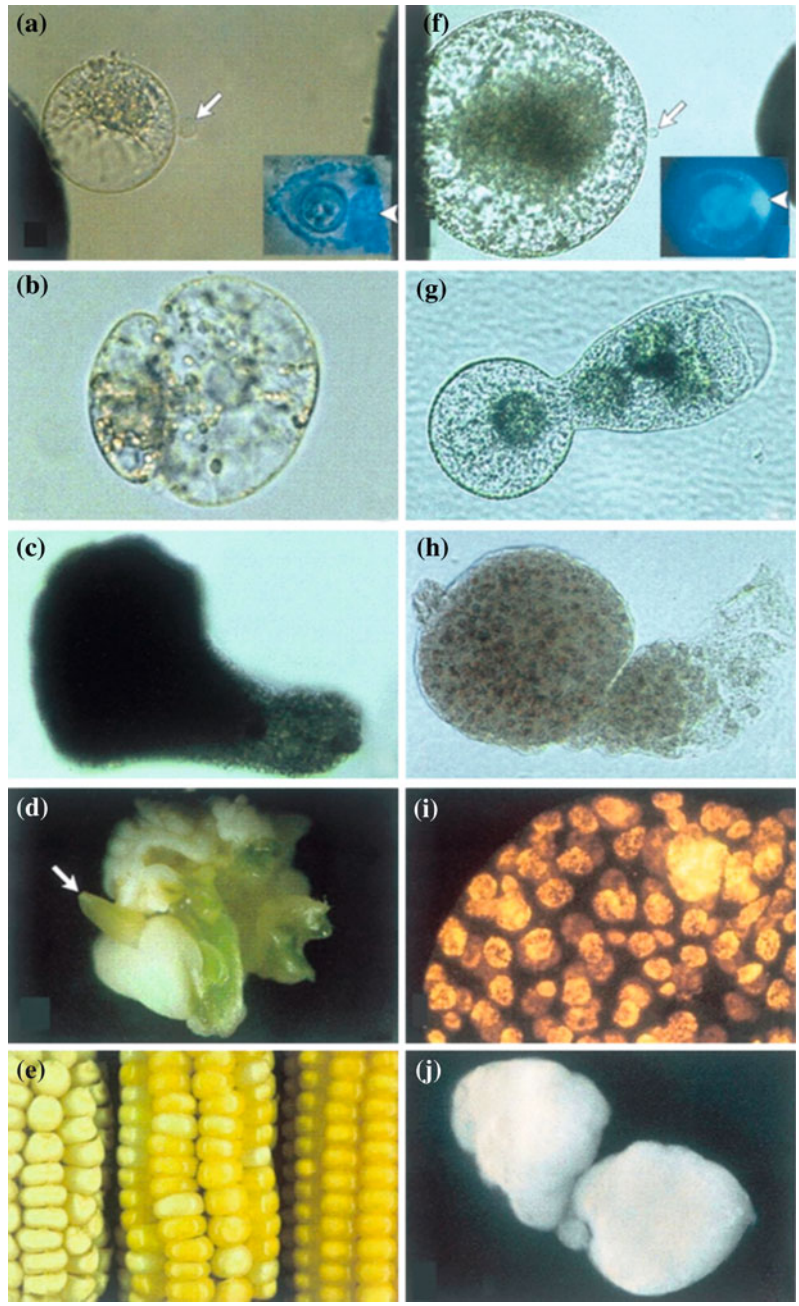

Fig. 13.6 In vitro fertilization (IVF) in maize. **a-e**. Embryogenesis and plant regeneration after IVF in maize. **a** Sperm (*arrow*) and egg cell aligned on one electrode before cell fusion. The inserted image shows karyogamy 1 hour after gamete fusion. *Arrowhead* indicates the sperm nucleus integrated in the egg nucleus. **b** Unequal division of the in vitro zygote. **c** A proembryo, 14 days after fusion. **d** Structure with a coleoptile (*arrow*), 30 days after fusion. **e** Cobs after selfing the plants derived from in vitro zygotes. The egg cell donor plant shows only white kernels (*left*), the sperm donor plant shows only yellow kernels (*right*) and the IVF-derived hybrid plant shows yellow and white kernels in the ratio of 3:1 (middle). **f–j** Endosperm development after IVF. **f** Sperm (*arrow*) and central cell aligned on one electrode before fusion. The inserted image shows karyogamy 2.5 h after sperm–central cell fusion. *Arrowhead* indicates the sperm nucleus integrated in the secondary nucleus. **g** Primary endosperm cell, 1 day after culture. **h** Feulgen-stained endosperm, 5 days after culture. **i** Three-dimensional survey of a section of 4-day-old endosperm, showing highly synchronized early prophases. **j** Endosperm, 11 days after in vitro central cell fertilization (*courtesy* Prof. Erhard Kranz, Germany)

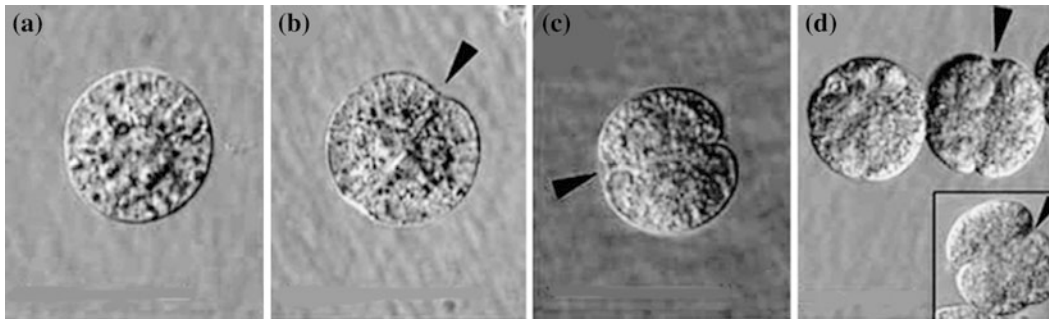

Fig. 13.7 Early embryonic development from in vitro zygote of rice. **a** A zygote, 12 h after gametic fusion. **b** A 2-celled embryo, 19 h after fusion. *Arrowhead* indicates the cleavage furrow. **c** An early embryo, 30 h after fusion. *Arrowhead* indicates a possible trace of the cleavage furrow. **d** Two globular-like embryos, 49 h after fusion. A possible trace of the cleavage furrow is indicated by *arrowhead*. The inset shows a globular-like embryo, 47 h after fusion, in which a possible trace of the cleavage furrow is clearly observed (*arrowhead*) (*courtesy* Prof. Takashi Okamoto, Japan)

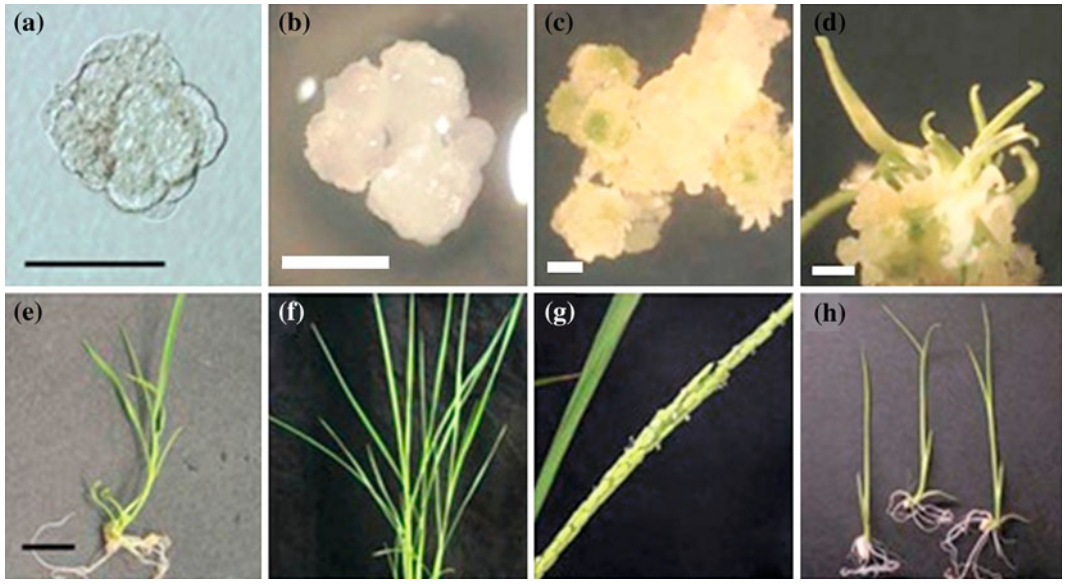

Fig. 13.8 In vitro fertilization in rice. Development and regeneration of globular-like embryos produced by IVF. **a** A cell mass, 5 days after gametic fusion, which developed from the globular-like embryo (Fig. 13.7d). **b** A white cell colony, 18 days after fusion. **c** A well-developed cell colony, 4 days after transferring the white cell colony (*B*) into regeneration medium (22 days after fusion). *Green spots* have appeared on the cell colony. **d** Regeneration of shoots from the callus, after 8 days of subculture of the white cell colony (as in **b**) and 26 days after fusion. **e** A plantlet, 12 days after subculturing a regenerated shoot on a hormone-free medium (43 days after fusion). **f** A regenerated plant, 42 days after transplantation to soil (85 days after fusion). **g** Flowers of a regenerated plant (100 days after fusion). **h** 13-day-old seedlings from seeds harvested from regenerated plants as in **g**. Bars: 50 μm in **a**, 1 mm in **b**, **c** and **d** and 1 cm in **e** (*courtesy* Takao Uchiumi, Japan)

divided by a typical unequal division, forming a small, richly cytoplasmic cell, and a large vacuolated cell just as the maize zygote formed by homologous fusion (Fig. 13.6b; Kranz et al. 1995). However, the wheat egg fertilized in vitro with its own sperm (Fig. 13.7b) or that of maize divided by equal division (Kranz et al. 1999). It has been possible to isolate the two unequal

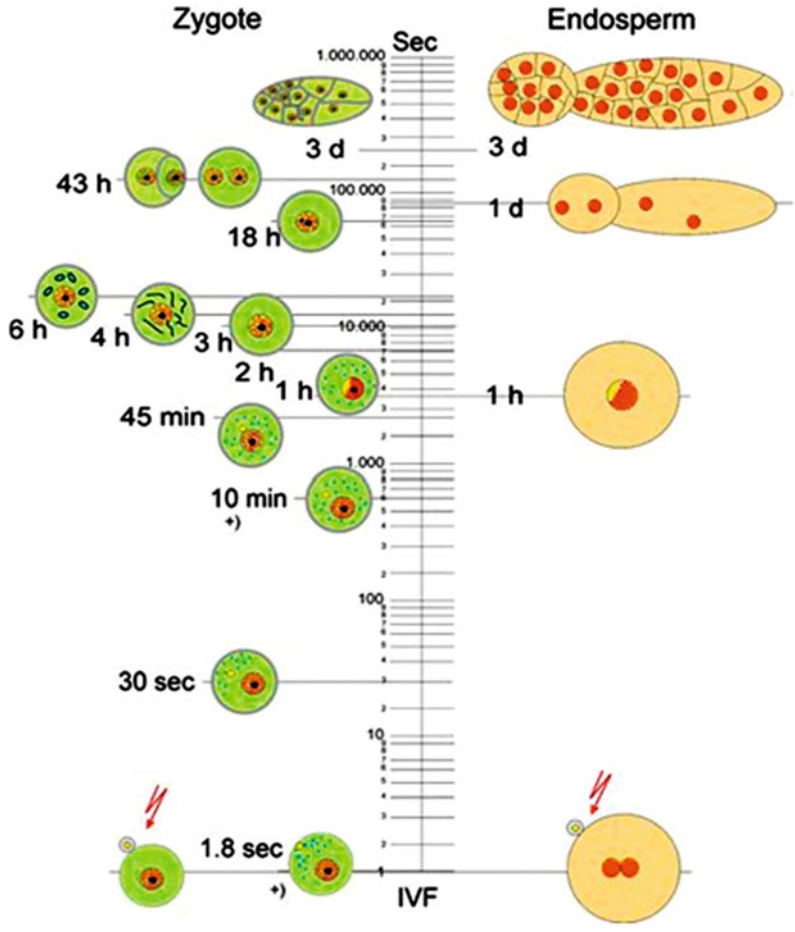

Fig. 13.9 In vitro fertilization in maize. Schematic representation of events in the zygote and early embryo and endosperm development after IVF. *Left.* Zygote and embryo development after electrofusion of the gametes. Ca^{2+} influx and cytosolic Ca^{2+} elevation in the zygote at various times after calcium-mediated gamete fusion are indicated by + sign. Ca^{2+} influx starts essentially immediately (1.8 s) after gamete fusion (Antoine et al. 2000). Subsequently, a cytosolic Ca^{2+} elevation occurs which reaches a maximum around 10 min after IVF (Antoine et al. 2001b). Ca^{2+} is indicated by dots. These results are included in the 30-s, 45-min and 1-h samples. Cell wall formation starts within 30 s (Kranz et al. 1995). Dots indicate Ca^{2+} influx and elevation. Karyogamy starts after 1 h (Faure et al. 1993). Appearance of gfp (*green* fluorescent protein) mRNA and decondensation of chromatin, reflecting paternal transcription, occurs after 4 h (Scholten et al. 2002), and translational activity occurs 6 h after IVF (Scholten et al. 2002). Nuclear division is over by 18 h, 2-celled embryo is formed within 43 h and multicellular embryo is formed within 3–4 days of fusion. *Right.* Primary endosperm cell and endosperm development after electrofusion of the gametes. Karyogamy starts within 1 h of fusion, syncytium is formed within 1 day and multicellular endosperm develops after 3–4 days (Kranz et al. 1998). An adhesion time of 10–20 min precedes calcium-mediated sperm–egg membrane fusion, during which sperm and egg membranes become strongly attached (Kranz and Lörz 1994). Depending on culture conditions applied and activity of the cells, the time course may vary. Also, it should be noted that female gametes from the same cob quite often are asynchronous with respect to their physiological status. Therefore, quite often a high number of cells is necessary to obtain statistically robust results of physiological experiments (*courtesy* Prof. Erhard Kranz, Germany)

daughter cells of the maize zygote and analyse the gene regulation in these cells with distinct fates and potential (Okamoto et al. 2005).

13.4 Applications

13.4.1 Basic Studies on Fertilization and Zygote Development

The success with IVF during the past decade-and-a-half provides system that facilitates more precise study of the process of gametic fusion, cellular and subcellular changes induced by fertilization that activate the egg cell and prepares the zygote to undergo the characteristic unequal division, forming a small, cytoplasmic cell (ca) and a large vacuolated cell (*cb*) with distinct developmental fate and the important developmental processes during early embryogenesis. Indeed, IVF has been successfully applied to observe and analyse post-fertilization events, including activation of egg on fusion with the sperm cell, karyogamy in the zygote (Faure et al. 1993), zygote development (Kranz et al. 1995), decondensation of paternal chromatin in zygote (Scholten et al. 2002), change in microtubule architecture in zygote (Hoshino et al. 2004) and identification of fertilization-induced/suppressed genes (Okamoto et al. 2005). Similar studies are also being extended to triple fusion and endosperm development through in vitro fertilization of the central cell.

Okamoto et al. (2005) identified some genes that are up- or down-regulated in the apical and basal cells of the 2-celled embryo of maize. The genes up-regulated in the apical and basal cells were already expressed in the early zygote.

13.4.2 Hybridization

The most important application of IVP and IVF is in wide hybridization where crosses are abortive because of prefertilization barriers. Several interspecific, intergeneric and interfamily crosses have been attempted through placental pollination with a fair amount of success. Marubashi and Nakajima (1985) produced fertile hybrids, with 96 chromosomes, by IVP of *Nicotiana tabacum* with the pollen of *N. rustica* followed by ovule culture. This sexually incompatible cross has also been successful through protoplast fusion, but all the somatic hybrids were aneuploid (Douglas et al. 1981). DeVerna et al. (1987) raised two interspecific hybrids by IVP of *N. tabacum* with *N. amplexicaulis*. This cross exhibits unilateral sexual incompatibility. Dhaliwal and King (1978) produced interspecific hybrids by IVP of *Zea mays* with the pollen of *Z. mexicana*.

Kranz et al. (1995) induced the fusion of maize egg cell with sperm cells of such genetically remote species as *Coix, Hordeum, Sorghum* and *Triticum*. In these crosses, the in vitro hybrid zygotes divided to form microcalli with the frequencies of 78 %, 50 %, 43 % and 24 %, respectively. The high frequency of hybrid zygote dividing to form microcalli makes the fusion of egg cells with sperm cells of different species or genera a potentially viable method to obtain hybrids that would be impossible to obtain through the conventional method of crossing. The IVF method of hybridization eliminates the influence of the sporophytic tissues of stigma, style and ovules.

13.4.3 Transformation

In vitro fertilization also provides a route to new and simpler transformation method, such as microinjection of DNA into the nucleus of an unfertilized egg cell.

13.5 Appendix

1. IVF Protocol for *Zea mays* (*after* Kranz 1999, 2001)

i) *Isolation of Egg and Central Cell*

a) Collect ears after silk emergence and sterilize the outer leaves with 70 % ethanol.
b) Dissect out 20–30 nucellar tissue pieces from the ovules under a dissecting microscope. The embryo sac should be visible in the nucellar tissue.
c) Collect the nucellar tissue pieces in one ml of mannitol solution (750 mosmol kg^{-1} H_2O) in 3-cm plastic Petri dishes and add 0.5 ml of enzyme mixture, containing 1.5 % pectinase, 0.5 % pectolyase Y23, 1 % cellulase Onozuka RS, 1 % hemicellulase and 530 mM mannitol (osmolality 570 mosmol kg^{-1} H_2O) and pH adjusted to 5. Incubate the plates at room temperature without shaking.
d) After 30 min, store the dishes in a refrigerator at 6 °C, or dissect out manually the embryo sac cells in the incubation dish with glass needles under microscopic observation. Using a micropump, transfer the cells to microdroplets (2 µl of 600 mosmol kg^{-1} H_2O mannitol droplet overlaid with 300 µl of autoclaved mineral oil) by microcapillary (tip opening of 100–200 µm for egg and 300 µm for central cell) on a siliconized and UV-sterilized cover glass.

(ii) *Isolation of Sperm Cells*

e) Overlay about 1000 pollen grains, in a 3.5-cm Petri dish, with 1.5 ml of mannitol solution (600 mosmol kg^{-1} H_2O). After the bursting of the pollen, pick the sperm cells with a capillary (tip opening of 20 µm) and transfer them into the microdroplet containing egg or central cells using a micropump.

(iii) *Gametic Fusion*

f) Fix two electrodes (50-µm-diameter platinum wire) to an electrode support under the condenser of the microscope. Adjust the electrodes to a crosshair position and lower these onto the cover slip and into one of the microdroplets. Sterilize the electrodes in light flame before use.
g) Align and fix the two gametes at one electrode. Prepare and adjust the electrodes carefully. By moving the microscope stage, first move an egg cell towards one of the electrodes. Finally, fix the egg cell to the electrode by dielectrophoresis (1 MHz, 70 V cm^{-1}). Using the same procedure, fix the sperm cell to the egg cell. Add 0.5–1.0 µl of mannitol solution of 520 mosmol kg^{-1} H_2O. Now, the final distance between the two electrodes is adjusted to about double the sum of the diameters of the two gametes.
h) Induce egg–sperm fusion by applying a single or a maximum of three negative DC pulses (50 µs, 0.9–1.0 $kVcm^{-1}$). With well-prepared electrodes, nearly 100 % fusion occurs. When no fusion occurs, lower the distance between the electrodes. Low fusion rate could also be due to very low turgor pressure of the gametes. In that case, reduce the osmolality of the fusion mixture. The central cell–sperm fusion is induced by a single or 2–3 negative DC pulses (50 µs, 0.4–0.5 kV cm^{-1}).

(iv) *Culture of In Vitro Zygote and Primary Endosperm Cells*

i) Gently moving the stage, remove the fusion product from the electrode. Move the electrode out of the droplet and transfer the fertilized egg (in vitro zygote) or central cell (in vitro primary endosperm cell), using microcappillary, to a 12-mm Millicell-CM insert plate with 100 µl of liquid ZMS medium (for composition see Table 13.2). Insert the plate into a 3.5-cm plastic petri dish containing 1.5 ml of a feeder suspension.
j) Next day, place the dish on a rotary shaker (50–70 rpm) and incubate the

cultures at 26 ± 1 °C under 16-h photoperiod and a light intensity of about 50 μmol $m^{-2}s^{-1}$.

(k) After 10–14 days, transfer the embryos (0.4 mm long), using a Pasteur pipette or a small blade, to 1.5 ml hormone-free RMS 1 regeneration medium (MS medium with 6 % sucrose and solidified with 0.4 % agarose) in a 3.5-cm plastic petri dish.

(l) After about 2 weeks, when a coleoptile and roots are formed, transfer the structures to 1.5 ml of RMS 2 medium (RMS 1 medium with 4 % sucrose).

(m) After another 1–2 weeks, transfer plantlet into a glass jar containing 50 ml of RMS 3 medium (RMS 1 medium with 1 % sucrose and macro and micro salts reduced to half concentration).

(n) After about 2 weeks, transfer the plants, with 15–20-cm-long leaves, to soil.

2. IVF Protocol for *Oryza sativa* (after Uchiumi et al. 2006, 2007)

(i) *Isolation of Egg and Central Cell*

(a) The experimental plants of rice (*Oryza sativa* cv. Nipponbare) are grown in green house under controlled conditions of light (13-h photoperiod with 150-μmol photons $m^{-2}s^{-1}$) and temperature (25 °C).

(b) Ovaries are collected from spikes before anthesis, and ovules are dissected out in 0.3 M mannitol solution using sharp forceps and 30G short-needle syringe under a dissecting microscope.

(c) The ovules are transferred to 3.5-cm Petri plates containing 3 ml of mannitol solution. The peripheral region of the ovule along the antipodal end of the ovary is gently cut with the tip of a 30G needle, and 0.5 ml of the mannitol solution (650 mosmol kg^{-1} H_2O) containing cell wall-degrading enzymes (0.5 % pectolyase Y-23, 1.5 % pectinase, 1 % cellulase Onozuka RS and 1 % hemicellulase) was added to the dish.

(d) After 10–15 min of incubation at room temperature, the egg cell and central cell become visible. At this stage, the egg cell could be manually isolated from the micropylar end of the ovule using glass needles under an inverted microscope. Longer incubation in the enzyme solution is damaging to the egg cell.

(e) The central cell is isolated from the enzyme-treated ovules by careful micromanipulation of the tissue with glass needle.

(ii) *Isolation of Sperm Cells*

(f) The anthers isolated from the flower buds just before anthesis are transferred to 0.3 M mannitol solution in 3.5 cm plastic Petri plates, and the anther tissue is torn with the help of a forceps to release the pollen grains. The pollen grains burst after 3–5 min releasing their contents including the two sperms (Fig. 13.3b).

(iii) *Gametic Fusion*

(g) An isolated egg cell and an isolated sperm cell are transferred to a 0.5–1.0 μl fusion droplet of mannitol solution (370 mosmol kg^{-1} H_2O) overlaid with mineral oil on a cover slip and electrofused.

(h) After aligning the egg cell and the sperm cell on one of the electrodes under a dielectrophoretic AC field of 1 MHz and 0.4 kV cm^{-1}, 0.5 μl of mannitol solution (520 mosmol kg^{-1} H_2O) containing 2 mM $CaCl_2$ is added to the fusion droplet. Addition of mannitol solution changes the shape of sperm to oblong and makes the attachment of the egg cell to the electrode more stable.

(iv) *Culture of In Vitro Zygote*

(i) The fusion product is washed twice by transferring it to fresh mannitol droplets (450 mosmol kg^{-1} H2O).

(j) The cleaned zygote is transferred onto the membrane of 12-mm Millicell-CM dishes, and the dish is placed in the centre of a 3.5 cm plastic dish filled with 3 ml of N_6Z medium (Cullen et al. 1998), modified by using commercial N_6 medium, and molality was adjusted to 450 mosmol kg^{-1} H_2O with glucose. To the outer dish, 50 µl of rice suspension culture is added as feeder cells. The composition of the N_6Z medium is given in Table 13.2.

(k) After overnight culture at 26 °C in dark without shaking, the cultures are continued with gentle shaking (40 rpm).

(l) After 110–124 h of the fusion, the Millicell-CM dish with the developing embryo is transferred to a clean 3.5 cm dish filled with 3 ml of fresh N_6Z medium without feeder cells. The cultures are maintained at 26 °C in dark with shaking (40 rpm.)

(m) After 18–19 days in culture, the cell colonies (Fig. 13.8b) derived from the in vitro zygote are transferred for plant regeneration to a medium containing MS salts and vitamins, 100 mg L^{-1}*myo*-inositol, 2 g L^{-1} casamino acid, 30 g L^{-1} sucrose, 30 g L^{-1} sorbitol, 0.2 mg l^{-1} NAA and 1 mg L^{-1} kinetin and gelled with 0.3 % gelrite. The cultures are incubated under light (80-µmol photons m^{-2} s^{-1}) at 30 °C.

(n) After 12–30 days, the differentiated shoots (Fig. 13.8c, d) are transferred to hormone-free medium containing MS salts, MS vitamins, 100 mg *myo*-inositol, 30 g L^{-1} sucrose and 0.3 % gelrite. The cultures are maintained under 13-h photoperiod with photon flux density of 55-µmol photons m^{-2} s^{-1}, at 28 °C.

(o) After 11–13 days, the plantlets (Fig. 13.8e) are transferred to soil.

Suggested Further Reading

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Parasexual Hybridization 14

14.1 Introduction

Protoplasts are single cells that have been stripped of their wall (Fig. 14.1a), exposing the plasmalemma. One gram of leaf may yield about a million protoplasts. A special property of the protoplasts is that when brought into close contact they tend to fuse with each other irrespective of the sources of the protoplasts. The technique of fusion of isolated protoplasts from somatic cells and regeneration of hybrid plants from the fusion products, called somatic hybridization, completely bypasses the sex, and thus allows combining genomes of two desirable parents, irrespective of their taxonomic relationship. In addition, somatic hybridization has a unique potential to combine both nuclear and cytoplasmic genes simultaneously unlike sexual hybridization or genetic engineering. The isolated protoplasts also have the ability to take up and incorporate foreign genetic material or purified DNA, which extends the realm of plant modification through cell fusion. Probably, the greatest contribution of protoplast fusion will be the production of cybrids and asymmetric hybrids (Li et al. 1999).

Real interest in genetic manipulation of somatic plant cells developed after Cocking (1960) demonstrated the feasibility of enzymatic degradation of plant cell walls to obtain large quantities of viable protoplasts. The area of protoplast technology gained momentum during 1980s and early 1990s due to the development of efficient methods to isolate and culture plant protoplasts, more effective fusion techniques, better heterokaryon selection strategies, and more powerful tools to analyze the hybrids. Since the first report of somatic hybrid production in the genus tobacco by Carlson et al. (1972), the technique of cell fusion has been extended to a large number of genera to produce symmetric allotetraploid somatic hybrids (with complete nuclear genomes of both the parents), asymmetric hybrids (part of the nuclear genome from the donor parent into the intact genome of the recipient parent), and somatic cybrids (nuclear genome of one parent with mitochondrial genome of the second parent). In late 1990s, the application of the protoplast technology had a slight setback due to rapid advances in the field of genetic manipulation of somatic cells by transferring specific genes in totipotent target cells by *Agrobacterium* or biolistic gun methods (Genetic Engineering; Chap. 15). However, during the past decade extensive work has been done on somatic hybridization in crops that include citrus, potato, rapeseed, rice, and tomato, and there is a continued interest to manipulate cytoplasmic genomes, particularly of mitochondria and to produce asymmetric hybrids through protoplast fusion. In *Citrus* alone, somatic hybrid plants from over 500 parental combinations and somatic cybrids from more than 50 combinations have been developed (Grosser et al. 2010).

The essential steps in the technique of somatic hybridization are: (1) isolation of protoplasts, (2) fusion of protoplasts, (3) culture of

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_14, © Springer India 2013

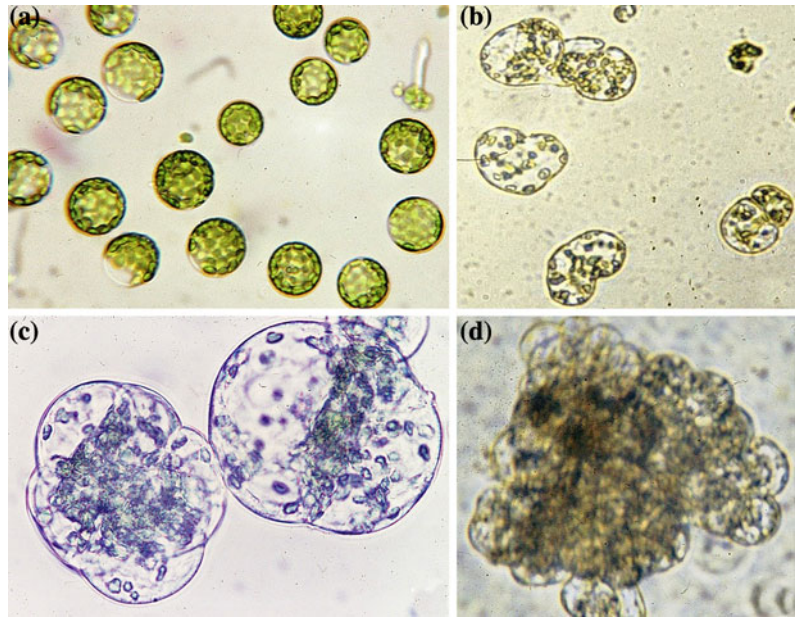

Fig. 14.1 Mesophyll protoplasts of *Trifolium repens*. **a** Freshly isolated protoplasts. **b** A sample from 6-days-old culture of protoplasts. Some of the protoplast-derived cells have undergone first division. **c**, **d** Cell colonies from 20 and 25-days-old cultures of protoplasts, respectively

protoplasts to raise full plants, (4) selection of hybrid cells, and (5) hybridity verification.

14.2 Protoplast Isolation

The isolation of protoplasts from higher plant cells mechanically, by incubating them in a plasmolyzing solution and cutting with a sharp knife, was reported as early as 1892 by Klercker. However, the yield of the protoplasts by this mechanical method was very poor and the technique was applicable only to vacuolated cells. In 1960, Cocking demonstrated that by using a concentrated solution of cellulase enzyme from the cultures of the fungus *Myrothecium verrucaria* a large number of protoplasts can be isolated from higher plant cells. However, real progress in this area was made after 1968 when cellulase and macerozyme enzymes became available commercially. Takebe et al. (1968) were the first to use commercial preparations of the enzymes to isolate protoplasts from mesophyll cells of tobacco. Cellulases, hemicellulases, and pectinases of bacterial/fungal origin are now produced by a number of companies and marketed under their brand names. Takebe et al. (1968) used the two enzymes sequentially. The tissue was first macerated with macerozyme to break it into single cells which were then subjected to cellulase treatment to digest the cell wall and liberate the protoplasts. However, the process of protoplast isolation can be considerably simplified by using the two enzymes simultaneously (Power and Cocking 1968). This one step method is now widely used to isolate plant protoplasts.

Protoplast isolation is now a routine process. With the available enzymes and techniques viable protoplasts can be isolated from virtually any plant organ or cultured cells, provided they have not acquired lignification (Davey et al. 2010). Protoplast isolation has been reported from mesophyll cells of in vivo and in vitro grown plants, aseptic seedlings, embryogenic and nonembryogenic suspension cultures, cotyledons, hypocotyls, and male and female gametes (see Chap. 13). Some of the factors that need to be standardized to obtain optimum yield of viable protoplasts are discussed below, and a couple of protocols to isolate protoplasts are given in Appendix.

14.2.1 Factors Effecting Protoplast Isolation

(i) *Source of material*. The most widely used tissue to isolate protoplasts are the young leaves from in vitro grown aseptic shoot cultures. Since the mesophyll cells in the leaves are loosely arranged, the enzymes have an easy access to the cell wall. Moreover, the leaves yield large number of relatively uniform cells. Where leaves do not yield culturable protoplasts, as in the case of cereals, their actively growing cell suspension cultures are used. To obtain totipotent protoplasts of *Citrus*, generally, embryogenic suspension cultures are used.

(ii) *Enzyme treatment*. Isolation of protoplasts requires at least two enzymes, viz. a pectinase enzyme to dissolve the middle lamella that binds the adjacent cells together and a cellulase enzyme to digest the cell walls and release the protoplasts. Some tissues may also require hemicellulase in addition to cellulase and pectinase to release protoplasts. Several brands of cellulase and pectinase enzymes of bacterial (*Trichoderma viridae*) and fungal (*Aspergillus niger*, *Rhizopus* sp.) origin are commercially available. Cellulase Onozuka RS, Cellulase R-10 (10 times more effective than Cellulase RS) and Macerozyme R-10 (all produced by Yakult Pharmaceutical Industry, Japan) have been most widely used. Macerozyme R-10 possesses high pectinase and hemicellulase activities. Pectolyase Y-23 (Seishin Pharmaceutical, Japan), a highly powerful macerozyme, in combination with cellulase, released protoplasts from mesophyll cells of pea within 30 min. Driselase (Kyowa Hakko, Japan), a mixture of cellulase, pectinase, laminarinase, and xylanase, have proved especially effective for isolating protoplasts from cultured cells.

The commercially available enzymes contain various impurities, including proteolytic enzymes, phenolics, and salts which may adversely affect the yield and viability of protoplasts. Therefore, some workers have recommended the purification of the enzymes by eluting through biogel, Sephadex G-25 filtration or treatment with activated charcoal (Li et al. 1999). However, most of the enzymes have been used in their crude forms as available in the market with satisfactory results. The combination and concentrations of the enzymes for a new system need to be optimized by several trials.

The activity of the enzymes is dependent on pH and temperature. The pH of the enzyme mixture is adjusted at 4.7–6.0. Although the maximum activity of the enzymes is at 40–50 °C but the tissues are incubated in enzyme solution at 25–30 °C as the higher temperatures may be injurious to the cells.

In practice, the leaves are shredded into thin strips and the callus is cut/broken into small pieces and incubated in the enzyme solution in dark. Gentle shaking of the incubation mixture (~40 rpm) may improve protoplast yields. Another factor that affects the yield of protoplasts is the relative volume of the enzyme solution to the amount of the tissue. Generally, 10 ml of enzyme solution for 1 g of tissue is satisfactory.

(iii) *Osmoticum*. Unlike cells, the isolated protoplasts are osmotically fragile. Therefore, right from the enzyme treatment to the culture of protoplasts, until they develop a substantial wall, a proper osmolarity is maintained around them. In a solution of suitable osmolarity (slightly hypertonic), the protoplasts appear completely spherical (Fig. 14.1a). A variety of solutes, ionic and nonionic, have been used as osmoticum for isolation and culture of protoplasts. However, the most commonly used osmotic stabilizer is mannitol, a metabolically inert sugar, in the range of 450–800 mM.

14.2.2 Purification of Protoplasts

After enzyme treatment of the tissue for adequate period, the incubation vessel is gently swirled or the leaf pieces are gently squeezed in the enzyme

solution to release the protoplasts. At this stage, the protoplast preparation is contaminated with undigested cells and tissues and debris of over-digested broken cells, which must be removed before their culture and manipulation.

The most commonly used purification method is filtration followed by centrifugation. The digestion mixture is passed through a metal or nylon sieve of pore size 30–100 μm to remove large debris. The filtrate is centrifuged at 100 × g for 5 min to pellet the protoplasts. The supernatant, containing light debris, is discarded. The pellet is resuspended in washing medium and washed thrice by repeated centrifugation at 50 × g for 3–5 min and resuspension. Alternatively, the pelleted protoplasts suspended in small volume of the enzyme solution or a protoplast washing medium is loaded over the sucrose pad (21 %) in a centrifuge tube and spun at 10 × g for 10 min. The debris settles to the bottom of the tube and the clean band of protoplasts can be pipetted out gently from the junction of the sucrose pad and the protoplast suspension medium and transferred to another centrifuge tube. They are washed by repeated centrifugation and resuspension as described above. Many scientists prefer to use discontinuous gradient of Percoll, a nondilysable polyvinylpyrrolidone (Li et al. 1999).

14.2.3 Viability of the Protoplasts

The most commonly used method to test the viability of isolated protoplasts is by staining with fluorescein diacetate (FDA). This and other methods to check the viability of cells/protoplasts are described in Chap. 4. When testing the viability of protoplasts the osmolarity of the staining solution must be properly adjusted.

14.3 Protoplast Fusion

Freshly isolated protoplasts will fuse if brought into intimate contact and held together for a few minutes. A reproducible and controlled fusion method, using $NaNO_3$ as the fusogen was first reported by Power et al. (1970). Although this method was employed by Carlson et al. (1972) to produce the first somatic hybrid, between *Nicotiana tabacum* and *N. langsdorffii* it suffers from low incidence of fusion. The two most widely used techniques to fuse plant protoplasts are chemical fusion by PEG (polyethylene glycol) and electric stimulation. By these methods any kind of protoplasts, even inter-kingdom, can be fused. These methods are described below.

14.3.1 PEG-Induced Fusion

Since the first reports by Kao and Michayluk (1974) and Wallin et al. (1974), PEG has achieved widespread acceptance as a fusogen of plant protoplasts. It induces high frequency heterokaryon formation with low toxicity to plant cells. It can fuse not only plant protoplast to plant protoplast but also plant protoplast to animal cell (Dudits et al. 1976), animal cell to animal cell (Ahkong et al. 1975), and animal cell to yeast protoplast (Ahkong et al. 1975).

Generally, a combination of the original PEG method described by Kao and Michayluk (1974) and high Ca^{2+}-high pH method of Keller and Melchers (1973) is used (see Protocol 3). Freshly isolated protoplasts from the two selected parents are mixed in appropriate proportions and treated with 15–45 % PEG (1,500–6,000 MW) solution. After 15–30 min, the protoplasts are gradually washed with the culture medium containing a high level of Ca^{2+} ions (50 mM $CaCl_2.2H_2O$) and pH adjusted to 9–10. Fusion actually occurs during elution with hypotonic washing solution.

PEG is a highly water soluble, nonionic surfactant, with slight negative polarity and forms hydrogen bonds with positively polarized groups of water, protein, carbohydrate and so on. When the PEG molecule chain is large enough it forms bridges between the surfaces of adjacent protoplasts, allowing their agglutination. PEG can also bind Ca^{2+} in the fusion mixture, and Ca^{2+} may further form a bridge between the PEG

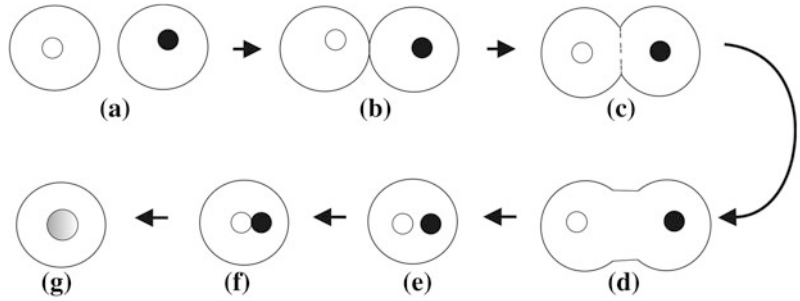

Fig. 14.2 Diagrammatized stages in the fusion of two protoplasts. **a** Two separate protoplasts. **b** Agglutination of two protoplasts. **c** Membrane fusion. **d** Dumbbell-shaped stage. **e**, **f** Formation of spherical heterokaryon. **g** Hybrid cell

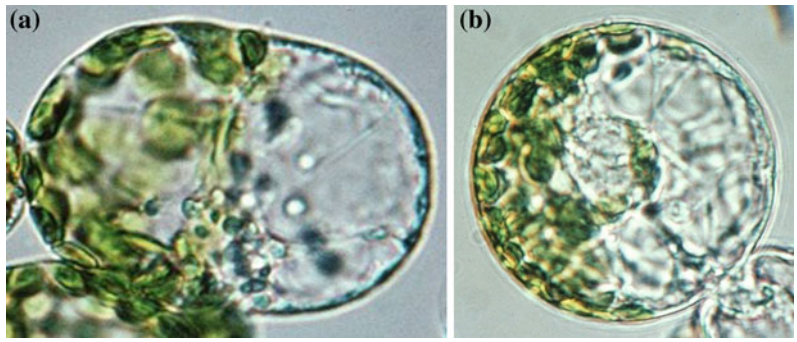

Fig. 14.3 Two stages in the fusion of a nonchlorophyllous protoplast from suspension cultures of *Petunia hybrida* with a green mesophyll protoplast of *P. parodii*. **a** Dumbbell-shaped stage, after the fusion of the adjacent plasma membrane of the two protoplasts. **b** The fusion body has become spherical and most of the chloroplasts are still restricted to one side (*courtesy* Power, U.K.)

chain and negatively polarized group of the membrane constituents and thus enhance the agglutination. During subsequent elution of the fusion mixture, the PEG molecules that are already bound to the cell membranes are forced away from the plasma membrane. Osmotic shock may also occur on cell membrane by the hypotonic washing solution used. These combined effects can cause disturbance and redistribution of electric charges in the adhering membranes leading to fusion (Kao and Michayluk 1989).

PEG treatment brings about agglutination of two or more protoplasts along large surface area, resulting in tight adhesion of the membranes (Fig. 14.2a, b). The closely apposed membranes fuse during elution of the PEG by the formation of one or more cytoplasmic channels in the region of close adhesion (Fig. 14.2c). The channels gradually expand and the fusion body, passing through the dumbbell-shaped structure (Fig. 14.2d), becomes spherical (Figs 14.2e, 14.3a, b). As the PEG is eluted, the fusing bodies are deplasmolyzed, and cytoplasmic streaming starts causing the mixing of the cytoplasms of the two cells. The fused protoplasts with two nuclei of different parents are called heterokaryons (Fig 14.2e, f) and those with nuclei of the same parent are called homokaryons. The fusion of nuclei in the heterokaryons during culture results into a hybrid cell (Fig. 14.2g).

Numerous factors are known to affect the frequency of PEG-induced fusion, including physiological status of the protoplasts, density of protoplasts, purity, concentration and molecular weight of PEG, concentration of Ca^{2+} ions, pH, osmolarity, and method of application of PEG solution and elution. PEG of molecular weight

higher than 1,000 (1,500–6,000), at a concentration from 15–45 %, is effective in bringing about high frequency fusion. PEG-induced fusion is enhanced by the presence of high level of Ca^{2+} ions (50 mM). The dilution after the PEG treatment should be gradual. Excessive washing of protoplasts after enzyme treatment leads to poor fusion, probably because of rapid synthesis of a new wall. Protoplasts from younger leaves and fast growing calli or cell suspensions exhibit better fusion. A 4–5 % protoplast suspension (protoplast volume/liquid volume) usually gives higher frequency of heterokaryon formation.

The major disadvantages of the chemical fusion method are: (a) the fusogen is toxic to some cell systems (Benbadis and deVirville 1982), (b) it produces random and multiple cell aggregates, and (c) the fusogen must be removed before culture.

14.3.2 Electrofusion

In the past two decades, electrofusion has become a competitive alternative to PEG-induced fusion of somatic cells for somatic hybridization (Sun et al. 2004; Taski-Ajdukovic et al. 2006; Thieme et al. 2008; Jiang et al. 2009; Sonntag et al. 2009) and gametic cells for in vitro fertilization (Chap. 13). Electrofusion is rapid (usually complete within 15 min), synchronous and convenient, and the treatments are relatively nontoxic to protoplasts, allowing fusion of defined protoplast pairs. Electrical fusion is more reproducible and often gives greater fusion frequency than chemical fusion (Davey et al. 2004). A versatile electrofusion instrument can be fabricated or a commercially available system can be used.

Senda et al. (1979) were the first to report electrically stimulated fusion of plant protoplasts. The method was further developed by Zimmerman and his co-workers (Zimmerman and Vienken 1982) leading to the production of the first automatic electrofusion system by GCA Corp., Precision Scientific Group, USA. It was 10,000 times more effective than any other method of protoplast fusion. Since then, this method has become very popular and several other companies have developed electrofusion apparatus (BTX, Inc, San Diego, California; BioRad, USA; Shimadzu Corporation, Japan; Ependorf, Germany). Electrofusion systems, with fixed or movable electrodes, have been used for protoplast fusion of a large number of plant species. The technique involves two steps:

(i) *Alignment of Protoplasts*. Prior to cell fusion, protoplasts are suspended in a medium of low conductivity (mannitol with $CaCl_2$) to stabilize plasma membrane. The protoplasts of the two parents, mixed in a ratio of 1:1, at a density of 5×10^5 ml^{-1}, are aligned in nonhomogenous, low voltage (100–200 V cm^{-1}), high frequency (about 1 MHz) AC field for 30–60 s. It induces the protoplasts to become dipole. The protoplasts undergoing dielectrophoresis are mutually attracted, and consequently bind together to form pearl chains parallel to the field direction. The fusion mixture contains a nonionic osmoticum (usually 0.5 M mannitol) to ensure appropriate osmolarity. Calcium ions, in the form of $CaCl_2.2H_2O$, at a concentration higher than 1.0 μM (usually 0.25–2.5 mM), is generally added to the fusion mixture for better alignment of protoplasts during dielectrophoresis.

(ii) *Fusion of Protoplasts.* The aligned protoplasts are fused by applying one or more 4–40 μs pulses of high voltage DC current (0.5–2 kV cm^{-1}) at an interval of 0.5–2 s. It causes reversible breakdown of the plasma membranes of the two protoplasts at several points in the contact zone, leading to the fusion of cell membranes. The fusion mixture is left undisturbed for 10–30 min for the fusion products to regain normal shape. Microelectrofusion of pre-selected pairs of protoplasts has been developed (Koop et al. 1983; Koop and Schweiger 1985) for somatic hybridization (Spangenberg 1990), in vitro fertilization through single gamete fusion (Kranz et al. 1991; Chap. 13 in this

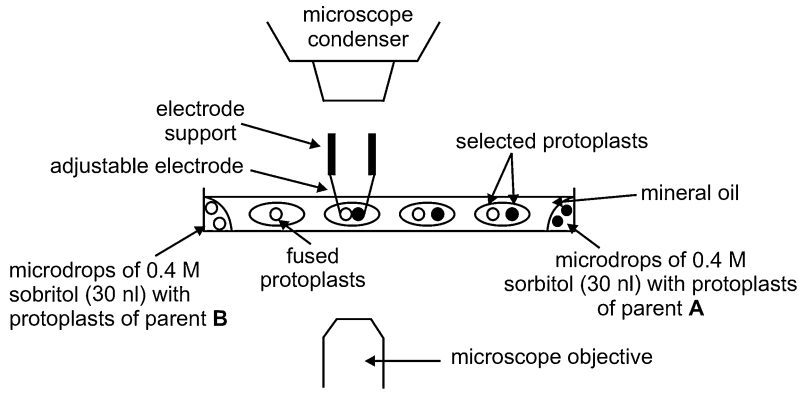

Fig. 14.4 Microelectrofusion of selected pairs of protoplasts (*based on the work of* Ŕakosy-Tican et al. 2001)

book) and organelle transfer either through microfusion of defined protoplast-cytoplast pairs (Spangenberg et al. 1991; Eigel and Koop 1992) or through transfer of plastids (Koop et al. 1992). In microelectrofusion, as developed by Koop et al. (1983), desired pair of protoplasts is transferred to a microdroplet containing low ionic strength fusion medium, and fusion is performed by introducing into the droplet a pair of platinum wire electrodes. Ŕakosy-Tican et al. (2001) carried out microelectrofusion of haploid protoplasts of wild type and streptomycin resistant mutant of *N. tabacum* in 35 mm plastic Petri dishes. The bottom of the plate was layered with 4 ml of 0.5 M mannitol, which was overlaid by purified mineral oil. With the help of a micromanipulator, mounted on Leitz Diavert inverted microscope, 20 microdroplets (about 30 ml) of 0.4 M sorbitol were arranged in a matrix formation in the center of the dish (Fig. 14.4). To each drop two protoplasts, one of each parent, were transferred using micropipette. The electrodes for fusion (made of entomological needles of 90 μm diameter) with a distance of approximately 500 μm between their tips, were lowered into the microdroplet containing the protoplast pair, so that the protoplasts were positioned between the tips. The electrodes were attached to a micromanipulator. The protoplasts were aligned by applying AC current with a frequency of 750 kHz and field strength of 84 V cm^{-1}. After 30–60 s, 1–3 rectangular DC pulses (4 ms each) of field strength 500–670 V cm^{-1} were applied to fuse the protoplasts. After 5–10 min of fusion treatment the fusion product was transferred, by micromanipulator, to nurse culture dish for individual cloning (Fig. 14.6). Microelectrofusion of preselected pairs of protoplasts combined with a reliable nurse culture method for individual fusion bodies is recommended as a good approach to controlled somatic hybridization and partial gene transfer (Yan et al. 2004).

For electrofusion the protoplasts should be of good quality. Poor quality protoplasts burst during electrofusion and release salts changing the conductivity of the fusion mixture, which is not good for fusion. For efficient electrofusion, the osmolarity of the fusion mixture should be proper and the strength, duration, and number of DC pulses should be optimized. Although increase in AC alignment voltage increases fusion efficiency but AC field greater than 125 V cm^{-1} causes lyses of protoplasts during DC pulse application (Grosse et al. 2010).

14.4 Protoplast Culture

The density of purified protoplasts is determined using hemocytometer and adjusted to the desired level before culture. A variety of methods have been used to culture freshly isolated protoplasts and after fusion treatment, which are similar to the methods used for cell culture (Chap. 4).

14.4.1 Culture Methods

(i) *Agarose Embedded Cultures*. This method is similar to the Bergmann's technique of cell plating, which allows following the development of specific individuals. The type and concentration of the gelling agent may influence protoplast development. Pure, low gelling temperature agarose, such as SeaPlaque (FMC BioProducts, USA) or Sigma type VII and IX are used extensively for protoplast culture. Alginate is a useful gelling agent for protoplasts that are heat sensitive, such as *Arabidopsis thaliana*. The semisolid medium with embedded protoplasts may be cut into sectors and transferred to liquid culture medium of the same composition in larger plates.

(ii) *In Liquid Medium*. The protoplasts are suspended in liquid culture medium at the desired plating density and dispensed into culture dishes (3, 5 or 9 cm diameter) as thin layer or microdroplets (50–100 μl). The dishes are sealed with an expandable gas permeable tape, such as Parafilm and Nescofilm. This method is preferred because: (a) the protoplasts of some species would not divide in semi-solid medium (Gosch et al. 1975; White and Bhojwani 1981), (b) the osmotic pressure of the medium can be effectively reduced after a few days of culture, (c) the density of the cells can be reduced or cells of special interest can be isolated after culturing them for a few days at high density, and (d) the medium can be changed if the degenerating protoplasts secrete substances that are toxic to the healthy protoplasts. Liquid medium also permits rapid diffusion of nutrients.

(iii) *Double Layer Method*. The protoplasts are suspended in liquid medium and overlaid on a thin layer of gelled medium of the same composition.

(iv) *Agarose Droplets or Beads*. Embedding the protoplasts in agarose beads or discs improved the plating and regeneration efficiencies in many species (Dons and Colijn-Hooymans 1989). Protoplasts of several recalcitrant species of Magnoliaceae and Liliaceae divided and regenerated plants when their protoplasts were trapped in agarose droplets in such a way that streaks of locally high cell densities were obtained (Binding et al. 1988). Protoplasts trapped agarose beads are covered with liquid medium.

14.4.2 Cell Wall Formation

Under optimal culture conditions protoplasts synthesize a wall within 24 h and lose their characteristic spherical shape. Cereal and woody plant protoplasts take little longer to regenerate a wall. Wall formation can be readily checked by staining with Calcofluor White ST. Generally, a proper somatic cell wall is necessary for the cell to divide by normal mitosis. In the absence of a proper wall nuclear divisions may occur but these are not followed by cytokinesis. If the regenerated wall is thin the cells may show budding.

14.4.3 Cell Division and Callus Formation

Time taken for the first cell division in protoplast cultures varies with the species, genotype, protoplast source, procedure of protoplast isolation, protoplast viability, medium composition, and culture conditions.

(i) *Plant material*. Generally, protoplasts from aseptic seedlings, embryogenic calli and

suspension cultures divide more readily than those from mesophyll cells of greenhouse grown plants. Mesophyll protoplasts of cereals and some other monocots have remained recalcitrant so far. In some cases, it was essential to pre-culture the leaves before protoplast isolation to obtain culturable protoplasts (Donn 1978; Kao and Michayluk 1980).

(ii) *Plating density*. Plating density of protoplasts is a critical factor for the division and sustained growth of the protoplasts. Protoplasts are generally cultured at a density of 5.0×10^4–1.0×10^5 protoplasts ml^{-1}. However, to follow the fate of individual protoplasts or the fusion products it is desirable to culture them at low density or individually.

Kao and Michayluk (1975) developed a complex 8p medium (Table 14.1) in which individually cultured protoplasts of *Vicia hajastana* regenerated a wall, underwent sustained divisions and formed callus. This medium also supported faster divisions of mesophyll protoplasts of alfalfa and pea at low plating density (<100 protoplasts ml^{-1}) than at higher densities. This medium in its various modifications has been successfully used to culture protoplasts of a range of other species. It is recommended that while using 8p medium the cultures should be incubated in dark or low light (50 lux).

Microdroplet method is another approach to culture protoplasts at low density. Gleba (1978) obtained whole plants of tobacco from protoplasts cultured individually in 0.25–0.5 µl droplets. Size of the droplet is critical for the division of single protoplasts. One protoplast per 0.25–0.5 µl droplet gives an effective plating density of $2–4 \times 10^3$ protoplasts ml^{-1}. This technique has been successfully used to culture hybrid cells of *Nicotiana glauca* + *Glycine max* (Kao 1977) and *Arabidopsis thaliana* + *Brassica campestris* (Gleba and Hoffmann 1978). The microculture system of Koop and Schweiger (1985) for individual protoplast culture is depicted in Fig. 14.5.

Nurse cell/feeder cell layer technique has also been used successfully for the culture of protoplasts or fusion products at low density or individually, as in the case of electrically fused single pair of protoplasts (Fig. 14.6). Protoplasts or cells capable of rapid division from the same genus or species can be used as nurse cells. Protoplasts or cells from embryogenic cell suspensions are preferred as nurse tissue. In such cases, the nurse cells must be separated physically from experimental protoplasts by a membrane with pore size of 0.2–12 µm, or the cells may be rendered incapable of dividing by X-ray or γ-irradiation. Individual protoplasts of barley, tobacco, and rape could be cultured successfully using feeder systems.

(iii) *Culture Medium*. Generally, MS and B_5 media and their modifications have been used for protoplast culture. Ammonium ions are toxic to the protoplasts of many species. Therefore, media lacking NH_4NO_3 or containing it at a low concentration are used for protoplast culture. Kao and Michayluk (1975) developed the 8p medium for the culture of protoplasts at low density. It contains several vitamins, organic nutrients, sugar alcohols, and undefined substances in the form of casamino acids and coconut milk (Table 14.1). This medium has been used successfully on a broad range of species, including legumes, cereals, ornamentals, and fruit trees.

Growth hormones, particularly auxins and cytokinins, are almost always required. For cereals, however, 2,4-D alone is either sufficient or better than in combination with a cytokinin. The nature and relative concentrations of the hormones may vary with the system and need to be optimized empirically. Culture requirements of cells and tissues of a plant may give clues to the composition of the culture medium for their protoplasts but this is not always true. For example, cereal protoplasts are more sensitive to phytohormones than their cells. Similarly, the growth regulator autonomy of

Table 14.1 Composition of 8p medium for culturing protoplasts at low density[a, b, c]

Constituents	Amount (mg L^{-1})	Constituents	Amount (mg L^{-1})
Mineral salts			
NH_4NO_3	600.00	KI	0.75
KNO_3	1,900.00	H_3BO_3	3.00
$CaCl_2.2H_2O$	600.00	$MnSO_4.H_2O$	10.00
$MgSO_4.7H_2O$	300.00	$ZnSO_4.7H_2O$	2.00
KH_2PO_4	170.00	$Na_2MoO_4.2H_2O$	0.25
KCl	300.00	$CuSO_4.5H_2O$	0.025
Sequestrene 330 Fe	28.00	$CoCl_2.6H_2O$	0.025
Sugars			
Glucose	68,400.00	Mannose	125.00
Sucrose	125.00	Rhamnose	125.00
Fructose	125.00	Cellobiose	125.00
Ribose	125.00	Sorbitol	125.00
Xylose	125.00	Mannitol	125.00
Organic acids (pH adjusted to 5.5 with NH_4OH)			
Sodium pyruvate	5.00	Malic acid	10.00
Citric acid	10.00	Fumaric acid	10.00
Vitamins			
Inositol	100.00	Biotin	0.005
Nicotinamide	1.00	Choline chloride	0.50
Pyridoxine–HCl	1.00	Riboflavin	0.10
Thiamine-HCl	10.00	Ascorbic acid	1.00
D-Calcium pantothenate	0.5	Vitamin A	0.005
Folic acid	0.2	Vitamin D_3	0.005
p-Aminobenzoic acid	0.01	Vitamin B_{12}	0.01
Hormones			
Soybean + Barley		Soybean + Pea or *N. glauca*	
2,4-D	1.00	2,4-D	0.2
Zeatin	0.1	Zeatin	0.5
NAA	–	NAA	1.00
Vitamin-free casamino acid	125 mg L^{-1}	–	–
Coconut water	10 ml L^{-1}	–	–

[a] *after* Kao and Wetter (1977)
[b] sterilized by filtration
[c] this medium is also recommended for cell culture at low density. In that case, the medium is modified to have 3 % sucrose, 1 % glucose, 250 mg L^{-1} casamino acid, and 20 ml L^{-1} coconut water. All other components are the same

the crown gall tissue cultures is lost on removing the cell wall and is restored at the multicellular stage (Scowcroft et al. 1973). The presence of antioxidants in the medium is either essential (*Beta vulgaris*; Kresn et al. 1990) or beneficial (*Lolium perenne*; Creemers-Molenaar and van Oort 1990) for the culture of protoplasts of some plants.

(iv) *Osmoticum*. Osmotic fragility is a fundamental property of protoplasts. Therefore, the presence of a suitable osmotic stabilizer in the medium is essential to begin with. After 4–5 days of culture it is gradually lowered by periodic addition of a few drops of fresh medium lacking the osmoticum until multicellular colonies are formed.

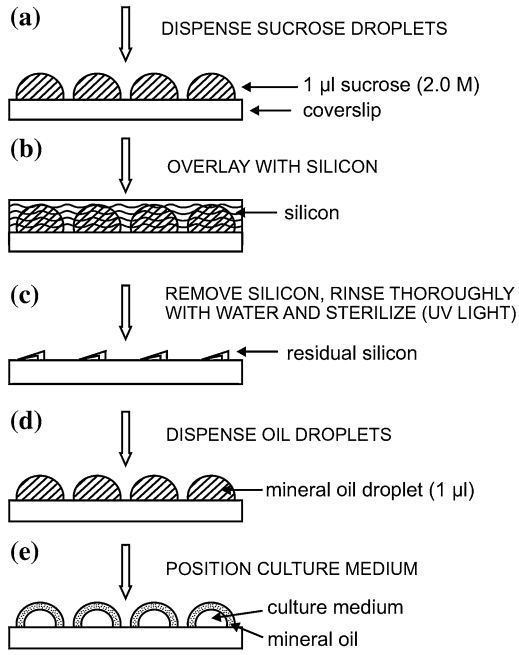

Fig. 14.5 Diagrammatized summary of the microdroplet method used for individual cell culture by Koop and Schweiger (1985). **a** 1 µl sucrose (2 M) drops are dispensed on a coverslip using a capillary pipette. **b** The sucrose droplets are overlaid with silicon. **c** The coverslip is thoroughly washed to remove the silicon and sterilized by UV irradiation. **d** One µl droplets of mineral oil are dispensed on the coverslip. **e** 15–100 nl of culture medium is injected into each oil droplet. Individual cells or fused protoplasts are planted in the medium with the help of a micropipette (*after* Koop and Schweiger 1985)

Generally, 500–600 mM mannitol is used as the osmotic stabilizer. In many cases glucose has proved superior to other osmotica. For potato, cassava, and sweet potato sucrose has been used as the osmoticum (Shepard and Totten 1977; Bidney and Shepard 1980; Shahin and Shepard 1980). For brome grass, sucrose proved better than glucose or mannitol (Michayluk and Kao 1975).

(v) *Physical Factors*. Very little attention has been paid to the effect of physical factors on protoplast culture. Several reports have described promotory effect of electroporation treatment to the isolated protoplasts on onset of cell division, plating efficiency, and regeneration potential of calli derived from treated protoplasts (Rech et al. 1987; Chand et al. 1988; Ochatt and Power 1992; Gupta et al. 1988). Heat shock treatment of rice protoplasts before culture doubled the incidence of cell division (Thompson et al. 1987). On the other hand, Muhlbach and Thiele (1981) reported enhanced plating efficiency following cold treatment of the freshly isolated protoplasts of *Pennisetum squamulatum*.

(vi) *Culture Conditions*. Freshly isolated protoplasts are very sensitive to light. Therefore, protoplast cultures are generally incubated in dark. Once the protoplasts have regenerated a proper wall and undergone a few divisions, they could be transferred to light. Protoplast cultures are generally maintained at 25–30 °C. Mesophyll protoplasts of *Lycopersicon esculentum* and *L. peruvianum* cultured at 25 °C either failed to divide or did so at very low frequency but at 27–29 °C they divided with a high plating efficiency (Zapata et al. 1977).

14.4.4 Plant Regeneration

Plant regeneration from freshly isolated protoplasts or after their fusion occurs via organogenesis or embryogenesis. The first report of plant regeneration from isolated protoplasts of *Nicotiana tabacum* was published by Takebe et al. (1971). By 1999, the totipotency of protoplasts was demonstrated in 368 species or subspecies of higher plants covering 161 genera and 46 families (Xu and Xue 1999). By the same time, 135 somatic hybrids and cybrids had been produced with definite breeding objective from 125 parental combinations involving 114 species (Li 1999).

The factors affecting regeneration from protoplast derived calli are similar to those described in Chaps 6 and 7. Interestingly, even if one of the fusion partners is regenerable it may be possible to obtain plants from the hybrid cells.

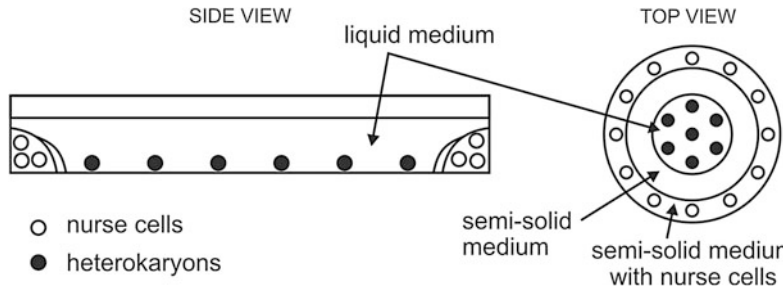

Fig. 14.6 Diagrammatized depiction of the nurse cell technique for the culture of electrically fused protoplasts at low density. The ring of nurse cells-containing (5×10^4 protoplasts ml^{-1}) K3 medium (Nagy and Maliga 1979) with 0.4 M glucose and 0.25 % agarose is separated from liquid medium containing fused protoplasts by a ring of cell-free agar medium. (*Based on the work of* Rakosy-Tican et al. 2001)

For example, interspecific hybrids in the genus *Citrus* have been produced mostly by fusing regenerable protoplasts from embryogenic callus/suspension cultures of one parent with nonregenerable mesophyll protoplasts of the other parent (An et al. 2008).

Some remote somatic hybrids, especially symmetric hybrids, may exhibit developmental abnormalities due to genomic incompatibility (Grosser et al. 2000). The shoots of interspecific somatic hybrids *Lupinus angustifolius* + *L. subcarnosus* and *Brassica carinata* and *Camelina sativa* (Narasimhulu et al. 1994) exhibited poor or no rooting. The somatic hybrids from the combination *Brassica oleracea* and *Matthiola incana* did not survive beyond 3 months after transfer to soil (Sheng et al. 2008).

14.5 Selection of Somatic Hybrids

In electrical fusion of one-to-one protoplasts, the selection of the hybrid cell is not a problem. The fusion body can be transferred to a suitable medium and cultured in isolation or at low density. However, after chemical fusion the fusion mixture is generally a heterogeneous mixture of homokaryons, heterokaryons, parental types, and a variety of nuclear cytoplasmic combinations. The frequency of the desirable fusion products is always much lower than the parental types. Being novel combinations several things may happen after the fusion treatment, further reducing the frequency of potential hybrid cell lines. Therefore, several strategies have been followed to select or enrich the population of hybrid cells. Of these, biochemical mutants and antibiotic and herbicide resistance are used very frequently. So far the biochemical mutants are concerned, chlorophyll or nitrate reductase deficient and albino mutants have been widely used. Complementation between the two parents mutually abolishes the deficiency, and only the hybrid cells are able to grow on minimum medium nonpermissive for the growth of parental protoplasts. Similarly, differences in resistance to specific antibiotics, amino acid analogs or herbicides could also expedite hybrid selection when fusion products are grown in a medium supplemented with the above-mentioned chemicals. If each of the fusion parents is resistant to a different antibiotic the medium containing both the antibiotics will not allow the parental protoplasts to grow but the hybrid cells will be able the grow through.

Genetic and/or metabolic complementation is often applied in asymmetric hybridization. The donor protoplasts are irradiated to fragment their chromosomes and the recipient protoplasts are treated with metabolic inhibitors, such as iodoacetate/iodoacetamide. Thus, both the parents are rendered incapable of dividing on their own. Even their homokaryons would be unable to divide. However, due to complementation the hybrid cells may divide. Complementation may also restore the regeneration potential of the

hybrid cells formed by the fusion of nonregenerative protoplasts (*Lupinus angustifolius* + *L. subcarmosus,* Sonntag et al. 2009).

In most of the reports on somatic hybridization in the genus *Citrus,* mesophyll protoplasts of one parent and embryogenic callus/suspension of the other parent have been used as the fusion partners that allows the recognition of freshly formed fusion products with green and nongreen cytoplasms (Wu et al. 2005; Anantha Krishnan et al. 2006; Cai et al. 2007; Olivares-Fuster et al. 2005). Furthermore, the media often used for their culture discourage the parental protoplasts from undergoing somatic embryogenesis. The result is a natural selection procedure for the fusion products, especially in interspecific and intergeneric fusions. Wu et al. (2005) hybridized Encore mandarin with Valencia sweet orange and Encore mandarin with Clementine mandarin by fusing protoplasts from their embryogenic calli. Under normal culture conditions, without employing a selection strategy, 90 % of the plants regenerated were somatic hybrids.

14.6 Characterization of Somatic Hybrids

Even after a passage through an effective selection method, it is desirable to confirm the hybridity of the plants regenerated from the fusion products because of the chances of escapes through the selection pressure. Morphological, cytological, and molecular methods have been applied for the purpose.

The intermediate expression of vegetative and floral characters is the simplest approach to check the hybrid nature of the plants. Cytological analysis reveals the morphology and number of chromosomes, which may be helpful if the fusion partners are distantly related with prominent differences in the number and morphology of their chromosomes but not for somatic hybrids between closely related plants. However, cytology in combination with *in situ* hybridization (ISH) techniques, such as GISH (Genomic *In situ* Hybridization) and FISH (Fluorescence *In Situ* Hybridization) may prove to be powerful tools not only to distinguish between the chromosomes of the two parents but also to detect introgression of parts of chromosomes of one parent into the genome of the other parent by recombination and chromosome rearrangement (Han et al. 2009). Analysis of tetraploid and hexaploid somatic hybrids between *Lycopersicum esculentum* and *Solanum lycopersicoides* (nightshade), using GISH, revealed that in tetraploids both the parents contributed equal number of chromosomes, but the hexaploid hybrids had four sets of tomato chromosomes and two sets of wild nightshade. Intergenomic translocations and chromosome rearrangement have also been detected by GISH in the somatic hybrids *Zea latifolia* + *Oryza sativa* (Liu et al. 1999), *Triticum aestivum* + *Avena sativa* (Xiang et al. 2003).

Use of molecular techniques such as isozyme analysis and DNA analysis by RFLP, RAPD, SSR, AFLP, and 5S rDNA spacer sequence have been used to characterize the somatic hybrids (Liu et al. 2005). Some of these techniques are briefly described in Chap. 12.

14.7 Consequences of Protoplast Fusion

The hybrid cells formed by the fusion of two unrelated protoplasts combine a set of three genomes from the parents, viz. nuclear genome, mitochondrial (mt) genome, and plastid (pt) genome. The hybrid cells may give rise to hybrid plants with full nuclear genomes of both the parents (Symmetrical hybrids; Fig. 14.7). However, very often interactions between the genomes of the two parents result in various combinations of nuclear and cytoplasmic genomes (Fig. 14.7). The nuclear genome of one of the parents may be partially or completely eliminated during successive cell cycles, before regeneration of plants, leading to asymmetric hybrid or cybrid formation, respectively. Somatic hybrid cells may also show segregation

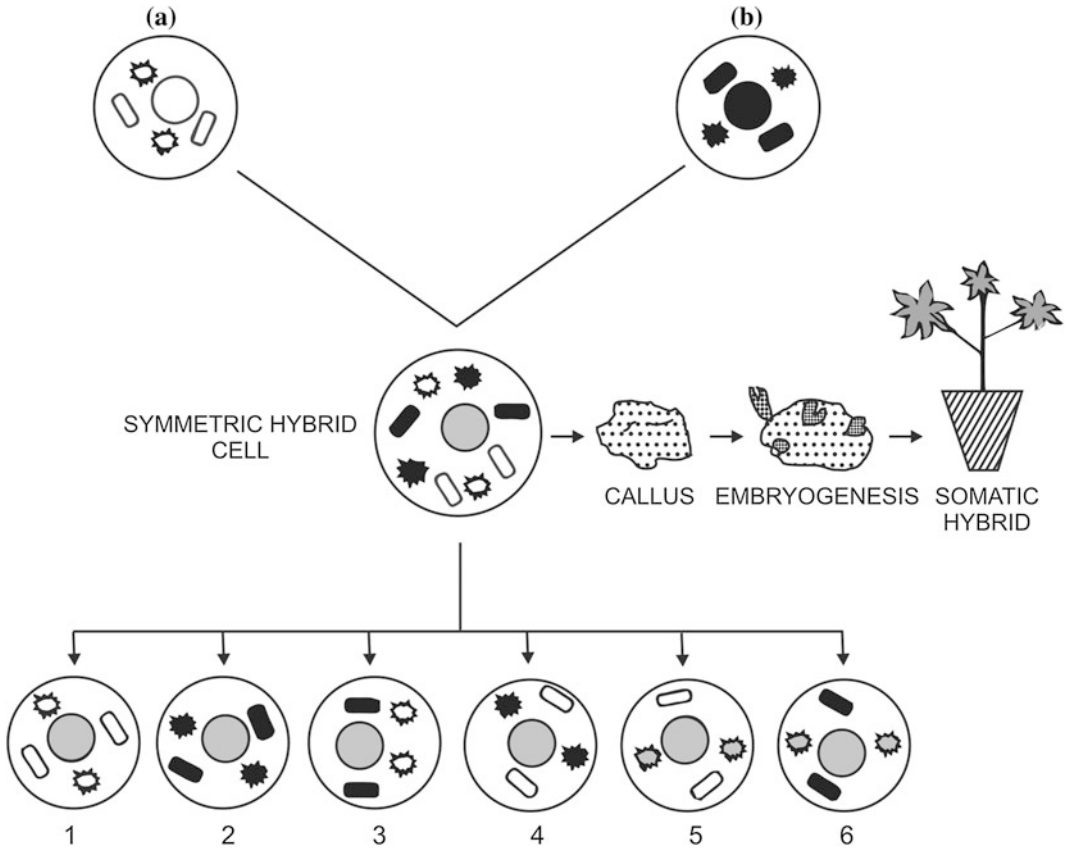

Fig. 14.7 Fusion of protoplasts of different species (**a** and **b**) results in the formation of a somatic hybrid cell, which may form symmetric hybrid plants. During culture the hybrid cell may show segregation of chloroplasts and mitochondria independent of each other. Assuming that the nuclear genome of the hybrid cell does not show any change, hybrids with various nuclear organelle combinations may be formed: both the organelles may be from the same parent (*1*, *2*), or chloroplasts of one parent and mitochondria of the other (*3*, *4*). The mitochondrial genomes of the two parents may undergo recombination and occur in combination with the chloroplasts of one or the other parent (*5*, *6*). c*ircles*, nuclei, *rectangles*, chloroplasts, *stars*, mitochondria; *blank*, parent a, *black*, parent b, *Grey*, recombinants

of cytoplasmic genomes. As a rule, the plastid populations show random segregation of one of the parental types, resulting in cells with one or the other type of plastids. Recombination of plastid genomes occurs only rarely. On the other hand, the mitochondrial genomes very often undergo interparental recombination. and after segregation one of the many recombined genomes is retained in a regenerated plant or its progeny. This behavior of nuclear and cytoplasmic genomes results in plants with a range of nuclear-mitochondrial-plastid genomic combinations.

14.8 Symmetric Hybridization

The most common target using somatic hybridization is the generation of symmetrical hybrids with complete genomes of both the fusion parents. Since the first report of interspecific hybrids of tobacco (Carlson et al. 1972), a large number of symmetric hybrids have been produced. Although combining unrelated genomes through protoplasts fusion often leads to partial or complete elimination of nuclear and/or cytoplasmic genomes of one of the parents there are

many examples where symmetric somatic hybrids with traits of agronomic importance from both the parents have been obtained (Cai et al. 1999).

Austin et al. (1985, 1986) produced tuber bearing amphidiploid (hexaploid) hybrids by fusing protoplasts of *Solanum tuberosum* and *S. brevidens;* the latter is a nontuber bearing wild species. The hybrid combined the genomes of both the parents and exhibited resistance to *Potato Leaf Roll Virus* (PLRV) and *Phytophthera infestans* contributed by the wild parent and tuber forming character of potato (Austin et al. 1985; Helgeson et al. 1986). Similarly, three of the four symmetric hybrids obtained by fusing the protoplasts of dihaploid *S. tuberosum* and diploid *S. circaeifolium* were fully resistant to the pathogen *P. infestans,* and all the four hybrids were highly resistant to the nematode, *Globodera pallida*. Sexual hybrids between the somatic hybrid (as female parent) and *S. tuberosum* set viable seeds, suggesting the potential of the hybrids in potato breeding (Mattheij et al. 1992). One of the six tetraploid somatic hybrids between *S. tuberosum* and *S. phureja* gave three times higher yield than the potato cultivar under field trials (Mattheij et al.; *cited in* Mattheij et al. 1992). Kameya et al. (1990) produced somatic hybrids between *S. melongena* and *S. integrifolium*, possessing total chromosome number of the two species, which exhibited high resistance to *Pseudomonas solanacearum* than either of the parents.

Somatic hybrids between Japanese radish and cauliflower showed resistance to clubroot, a serious disease in cauliflower. The selfing progenies of the somatic hybrid showed stable and perfect resistance to clubroot over three generations. The backcross progenies also showed resistance to the disease (Hagimori 1995; Hagimori et al. 1992). Somatic hybrids between chrysanthemum (*Dendranthema* x *grandiflorum*) and wormwood (*Artemisia sieversiana*) were more resistant to rust caused by *Puccinia horiana* than chrysanthemum (Furuta et al. 2004). The backcross progenies of the somatic hybrids between rapeseed and *Sinapsis arvensis* or *Arabidopsis thaliana* showed significantly higher resistance to blackleg or stem canker than rapeseed, indicating stable inheritance of disease resistance from the somatic hybrid to the progeny.

Symmetric somatic hybridization is being applied to produce allotetraploids of scion varieties of *Citrus* for sexual crossing with diploids to obtain seedless triploids (see Sect. 14.11). For more examples of symmetric and asymmetric hybrids produced with specific breeding objectives see Li et al. (1999).

14.9 Asymmetric Hybridization

Combination of complete genomes of two alien parents is neither desirable nor always stable. When a cultivated species is crossed with a wild species, the hybrid contains many undesirable genes of the wild parent, which must be removed by recurrent back crossing. Moreover, combining alien genomes may cause genetic imbalance and interfere with normal development of the hybrid. Many asymmetric hybrids showed higher regeneration and rooting capacity as well as increased fertility as compared to symmetric hybrids (Bates et al. 1987; Fahlson et al. 1994; Hull et al. 2002; Skarzhinskaya et al. 1996; Forsbera et al. 1998). Therefore, partial nuclear genome transfer of the donor parent (asymmetric hybridization) is considered more desirable.

Spontaneous occurrence of asymmetric hybrids by selective elimination of chromosomes of one of the parents following protoplast fusion of distantly related plants prompted search for methods for directed elimination of chromosomes of the donor plant, to synthesize fertile asymmetric hybrids. This has been possible by fragmentation of the chromosomes of the donor parent by X-ray or γ-irradiation before fusion. Dudits et al. (1980) produced the first intergeneric asymmetric hybrid between X-ray irradiated parsley (*Petroselinum hortense*) and

normal tobacco protoplasts. Following this approach, interspecific transfer of resistance to various diseases has been achieved, particularly in the genera *Brassica* (Schieder et al. 1991; Itoh et al. 1991; Sjodin and Glimelius 1989), *Nicotiana* (Bates 1990), rice (Kinoshita and Mori 2001; Yan et al. 2004), *Helianthus* (Valconen and Rokka 1998; Collonier et al. 2003; Furuta et al. 2004), and *Citrus* (Grosser et al. 2000). UV radiation is being increasingly preferred over γ-irradiation because of its easy access, convenience for causing chromosome breakage and better results (Hall et al. 1992). Irradiation-induced chromosome elimination may be affected by genotype, irradiation type, and dosage, phylogenetic relationship between the fusion partners, physiological status of the irradiated material and ratio between DNA content of the donor and the recipient parents.

The somatic hybrids raised through fusion of normal hypocotyl protoplasts of *Helianthus annuus* with UV irradiated protoplasts of *H. maximiliani* exhibited morphological characters of *H. maximiliani* but did not show any band in the zymogram and RAPD profiles characteristic of the wild species (Taski-Ajdukovic et al. 2006). Asymmetric somatic hybrids produced by fusion of UV-irradiated protoplasts of common wheat with the normal protoplasts of *Arabidopsis thaliana* formed green wheat-like plants without any chromosome of *A. thaliana.* However, isozyme pattern and RAPD analysis of the hybrid calli confirmed their hybrid nature, and GISH analysis revealed that 1–3 small chromosome fragments of *Arabidopsis* got introgressed into the terminals of wheat chromosomes (Deng et al. 2007).

In an attempt to transfer high erucic acid trait from *Crambe abyssinica* to *Brassica napus*, Wang et al. (2003) fused hypocotyl protoplasts of *B. napus* with UV-irradiated mesophyll protoplasts of *C. abyssinica*. Of the 124 shoots regenerated from the calli formed by the fused protoplasts 20 were asymmetric hybrids as confirmed by DNA content and AFLP analyses. Two of these hybrids with 38 chromosomes, like *B. napus* contained higher erucic acid content and showed higher seed set.

Several fertile asymmetric somatic hybrids have been produced between *Oryza sativa* and wild *Oryza* species (Kinoshita and Mori 2001; Yan et al. 2004). Yan et al. (2004) transferred bacterial leaf blight resistance, a multigenic trait, from *O. meyeriana* (a wild species) to an elite japonica cultivar of rice by asymmetric somatic hybridization. The isolated protoplasts of *O. sativa* and *O. meyeriana* were subjected to iodoacetamide (2.5 mM for 15 min) and X-ray (60 krad) treatments, respectively, to render them incapable of dividing. Iodoacetamide (IOA) is a metabolic inhibitor, which physiologically impairs the protoplasts for division by affecting the metabolic processes of the cell. Iodoacetate can also be used for this purpose. Thus, after fusion none of the parental type protoplasts divided but the hybrid cells through functional complementation were able to divide and regenerate plants. The chromosome number of the hybrids was the same as *O. sativa* ($2n = 24$) but they showed high resistance to bacterial blight, suggesting integration of some genetic material from *O. meyeriana* into *O. sativa* genome through chromosome recombination or substitution.

Microprotoplast-mediated chromosome transfer (MMCT) is another promising tool that allows partial genome transfer from a donor species that cannot be hybridized sexually with the recipient species and produce monosomic additional lines (MAL) without intensive or tedious backcrosses. In this technique, established by Ramulu et al. (1995, 1996), complete protoplasts of the recipient parent are fused with microprotoplasts of the donor parent. Microprotoplasts are prepared by treating the cultured cells in early log phase with an inhibitor of DNA synthesis (e.g., 10 mM hydroxy urea) for 24 h, followed by treatment with a microtubule inhibitor (e.g., 32 μM amiprophos-methyl) for 48 h to fragment the nucleus into micronuclei each with one or more chromosomes. The microtubule inhibitor and cytochalasin B (20 μM) are also added to the wall degrading enzyme mixture during protoplasting. After enzyme treatment the sample is filtered through 297 and 88 μm nylon meshes. The purified and

washed dense suspension of mononucleate and micronucleated protoplasts are loaded on a continuous iso-osmotic Percoll gradient and subjected to high-speed centrifugation (1,00,000 g) for 2 h. It results in pinching off of microprotoplasts, each with one or more micronuclei. By filtration through nylon sieves of decreasing pore size (30–5 μm) smaller sub-diploid microplasts are obtained. There is direct correlation between the size of microprotoplasts and the number of chromosomes in its micronucleus. The procedure to produce microprotoplasts is summarized in Fig. 14.8. Ramulu et al. (1996) obtained MAL containing whole genome of tomato (*L. peruvianum*) and one chromosome of potato (*S. tuberosum*). Following this approach, Binsfeld et al. (2000) produced hybrid plants of common sunflower (*Helianthus annuus*) containing 2–8 chromosomes of maximilian sunflower (*H. maximiliani*) or giant sunflower (*H. giganteus*). MAL may alleviate somatic incompatibility because addition of a single chromosome to the whole genome of another species could be much better tolerated than combining of whole genomes from two divergent species.

14.10 Cybridization

Cybridization refers to combining nuclear genome of one parent with mitochondrial and/or chloroplast genome/s of another parent. In sexual hybridization, cytoplasmic genomes are generally contributed by only the female parent. However, in somatic hybridization of normal protoplasts extranuclear genomes from both the parents are combined. Interparental recombination of mitochondrial genomes and independent assortment of chloroplasts and mitochondria following cell fusion result in plants with novel combinations of nuclear-plastid-mitochondrial genomes (Fig. 14.7).

In somatic hybrids or cybrids the chloroplast genome is generally from one of the parents as those of the other parent are lost at an early stage. However, co-existence of chloroplasts from both the parents has been noted in some cases (Primard et al. 1988; Mohapatra et al. 1998; Moreira et al. 2000; Cheng and Xia 2004). In some intergeneric crosses chloroplast DNA recombination has also been reported (Thanh and Medgyesy 1989; Baldev et al. 1998; Zhou et al. 2001, 2002). Unlike the chloroplast genome, mitochondrial genomes in most of the somatic hybrids show extensive recombination and rearrangement (Yamagishi et al. 2002, Leino et al. 2003; Takami et al. 2004; Xu et al. 2004). Nuclear-cytoplasmic combinations may be compatible or incompatible. Cybrid plants of tobacco and *Atropa belladonna* were green when they contained nuclear genome of tobacco and chloroplast genome of *A. belladonna* but became chlorophyll deficient if they contained *A. belladonna* nuclear genome and chloroplast genome of tobacco (Kushnir et al. 1987; 1991). Nuclear-cytoplasmic incompatibility was also observed in the cybrids between tobacco and *Hyoscyamus niger* (Zubki et al. 2001).

Irradiation of the donor protoplasts, as in the case of asymmetric hybrid production, may also cause complete loss of donor chromosomes, in which case only cytoplasm from the donor is transferred to the somatic hybrids resulting in cybrid formation. However, desirable cybrids can be produced with relatively high frequencies by donor-recipient or cytoplast fusion methods. In the former, the nucleus of the donor protoplasts is inactivated (rendered incapable of dividing) by X-ray or γ-irradiation and fused with the normal protoplast of the recipient parent. Alternatively, the protoplasts of the recipient parent are treated with metabolic inhibitors, such as iodoacetate or iodoacetamide, and fused with the irradiated donor parent. In such combinations, the parental protoplasts are unable to divide but due to metabolic complementation the fusion products may divide and form tissue in which the cells contain the nucleus of the recipient and cytoplasm of the donor parent (Fig. 14.9). The nucleus of the donor parent can also be avoided by fusing their cytoplasts (enucleated sub-protoplasts) with normal protoplasts of the recipient parent.

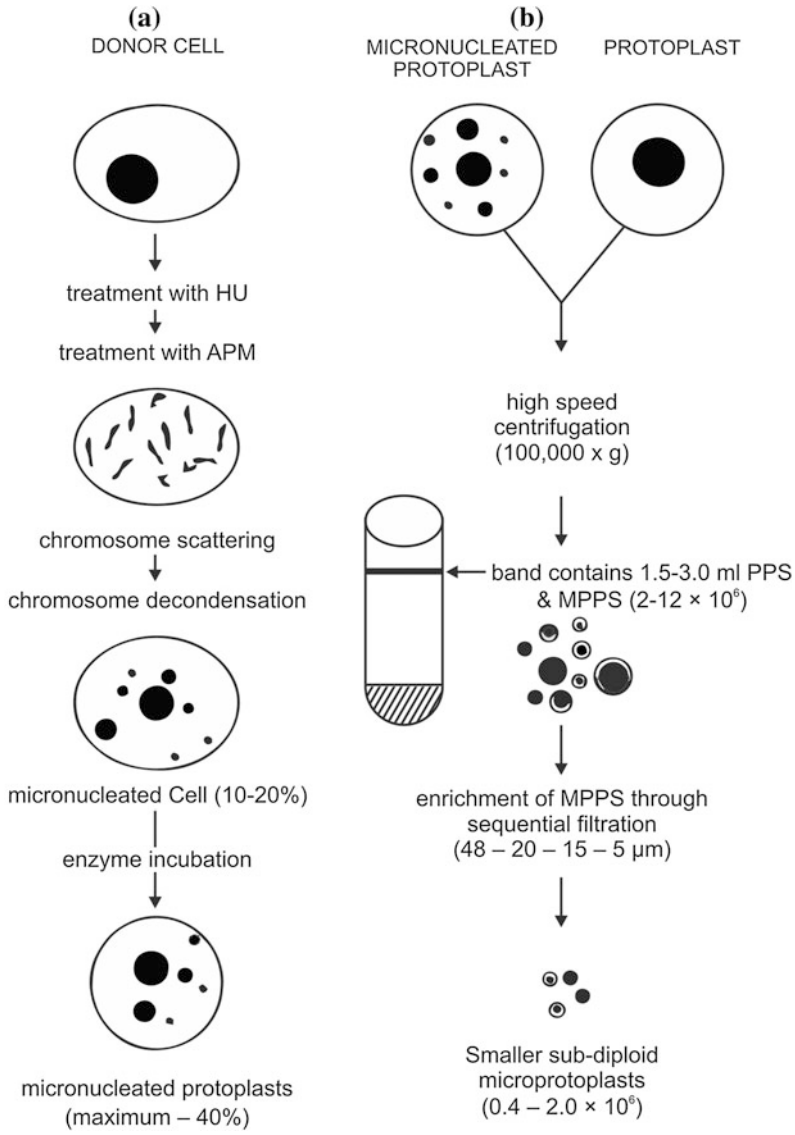

Fig. 14.8 Diagrammatized summary of the stages involved in the preparation of microprotoplasts (MPPS). **a** The cells from suspension cultures in the early log phase are treated with hydroxy urea (HU; 10 mM) for 24 h, followed by treatment with mitotic spindle inhibitor, such as amiprophos-methyl (APM; 32 µM) for 48 h. These treatments cause chromosome scattering, followed by chromosome decondensation and formation of micronuclei of various sizes with proportionate amount of the genome. **b** The micronucleated protoplasts obtained from micronucleated cells are subjected to high-speed centrifugation. The individual protoplasts (PPS) and MPPS, enriched by gradient centrifugation are filtered through a series of nylon sieves of decreasing pore size to enrich the population of MPPS with one micronucleus. These MPPS are fused with normal protoplasts of the recipient parent to produce asymmetric hybrids (*after* Ramulu et al. 1995)

Cybridization has been successfully used to make intergeneric and interspecifc transfer of cytoplasmic traits, such as male sterility and disease and herbicide resistance in tobacco, petunia, rice, *Brassica*, and *Citrus*. A major objective in somatic cybridization in citrus has

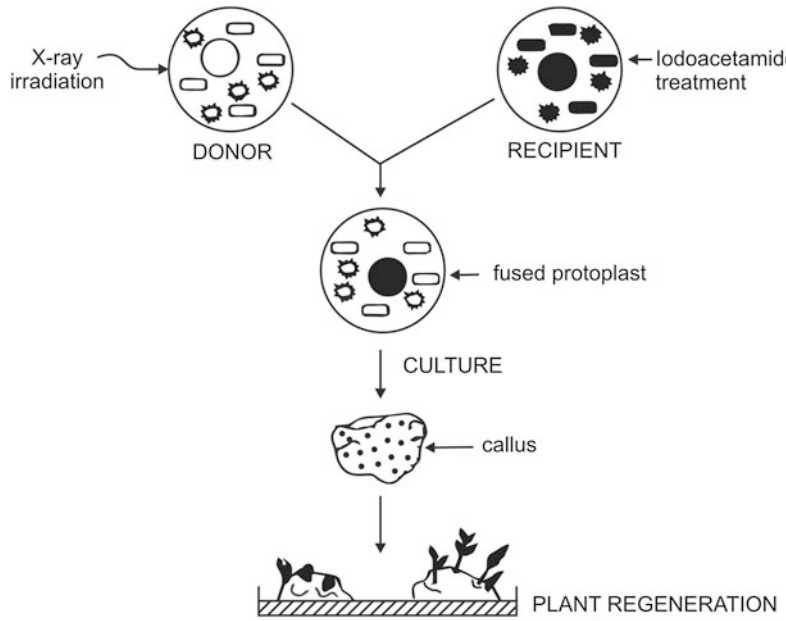

Fig. 14.9 Cybridization by fusion of irradiated protoplast of one parent (Donor) with iodoacetamide-treated protoplasts of the other parent (Recipient). It results in a hybrid cell with nuclear genome of the recipient and cell organelles of the other parent (Cytoplasm Donor)

been to transfer cytoplasmic male sterility (CMS) to facilitate conventional breeding or to produce seedless fruits (Grosser et al. 2010)

Jourdan et al. (1989) synthesized atrazine resistant CMS *B. napus* by cybridization between CMS (ogura) *B. oleracea* and atrazine-resistant *B. campestris*. CMS transfer via protoplast fusion has been accomplished in several combinations, such as sunflower + chicory (Rambaud et al. 1993; Varotto et al. 2001), *A. thaliana* + *B. napus* (Leino et al. 2003, 2004), *R. sativus* + *B. oleracea* (Kameya et al. 1989), tobacco + *Petunia hybrida* (Dragoeva et al. 1997), *B. juncea* + *Moricanda arvensis* (Kirti et al. 1998), and *R. sativus* + *B. napus* via cytoplast-protoplast fusion (Sakai and Imamura 1990).

14.11 Applications to Crop Improvement

Due to reproductive incompatibilities the agronomically important genes, such as resistance to biotic or abiotic stresses, from close and distant wild relatives cannot be efficiently employed in crop improvement programs. In contrast, many intergeneric, intertribal, or interfamily somatic hybrids have been produced via protoplast fusion and some of them are fertile, and therefore can be used as bridging materials for breeding (Liu et al. 2005).

Despite the rapid and impressive advancements in the field of genetic engineering leading to the release of several improved transgenic crop varieties interest in genetic manipulation of crop plants by cell fusion has continued, especially in the crops *Brassica*, *Citrus*, potato, rice, and sunflower, because of the obvious advantages offered by somatic hybridization. The major limitations of genetic engineering are: (i) difficulty in transferring polygenic traits, such as yield and resistance to biotic and abiotic stresses, impeding the wide application of genetic engineering, (ii) it has not been able to effectively manipulate extranuclear traits which can be combined by somatic hybridization, or cybridization, and (iii) public acceptability of genetic engineering is a subject of serious debates as it has integrated nonplant genes into plants, besides the unwanted reporter or selection marker genes. In these respects, somatic hybridization merits over genetic engineering. A unique advantage of somatic hybridization is in generating novel combinations of nuclear and/or cytoplasmic genomes by way of cybridization,

Table 14.2 Some somatic hybrids produced during the last decade

Species	Desirable trait	Reference
Brassica juncea + *B. oleracea*	Turnip Mosaic Virus resistance	Chen et al. (2005)
B. oleracea + *Matthiola incana*	Generate breeding material	Sheng et al. (2008)
B. napus + *Crambe abyssinica*	Oil quality	Wang et al. (2003)
Citrus unshiu + *C. sinensis*	Produce alloteraploids for breeding	An et al. (2008)
(*C. nobilis* x *C. deliciosa*) + *C. sinensis*	Produce alloteraploids for breeding	Wu et al. (2005)
(*C. nobilis* x *C. deliciosa*) + *C. clementina*	Produce alloteraploids for breeding	Wu et al. (2005)
Helianthus annuus + *H. maximiliani*	White rot resistance	Taski-Ajdukovic et al. (2006)
Lupinus angustifolius + *L. subcarnosus*	Establishment of protocol for somatic hybridization	Sonntag et al. (2009)
Nicotiana tabacum +*N. tabacum*	Resistance to antibiotics	Rákosy-Tican et al. (2001)
O. sativa + *O. meyeriana*	Bacterial blight resistance	Yan et al. (2004)
Solanum tuberosum + *S. brevidens*	Tuber soft rot and Early blight resistance	Polgar et al. (2000)
S. tuberosum + *S. pinnatisectum*	Late blight resistance	Szczerbakowa et al. (2005)
S. tuberosum + *S. rickii*	Antibiotic resistance	Matveeva et al. 2008
S. tuberosum + *S. tarnii*	Potato Virus Y and Late blight resistance	Thieme et al. (2008)
S. tuberosum + *S. verneï*	Plastome recombination	Trabelsi et al. (2005)
Triticum aestivum + *Arabidopsis thaliana*	Establishment of protocol for somatic hybridization	Deng et al. (2007)

which is not possible through sexual hybridization (Sect. 14.10).

According to Li et al. (1999), 135 somatic hybrids and cybrids, involving 114 species, have been produced with specific breeding objectives, including transfer of resistance to diseases, pests, frost and drought, cytoplasmic male sterility, and quality improvement traits. Liu et al. (2005) have listed 90 intergeneric combinations from which somatic hybrids have been obtained. In *Citrus* alone somatic hybrids and cybrids have been produced from 500 and 50 sexually compatible and incompatible parental combinations, respectively, with the objectives of improvement of scion and stock cultivars (Grosser et al. 2010). Some examples of somatic hybrids and cybrids produced since 2000 are included in Table 14.2. For more examples refer to Li et al. (1999), Liu et al. (2005) and Grosser et al. (2000).

Somatic hybridization in citrus is proving to be a valuable technology contributing to improvement of scion and rootstock cultivars in various ways (Grosser et al. 2000, 2010; Grosser and Gmitter Jr 2005). One of the objectives of scion improvement is to produce easy to peel, seedless, and triploid cultivars. Triploids can be produced directly by somatic hybridization of haploid and diploid parents which cannot be crossed sexually (Ollitrault et al. 2000). However, this approach is of limited application because of the difficulty in obtaining haploids. A more practical and popular approach to produce seedless triploids is to cross diploids with tetraploids. Autotetraploids produced by colchi diploidization of diploid cultivars generally exhibit poor and stunted growth. Therefore, vigorously growing allotetraploids, produced by symmetric somatic hybridization of elite diploid cultivars, have been mostly used as the pollen parents in the crosses with elite diploids to produce triploids for the improvement of grapefruit/pummelo, acid fruits (lemon/lime), and mandarin (Grosser et al. 2000, 2010; Grosser and Gmitter Jr. 2005, Wu et al. 2005;

An et al. 2008). Several of the tetraploid somatic hybrids are producing seedless or nearly seedless fruit and a few of them are of cultivar quality, which may be released as fresh fruit cultivars in their own right (Grosser and Gmitter Jr. 2005). Another approach being tried to produce seedless citrus fruit varieties is by transferring CMS from Satsuma mandarin into commercially important seeding citrus cultivars via somatic cybridization.

Most of the fruit trees comprise two parts, scion and rootstock. Rootstock affects the adaptability, yield, size, and resistance of the grafted trees. The desirable traits are often present in distantly related species which are sexually and/or graft incompatible. This and some other barriers associated with traditional breeding, such as heterozygosity, long juvenility, and large tree size can be overcome by somatic hybridization. Two approaches are being followed for rootstock improvement in citrus. One is complementary combination of diploid rootstocks to combine their elite traits into novel tetraploid rootstocks. This has been accomplished for numerous combinations and over 70 such somatic hybrids are under commercial field trials. The second strategy is to cross the citrus rootstock cultivars with the sexually incompatible genera, such as *Citropsis, Atlantia, Clausena and Murraya*. Most of the citrus somatic hybrids are tetraploids which are effective in reducing tree size (Grosser and Chandler 2003). Yield and tree size data from field trials demonstrated that somatic hybrid rootstock can produce adequate yield and high quality sweet orange fruits on small trees.

14.12 Concluding Remarks

In vitro technology has contributed significantly to enlarge the scope of wide hybridization for genetic upgradation of crop plants by providing methods to overcome pre-zygotic and post-zygotic barriers of sexual incompatibility (Chaps. 10 and 13). Genetic engineering (Chap. 15) is a precise, aim-oriented sophisticated technology for genetic manipulation of crop plants. However, its applications are limited due to lack of target genes, difficulty in transferring polygene traits (e.g. yield, stress resistance). Moreover, presence of the selection or reporter marker genes in the transgenic plants poses negative impact on public acceptability of the transgenic products. In this regard, somatic hybridization is a promising technique for the introgression of alien genes, including polygenic traits into crop plants and the technique has advanced from the academic stage to field applications. A unique advantage of somatic hybridization is in creating new combinations of nuclear and/or cytoplasmic organelles, leading to more variation enrichment of current gene pool. Asymmetric somatic hybridization to transfer part of the gene of the donor parent and cybridization to transfer cytoplasmic traits has considerably enhanced the importance of somatic hybridization. In terms of biosafety, somatic hybridization has an edge over genetic engineering because in the latter some of the genes are alien to plants, derived from other kingdoms.

It has been well documented that protoplast fusion can be used to create useful bridging material for breeding. However, it must be appreciated that genomic incompatibility following protoplast fusion continues to be a serious bottleneck in somatic hybridization.

14.13 Landmarks in the History of Somatic Hybridization

1960	Enzymatic isolation of large number of protoplasts (Cocking)
1971	First report of plant regeneration from protoplasts (Takebe et al.)
1972	First somatic hybrid plant production (Carlson et al.)
1974	High frequency protoplast fusion obtained using polyethylene glycol as the fusogen (Kao and Michayluk; Wallin et al.)
1978	First intergeneric somatic hybrid plant produced (Melcher et al.)
1978–1980	First asymmetric somatic hybrid or cybrid plants produced with transferred cytoplasmic male

	sterility, using ionizing radiation to reduce genomic contribution from the donor (Zelcer et al. 1978; Aviv et al. 1980)
1979–1981	A versatile electrofusion method developed (Senda et al. 1979; Zimmermann and Scheurich 1981).
1979–1984	A highly efficient method for mass selection of hybrid cells developed using fluorescence-activated cell sorting (Galbraith and Galbraith 1979; Harkins and Galbraith 1984)
1987	Individual selection, culture, and manipulation of protoplasts achieved using computer-directed system (Schweiger et al.)
1990	First release (to Canadian tobacco industry as quoted by Brown and Thorpe 1995) of a commercial cultivar derived from protoplast fusion (Douglas et al. 1981, Pandeya et al. 1986)
1995	First inter-kingdom (*Nicotiana*-mouse) hybrid plant produced with introduced animal trait (mouse immunoglobulin G) in the leaves (Makankawkeyoon et al.)

14.14 Appendix

1. Isolation and culture of hypocotyl protoplasts from aseptic seedlings of *Brassica napus* cv *isuzu.* (*after* Chuong et al. 1985)
   (i) Raise aseptic seedlings of *B. napus* on a medium containing 0.2 % sucrose and 0.8 % agar, in the dark, at 25 °C.
   (ii) Excise 150–200 hypocotyl hooks (2–3 cm long) from 2 days old seedlings and plasmolyze them for 1 h in CPW 13 M solution[1] supplemented with (in mg L^{-1}) ampicillin (400), gentamycin (10) and tetracyclin (10).
   (iii) Transfer the hypocotyl pieces to the enzyme solution containing 2 % Rhozyme HP-150, 4 % meicelase, and 0.3 % macerozyme R-10 in CPW 13 M in a Petri dish and incubate at 25 °C in dark on a shaker at 60 rpm.
   (iv) After 12 h, gently agitate the enzyme mixture by pumping in and out of a pipette several times to enhance the release of the protoplasts.
   (v) Filter the enzyme mixture through two layers of a nylon mesh (60 μm pore size on top of 44 μm).
   (vi) Transfer the filtrate to a centrifuge tube and spin at 100 g for 3 min.
   (vii) Suspend the pellet in CPW 13 M and spin again. Repeat this washing process three times.
   (viii) Finally, suspend the protoplast pellet, at a density of 2×10^5 protoplasts ml^{-1} in Lichter's medium,[2] supplemented with 13 % w/v sucrose, 5 g L^{-1} Ficoll 400, 0.5 mg L^{-1} BAP, 1 mg L^{-1} NAA, 0.5 mg L^{-1} 2,4-D with pH set at 5.7. Plate them in Petri dish (2.5 ml of protoplast suspension per 15 × 60 mm Petri dish) and incubate in dark at 25 °C.
   (ix) After 4–6 weeks, transfer the floating microcalli to MS medium containing 200 mg L^{-1} casein hydrolysate, 5 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 0.6 % agarose, with pH set at 5.7.
   (x) After 3–4 weeks shoot buds may differentiate.
2. Isolation and culture of protoplasts from embryogenic suspension cultures of rice

[1] *CPW 13 M*: (mg L^{-1}) KH_2PO_4 (27.2), KNO_3 (101), $CaCl_2.2H_2O$ (1480), $MgSO_4.7H_2O$ (246), KI (0.16), $CuSO_4.5H_2O$ (0.025), Mannitol (13 %), pH 5.8.

[2] *Composition of the Lichter's medium* (mg L^{-1}): $Ca(NO_3)_2,.4H_2O$ (500), KNO_3 (125), $MgSO_4.7H_2O$ (0.25), KH_2PO_4 (125), $MnSO_4.4H_2O$ (25), H_3BO_3 (10), $ZnSO4.4H_2O$ (10), $Na_2MoO_4.2H_2O$ (0.25), $CuSO_4.5H_2O$ (0.025), $CoCl_2.6H_2O$ (0.025), EDTA (0.037), $FeSO_4.7H_2O$ (0.028), glycine (2), myo-inositol(100), nicotinic acid (5), pyridoxine.HCl (0.5), thiamine.HCl (0.5), folic acid (0.5), biotin (0.05), glutathione (30), L-glutamine (800) and L-serine (100).

(*Oryza sativa* cv. Taipei 309). (*after* Davey et al. 2010)

Isolation

(i) Surface sterilize the dehusked seeds of rice and, after three washings in sterile distilled water, culture them on LS2.5 medium (Linsmaier and Skoog's medium) containing 1.0 mg L^{-1} thiamine HCl and 2.5 mg L^{-1} 2,4-D, and gelled with 0.8 % SeaKem Le agarose (FMC BioProducts, USA). Seal the dishes with Nescofilm or Parafilm and incubate in the dark at 28 ± 2 °C.

(ii) After 28 days, excise yellow/white compact and nodular embryogenic callus and transfer ca 1 g aliquots to fresh LS2.5 medium of the original composition.

(iii) Transfer 1–2 g (F.W.) of the embryogenic callus to 100 ml Erlenmeyer flasks each containing 25 ml of LS2.5 liquid medium and incubate the cultures on an orbital shaker (120 rpm) in the dark, at 27 °C.

(iv) Every 5 days remove the flasks from the shaker and allow the cells to settle. Carefully remove 80 % of the spent medium by decanting or using a sterile pipette, avoiding any loss of cells and reduction in cell density, and replenish it with fresh medium.

(v) After 42 days, transfer the cell suspensions to 250 ml flasks and add 15 ml of fresh medium to each flask. Every 5 days, allow the cells to settle, remove 30 ml of spent medium and replace it with fresh medium.

(vi) At the third subculture, pass the cell suspensions through a sieve of 500 μm pore size. Discard the large aggregates and retain the suspensions.

(vii) After 90–120 days, transfer the cells to the same volume of AA2[3] liquid medium. Transfer the cultures every 7 days to new liquid medium by mixing 1 volume of cell suspension with three volumes of new medium.

(viii) Filter the cell suspension through a nylon sieve of pore size 500 μm into a preweighed 9 cm Petri dish and pipette out the liquid medium, leaving the cells in the dish. Reweigh the Petri dish and add appropriate volume of enzyme mixture (10 ml of enzyme solution g^{-1} FW of cells), containing 0.3 % (w/v) Cellulase RS, 0.03 % (w/v) Pectolyase Y23 and 0.05 mM MES in CPW13 M solution, pH 5.6.

(ix) Seal the Petri dish with Nescofilm or Parafilm M and incubate them on an orbital shaker at slow speed (30 rpm), for 16 h in the dark, at 27 ± 2 °C.

(x) Filter the protoplast suspension through sieves of 64, 45, and 30 μm pore size to remove undigested cell clumps, and transfer the protoplast suspension to a sterile 15 ml centrifuge tube. Wash the protoplasts three times by gentle centrifugation (at 80 g for 10 min each) and resuspension in CPW 13 M solution.

(xi) Suspend the protoplasts in a known volume (e.g. 10 ml) of CPW13 M solution and count protoplasts using a hemocytometer. Stain an aliquot of the protoplast suspension with Tinopal or Calcofluor White to confirm that the cell walls have been digested completely.

(xii) Check the viability of the protoplasts by staining with fluorescein diacetate and observing under UV illumination.

(xiii) Resuspend the protoplasts in normal strength liquid K8p medium (Gilmour et al. 1989) + 2,4-D (3 mg L^{-1}) at a density of 5.0×10^5 ml^{-1} in 15 ml screw-capped centrifuge tubes. Heat shock the protoplasts by placing the tubes in a water bath at 45 °C and, after 5 min, plunge the tubes into ice for 30 s.

[3] AA2 composition (mg L^{-1}): MS salts (NH_4NO_3 and KNO_3 replaced by KCl) + myo-inositol (20) + nitotinic acid (0.5) + pyridoxine HCl (0.1) + thiamine HCl (0.4) + L-glutamine (876) + L-aspartic acid (266) + L-arginine (174) + glycine (7.5) + 2,4-D (2) + sucrose (20,000).

(xiv) Pellet the protoplasts by centrifugation at 80 g. Remove the supernatant and resuspend the pelleted protoplasts in fresh K8p liquid medium (normal strength). Repeat this procedure. Pellet the protoplasts.

(xv) Mix equal volumes of liquid K8p medium (double strength) with 2.4 % SeaPlaque agarose at 40 °C. Carefully resuspend the protoplasts at a density of 3.5×10^5 ml^{-1} in the resulting K8p agarose culture medium.

(xvi) Immediately dispense 2 ml aliquots of the protoplast suspension in K8p agarose medium into 3.5 cm diameter Petri dishes. Allow the medium with the suspended protoplasts to gel for at least 1 h. Seal the dishes with Nescofilm or Parafilm M and incubate the cultures in the dark at 27 ± 2 °C.

(xvii) After 14 days, divide the agarose layers from each dish into quarters with a sterile scalpel. Transfer each quarter to a separate 5 cm diameter Petri dish. Add 3 ml of K8p liquid medium (normal strength) to each dish. Incubate the cultures in the dark at 27 ± 1 °C until cell colonies develop from the embedded protoplasts.

(xviii) Mix MSKN (MS + 2 mg L^{-1} NAA + 0.5 mg L^{-1} zeatin +3 % sucrose) liquid medium (double strength) with 2.4 % SeaPlaque agarose at 40 °C. Immediately dispense 20 ml aliquots of the medium into 9 cm diameter Petri dishes. Allow the medium to gel for at least 1 h. Transfer the protoplast-derived cell colonies to the plate, seal it and incubate as in step (xvi).

(xix) Mix MSKN liquid medium (double strength) with 0.8 % SeaKem Le agarose (8 g L^{-1}) at 40 °C. Immediately dispense 20 ml aliquots into autoclaved screw-capped 50 ml glass jars. From 7 to 14 days-old-cultures in step (xviii), transfer somatic embryo-derived shoots with coleoptiles and roots to the jars (one shoot per jar). Incubate at 25 ± 1 °C in the light (50 μmol m^{-2} s^{-1}, 16 h photoperiod, 'Daylight' fluorescent tubes).

(xx) Remove rooted plants from the jars and gently wash their roots free of semisolid culture medium. Transfer the plants to compost in 9 cm diameter pots, water the plants and cover with polythene bags. Stand the pots in trays containing water to a depth of about 10 cm in a controlled environment room (27 ± 2 °C with 12 h photoperiod, 180 μmol m^{-2} s^{-1}, 'Daylight' fluorescent tubes).

(xxi) After 7 days, remove one corner from each bag and a second corner 3 days later. Continue to open gradually the top of the bags during the next 10 days. Remove the bags after 21 days.

(xxii) Maintain the protoplast-derived plants in a controlled environment room at 27 ± 1 °C with an 18 h photoperiod provided by mercury vapour lamps (310 μmol m^{-2} s^{-1}). Transfer to glasshouse/field conditions as appropriate.

3. PEG (Polyethylene glycol)-induced fusion of protoplasts (the protocol may be slightly modified to suite the system under investigation). (after Kao 1976)

(i) Mix freshly isolated protoplasts from the two parental sources (while still in the enzyme solution) in a ratio of 1:1. Filter the suspension through a sieve of pore size 62 μm, collect the filtrate in a centrifuge tube and spin at 50 g for 6 min to sediment the protoplasts.

(ii) Remove the supernatant with a Pasteur pipette and wash the protoplasts with 10 ml of solution-I (500 mM glucose, 0.7 mM $KH_2PO_4.H_2O$ and 3.5 mM $CaCl_2.2H_2O$, pH 5.5).

(iii) Resuspend the washed protoplasts in solution-I to make a suspension with 4–5 % (v/v) protoplasts ml^{-1}.

(iv) Pipette 4–6 drops (40 µl) of protoplast suspension onto a Petri dish (6 cm diameter) and leave it undisturbed for 5–10 min, so that the protoplasts settle on the bottom of the Petri plate.

(v) Add, drop by drop, 60 µl of the fusion mixture (40 % PEG 1,500, 10.5 mM $CaCl_2.2H_2O$, 0.3 M glucose, pH 6) to each drop and incubate the plate in the dark at room temperature (24 °C).

(vi) After 10–20 min, add two 0.5 ml aliquots of solution-II (50 mM glycine, 50 mM $CaCl_2.2H_2O$, 300 mM glucose, pH 9–10.5) at 10 min interval. After another 10 min add 1 ml of the protoplast culture medium.

(vii) Wash the protoplasts five times at 5 min intervals with the culture medium. At the end of each washing do not remove the entire medium; leave behind a thin film of the medium over the protoplasts and add fresh medium. If the protoplasts of the two parents are distinguishable, it may be possible to identify the heterokaryon formation at this stage.

(viii) Finally, add 2–2.5 ml of the culture medium and gently swirl the plate to make a thin layer of the medium. Seal the Petri plate with Parafilm and culture in dark for 4–5 weeks.

4. Microelectrofusion of tobacco protoplasts (*based on* Rákosy-Tican et al. 2001)

(i) Take a 33 mm diameter Petri dish and cover its bottom with 1 ml of mineral oil, purified by washing with 1 N HCl and 1 N NaOH, followed by washing with distilled water until the pH of the water is 7.

(ii) Using a micropipette of bore diameter of 100 µm, attached to a micromanipulator, place 20 microdroplets (30 nl) of 0.4 M sorbitol in a matrix formation in the center of the bottom of the dish.

(iii) Along the wall of the dish place microdroplets of equimolar sorbitol solution containing protoplasts of the two parents separately and blank droplets to maintain humidity inside the dish. The design of the microelectrofusion chamber is depicted in Fig. 14.4.

(iv) To each sorbitol droplet inject two protoplasts, one of each parent, using micropipette.

(v) Lower the electrodes, with a distance of 500 µm between their tips, into the drops, so that the pair of protoplasts is positioned between their tips. The electrodes, made of entomological needles of 90 µm diameter, are attached to another micromanipulator and sterilized by immersion in 70 % ethanol.

(vi) Apply AC electric field of 84 V cm^{-1} and 750 kHz frequency to align the protoplasts.

(vii) After 30–60 s, apply 1–3 rectangular DC pulses of 500–670 V cm^{-1} of 4 milli seconds to fuse the aligned protoplasts.

(viii) Leave the protoplasts undisturbed for 5–10 min to regain a spherical shape. Transfer the fusion product, by micromanipulation, to nurse cultures[4] for individual cloning (Fig. 14.6), seal the plate with Parafilm and incubate in dark at 26 °C.

(ix) Transfer 1–2 mm cell colonies to RMO[5] medium and incubate in light

[4] Nurse culture medium (mg L^{-1}): $NaH_2PO_4.H_2O$ (150), $CaHPO_4$ (50), $CaCl_2.2H_2O$ (900), KNO_3 (2500), NH_4NO_3 (250), $(NH_4)_2SO_4$ (134), $MgSO_4.7H_2O$ (250), $FeSO_4.7H_2O$ (27.8), Na_2.EDTA (37.3), KI (0.75), H_3BO_3 (3), $MnSO_4.H_2O$ (10), $ZnSO_4.7H_2O$ (2), $Na_2MoO_4.2H_2O$ (0.25), $CuSO_4.5H_2O$ (0.025), $CoCl_2.6H_2O$ (0.025), Inositol (100), Nicotinic acid (1), Pyridoxine.HCl (1), Thiamine.HCl (10), 2,4-D (0.1), BAP (0.2) NAA (1), Xylose (250), Sucrose (0.4 M), Glucose (0.4 M) and SeaPlaque Agarose (0.25%).

[5] RM (mg L^{-1}): NH_4NO_3 (1650), KNO_3 (1900), $CaCl_2.2H_2O$ (440), $MgSO_4.7H_2O$ (370), KH_2PO_4 (170), Na_2.EDTA (37.3), FeSO4.$7H_2O$ (27.8), H_3BO_3 (6.2), $MnSO_4.4H_2O$ (22.3), $ZnSO_4.4H_2O$ (8.6), KI (0.83), $Na_2MoO_4.2H_2O$ (0.25), $CuSO_4.5H_2O$ (0.025), $CoCl_2.6H_2O$ (0.025).

(37.8 μmol m^{-2} s^{-1} and 16 h photoperiod) at 26 °C.

(x) For shoot differentiation and elongation transfer the well-developed calli to RM medium containing BAP (1 mg L^{-1}) and NAA (0.1 mg L^{-1}) and gelled with 0.8 % Difco agar. Incubate the cultures in light (3,000 lux).

(xi) The shoots are rooted on semi-solid medium with RM salts (the concentrations of KNO_3, NH_4NO_3, and $MgSO_4$ reduced to one-fifth) and 3 % sucrose. The cultures are incubated at 20 °C, under 16 h photoperiod.

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Genetic Engineering 15

15.1 Introduction

The traditional plant breeding, based on sexual crossing of two desired parents, made unimaginable increase in crop productivity and quality. However, a serious limitation of this approach to crop improvement is reproductive isolation of two species. Moreover, in crossing two parents to combine desirable traits there is mixing of other characters also, which must be excluded by a careful and time-consuming process of selection in the segregating progeny over several generations.

The developments in the understanding of plant processes during the past three decades have led to the perfection of tools and techniques that allow the transfer of specific genes without disrupting the elite phenotype of the recipient plant. This highly sophisticated method of genetic modification of crop plants, variously referred to as *recombinant DNA (rDNA) Technology, Genetic Transformation, Genetic Engineering* (*GE*)*, Molecular Breeding or Precision Breeding*, is not limited by sexual reproductive isolation. Required genes from any source, bacteria, viruses, fungi, animals, or completely unrelated plants, can be inserted in a functional form into the genome of a well-established plant cultivar, and their expression can be regulated, so that it is expressed throughout the plant or in a specific organ or specific tissue of an organ.

Genetic engineering can be simply defined as deliberate alternation of the genome of an organism by introducing one or a few specific foreign genes. The genetically engineered crop plants are referred to as *Transgenic Crops* or *Genetically Modified (GM) crops*, and the gene introduced is termed *transgene*. The transgenic technology is one of the most powerful methods for crop improvement and has already produced improved crop varieties (Table 15.1).

GM crop varieties for large-scale cultivation were first introduced in the US in 1995–1996, and since then several GM cultivars of alfalfa cotton, canola (*Brassica napus*), maize, rice, papaya, sweet corn, and various types of squashes (*Cucurbita* sp.) have been released for commercial cultivation. Insect-pest tolerance, herbicide tolerance, and improved nutritional attributes are some of the desirable traits in the GM crops under cultivation today.

Responsible use of biotechnology in agriculture is considered imperative for future food, livelihood, and economic security of developing countries, including India (Narayanan 2002). In 2002, India took a significant step towards adoption of GM crops by introducing genetically altered cotton varieties, carrying the insecticidal protein gene from the soil bacterium *Bacillus thuringiensis* for commercial cultivation.

Successful creation of transgenic plants requires tools of biotechnology and those of conventional breeding. Thus, genetic engineering supplements but not supplants breeding. Genetic

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_15, © Springer India 2013

Table 15.1 Some GM crops under large-scale cultivation

Crop	Transgenic trait	Transgene
Soybean	Resistance to glyphosate or glufosinate herbicides	Glyphosate resistant bacterial *EPSPS* gene, *Bar* gene
Maize	Resistance to glyphosate or glufosinate herbicides; Insect resistance; vitamin-enriched maize with 169× increase in β-carotene, 6× in vitamin C and 2× in folate	New genes, some from *Bacillus thuringiensis*,
Cotton (seed oil)	Pest-resistance	*Cry* gene
Alfalfa	Resistance to glyphosate or glufosinate herbicides	Glyphosate resistant bacterial *EPSPS* gene; *Bar* gene.
Papaya	Resistance to the papaya ringspot virus	PRSV coat protein gene
Tomato	Suppression of polygalacturonase (PG) to delay fruit ripening	Antisense construct based on *pTOM6* for Polygalacturonase enzyme
Rapeseed (Canola)	Resistance to glyphosate or glufosinate; high laurate in oil	New genes added/ transferred into plant genome
Sugar cane	Resistance to certain pesticides, high sucrose content	New genes added into plant genome
Sugar beet	Resistance to glyphosate, glufosinate	New genes added into plant genome
Rice	Rich in Vitamin A (β-carotene)	"Golden rice" with two transgenes, *psy* gene from daffodil or maize and *crt I* from *Erwinia uredovora*
Squash (Zucchini)	Resistance to watermelon, cucumber and zucchini yellow mosaic viruses	Virus coat protein gene
Sweet Peppers	Resistance to virus	Virus coat protein gene

engineering involves a number of molecular, biochemical, and tissue culture techniques. The initial step of gene insertion into plant cell (Sect. 15.2) is only the beginning of a long and cumbersome process of genetic engineering that involves cell selection (Sect. 15.3.1) and tissue culture followed by plant regeneration and acclimatization (Sect. 15.4). If the goal is the development of a commercial variety, the transgenic plants and their progeny are subjected to a series of tests and molecular analyses (Sect. 15.3.2) to check their genetic stability and assure field performance together with the product quality and safety in order to comply with both market demands and the relevant regulatory processes (Sect. 15.6). This chapter describes the essential steps involved in genetic engineering and issues related to it. The various aspects of genetic engineering have been recently reviewed by Lin et al. (2007), Sticklen (2008), Lemaux (2008, 2009), Curtis (2010), Altpeter and Sandhu (2010), Hogg et al. (2010), and Lacorte et al. (2010).

15.2 Gene Transfer

Two approaches have been used for gene insertion into plant cells: (1) Vector-mediated gene insertion (*Agrobacterium* mediated, agroinfection, and viral vectors), and (2) Direct Gene Transfer (Pollen mediated, imbibition, microinjection, particle bombardment, electroporation, ultrasound-induced, PEG-induced, silicon carbide fibre-mediated, Laser-mediated, and Liposome-mediated DNA uptake). Of these, *Agrobacterium* mediated and particle bombardment induced gene insertion, which have been most popular, are described here. While both of

these methods are quite effective, each has its own advantages and limitations.

15.2.1 *Agrobacterium* Mediated Transformation

For over a century it has been known that the Gram-negative soil bacterium, *Agrobacterium tumefaciens,* causes the crown gall disease in plants (Smith and Townsend 1907). However, it was only in 1970s that scientists discovered that this bacterium causes the symptoms as a result of transferring a piece of its genetic material, called "transfer DNA" (T-DNA) into the plant genome (Chilton et al. 1977). This is the only example known of natural inter-kingdom gene transfer and therefore *Agrobacterium* has been rightly called "Natural Genetic Engineer". This discovery formed the basis for researchers to develop tools to understand plant development and crop improvement by transfer of agronomically important traits. Wounding of the host tissue by scoring with scalpel or cutting the tissue into small pieces enhances the delivery of the bacterium which infects through the wound.

T-DNA is a part of extra-chromosomal plasmid called "Tumour inducing plasmid" (*Ti* plasmid; Fig. 15.1a). It encodes genes responsible for the synthesis of opines, special types of amino acids required for the growth of the bacterium strain inciting the disease. In addition, T-DNA carries genes for the production of auxin and cytokinin (oncogenes) which are responsible for plant cell proliferation. T-DNA is bordered on both sides by 25 base pairs (bp) direct repeat sequences (Fig. 15.1b). These border sequences are the only part of the plasmid required in *cis* for T-DNA transfer to the plant genome. Just outside the right border virulence (*vir*) genes are clustered together into *vir* region. A series of chemical signals from plant activate the *vir* genes of the bacterium leading to excision of T-DNA and its transfer to the genome of the target plant cells. Acetosyringon released by some plant cells activates the *vir* genes of bacteria. Addition of this compound exogenously has led to routine transformation of plants that were once thought to be not susceptible to *Agrobacterium*-mediated transformation. Different *vir* gene products are involved in the excision of T-DNA, coating it to prevent its degradation during its passage through the cytoplasm of the plant cells, and directing the T-DNA out of the bacterium and its delivery to the nucleus of the host plant cell. To perform their function of T-DNA transfer, the *vir* genes need not be physically linked to T-DNA; they can also function in *trans*. Removal of all the genes within the T-DNA does not impede the ability of *Agrobacterium* to transfer this DNA but does prevent the formation of tumors. Ti plasmids and their host *Agrobacterium* strains that are made nononcogenic by removing the genes for auxin and cytokinin synthesis are termed "disarmed".

The production of transgenic plants by *Agrobacterium*-mediated transformation has become the method of choice as it results in the transfer of low number of copies of genes into the plant nucleus, giving a reduced frequency of gene silencing caused by high gene dosage. Initially, this method of gene transfer was effective in transformation of dicots but significant improvement in tissue culture techniques, discovery of super virulent strains of *Agrobacterium* and the engineering of novel vectors over the years have enabled this natural vector system to be used for the production of transgenic plants from a wide variety of plant species, including monocots.

15.2.1.1 T-DNA Vectors

The basic features of vectors for plant transformation are: (1) the plasmid, to be used in *Agrobacterium*-mediated transformation, should be able to replicate in, both, *E. coli* (in which its manipulation is carried out) and *Agrobacterium,* (2) additional selectable markers need to be included so that the successfully transformed cells/plants can be recognized, (3) border sequences need to be incorporated into the design of plasmid vectors for *Agrobacterium*-

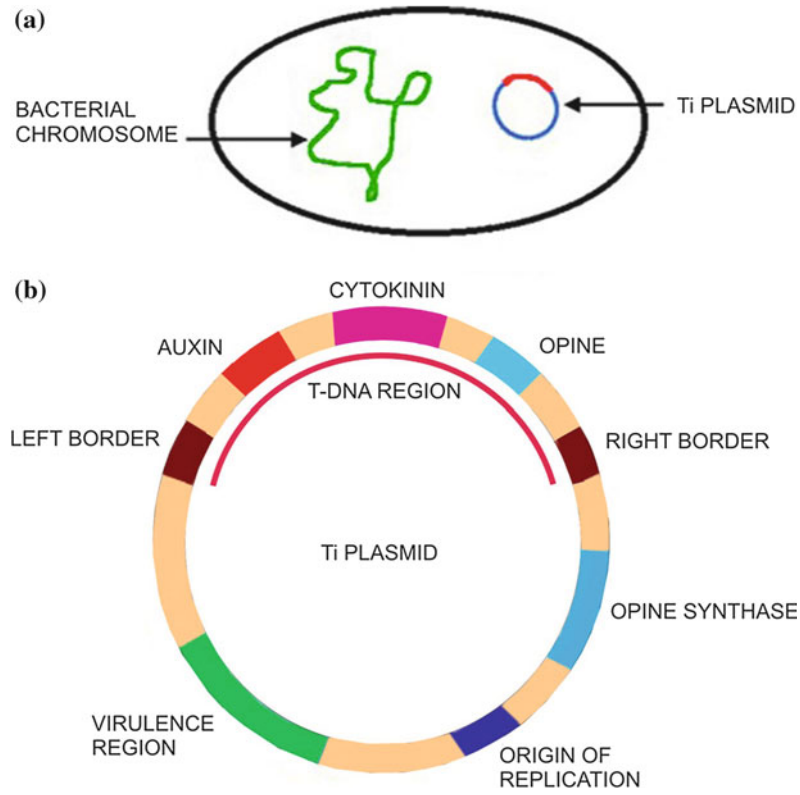

Fig. 15.1 **a** Diagram of a cell of *Agrobacterium tumefaciens* harboring *Ti* plasmid. **b** Structure of the *Ti* plasmid

mediated transformation to ensure integration of the genes of interest into the host plant's genome, and (4) the genes (especially from prokaryotes or non-plant eukaryotes) to be integrated into the genome of the host plant may need to be made 'plant like' by use of appropriate promoters and terminators to ensure that expression of the genes occurs.

One of the first vectors developed for *Agrobacterium*-based genetic transformation of plants was created by disarming the *Ti* plasmid and replacing the oncogenes by the genes of interest (*goi*) to be transferred to the plants. Such vectors in which the *vir* genes and the T-DNA carrying the *goi* are present on the same plasmid are called "co-integrated vectors" or "*cis* vectors". These vectors are effective but are difficult to isolate and manipulate in vitro due to their large size ($\sim$150 kb) and instability.

The discovery, in 1983, that the *vir* genes present on a separate plasmid are able to process and export the T-DNA region on another plasmid present within the same *Agrobacterium* cell (Hoekema et al. 1983; De Framond et al. 1983) led to the evolution of "Binary Vectors" (Fig. 15.2), the most important tool in gene transfer (Lee and Gelvin 2008). In binary vector systems, T-DNA is located on the binary vector; the nonT-DNA region of the vector containing origins of replication that could function both in *E. coli* and *Agrobacterium tumefaciens* and antibiotic resistance gene, used for selection of binary vector in the bacteria, is known as *vector backbone sequences*. The other plasmid, carrying the *vir* genes is called the *vir helper plasmid*. The genes of interest could be easily cloned into small T-DNA region within the binary vectors specially suited for the purpose. After characterization and verification of the construct in *E. coli*, the T-DNA binary vector is transferred into appropriate *Agrobacterium* strain harboring a *vir* helper plasmid, by electroporation, freeze–thaw procedure or by mating of two strains of *E. coli* (one helper and the other donor) with a recipient *Agrobacterium* strain by a technique known as "triparental mating" (for details see

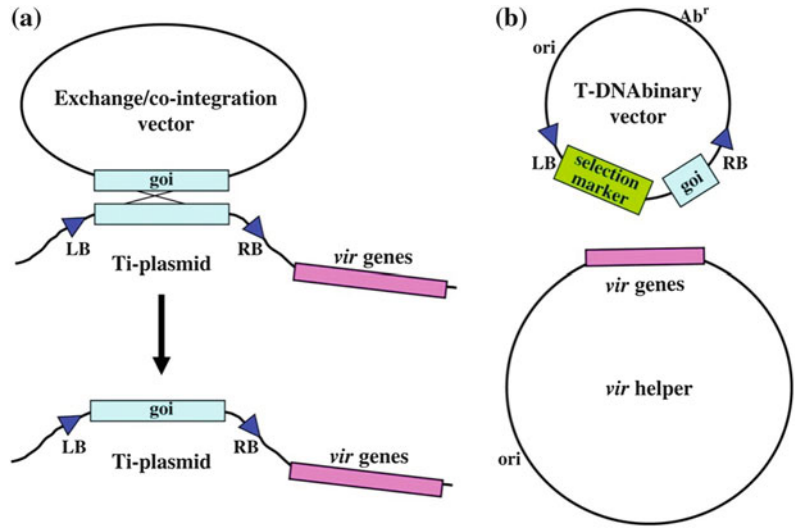

Fig. 15.2 Schemetic diagramme of co-integration (**a**) and T-DNA binary vector systems (**b**) to introduce genes into plants using *Agrobacterium*-mediated genetic transformation. (Ab^r antibiotic resistance, *goi* gene of interest, *LB* left border, *RB* right border, *vir* virulent, *ori* origin of replication) (*after* Lee and Gelvin 2008)

Wise et al. 2006). During the past 27 years, both T-DNA binary vectors and disarmed *Agrobacterium* strains harboring *vir* helper plasmid have become more sophisticated and suited for specialized purposes (Curtis and Grossniklaus 2003; Curtis 2008). Mid 1990s saw the development of the "super binary system" which enabled enhancing the transformation frequency by employing additional *vir* genes. This was achieved by inserting a DNA fragment containing *virB, vir C*, and *vir G* genes from pTiB0542 into a small T-DNA-carrying plasmid.

T-DNA binary vectors have greatly simplified the generation of transgenic plants. No longer are complex, sophisticated microbial genetic regimens required to integrate *goi* into T-DNA regions located on large, cumbersome Ti- or Ri- plasmids. However, the use of multicopy binary vectors may have exacerbated two common problems associated with plant transformation, viz. multiple integrated transgene copy number and vector backbone integration (Lee and Gelvin 2008).

The salient features of a binary vector for genetic engineering of plants are:

1. T-DNA left border (*LB*) and right border (*RB*) repeat sequences (containing 25 bp) define and delimit T-DNA. In plants, the T-DNA strand is frequently chewed back, probably by exonucleases, which are more frequent from the unprotected 3′ (*LB*) end than from 5′ (*RB*) end of the strand. Therefore, the *goi* is placed near the *RB* and the selection marker gene near the *LB*.
2. A plant-active selectable marker gene, such as an antibiotic or an herbicide resistance gene is present. The most commonly used antibiotics are kanamycin or hygromycin and the most commonly used herbicides are Basta or Bialophos. Earlier binary vectors, such as pBIN19, had few restriction endonuclease cloning sites in a *lacZ* complementation region. In promoters and poly A addition signals flank these sites. The recent binary vectors contain multiple rare-cutting restriction endonuclease or homing endonuclease cloning sites. Binary Vectors are now available that contain Gateway sites to facilitate inserting of genes or exchange of gene cassettes from other vectors (Curtis and Grossniklaus, 2003).
3. It has origin(s) of replication (ori) to allow maintenance in *Agrobacterium* and *E. coli* (in which initial constructs are generally made).
4. It carries antibiotic resistance genes within the chromosome and within the backbone sequences for selection of the binary vector in *E. coli* and *Agrobacterium*.

For the details of vectors available, their construction, the marker genes which they carry

and their limitations see Veluthambi et al. (2003), Komari et al. (2006), Dafny-Yelin and Tzfira (2007), Lee and Gelvin (2008).

15.2.1.2 Method for *Agrobacterium*-Mediated Transformation

For *Agrobacterium*-mediated gene transfer several methods have been employed. Earliest studies involved co-cultivation of freshly isolated protoplasts with the bacteria carrying the vector. However, despite the success of this method, it has not been popular due to the tedious and time-consuming process of protoplast isolation and plant regeneration from them. A major advancement was made when Horsch et al. (1985) reported transformation of tobacco by co-cultivation of leaf discs with the bacteria (Fig. 15.3). This system relies on the injured cells along the margin of the disc that get infected with the bacterium and transformed, and eventually differentiate shoots. Refinement in the technique of leaf disc method allowed various seedling explants (hypocotyl, stem, petiole segments, and cotyledons) to be used as the target tissue depending on the ease to regenerate plants from the explant in different species (Curtis 2010).

The explant co-cultivation essentially involves incubation of the surface sterilized explants from green house grown plants or from 21 to 28 days old aseptic seedlings with freshly grown bacteria for 24–48 h to permit the bacteria to invade, infect, and transform plant cells. In the ornamental *Kalanchoe laciniata*, 7 days incubation with bacteria greatly increased frequency of transformed shoots as compared to shorter co-culture period (Jia et al. 1989). The explants are then transferred to selection medium containing appropriate antibiotic/s, such as cefotaxime, claforan, carbenicillin, triacillin, and vancomycin, to kill the bacteria. Under proper selection pressure untransformed cells are unable to grow while the transformed cells grow and form callus. The transformed cells are suitably manipulated using standard tissue culture protocols to regenerate plants. Sometimes the shoots may regenerate directly from the transformed cells without a major callus phase. Some of the factors that need to be optimized to achieve high frequency transformation by this method are, (i) the nature of the explant, (ii) size of the explant, (iii) number of bacteria in the inoculum, (iv) preculture of the explants for a couple of days, (v) nurse culture, (vi) presence of a supervirulent plasmid within the *Agrobacterium*, and (vii) pH of the co-cultivation medium. Many dicotyledonous plants could be transformed using the co-cultivation method but individual plant species require specific modifications to achieve optimal transformation efficiency.

The monocots, particularly cereals, are not susceptible to infection by *Agrobacterium*, and therefore the standard protocol for co-culture of explants is not effective. Overexpression of *vir* genes by the addition of acetosyringone to the co-cultivation medium and the development of embryogenic system for cereals have made it feasible to transform cereals by the co-cultivation method. The first monocot plant to be transformed using *Agrobacterium* was rice (Chan et al. 1993; Hiei 1994), and later it has been possible with maize and wheat (Ishida et al. 1996).

A simplified floral dip method has been developed for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, the model system for plant genomics (Cough and Bent 1998; Sivamani et al. 2009). The *Arabidopsis* plants, at or just before flowering, are immersed in a suspension of *Agrobacterium* containing the wetting agent 'Silwet', which reduces surface tension and allows good access of the bacterium to the cracks, crevices, and pores on the plant body. After the dipping treatment (ca 5 s), the plants are maintained under high humidity for a few days and allowed to, eventually, flower and set seeds. Since *Arabidopsis* produces so many seeds and the plant is so small, seeds can be easily planted on selective media and seedlings/plants can be screened for a certain character or phenotype to recover whole transgenic plants. In this method, the egg is targeted for transformation leading to the production of transgenic seeds. Usually, each seed is from a different transformation event. This technique has been successfully extended to *Medicago truncatula*

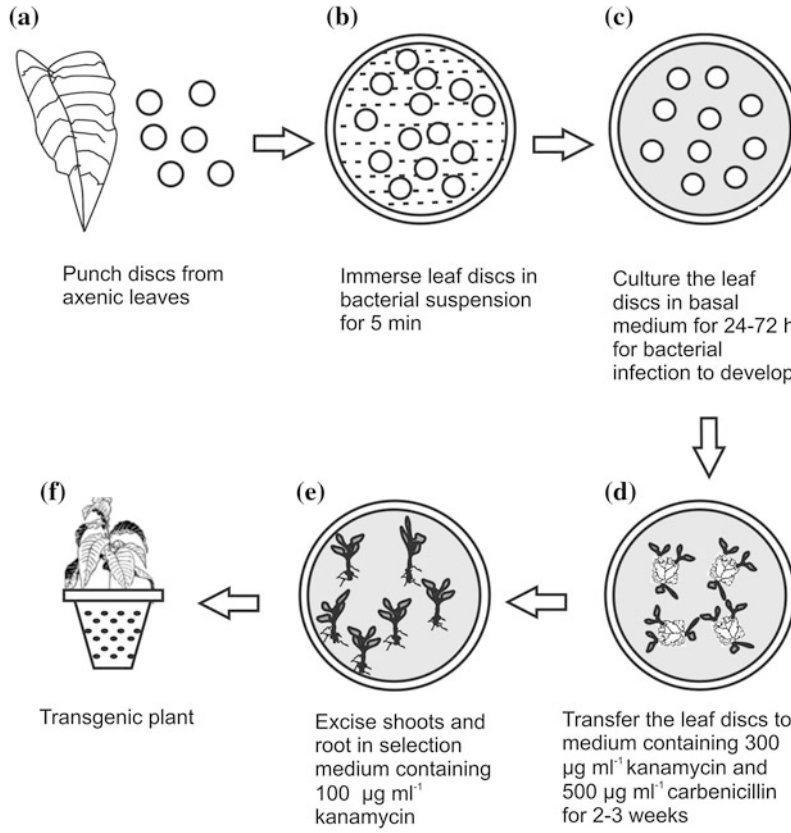

Fig. 15.3 Diagrammatic summary of the *Agrobacterium*-mediated co-culture system of transformation

(Trieu et al. 2000), radish (Curtis and Nam 2001), and pakchoi (Qing et al. 2000).

Agrobacterium-mediated transformation is a very successful method for obtaining transgenics and has been successfully applied to a large number of economically important plants. However, it has a lot of IPR issues attached to it. To offset this problem, Jefferson et al. have developed, what is called "TransBacter", by inserting a disarmed Ti plasmid and a binary vector in bacteria other than *Agrobacterium*, such as *Rhizobium*, *Sinorhizobium,* and *Mesorhizobium* (Brutehaerts et al. 2005). These TransBacter (www.cambia.com), which could be used to transfer transgene to a number of plant species, is available under an "open license" arrangements (www.bios.org).

15.2.2 Direct Gene Transfer

The biolistic method of direct gene transfer was invented by John Sanford (Sanford et al. 1987) in an attempt to overcome the host limitations of *Agrobacterium*-mediated transformation. It is easy to use and is adaptable to a wide range of plant cells and tissues with high transformation efficiency, which make it a popular system of choice for many crops. Most of the acreage under transgenic crops represents the success of particle bombardment method of gene delivery (Finer and Dhillon 2010). It is the only method to transform plastids and mitochondria. For comprehensive reviews on this technique, variously termed as biolistic method, particle bombardment, microprojectile bombardment, and

particle gun method, *see* Taylor and Fauquet (2002), Altpeter et al. (2005), and Altpeter and Sandhu (2010).

Basically, the particle bombardment technique involves the use of accelerated DNA-coated particles fired directly into the cells (Figs. 15.4, 15.5). In the first experiment, DNA-coated tungsten particles were utilized as microcarrier adhered to a plastic macroprojectile. Initially, the biolistic devise (model BPG), in which the macrocarrier was propelled by gun powder cartridge, was marketed by the Biolistics Inc. Later, DuPont Inc. developed the PDS-1000 devise which was, subsequently, modified to PDS-1000/He instrument (Fig. 15.4) by Biorad Laboratory. In the most commonly used biolistic devise, inert Helium (He) gas is used as the accelerator force. A simple, cost-effective alternative to PDS-1000He, called particle inflow gun, was developed by Finer et al. (1992).

PDS-1000/He biolistic particle delivery system (Fig. 15.5), introduced in the 1990s, is the most widely used device for transient gene expression studies and the generation of transgenic plants by direct gene transfer. It uses high-pressure He gas released by a rupture disc and partial vacuum to propel a macrocarrier plastic sheet loaded with DNA coated tungsten or gold microcarriers toward target tissues at high velocity (Fig. 15.5). A stopping screen arrests the macrocarrier after a short distance, and the DNA-coated microcarriers, shed from the macrocarrier, continue traveling, and penetrate the target tissue to affect gene transfer (Fig. 15.5). The launch velocity of macrocarriers for each bombardment is dependent upon the He pressure which is typically adjusted to 6.2–8.9 MPa (900–1,300 Psi). The critical factors to maximize transformation efficiency by this system include size and density of microparticles, distance between rupture disc, macrocarrier and the target tissue, helium pressure, osmoticum treatment, and time of pre-culture of the target tissue. Gold particles of 1.0 μm diameter are most widely used for gene transfer to the nucleus and particles of 0.6 μm diameter for gene transfer to plastids.

The bombarded particles penetrate the plant cell wall and reach directly into the nucleus or adjacent to it. The DNA gets integrated into the chromosomes and becomes a permanent addition to the plant genome.

Precipitation conditions, including the concentration of $CaCl_2$ and spermidine, used to coat microprojectiles, must be optimized. Of late, protamine has been suggested as an alternative

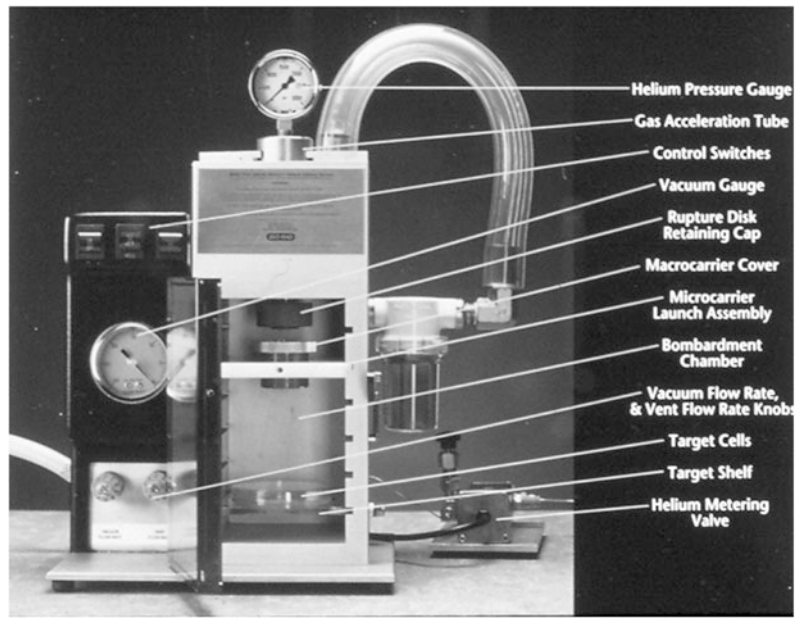

Fig. 15.4 Biolistic PDS-1000/He biolistic gun. (*courtesy* Biorad Laboratories, USA)

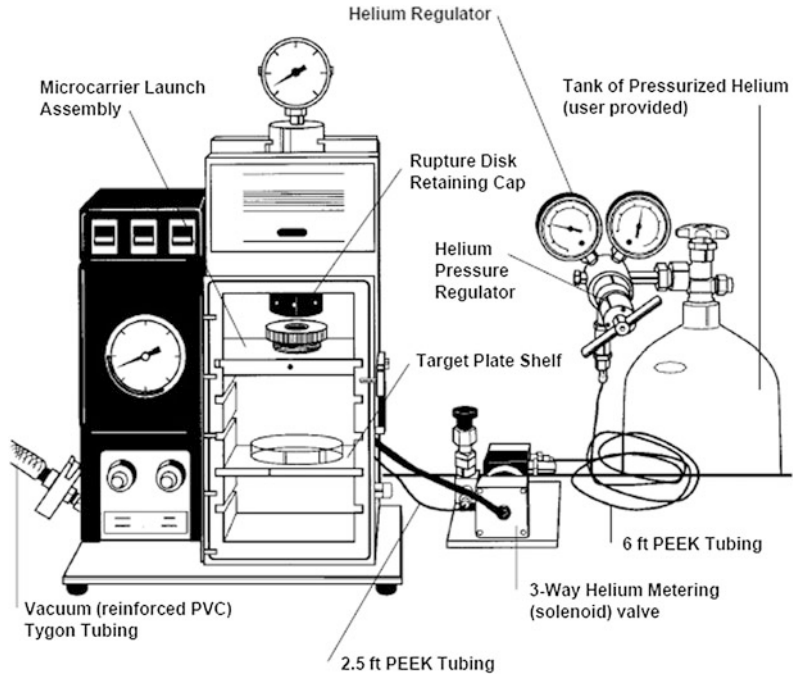

Fig. 15.5 Diagrammatic representation of Biolistic PDS-1000/He biolistic gun (*courtesy* Biorad Laboratories, USA)

to spermidine since it supports better DNA protection and results in greater transient and stable transformation frequencies.

There is no well-established method currently available to achieve transformation in a way that copy number, integration site or gene expression is accurately predetermined. *Agrobacterium*-based transformation tends to generate less complex integration pattern, but the presence of sequences outside the T-DNA is frequently observed. In biolistic method the main limitation is randomness of DNA integration and high copy number of the introduced genes. It also generates complex patterns and concatemers, particularly if intact plasmids are used for transformation. However, if a fragment is used, single copy integration events are more common (Lacorte et al. 2010). Another drawback with the biolistic method of gene insertion is that it requires expensive equipment, and the isolated DNA is not stable for long outside the nucleus.

There are many other methods that have been successfully used for direct gene transfer (uptake by protoplasts, uptake by pollen, pollen tube pathway, floral tiller injection, electroporation of whole tissue or germinating pollen, seedling infiltration, silicon carbide whiskers, laser micropuncture, and myofibril arrays) but with little follow up data, suggesting that these methods are not of wide applicability or are not effective in genetic transformation of plants (Fineraw and Dhillon 2008).

15.3 Selection and Identification of Transformed Cells/Plants

15.3.1 Selection

Following gene insertion either by *Agrobacterium* or directly by the biolistic method, generally only a very small population of cells in the tissue is transformed. It is, therefore, important to apply a powerful selection system to identify the transformed cells and multiply them. Various marker genes have been used to identify the transformed cells or plants, which fall under two categories: (a) *Selectable marker genes*, and (b) *Nonselectable marker* or *reporter genes* (Miki 2008). In practice, the marker gene is closely linked to the gene of interest in the plant transformation vector.

The presence of selectable marker gene gives the transformed cells selective advantage over the nontransformed cells when grown in media containing the selecting agent. For successful selection, the target plant cells must be susceptible to relatively low concentration of selecting agent in a nonleaky manner. The compounds that inhibit the growth but do not kill the wild-type cells are preferred as selecting agents.

The most commonly used selectable plant marker genes include *nptII* (Neomycin phosphotransferase) and *hpt* (hygromycin phosphotransferase) genes that confer resistance to the antibiotics kanamycin and hygromycin, respectively (Miki and McHugh 2004). In some cases, the gene of interest itself can act as selectable marker, such as herbicide resistance. The most commonly used herbicide resistance gene for selection is the *bar* gene which imparts resistance to the herbicide phosphinothricin. Selection in the presence of toxic agents, which is the most common type of selection, is called negative selection. Positive selection on the basis of novel carbon utilization pathway provides important alternative to antibiotic-based selection strategy (Roa-Rodriguiz and Nottenburg 2003). Phosphomannose isomerase (PMI) enzyme that catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate, is not found in plants. Therefore, the nontransformed cells are not able to survive on a medium with mannose as the sole carbon source while the transgenic cells with the PMI gene survive and grow. This system has been used in monocots, such as maize, rice, and sugarcane.

Some of the Reporter genes used to identify the transformed cells are *gusA* (β-Glucuronidase), *lux* (Luciferase—bacterial), *luc* (Luciferase—firefly), and *gfp* (Green fluorescent protein). In these cases, the transformed cells do not have any selective advantage over the nontransformed cells but can be separated out based on visual identification after or without special treatment and re-cultured. *gusA* is the most widely used reporter gene in plants (Jefferson et al. 1987). It codes for the enzyme β-Glucuronidase which upon staining with 5-bromo-4-chloro-3-indolyl glucuronide (*X-gluc*) gives blue color. *gfp* from Pacific jellyfish is becoming increasingly popular now because it fluoresces in UV/blue light and does not require an external substrate, and is a nondestructive assay.

Once a transgenic plant has been selected, the marker gene is no longer required but is usually retained. Mainly due to consumers concern, efforts are being made to eliminate the marker gene from the nuclear and plastid genomes after selection.

15.3.2 Analysis of Putative Transformants

Presence of gene is one thing and its expression is another. Only whole plant analysis for the particular plant and phenotypic expression of the trait would establish whether the transgene is present in the putative transformant or not, and if present whether the expression of such a gene is complete or not. Many a times even after stable integration of the transgene, its function is lost due to "gene silencing".

The calli and plants regenerated from the selected cells are subjected to biochemical analyses (Zale 2008, LcCorte et al. 2010). The cells and plants recovered after selection and polymerase chain reaction (PCR) positive are called putative transgenics. The putative transgenics are subjected to DNA hybridization (southern blot) technique to assess the integration of the transgene into the plant genome and the number of copies of the transgene present. To analyze how much of transgene is transcribed, RNA hybridization (northern blot) analysis is done. Eventually, the plants are subjected to enzyme-linked immunosorbant assay (ELISA) to assess how much transgenically expressed protein is produced. Real-time reverse transcriptase PCR (RT-PCR) technique is also applied for detection and quantification of mRNA.

For sexually reproducing species, genetic segregation of the transgene in T_2 progeny will provide additional evidence of its Mendelian

inheritance. The original transgenic plant is called T_0 generation. The transgene, when integrated stably, behaves normally and follows Mendelian inheritance. The transgene can be transferred from the transgenic plant to other cultivars of the species by crossing the two. It is also possible to pyramid two transgenes for different characters in the same plant. Some promoters cause the genes to which they are linked to be expressed all the time (*constitutive promoters*), whereas others allow expression only at certain stages of plant growth (*e.g.* flowering), or in certain plant tissues (*e.g.,* roots, shoots, tapetum, or fruits) (*tissue specific promoter*). Some promoters are weak, whereas others are strong, and this determines how much of the gene product is made. By controlling gene expression, the plant's energy resources can be directed toward growing or synthesizing valuable molecules like starch or pharmaceuticals. At present, in *Bt* maize or *Bt* cotton, the *cry* gene (which makes the plant insect resistant) is switched on all the time, making it more likely that insect pests resistant to *Bt* will arise. Therefore, efforts are being made to link expression of the *Bt* gene to a promoter that switches the gene on to synthesize the insecticide only when the plant is wounded by an insect attack (*inducible promoters*).

15.4 Regeneration of Transformed Plants

Generally, the desired product of genetic transformation is the regenerated plant expressing the introduced gene. Therefore, it is important that the explants selected for transformation exhibit high regeneration potential, and an efficient protocol to achieve it has been developed. The regeneration frequency of transformed cells is influenced by, besides the factors discussed in Chaps. 6 and 7, the method of transformation (infection by *Agrobacterium*, or trauma of cells by particle bombardments) and the presence of selection agent (an antibiotic) and other antibiotics used against *Agrobacterium* in the culture medium. Besides the frequency of regeneration, the nature of regeneration is also critical in the recovery of transformed plants. Transformed plants are obtained only if regeneration occurs within the region of the explant where the cells can be in contact with the *Agrobacterium* or other instruments of gene transfer. Where regeneration from isolated cells or callus is not well defined or not possible, tissues such as intact explants (intact petioles and embryogenic calli) are used.

15.5 Applications

Genetic engineering is a useful technique for both basic and applied areas of plant sciences. A large number of transgenic crops have been developed and progressed to the stage of commercialization. The transgenic crops released for large-scale cultivation so far are created, largely, for resistance to herbicides and pests (Table 15.1).

15.5.1 Herbicide Resistance

Weeds compete with crop plants for water, nutrients and light and cause considerable decline in the yield and quality of their economic products. Chemical herbicides, which selectively kill the weeds, are widely used for weed management. The choice of herbicide depends on its effectiveness against the problem weed, the crop sensitivity, herbicide treatment, and often on its effect on the environment. The herbicides generally work by targeting metabolic steps that are vital for plant survival. Since these herbicides may also cause considerable injury to the crop plants at the application rate required to eliminate the weeds, considerable work has been done to create herbicide resistant/tolerant crop plants (Korth 2008).

Glyphosate is an active ingredient of the herbicide "RoundUP^{TM}". When sprayed on the plants it is rapidly translocated to the growing tips causing cessation of growth in shoot tips and root tips. At molecular level, it binds to and

inhibits the enzyme 5-enolpyruvateshikimate-3-phosphate synthase (EPSPS), which is active in the shikimate pathway leading to the synthesis of metabolites, including aromatic amino acids (tyrosine, phenylalanine, and tryptophan) (Fig. 15.6). By inserting a bacterial gene encoding glyphosate resistant EPSP synthase (CP4 EPSPS from *Agrobacterium*), it has been possible to create glyphosate resistant varieties of soybean (Hinchee et al. 1988) and *Brassica napus* (Hinchee et al. 1993), termed 'RoundUp Ready'. The 'RoundUp Ready' soybeans were one of the first transgenic crops to be approved for commercial cultivation in 1996. Now this trait has been engineered into a large number of crops (Table 15.1) growing in several countries.

The alternative strategy employed to produce herbicide resistant transgenic plants is to engineer the expression of a gene that inactivates the herbicide. For example, Glufosinate (Liberty^{TM}), another broad spectrum herbicide, kills plants by inhibiting the plant enzyme glutamine synthase (GS) responsible for the synthesis of glutamine. In the absence of GS enzyme, the endogenous ammonium attains a toxic level killing the plant. The *bar* gene encoding the *phosphinothricine acetyl transferase* (PAT) enzyme from Glufosinate resistant *Streptomyces hygroscopicus* bacteria was deployed to create Glufosinate resistant crop plants. The PAT enzyme degrades Glufosinate. Several crop plants (alfalfa, canola, cotton, maize, and rice tomato) resistant to this herbicide have been produced by inserting the *bar* gene. Another bacterial gene that inactivates the herbicide Bromoxynil (Buctri^{TM}) has been used to create varieties of tobacco and potato resistant to Buctri. Bromoxynil kills plants by inhibiting the Photosystem II, a crucial component of photosynthesis.

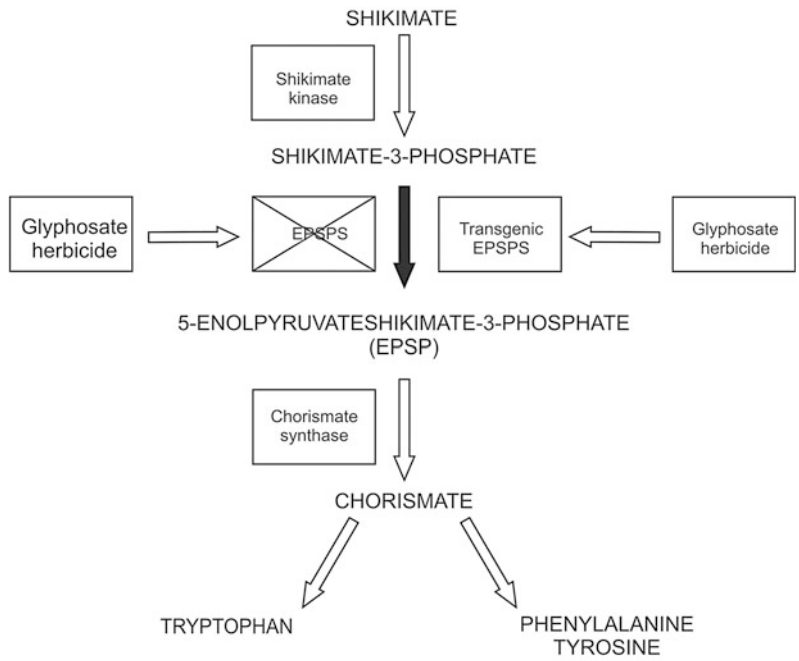

Fig. 15.6 Production of glyphosate (roundup herbicide) resistant transgenic plants. Glyphosate herbicide inhibits the activity of the native 5-enolpyruvateshikimate-3-phosphate synthase (EPSPS) enzyme, and thus blocks the synthesis of the essential aromatic amino acids, tryptophan, phenylalanine, and tyrosine. However, in the transgenic plants, the herbicide resistant EPSPS is unaffected by the herbicide and continues to produce the essential amino acids even after the application of the herbicide

15.5.2 Insect Resistance

Many insects cause considerable damage to agriculture production in the field and during storage. The insects of the orders Coleoptera (beetles), Lepidoptera (moths and butterflies), and Diptera (flies and mosquitoes) are the most serious plant pests. Even though chemical insecticides are used for their control, these insects continue to be major pests of important food and fiber crops (Jenkins 2004). Development of pest resistant varieties and agronomic management are some of the approaches adopted to combat this problem. Synthetic chemicals are most effective but are costly, and their indiscriminate use has harmful effects on beneficial insects, birds, fishes, and mammals. Insect resistance genes for most pests are either not available or barriers to hybridization do not allow their transfer to established crop cultivars. Therefore, there has been increasing interest in the use of biopesticides. Insecticides based on crystalline proteins from the common, Gram-positive soil bacterium, *Bacillus thuringiensis* (*Bt*), have been used successfully and safely for over 50 years. They are not toxic to humans or other higher animals. However, a major problem in using *Bt* sprays to control insect attack on plants is the high cost of production of *Bt* insecticides and instability of the Protoxin crystal proteins under field conditions, necessitating multiple sprays. To obviate this problem insect resistant transgenic plants have been developed.

Genes for several proteins have been tested to engineer insect resistant transgenic crops and reported to inhibit insect growth or cause higher insect death rates. These include genes for protease inhibitors, which interfere with insect digestion; Lectins, which kill insects by binding to specific glycosylate proteins, and chitinase enzymes that degrade chitin found in the cuticle of some insects. However, none of these genes have been as effective and widely used as genes coding for *Bt* endotoxin from the sporulating *B. thuringiensis*. This bacterium produces nontoxic protoxin which exists as crystalline protein. When consumed by the insect larvae it gets hydrolyzed in the alkaline medium of its gut to yield the toxic protein. In sensitive insects, the toxin molecule binds to specific protein receptors on the epithelial membrane of the mid gut, causing holes in the membrane. This paralyses the midgut and mandible, leading to death of the insect by starvation and septicemia.

The genes that code for the protoxin are called *Cry* genes. Different strains of *B. thuringiensis* produce *cry* proteins of different structure, which are effective against different insects. For example, *Cry1* and *Cry2B* proteins are specifically toxic to Lepidoptera, *Cry2A* proteins to Lepidoptera and Diptera, *Cry3* proteins to Coleoptera and *Cry4* proteins to Diptera (Stotzky 2004). As only a part of the *Cry* gene codes for the entire insecticidal activity, truncated *Cry* genes have been used to engineer insect resistance in crop plants. In some cases, the expression of the truncated genes could be dramatically enhanced by replacing some DNA sequences of *Bt Cry1* gene by synthetic sequences.

The first reports of engineering *Bt* toxin gene into tobacco (Adang et al. 1987; Barton et al. 1987; Vaech et al. 1987) and tomato plants (Fischoff et al. 1987) were published in 1987. However, Monsanto was the first company to produce transgenic cotton, maize, and potato plants that expressed *Bt* toxins sufficient for field control of the targeted insect pests (Perlak et al. 1990; McIntosh et al. 1990). It was achieved by modifying the DNA sequence of a specific *Bt* toxin gene (Perlak et al. 1990). Most of the insect resistant transgenic crop species that have been commercialized so far express one of the various *cry*stalline proteins from *B. thuringiensis*. Over 240 insecticidal c*ry* proteins produced by various strains of *B. thuringiensis* have been classified (Jenkin 2004). At least 10 *cry* genes encoding different *Bt* toxins (c*ry1Aa, cry1Ab, cry1Ac, cry1Ba, cry1Ca, cry1H, cry2Aa, cry2A, cry3A, cry6A, and cry9c*) have been genetically engineered into 26 plant species (Jenkins 2004). However, codon optimized genes have been introduced only in alfalfa, broccoli, cabbage,

cotton, maize, and tobacco (Schuler et al. 1998). Transgenic Bollard cotton with c*ry1Ac* gene was the first to be grown commercially in 1996. A stable inheritance of the transgene has been demonstrated.

In April 2000, the Government of India approved three *Bt* cotton hybrids for commercial cultivation, and in 2005 *Bt* cotton was grown on 1.3 million hectares of land. This is the only transgenic crop so far cleared for cultivation in India (Ahuja 2008).

One of the major problems with *Bt* transgenic crops is the rapid appearance of resistant pests. To offset this problem, more than one slightly different *cry* genes can be introduced into the same plant by crossing different transgenic lines. This method, called pyramiding of genes, can considerably delay, if not prevent, the pests from adapting to this mechanism (Cohen 2004). 'Bollard II' cotton has been developed by staking *cry2Ab* with *cry1AC*. Similarly, 'Wide-Strike' cotton and 'Herculex RW' maize are produced by transferring two *cry* genes each, viz. *cry1Ac* + *cry1Fa* and c*ry34Ab* + *cry35Ab*, respectively.

Another strategy being used to overcome the problem of insects becoming resistant to the *Bt* toxin is to grow non-*Bt* crop around the *Bt* field, as 'refuge'. The refuge plants, which are of the same cultivar as the transgenic crop, allow maintaining sufficient population of homozygous susceptible insects (SS). The rare homozygous resistant insects (RR) that survive on *Bt* plants mate with susceptible (SS) insects rather than with each other. As a result, all offsprings are heterozygous (RS) which get killed by high dose of toxin in the *Bt* plants.

15.5.3 Disease Resistance

Plant pathogens, such as viruses, bacteria, and fungi, are constant threat to agricultural crop production. The most widely used strategy to control bacterial and fungal diseases has been seasonal applications of agrochemicals. However, chemical protection against bacterial pathogens has not developed as well as that against fungi, and it is not applicable to viruses. The intensive use of agrochemicals against fungal pathogens has led to the development of resistance in various pest and pathogen populations and caused considerable damage to the environment. Therefore, the focus is on strategies that allow for crop production with minimal use of agrochemicals. The most effective strategy to check crop losses due to pathogen attack is to breed resistant crop cultivars. However, there are many pathogens for which no effective sources of disease resistance have been identified.

Most effective resistance is conferred by a single gene (*R* gene), the product of which recognizes the activity of a single virulence factor from the pathogen (*Avr* gene). Indeed, conventional breeding has been used to produce disease resistant plants but the main limitation of this approach is that it is a lengthy process to breed the trait into useful cultivars, and it is difficult to remove unwanted and undesirable genes linked to the *R* gene. In contrast, by genetic engineering disease resistance can be induced by inserting a specific resistance gene. Therefore, there has been interest in producing disease resistant transgenic plants.

A range of strategies have been tried to enhance resistance in plants against pathogens but none of the disease resistant cultivars, excepting virus resistant lines, have been commercialized. For example, to breed rice plants resistant to bacterial blight (caused by *Xanthomonas oryzae*), a destructive disease of rice in Asia and Africa, the *R* gene *Xa21* from a wild rice variety (*Oryza longistamminata*) was introduced into domesticated rice using particle bombardment (Ronald 1997). The *Xa21* gene has since been incorporated into several important rice varieties, but these transgenic lines are still not under large-scale cultivation (Korth 2008).

In nature, the plants produce special class of proteins in response to infection by pathogenic microorganism called "pathogenesis related proteins (PRP)" which correspond to hydrolytic

enzymes such as chitinase and β-1,3 glucanase that can hydrolyse chitin and β-1,3 glucan, the two main components of most fungal walls. PR proteins from different sources, including plants, bacteria and fungi, have been engineered into plants. The hydrolytic enzymes produced with these transgenes degrade the polymers in the cell wall of many fungi without affecting the plant performance. Constitutive expression of the defense proteins is designed to provide barrier against initial attack and do not allow establishment of the pathogen. Wound inducible promoters have been used to regulate rapid and localized expression of the gene to pathogen attack. One of the earliest reports on this is the transgenic tobacco lines containing chitinase gene from the bacterium *Serratia marcescens*. Similarly, cucumber plants transformed with rice chitinase sequence *RCC2* driven by a *35S* promoter showed a range of responses to the fungus *Botrytis cinerea*. Some of the lines showed complete resistance against the fungus.

Apple plants expressing the transgene for exochitinase and/or endochitinase from *Trichoderma harzianicum* showed resistance to the fungus *Venturia inaequalis* which causes scab. The degree of resistance showed positive correlation with the level of expression of the proteins. If both the genes were introduced into a plant the proteins acted synergistically. However, the presence of endochitinase reduced plant growth. Moreover, this strategy does not work with disease caused by fungi that lack chitin in their walls such as *Phytophthera* sp.

Another strategy tried to impart resistance against fungal diseases is the use of ribosome-inactivating protein (RIP) which inhibits protein synthesis by rRNA.

15.5.4 Virus Resistance

Cross protection, in which the presence of a mild strain of virus in the plant protects it from subsequent infection by a more severe strain of a related virus, has become a common practice to protect crop losses by viral infection. The coat protein of the pre-existing virus encapsulates the RNA of the superinfecting virus, and thus renders it noninfectious. The disadvantage of this approach is: (a) the protecting virus may mutate to more severe form, (b) the protecting virus may cause low but significant drop in yield of the host plant, (c) the superinfecting virus may act synergistically with the protecting virus, and (d) in cross protection the protecting virus should be applied every growing season. These problems can be offset by genetically engineering coat protein-mediated resistance (CP-MR) in plants.

Transgenic virus resistance is the most advanced of the applications of biotechnology for the management of plant pathogens (James 2009). The feasibility of protein-dependent resistance (PDR) to virus was first examined by the expression of the TMV CP gene in tobacco plants (Abel et al. 1986). When challenged with TMV, transgenic plants expressing the TMV CP either did not display symptoms of TMV infection or showed a delay in symptom development. The selfed progeny of the transgenic plants as well expressed protection against TMV infection. As this type of resistance is related to the CP protein expression level, it has been termed CP-MR (Beachy 1999).

The first report of field expression of CP-mediated virus resistance in tomato plants and their sexual progeny was published by Nelson et al. (1988). It did not affect the yield adversely. Thereafter, CP-MR to virus infection has been achieved in several crops, such as *Alfalfa Mosaic Virus* (AlMV)-resistant tomatoes (Tumer et al. 1991) and PVX and PVY-resistant potato (Kaniewski et al. 1990).

The mechanism of protein-mediated resistance requires a direct correlation between the coat protein expression level and virus resistance level. Surprisingly, in later studies it was observed that high virus resistance levels in transgenic lines are not always associated with viral coat protein expression (Pang et al. 1992; deHaan et al. 1992). Moreover, the coat protein RNA level was also very low or absent in these resistant plants. For example, transgenic

potatoes showing field resistance to PVY did not accumulate detectable levels of PVY coat protein (Kaniewski et al. 1999). It soon became clear that in these transgenic plants the CP mRNA triggered post-transcriptional gene silencing (PTGS), and provided RNA-mediated resistance to virus through the short interfering RNA (siRNA) pathway (see Sect. 15.5.11). The alternative RNA-based mechanism for virus resistance is the formation of another category of small RNAs (~21–25 nucleotides) from the silenced mRNAs, called microRNA (miRNA). The miRNA pathway downregulates endogenous gene expression in plants. This pathway has been used to design artificial miRNAs (amiRNAs) with sequences complementary to viral sequences and has been successfully applied to generate virus resistance. This novel anti-viral strategy, which has the advantage of reducing possible biosafety risks associated with protein- and RNA-based strategies, is a first step toward designing environmental friendly virus resistance in transgenic crops (Niu et al. 2006; Qu et al. 2007).

Protein-based and RNA-based virus resistance works only against viruses closely related to the virus from which the CP transgene is derived. Therefore, efforts are being made to achieve resistance against several viruses by co-expression of 2–3 amiRNAs in the same plant (Niu et al. 2006).

A well-known example of engineering virus resistance in plants by the RNA interference (RNAi) mechanism is the development of *Papaya Ringspot Virus* (PRSV)-resistant papaya (Gonsalves 1998). In 1992, the papaya plantation in Hawaii got infected with the PRSV and by 1995 almost 95 % of the plantation was severely affected by the disease. The conventional breeding and selection did not help to save the crop. Fitch et al. (1992) transformed papaya independently with two constructs, one carrying the full length coat protein gene under the control of 35S promoter (pSA1175) and the other with inverted repeats of the coat protein coding region (pSA1304). Five independent transgenic lines showing integration of the PRSV CP gene into the plant genome were obtained. All the four lines with pSA1304 construct were fully resistant to the virus, whereas the fifth line, with pSA1175, remained sensitive to the virus. The pSA1304 construct imparted virus resistance through the RNAi mechanism. In 1998, two transgenic varieties of papaya, viz, 'SunUP' and 'Rainbow' were released for commercial cultivation. The introduced trait has been stable over the years, and has been bred into other commercially important varieties. A similar approach has been successfully followed to produce CMV resistant transgenic squash (*Cucurbita* sp.).

15.5.5 Nutritive Quality of Food

The nutritive value of foods can be enhanced by inserting gene/s for the new trait/s. A well-known example of this is the Golden rice variety that produces high levels of β-carotene or vitamin A in the endosperm (Ye et al. 2000).

Rice is the major staple food for one-third of the world population. It is a good source of calories but lacks or is very poor in vitamins and proteins. Dietary β-carotene (pro-vitamin A), converted into vitamin A, plays an important role in the normal development of humans. Deficiency of vitamin A leads to night blindness or total blindness in children, besides diseases such as measles and malaria. Rice plants normally do not contain β-carotene.

Rice plants produce geranylgeranyl diphosphate (GGPP) but lack the enzymes to convert it into β-carotene (Fig. 15.7). Transgenic rice was produced by high level tissue specific expression of two genes, viz., *psy* gene, encoding phytoene synthase (converts GGPP into phytoene) from daffodil and *crt I* gene, encoding phytoene desaturase, (catalyzes the conversion of phytoene to lycopene) from the bacterium *Erwinia uredovora.* Finally, lycopene is converted into β-carotene by an endogenous lycopene β-cyclase. Both the transgenic enzymes were

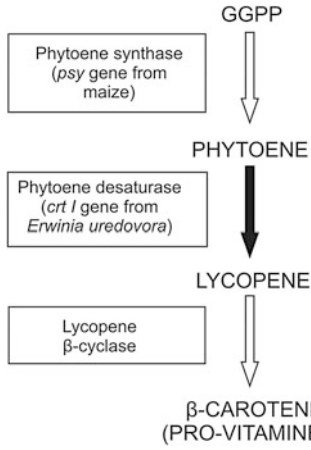

Fig. 15.7 Transgenic golden rice production. It involves insertion of two transgenes, one plant gene (*psy*) that converts native geranylgeranyl diphosphate (GGPP) into phytoene, and the other (*crt I)* from a bacterium which catalyzes the conversion of phytoene into lycopene. The conversion of lycopene to *β*-carotene (pro-vitamin A) is brought about by the native lycopene *β*-cyclase

modified by addition of a transit peptide and were targeted to the plastids where GGPP is synthesized. The endosperm in the seeds of these GM plants appeared yellow due to the accumulation of *β*-carotene, and therefore the variety is known as "Golden Rice". However, the golden rice produced initially (Golden Rice 1) synthesized *β*-carotene in low amounts (1.6 μg/endosperm). Substitution of *psy* gene from daffodil by *psy* gene from maize-enhanced *β*-carotene accumulation by 23 times (Golden Rice II; Paine et al. 2005). Originally, the golden rice trait was introduced in the japonica cv. Taipei 309. However, more than 90 % rice eaters consume indica rice. Therefore, the Golden rice trait has been transferred to indica cultivars.

Significant increase of essential amino acids, including lysine, tryptophan, tyrosine, and sulfur-containing amino acids in potato tubers could be achieved by inserting a nonallergenic seed albumin gene *AMA1* from *Amaranthus hypochondriacus* (Chakraborty et al. 2000). Some examples of improvement of nutritive quality of foods based on RNAi technology are described in Sect. 15.5.11.

Maize with increased lysine content due to the suppression of lysine catabolism in endosperm (Houmard et al. 2007) and the subsequent insertion of the bacterial dihydrodipicolinate synthase gene *CordapA* from *Corynebacterium glutamicum* was obtained by Frizzi et al. (2008). This procedure led to a 100-fold enhancement in lysine content in maize seeds as compared to untransformed plants. Attempts are being made to obtain transgenic coffee plants with caffeine-free seeds by RNAi technology (Ogita et al. 2003, 2004; Ashihara et al. 2006).

15.5.6 Abiotic Stress Tolerance

Abiotic stresses like drought, high and low temperatures, freezing injury, and salinity cause considerable losses to agriculture production worldwide by affecting plant growth and development. Many potential candidate genes have been identified and cloned (Table 15.2). However, despite enormous research in this area, no report on stability and commercial viability of such transgenics is available (Mittler and Blumwald 2010). This still remains a challenge area for plant biotechnologists and breeder's alike.

15.5.7 Male Fertility Control

Natural male sterility may be cytoplasmic or nuclear. In hybrid seed production mostly CMS lines have been used which may be unstable under certain environmental conditions or pose

Table 15.2 Genes for abiotic stresses

Abiotic stress	Gene
Drought	Gene for mannitol dehydrogenase (from *E. coli*), proline
Salt tolerance	HaI_2 gene Glyoxalase1, Glyoxalase2, DREB
Heavy metals (Hg, Al, Cd)	Gene for exclusion of metals, genes for storage of metals by plants (Phytoremediation)
Cold tolerance	Glycerol-3-phosphate acyltransferase (*Arabidopsis*) Rob5
Frost tolerance	Antifreeze protein genes from fish

other problems, such as linkage of CMS character with sensitivity to *Helminthosporium maydis* in maize.

It has been possible to induce nuclear male sterility in a wide range of plants by inserting a dominant chimeric gene comprising of the 5′ regulatory region of *TA29* (*pTA29*) promoter from tobacco and ribonuclease coding *barnase* gene from *Bacillus amyloliquefaciens* (Mariani et al. 1992; Raynaerts et al. 1993). *pTA29* gene is characterized by extreme cell specificity in the tapetal cells of young anthers (Goldberg 1988). The presence of the chimeric gene in the plant cells causes premature breakdown of tapetum, resulting in arrest of microspore development.

Restorer lines for *barnase* male sterile lines could be engineered by introducing another chimeric gene comprising *pTA29* promoter fused with the bacterial gene *barstar*, which inactivates the protein produced by barnase gene. Figure 15.8 summarizes the strategy used by Mariani et al. (1990, 1992) to engineer fertility control in tobacco and some other flowering plants.

More recently, RNAi technology has been employed to induce male sterility. Total 10 out of 13 tobacco lines transformed with a hairpin RNAi construct containing TA29 promoter were male sterile. These transgenic male sterile plants were otherwise indistinguishable from the non-transgenic tobacco plants and set seeds on cross pollination with nontransgenic plants (Nawaz-ul-Rehman et al. 2007).

15.5.8 Parthenocarpy

Parthenocarpy refers to the formation of fruits without fertilization. Seedlessness of such fruits is a highly desirable trait for their consumption as fresh fruits or for juice and jam industry. Parthenocarpy also offers the possibility of improving fruit quality and productivity in many crop plants grown for their fruits (Spena and Rotino 2001). The cultivated plantain and banana are highly sterile polyploid plants which develop parthenocarpic fruits. Triploid watermelons produced by crossing tetraploids and diploids also produce parthenocarpic seedless fruits. Parthenocarpy can be experimentally induced by treatment with hormones, especially auxin.

It has been possible to genetically engineer the parthenocarpy trait by inserting a gene that enhances auxin synthesis in the plant tissues. Rotino et al. (1997) and Ficcadenti et al. (1999) induced parthenocarpy in tobacco and several lines of eggplant and tomato by introducing the chimeric gene *DefH9-iaaM*. The *iaaM* gene from *Pseudomonas syringae* pv savastanoi increases auxin synthesis in plant tissues and organs of the transgenic plants and the ovule specific promoter and regulatory gene *DefH9* from *Antirrhinum majus* regulates the expression of *iaaM* only in the ovules without affecting vegetative growth.

The transgenic plants of eggplant carrying the gene *DefH9-iaaM* produced fruits with viable

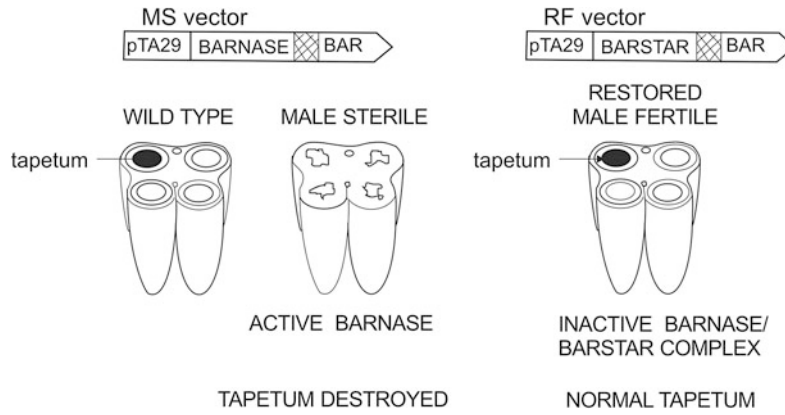

Fig. 15.8 Schematic representation of genetic engineering for male fertility control using barnase and barstar genes. (*courtesy* Dr A. Reynaerts, Belgium)

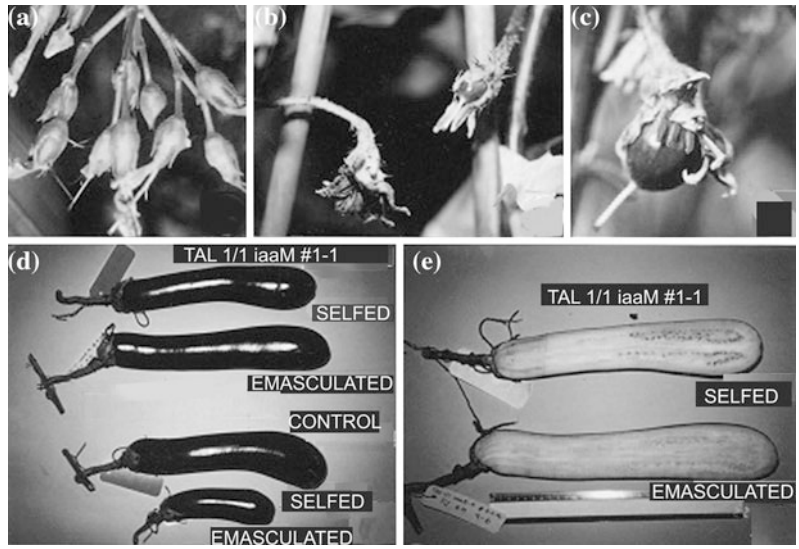

Fig. 15.9 **a** Tobacco. **b–e** Eggplant. **a** Tobacco capsules derived from emasculated inflorescence of a T_2 plant with the transgene *DefH9-iaaM*. **b** Comparison of eggplant flowers 10 days after flowering under adverse environmental conditions. An aborted hand pollinated flower of an untransformed plant (*left*) and a small fruit developed from an emasculated flower of a transgenic *DefH9-iaaM* plant (*right*). **c** Early stage of fruit development in transgenic parthenocarpic eggplant, showing healthy anthers. **d** Fruits from selfed and emasculated flowers of transgenic parthenocarpic (*top*) and untransformed control (*bottom*). **e** Longitudinal halves of fruits from hand pollinated (*top*) and emasculated (*bottom*) flowers of transgenic plants. Both, selfed and emasculated flowers of transgenic eggplant produce comparable fruits. (*after* A. Spena and G.L. Rotino, 2001)

seeds when pollinated and parthenocarpic fruits in the absence of pollination (Fig. 15.9). Thus, in greenhouse eggplant fruits could be produced round the year. The parthenocarpic trait was transmitted to the progeny in a Mendelian fashion as a dominant trait.

15.5.9 Plants as Bioreactors

Plants can be used as bioreactors and cost-effective alternatives to microbial and animal systems to produce biomolecules (Sharma et al. 2004). Technologies have been developed to engineer plants capable of producing a wide variety of commercial products, including products for pharmaceutical applications, such as vaccines, antigens, antibodies, and other therapeutic proteins. Two examples of human protein production in plants are human serum albumin in tobacco and potato and human insulin in tobacco. In both these cases, the protein produced appears to be fully effective in humans. However, one cannot raise his/her insulin level by eating transgenic tobacco leaves, as the protein in most cases will be broken down to amino acids before it reaches the blood stream. Therefore, in these cases one cannot escape the practice of protein isolation and purification before transgenic leaves are converted into drugs. Although during the past 15 years, expression of many different vaccine antigens in plants has been reported and many of these stimulated antibody responses when eaten by humans or animals, but low expression of antigens in the transgenic plants limit the dose that can be delivered in crude material (Mason 2004).

A new approach to genetic enhancement of cell cultures for the production of phytochemicals is biochemical engineering of enzymes of their biosynthetic pathways. The first example of successful modification of a medicinal plant for its secondary metabolite is the introduction of cDNA encoding hyoscyamine-6β-hydroxylase

(h6h) from *Hyoscyamus niger* into *Atropa belladonna* which normally produced hyoscyamine as the main alkaloid and very little of scopolamine (Yun et al. 1992). The hairy root cultures of the transgenic *A. belladonna* exhibited increased hyoscyamine hydroxylase activity and produced five times more scopolamine than the wild-type hairy roots (Hashimoto et al. 1993). Root cultures of *H. muticus* carrying the transgene *h6h* produced over 100 times more scopolamine than the wild-type control (Jouhikainen et al. 1999).

15.5.10 Biofuel

Increasing demand and shrinking reserves of petroleum and the negative environmental impacts of its extraction, refining, transportation, and uses as fuel is turning world attention to biofuel. To some extent, starch and sugar-derived ethanol is being produced and used as transportation fuel. Brazil produces relatively cheap ethanol by fermentation of cane sugar to meet one-quarter of its requirement for ground transportation. The United States produces ethanol from maize. However, these sources are not commercially viable to meet the increasing demand (Sticklen 2008). Enzymatic conversion of cellulosic biomass, which is available in abundance and is renewable, is being considered as promising alternative. The production of microbial cellulases for the purpose is still not cost-effective. Therefore, efforts are being made to genetically engineer plants to produce cellulases and hemicellulases within the plant biomass.

Transgenic plants of rice and maize that synthesize endocellulase E1 have been produced, but the level of total soluble protein (TSP) in these plants is insufficient (5 and 2 %, respectively) for complete hydrolysis of cell wall polysaccharides. It must be raised to 10 % of TSP (Sticklen 2008). There are many other related issues to be resolved before biofuel from GM crops becomes reality.

15.5.11 RNA Interference (RNAi) Based Improvement of Plant Products

More than a decade ago, Napoli et al. (1990) attempted to overexpress chalcone synthase (*CHS*) gene in petunia using a transgene sequence that was identical to the sequence of the natural *CHS* gene responsible for the purple color. To achieve this, *CHS* gene driven by the constitutive promoter known to give high levels of gene expression was used with the expectation that with additional copies of CHS gene deeper purple flowers will be produced. However, the unexpected happened. The CHS overexpressing transgenic plants produced fully or partially white flowers instead of deep purple flowers. The CHS messenger RNA (mRNA) levels in the white sector, as compared to the purple sector, were drastically reduced for, both, the CHS transgene and the native petunia *CHS* gene.

Similarly, for several years it was believed that a transgenic plant expressing viral coat protein for a specific virus had higher resistance to the virus, because the viral coat protein prevented the uncoating of the infecting virus, and thus interfered with virus replication. However, the work of Lindbo et al. (1993) resulted in an alternative explanation, according to which high level of viral mRNA expressed from plant transgene resulted in virus resistance. Transcription rates of the transgene were similar in both virus infected and uninfected plants but no transgene viral mRNA accumulated, indicating that resistance was at the post-transcriptional level. According to the model proposed by these workers, an RNA-dependent RNA polymerase (RdRP) produced an antisense RNA that led to the degradation of, both, the viral RNA and the transgene viral RNA.

RNA-mediated gene silencing involves suppression of gene expression by degradation of homologous RNAs (Dijk and Cerutti 2004). This strategy might have been evolved as a defense response in eukaryotes against intracellular parasites such as viroids, viruses, and transposable elements. The key steps in inducing RNA-

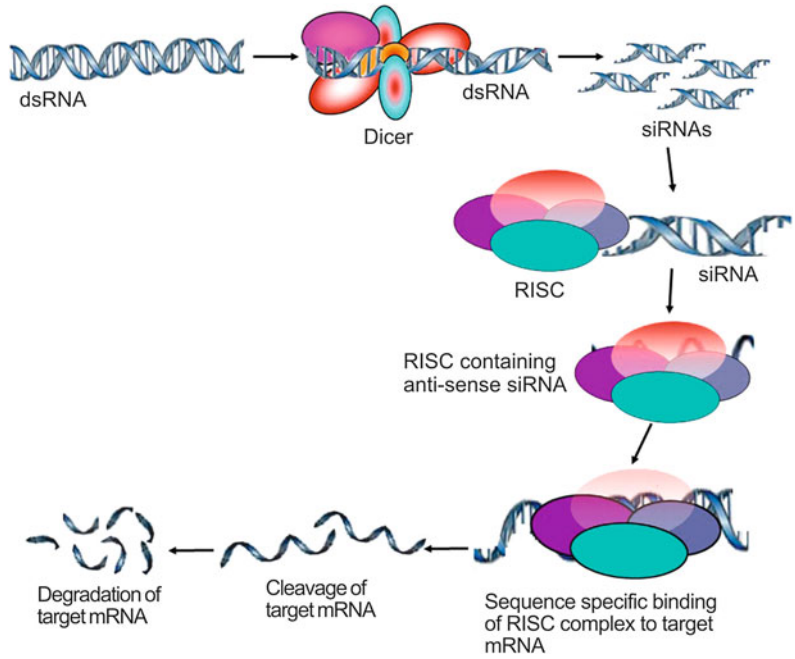

Fig. 15.10 Schematic representation of RNA-based gene silencing. For details see text (*dsRNA* double stranded RNA, *RISC* ribosome-induced silencing complex *siRNA*, small interfering RNA). (*courtesy* Dr R.K. Bhatnagar, ICGEB, New Delhi)

mediated gene silencing is the formation of double-stranded RNA (dsRNA; ~200 nucleotides) by the host RNA-dependent RNA polymerase (RdRP), which is recognized and cleaved by the multidomain RNase III enzyme, called DICER, to yield small (21–25 nucleotides long) interfering RNAs (siRNAs). The siRNAs are transferred to the RNA-induced silencing complex (RISC). The RISC unwinds the double-stranded siRNA and uses the antisense strand to identify and degrade complimentary mRNAs (Fig. 15.10).

RNA interference (RNAi) is considered to be the most effective strategy for the suppression of gene expression (silencing) at the post-transcriptional stage in plants and other organisms (Agarwal et al. 2003, Rukavtsova et al. 2010). However, it is not exclusively a post-transcriptional process. It may also act at the transcriptional level, by inducing direct methylation of homologous DNA or local DNA modification rendering chromatin into heterochromatin (Dijk and Cerutti 2004). Another class of noncoding transcripts, such as microRNAs (miRNAs), which have complex duplex structure, are cleaved by DICER to yield short temporal RNA (stRNAs) similar to siRNAs.

The strategy of using dsRNA to silence gene, called RNAi technology, has revolutionized the possibilities for creating custom "knock downs" of gene activity in plants and animals. Employing this approach, several novel crop plants, such as nonallergenic peanut, decaffeinated coffee, nutrient fortified maize (Sect. 15.5.5), improved quality cotton oil (Sect. 15.5.11.2), male sterile tobacco (Sect. 15.5.7), and blue colored rose (Sect. 15.5.11.2), have been developed. Some of these products have already reached the market. Constitutively expressed RNAi can be used to silence genes throughout the plant development or can be expressed conditionally to provide temporal control over the onset of gene silencing. Tissue and organ specific RNAi vectors have proved to be useful for targeted gene silencing in specific plant tissues and organs with minimal interference with the normal plant life cycle. RNAi is considered to be one of the most prospective strategies for the construction of transgenic plants characterized by high biological safety due to the absence of foreign proteins. Greater understanding of the mechanism involved in RNA silencing has allowed the design of better constructs Table 15.3.

Table 15.3 Some examples of novel plant traits engineered through RNAi technology

Trait	Target gene	Host plant	Application
Enhanced nutrient content	*Lyc*	Tomato	Increased concentration of lycopene (carotenoid antioxidant)
	DET1	Tomato	Higher flavanoid and β-carotene contents
	SBEII	Wheat, Sweet potato, Maize	Increased amylase for glycemic management and digestive health
	FAD2	Canola, Peanut, Cotton	Increased oleic acid content
	SAD1	Cotton	Increased stearic acid content
	ZLKR/SDH	Maize	Lysine fortified maize
Reduced alkaloid production	*CaMXMT1*	Coffee	Decaffeinated coffee
	COR	Opium poppy	Production of non-narcotic alkaloid instead of morphine
	CYP82E4	Tobacco	Reduced levels of the carcinogen nornicotine cured leaves
Heavy metal accumulation	*ACR2*	*Arabidopsis*	Arsenic hyper-accumulation for phytoremidiation
Reduced polyphenol production	s-cadinene synthase gene	Cotton	Lower gossypol level in cotton seeds for safe consumption
Ethylene sensitivity	*LeETR4*	Tomato	Early ripening tomatoes
	ACC oxidase gene	Tomato	Longer shelf life due to slow ripening
Reduced allergenicity	*Arah2*	Peanut	Allergen-free peanut
	Lolp1, Lolp2	Ryegrass	Hypo-allergenic ryegrass
Reduced production of lachrymatory factor synthase	Lachrymatory factor synthase gene	Onion	'Tearless' onion

15.5.11.1 Delayed Fruit Ripening

One of the first GM products to reach the market, in 1994, was tomato *var* FlavSavr, showing delayed softening of fruits, produced by Calgene, USA and Tomato paste from GM tomatoes produced by Zeneca Co., UK. Fruit ripening is an active process. In climacteric fruits, such as tomato, fruit ripening is characterized by a burst of respiration, ethylene production, changes in color, flavor, and tissue softening. The hormone ethylene triggers tomato ripening, which involves change of color from green to red, due to the synthesis of lycopene pigment, and flavor due to breakdown of starch to form sugar and a large number of secondary metabolites, and softening of the fruit tissues by the activity of cell wall degrading enzymes polygalacturonase (PG) and pectin methylesterase (PME).

The PG enzyme is synthesized de novo during fruit ripening and induces breakdown of pectin material that cements the neighboring cells, resulting in softening of the tissue which makes the fruits more susceptible to rottening by fungal infection. Almost simultaneously, Smith et al. (1988) in the UK and Sheehy et al. (1988) in the USA reported that introduction of an antisense construct (short synthetic pieces of DNA that are designed to bind to their target mRNA and inhibit the translation of, splicing of, or degradation of the DAN–RNA hybrid by RNase) based on the gene *pTOM6* for PG enzyme considerably reduced the PG activity and delayed fruit softening without affecting other ripening events. Thus, the fruits allowed to ripen on the GM vines developed full flavor and color and taste but remained firm. Unmodified tomatoes are, normally, picked while still green and firm for easier handling and extended shelf life. Then, the fruits are ripened by the application of ethylene gas. Such fruits develop red

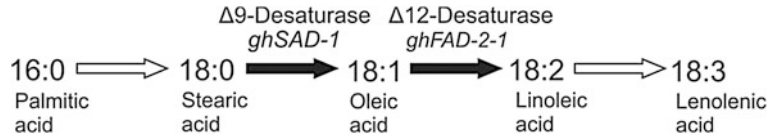

Fig. 15.11 Schematic representation of steps involved in the biosynthesis of fatty acids in cotton

color and soften but do not acquire the normal flavor of tomatoes.

Similarly, the activity of the PME enzyme in the tomato fruits could be reduced by antisense gene, which resulted in fruits with enhanced serum viscosity due to increased soluble pectin. The tomato paste from such fruits appeared glossy. The two characters, viz, low PG and low PME could be combined in a single plant by two successive transformation events or by crossing transgenic plants independently transformed with the two genes. This resulted in significant increase in soluble solids.

Fruit ripening in tomato could also be delayed by lowering the synthesis of ethylene hormone by antisense technology. The antisense of the gene *pTOM13* reduced the level of ACC oxidase enzyme required for the synthesis of ethylene.

Despite their initial success, neither the Zeneca nor the Calgene low PG tomato products can now be found in the market. The commercial production of FlavrSavr tomato, which was started in 1994, was stopped in 1997 for economic reasons. The Zeneca tomato paste, clearly labeled as GM product, sold well when it was first introduced in 1996. It was 20 % cheaper than the conventional tomato paste. At one stage, the paste from GM tomatoes outsold normal tomato paste, but in 1998 it had to be removed from the shelves of supermarkets because of anti-GM backlash following the report of Ewen and Pusztai (1999) that feeding GM potatoes carrying lectin GNA gene to rats caused proliferation of gastric mucosa cells.

15.5.11.2 Improvement of Cotton Oil

RNAi technology has been applied to improve the quality of cotton oil (Liu et al. 2002). The natural oil from cotton seeds contains 26 % palmitic acid (16:0), 15 % oleic acid (18:1), and 58 % linoleic acid (18:2) (Fig. 15.11). Δ12-Desaturase enzyme (FAD2-1) catalyzes the transformation of oleic acid into polyunsaturated fatty acids. The inhibition of the Δ12-Desaturase enzyme by RNAi resulted in increased oleic acid content in the oil up to 77 % as against 15 % in the untransformed plants. Similarly, inhibition of the Δ9-Desaturase enzyme (SAD-1), which catalyzes the conversion of stearic acid to oleic acid, increased stearic acid content up to 40 % as compared to 2–3 % in the parent plants (Liu et al. 2002). The phenotypes showed stable inheritance. Intercrossing of transgenic cotton plants characterized by the inhibited expression of *FAD2-1 and SAD-1* yielded some F_1 lines (7/36) with high content of both stearic and oleic acids in the oil (up to 40 %). The modified cotton seed oil with high oleic acid and high stearic acid are nutritionally superior, as these fatty acids are good for human heart. However, the cotton seed contains gossypol and a toxic terpenoid. Gossypol is also produced in the vegetative parts of the cotton plant where it acts as a defense against insects and other pathogens. By using a construct containing the *δ*-cadinene synthase gene of gossypol synthesis under the control of a seed specific promoter, Sunilkumar et al. (2006) achieved seed-specific reduction of gossypol by RNAi technique. It did not disturb the gossypol content in the vegetative tissues. These cotton plants are, thus, expected to have similar insect and pathogen resistance as the wild-type cotton.

15.5.11.3 Flower Color

To date, the usual selection has failed to obtain blue tinted rose flowers due to the absence of flavanoid 3′5′-hydroxylase (*F3′5′H*), the key enzyme for the biosynthesis of delphinidin,

which is the major constituent of violet and blue flowers. Japan-based Suntory Co. and Australia-based Florigene Co. have produced the world's first blue roses by introducing viola *F3′5′H* gene in rose cultivars (Katsumoto et al. 2007). For more exclusive and dominant accumulation of delphinidin, the endogenous dihydroflavonol 4-reductase (*DFR*) gene, which encodes red color, was silenced by RNAi technology, and *DFR* of *Iris* x *hollandica* was introduced. The transgenic roses with overexpression of viola *F3′5′H* gene and Iris *DFR* gene, and downregulation of the native *DFR* gene, resulted in accumulation of delphinidin in the petals, and the flower thus had blue hues. The introduced trait was heritable in the progeny. In 1994, the Florigene Co., Australia produced transgenic cultivar of carnation 'FLORIGENE Moondust^{TM,} with mauve colored flowers, and in 1997 they created another transgenic cultivar of carnation 'FLORIGENE Moonshadow^{TM,} with a richer true purple color.

15.6 Biosafety

The final stage of genetic engineering is the field trial of the transgenic plants, not only to establish their agronomic superiority over the parent cultivar, but also to ensure that it is safe both ecologically and for human and animal consumption. The GM cultivars are expected to be the same as those produced by the conventional methods of plant improvement except that in the former the transgenes are often derived from completely unrelated organisms, such as viruses, bacteria, insects, and human beings. The GM plants also carry the unwanted marker genes in addition to the gene of the desirable trait. Moreover, in genetic engineering the gene could be manipulated outside the cell before inserting it into the recipient cell or the transgene could be a novel gene synthesized in the laboratory.

The greatest concern associated with the GM crops is their potential impact on environment due to the escape of transgene to other plants in the area, and thus threaten the ecosystem. Therefore, in most of the countries environmental studies are required before the approval of a GM crop for commercial cultivation. Another serious concern related to the GM crops is the appearance of toxins and allergens in GM foods and feeds or reduction in the nutrients due to the introduction of a new gene. The transgenic soybean expressing Brazil nut seed storage protein was not allergenic to the individuals allergic to the Brazil nut (Nordler et al. 1996). Allergenicity of nonfood and nonfeed plants consider dermal and/or respiratory exposure to farm workers and the impact of dietary exposure to the wildlife.

To ward against the potential risks from GM crops, in most of the countries research in the area of genetic engineering and commercialization of its products is governed by GMO-safety regulations, and committees have been constituted to monitor and grant permission for the cultivation of GM crops and marketing of products derived from them. In the USA, which has seen a widespread adoption of GM maize, cotton, soybean, and more recently alfalfa crops, three Federal Agencies look after the regulatory control of GM food crops and products made from them: (i) the Animal and Plant Health Inspection Service Division of USDA overlooks the environmental impact of the transgenic plants and issues permit after the assessment, (ii) the Food and Drug Administration (FDA), operating within the Department of Health and Human Services (HHS), is concerned with the safety of food and feed. It focuses on the occurrence of toxins and allergens in any way induced due to transgenosis, and (iii) the Environmental Protection Agency (EPA) evaluates food safety and environmental issues associated with new pesticides and pesticidal products, and therefore evaluates GM plants with altered pesticide trait such as *Bt* crops and virus coat protein mediated virus resistant plants.

The Indian biosafety regulatory system is one of the most stringent in the world. India has laid down detailed biosafety regulations and guidelines for transgenic research which have been notified in government Gazette through notification number 621, dated 5 December 1989. This necessitates biosafety assessment at three stages, viz, (i) Recombinant DNA Advisory

Committee (RDAC), Review Committee on Genetic Manipulation (RCGM), and Genetic Engineering Appraisal Committee (GEAC) at the national level, (ii) Institutional Biosafety Committee (IBSC) at the institutional level, (iii) State Biotechnology Co-ordination Committee (SBCC) and District Level Committee (DLC) at the state level (http://www.dbtindia.nic.in). The regulatory setup oversees the development of GM crops from the research stage to large-scale commercial use through a three tier system. All GM plants require evaluation in the open environment. The Department of Biotechnology, Government of India (1990, 1998) has been playing a major role in developing guidelines for field evaluation. The violation of regulatory procedures attracts penal action. In 2005, 16 Research Institutes and eight private companies were involved in genetic manipulation of 19 crops by rDNA technology. However, so far only one GM crop, viz, *Bt* cotton, has been released for commercial cultivation (Tripathi and Behera 2008; Ahuja 2008). On 9th February 2010, the Indian Environment Minister imposed moratorium on the cultivation of GM Foods for as long as it is needed to establish public trust and confidence.

Many countries, especially in the European Union, where GM crops have not been introduced so far, demand freedom to choose between foods derived from GM crops and organically produced plants. This requires a labeling system as well as reliable separation of GM and nonGM crops at field level and throughout the whole production chain.

Scientists have often questioned the special concern about GM crops over traditionally modified crops which are regarded as risk free (Lemaux 2008, 2009). Their argument is that in order to meet the demand for food in the developing world, a second green revolution with increased use of GM crops is needed, and therefore they should be allowed to use GE for the benefit of those who need it without over restrictive regulations.

15.7 Concluding Remarks

Genetic engineering allows geneticists to introduce genetic variation in plant species that does not occur in its germplasm pool. Genetic engineering can be used either to introduce a completely novel trait or to manipulate a known biochemical pathway. Initially, the progress in the area of genetic engineering was slow because of the recalcitrance of many crop plants to infection by *A. tumefacience* and/or regeneration from tissue cultures. However, these problems have been largely overcome.

It must be very clear that GE is a supplement not a supplant to plant breeding. Creation of successful transgenic plants requires tools and techniques of, both, biotechnology and conventional breeding. During the last three decades these tools and techniques have been considerably refined and transgenic plants have been produced with diverse economic traits, and some of the transgenic products have been commercialized. More GM crops are being developed with novel traits, such as controlled fruit ripening, flower color, increased protein content, reduced allergenicity, altered starch and fatty acid profile, higher vitamin, and mineral content and tolerance to environmental stress.

GE technologies have advantages over classical breeding not only by enlarging the scope of genes and the types of mutations to be manipulated but also by the ability to control the spatial and temporal expression patterns of the genes of interest. Tissue or organ specific RNAi vectors have recently proved to be useful for targeted gene silencing in specific plant tissues and organs with minimal interference with the normal plant life cycle. New generation RNAi vectors have been developed with high silencing accuracy and fewer side effects in plants (Tang et al. 2007). Unlike traditional plant breeding, in GE only the gene of interest or a part of it or a synthetic gene is introduced, and therefore it is also referred to as precision breeding or molecular breeding.

A major set-back in the commercialization of GM crops has been the apprehension of public. Whereas the US and many developing countries have accepted GM crops for large-scale cultivation, most of the European countries, including the UK, have not accepted the introduction of GM crops or their products. For example, the tomato paste from transgenic fruits was withdrawn from the supermarket shelves because of anti-GM backlash following the report that feeding GM potatoes, carrying lectin GNA gene, to rats caused proliferation of gastric mucosa cells (Even and Puszati 1999).

15.8 Appendix

1. *Agrobacterium*-mediated leaf disc method of transformation of tobacco. (*after* Horsch et al. 1985).
   (i) Immerse tobacco seeds in 10 % 'Domestos' bleach solution for 20–30 min and rinse three times in sterile distilled water. Place the seeds on a 64 μm nylon mesh during surface sterilization for easy handling.
   (ii) Sow the seeds on MSB medium containing MS salts, B_5 vitamins and 3 % sucrose and gelled with 0.8 % agar (20 mL medium/9 cm Petri plate). Incubate the plates at 24–28 °C, under 16 h photoperiod with a light intensity of 48 μmol m^{-2} s^{-1} (daylight fluorescent tubes) for 7 days, when the cotyledons are fully expanded.
   (iii) Transfer individual seedlings to magenta boxes (one per box), each containing 40 ml of culture medium as in step (ii). Incubate the culture for 3–4 weeks under the same conditions as in step (ii) until plants have developed 4–5 leaves suitable for preparing leaf discs.
   (iv) Excise leaves from the in vitro plants or 28 days old greenhouse grown plants and place them on a sterile tile. Using a cork borer or a scalpel, excise 1 cm diameter discs and transfer them, with abaxial surface down, onto MSB medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA (MS104; 7–8 discs per plate). Place a single filter paper over the discs to help keep them flat on the medium. Incubate under conditions as in (ii).
   (v) Take an overnight liquid culture of *Agrobacterium tumefaciens* and dilute it 10 times with MSB liquid medium (2 ml of bacterium culture and 20 ml MSB medium in 9 cm Petri plates).
   (vi) Float the discs in bacterial suspension, making sure that the wounded surface of the explants is immersed in the suspension. After 5 min, blot the explants on a sterile filter paper, and transfer the explants back to MS104 medium and incubate at 24–26 °C at a low light intensity (24–48 μmol m^{-2} s^{-1}) for 2 days.
   (vii) Transfer the explants to the selection medium (MS104 + 500 mg L^{-1} carbenicillin and 300 mg L^{-1} kanamycin sulfate.
   (viii) Shoots should be visible from the wounded edges of the discs after 18–21 days of inoculation. Putatively transformed shoots should emerge from kanamycin-resistant calli. After 28 days of inoculation, regenerated shoots should be large enough (ca 1 cm in height) to be excised and transferred to rooting medium (MSB + 500 mg L^{-1} carbenicillin + 300 mg L^{-1} kanamycin sulfate).
2. Floral-dip method of transformation of radish. (*after* Curtis and Nam 2001).
   (i) Sow seeds (1 seed/3 cm^2) in a deep seed tray (12 cm depth containing peat-based compost and maintain in a glasshouse under natural daylight, supplemented with 16 h photoperiod with a light intensity of 61 μmol m^{-2} s^{-1} (daylight fluorescent tubes) at 26 °C (day) and 18 °C (night).

(ii) Transfer 3 to 4-week-old seedlings, individually, to deep pots (20 cm diameter, 30 cm depth) containing new compost to encourage plants to develop long tap roots. Maintain the plants under glasshouse conditions for 10 days to aid recovery.

(iii) At the six leaf stage, transfer the plants to a cold chamber set at 4 °C, 16 h photoperiod and light intensity of 45 μmol m^{-2} s^{-1} (daylight fluorescent tubes) for 10 days to promote bolting. Return the plants to the glasshouse under conditions as in step (i).

(iv) Plants and with single thick stems and numerous immature floral buds are ideal for the transformation treatment.

(v) Four days before floral dip treatment, take a loop-full of a glycerol stock of *Agrobacterium* strain AGL1 carrying pCAMBIA3301 and streak onto the agar (14 g L^{-1}) solidified YEP medium (10 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl) containing 50 mg L^{-1} kanamycin and 100 mg L^{-1} rifampicin. Incubate the cultures in the dark at 28 °C for 2 days.

(vi) Transfer a loop-full of bacteria to a 50 mL falcon tube containing 10 mL of bacterial culture medium (YEP medium + 50 mg L^{-1} kanamycin sulfate + 50 mg L^{-1} rifampicin). Place the culture on an orbital shaker at 1,800 rpm and maintain in dark at 28 °C overnight.

(vii) Transfer 10 ml liquid bacterial culture to a 1 L flask containing 500 ml of bacterial culture medium and incubate for 12–16 h as described in step (vi) until the OD reaches 1.0 at 600 nm.

(viii) Pellet the bacterial culture by centrifugation at 3,500 g, for 20 min at 4 °C. Resuspend the pellet in 500 mL of inoculation medium (50 g L^{-1} sucrose + 0.05 % v/v Silwet L-77, pH 5.2).

(ix) Remove any floral buds which show petal color and carefully submerge the inflorescence into the inoculation medium and gently swirl for 5 s. Cover the inflorescence with a ploythene bag.

(x) Remove the bag next morning and allow the plants to grow under conditions as before. Hand pollinate all flowers using a fine paint brush to aid seed-set.

Suggested Further Reading

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Production of Virus-Free Plants 16

16.1 Introduction

Pests and pathogens cause considerable damage and economic losses to agricultural and horticultural crops. A good amount of this damage is caused by virus infections. The crop plants, such as potato, sweet potato, banana, cassava, sugarcane, potato, horticultural crops (*e.g.* citrus, pome and stone fruits) and ornamentals, which are generally propagated by vegetative means, are particularly prone to losses caused by viruses that are transmitted from generation to generation. A rough estimate of annual global losses of agricultural produce by virus infection is to the tune of US$ 6×10^{10} billion (http://www.microbiologybytes.com/virology). The losses could be in terms of yield and quality of fruits and flowers, vigour and longevity of the productive life of the perennial crops, increased susceptibility of the host plant to other phytopathogens and the severity of the damage caused by them (Waterworth and Habidi 1998). Virus infection of the ornamentals, causing change in colour and reduced flower size, affects adversely the consumers' acceptability. Virus infection is also known to reduce the rate of clonal propagation. The rate of in vitro multiplication (micropropagation) of raspberry cv. 'Malling Landmark' infected with *Tobacco Streak Virus* (*TSV*), *Tomato Ringspot Nepovirus* (*TomRSV*) or *Raspberry Bushy Dwarf Idaeovirus* (*RBDV*) was less than half that of virus-free cultures (Tsao et al. 2000).

Grapevine alone is infected by about 53 viruses (Martelli 2006), and the estimated annual loss due to single viral disease, viz., the *Grapevine Fanleaf Virus* (*GFV*) is US$ 1.5 billion (Komar et al. 2007). *Potato Leafroll Virus* (PLRV) and *Potato Virus Y* (PVY) are among the most damaging potato viruses (Loebenstein 2000; Brunt 2001), and PLRV alone can reduce the total yield by almost 60 % and that of marketable yield by as much as 88 % (Hamm and Hane 1999). *Sugarcane Mosaic Potyvirus* (SCMV), one of the most widespread viral diseases of sugarcane, causes 50 % losses due to which a large number of traditionally high-yielding sugarcane varieties have gone out of cultivation (Singh et al. 1997; Viswanthan and Mohanraj 2001).

The majority of plant viruses are transmitted by vector organisms that feed on the plant. The most common vector organisms of plant viruses are insects, particularly aphids (Wang et al. 2006). Seeds and pollen, once thought to be comparatively free of viruses, are now known to transmit a large number of viruses (Mink 1993).

Whereas fungal and bacterial diseases can be controlled by the application of fungicides and bactericides, control of viral diseases is a serious problem as commercial chemical control methods are either not available or are not economical. Eradication of viruses and other pathogens is highly desirable to optimize the yield, to facilitate the movement of plant materials across international boundaries and for long-term

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_16, © Springer India 2013

germplasm storage, ex vitro or in vitro (Button 1977; Sediva et al. 2006).

Traditionally, thermotherapy of the infected plants has been used to obtain virus-free plants. However, it is not only a cumbersome process but also not all viruses are eliminated by this method, and many host plants are thermosensitive. The knowledge of the gradient of virus distribution in the shoot tips enabled Holmes (1948) to raise virus-free plants from infected individuals of *Dahlia* by shoot-tip cuttings. Morel and Martin (1952) further refined the technique and developed the meristem-tip culture technique to eliminate viruses. This in vitro technique soon became the most popular technique to eradicate virus from infected plants and is being widely used for the purpose. This chapter describes the technique of meristem-tip culture for virus eradication (in vitro therapy) after a brief introduction to thermotherapy at whole-plant level (in vivo thermotherapy).

The term 'virus-free' is used quite indiscriminately in the literature. A plant could be infected with more than one known type of virus and/or with the viruses that are not yet discovered. Thus, a plant could be called 'virus-free' only for a specific known type of virus for which it tests negative, as it may still be infected with other known and not yet known viruses. Alternative terminologies such as 'specific virus-free' or 'specific pathogen-free' have also been used (Hollings 1965; Langhans et al. 1977; Murashige 1980). It must be remembered that not all the plants obtained through meristem culture, thermotherapy, chemotherapy or cryotherapy would be free of virus. It is, therefore, necessary that the apparently 'virus-free' planting material obtained from any of the methods is tested for the presence of specific virus(es) through various tests (Sect. 16.5) by an accredited laboratory, and only those tested negative for the virus of concern are labelled free of specific virus/es and further multiplied for planting. Other plants may be destroyed or reused for virus elimination.

16.2 In Vivo Thermotherapy

One of the earliest practices, started by the Scots, to kill plant pathogens was to treat bulbs with hot water before planting. Thermotherapy has been used effectively for a long time to obtain virus-free plants from infected plants of diverse species. Many viruses are killed or inactivated at higher temperatures without causing serious injury to the host plant. High temperatures reduce replication of viruses significantly and may be inhibiting the synthesis of the virus coat protein and virus-encoded movement proteins, which help in cell-to-cell movement of viruses. Thermotherapy is usually effective against isometric and thread-like viruses and mycoplasmas, but is ineffective against many other viruses.

Heat therapy could be given either by hotwater or by hotair treatment. Hotwater treatment has been found to be useful for removing pests and eliminating pathogens such as bacteria and fungi and rarely, if ever, viruses. For virus elimination, hot-air treatment is more effective (Hollings 1965). Conventionally, the whole infected plants are shifted to a thermotherapy chamber and exposed to a temperature of 30–40 °C for a suitable period, varying from few minutes to several weeks. The treated plants are then transferred to normal temperature in a greenhouse. This method is referred to as in vivo thermotherapy. Grapevines were rid of the *Grape Fanleaf Virus* by subjecting soft cuttings from the infected plants to 37 °C in a thermal box under relative humidity of 80 % and light intensity of 22 μmol m^{-2} s^{-1} for 45 days. The new shoots arising from these treated plants were rooted and cultivated for 12 months in a greenhouse. These plants were found to be free of virus (Krizan et al. 2009). The temperature of the air is gradually raised during the first few days until the desired temperature is achieved. If the host plant is sensitive to continuous exposure to high temperature required to inactivate the viruses, diurnal cycle of low and high temperature may

be tried. During thermotherapy, the plants should have adequate carbohydrate reserve and it should be prevented from desiccation by maintaining high humidity (80–90 %).

Although whole-plant thermotherapy is less labour intensive, it is not the preferred method of virus elimination. The main drawbacks of this method are as follows: (i) not all viruses are sensitive to high temperature, (ii) low percentage of plant survival after heat treatment, (iii) prolonged thermotherapy may also inactivate the resistance factor(s) of the host plant (Walkey 1978), and (iv) high temperature with high humidity promotes infestation by thrips and other insects and pathogens such as fungi and bacteria.

Since late 1960s, meristem-tip culture has become the most popular method of virus elimination. Many of the viruses that could not be eliminated by thermotherapy alone have been eradicated by meristem-tip culture alone or in combination with thermotherapy and/or chemotherapy. The additional advantages of meristem-tip culture are as follows: (i) the potential of removing fungal and bacterial infections from the donor plant; (ii) in vitro clonal propagation with high genetic fidelity; (iii) the practical propagule for cryopreservation and other techniques of germplasm storage; (iv) easily acceptable by quarantine regulations for international exchange; and (v) the technique is suitable for precise in vitro multiplication of chimeras.

16.3 In Vitro Therapy

16.3.1 Meristem-Tip Culture

Transmission of pathogens in clonally propagated plants is very rapid. The plant materials when subjected to various steps of tissue culture get cleaned of majority of the pathogens such as bacteria and fungi. However, virus elimination requires the specialized technique of meristem-tip culture.

The distribution of viruses in plants is uneven. The apical meristems of infected plants are either free or carry a very low concentration of viruses (Quack 1977; Wang and Hu 1980). The virus titre in the plant increases as the distance from the meristem-tip increases (Holms 1948; Kassannis 1957). Several reasons have been proposed for the lack of viruses in the meristem-tip (Wang and Hu 1980): (i) Virus multiplication is dependent on the metabolism of the host plant. High metabolic activity in the actively dividing meristematic cells does not allow virus replication, (ii) The rapid spread of viruses in the plant is through the vascular system which is absent in the meristem. Those viruses invading non-vascular regions move from cell to cell via the plasmodesmatal connections, which is rather slow to keep pace with rapidly growing tip region, (iii) A high endogenous auxin level in the shoot tips may be inhibitory to the viruses, and (iv) The meristem is probably protected by certain 'virus inactivating systems'.

Holmes (1948) applied, for the first time, the knowledge of gradient of virus distribution in the shoot tips to raise virus-free plants from infected plants of *Dahlia* through shoot-tip cuttings. On the same principle, Morel and Martin (1952) developed the technique of meristem-tip culture to eradicate *Dahlia Mosaic Virus* (*DMV*) from *Dahlia* plants. They cultured 100-μm-long meristem-tips from the infected plants on a nutrient medium and obtained virus-free shoots. These shoots failed to root and had to be grafted onto healthy seedling rootstock to recover full virus-free *Dahlia* plants. Since then, the meristem-tip culture has developed into the most efficient technique for virus eradication and has been successfully applied to produce virus-free plants of a wide range of crop plants (Bhojwani and Razdan 1996).

The technique of meristem-tip culture involves isolation of meristematic tip (Fig. 16.1) under aseptic conditions and its culture on a suitable medium under optimal conditions, clonal multiplication of the surviving tips, virus

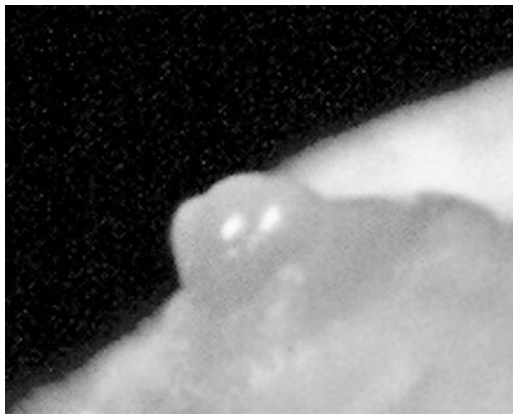

Fig. 16.1 Exposed shoot meristem after removing the leaf primordia

indexing the regenerated shoots/plants using suitable methods and selecting virus-free plants and transferring the plants to insect-proof net-house or glasshouse for their periodic check for freedom from virus. Some of the factors that contribute towards the success of the technique are described below.

16.3.1.1 Meristem-Tip Isolation

In practice, 1 to 2cm long shoots are collected from the donor plants grown in a greenhouse or in the field and surface sterilized using a suitable surface sterilant. Care should be taken not to use harsh sterilants or over sterilize, lest the shoots are killed. Better retrieval of sterile shoots can be expected by pretreating the stock plants regularly with fungicides and bactericides. The shoots are taken to a laminar air flow chamber, and after washing it with sterile water to remove remnants of the sterilant, the unwanted tissue and easily visible small leaves are removed with a sharp scalpel. Further operations are performed with the aid of a simple stereoscopic microscope (40–60x). Holding the shoot tip by one hand with the help of a pair of forceps, individual leaves are removed to expose the shoot tip. After each cut, the forceps and scalpel or needle are flame sterilized and cooled to prevent contamination of young, inner tissue with virus particles from the older tissue. When the shiny meristematic dome (Fig. 16.1) becomes visible, a clean cut is given below the first pair of youngest leaves with a sharp scalpel. The meristem-tip is lifted on the tip of the same scalpel and transferred to the culture medium. The orientation of the meristem-tip on the medium does not appear to be critical (Wang and Hu 1980).

Size of the explants is critical for the success of the technique. Whereas the size of the explant and virus elimination is inversely related, the survival of the explant is directly related to its size. Very small explants result in better virus elimination but show poorer survival, and larger explants show better survival but poorer virus elimination. The danger with very small explants is the formation of callus and introduction of genetic variation in the plants regenerated from them. Sometimes the smaller explants may produce only root or else pose a problem of rooting of the shoots. Therefore, the explants should be small enough to eliminate viruses and other phytopathogens (bacteria, phytoplasma), yet large enough to develop directly into a shoot. In *Dahlia* and grapes, 400- to 500μm-long meristem-tips yielded both a high percentage of virus-free plants and a formation of complete plants (Sadiva et al. 2006; Sim 2006). On the other hand, rose meristems ranging from 200 to 800 μm could yield virus-free plants (Golino et al. 2007).

The presence of 1–2 leaf primordia appears to be essential for the normal development of complete plant from apical meristem. It has been suggested that these primordia provide the necessary auxin and cytokinin to the growing apex. Smith and Murashige (1970) and Shabde and Murashige (1977) demonstrated the regeneration of full plants from apical meristems (without a leaf primordium) of several plants, but it is not a practical method for virus elimination. Murashige (1980) concluded 'the chances of eradicating viruses by culturing the meristem may not be substantially higher than that by a properly executed culture of larger meristem-tips'.

16.3.1.2 Physiological Condition of Explants

Meristem-tips should be derived from actively growing buds. Terminal buds are generally preferred for isolation of meristems. In chrysanthemums (Hollings and Stone 1968) and carnations (Stone 1963), meristem-tips from apical buds gave better results than those from axillary buds. However, such a difference in response was not observed in strawberry (Boxus et al. 1977). To increase the overall number of virus-free plants, it is desirable to make use of the axillary buds which are available in much larger numbers. For temperate trees, the best time to initiate cultures is spring when the plants are in active state of growth. The stems of *Prunus* species yielded culturable meristem-tips only after 6 months of exposure to 4 °C (Boxus and Quoirin 1974). The success of meristem-tip culture finally depends on the rootability of the shoots and their freedom from viruses. The efficiency of virus elimination and rooting is dependent on the season.

16.3.1.3 Culture Medium

Right choice of the culture medium is critical for the success of meristem-tip culture. In early studies on meristem-tip culture, modifications of the White's (1943) and Gautheret's (1959) basal media were used. However, these media proved to be deficient for healthy growth of shoots. The Gautheret's medium required five-fold increase in the level of K^+ ions for rapid and sturdy growth of potato shoots. On the original medium, the meristem-tips did not grow more than 1 mm and the shoots appeared chlorotic (Morel et al. 1968). MS (Murashige and Skoog 1962) basal medium has proved to be better than all previously used basal media for meristem-tip culture (Kartha 1975; Wang and Hu 1980; Balamuralikrishnan et al. 2002; Sim 2006; Sediva et al. 2006; Stace-Smith and Mellor 1968).

The basal medium generally requires to be supplemented with growth regulators such as auxin, cytokinin and/or gibberellin for the optimal development of the meristem-tip. The type of growth regulator and its concentration is dependent on the size of explant, plant species and, may be, the season of meristem-tip isolation. Meristems larger than 500 μm may develop into complete plants even in the absence of a growth regulator (Hakaart and Versluijs 1988), but generally, addition of a small amount (0.1–0.5 mg L^{-1}) of auxin or cytokinin or both to the medium is desirable. In higher plants, auxin is synthesized in the young leaf primordia and continues to be produced in leaves as it grows, though the site of production within the leaf changes (Aloni et al. 2003). Cytokinins play a positive role in the development of the shoot meristem (Werner et al. 2003). Meristem cultures of *Dahlia* and *Helleborus* required only a cytokinin (Sediva et al. 2006; Poupet et al. 2006), while those of peanut and chrysanthemum required both auxin and cytokinin (Morris et al. 1997; Verma et al. 2004). The most widely used auxin is NAA, although IAA and IBA have also been used. The auxin 2,4-D should be avoided as it promotes callusing.

The well-established protocols for meristem culture of potato recommend the use of gibberellin. For the culture of excised meristem-tip (200–500 μm) of cassava, GA_3 in combination with BAP and NAA was essential (Kartha et al. 1974). In *Dahlia*, GA_3 suppressed callusing of the meristem-tip of explants and favoured better growth and differentiation of shoots. However, in most other cases, GA_3 did not have an appreciable effect.

Meristem-tip cultures can be raised in both liquid and agar-gelled media. However, gelled medium is preferred because of the ease of handling. Liquid medium is recommended where agar medium induces callusing of the meristem. With liquid culture, the technique of filter paper bridge can be used.

16.3.1.4 Storage conditions

Meristem-tip cultures are generally stored in light of varying intensities (100–600 lux). In some cases, such as *Lolium multiflorum* (Dale 1980) and potato (Wang and Hu 1980), meristem growth had an absolute requirement for

light. The plants that produce polyphenols need to be incubated in dark during the initial 2–4 weeks and later transferred to light (Minas 2007). Meristem-tip cultures have generally been stored at 21–25 °C.

16.3.1.5 Genotype

Virus elimination by meristem-tip culture is also dependent on the genotype of the host plant. Only six out of 12 varieties of roses regenerated plants from 0.2–0.8-mm meristem-tips, and the frequency of plants free of *apple mosaic virus* (*ApMV*) and *prunus necrotic ringspot virus* (*PNRSV*) among the responding explants varied from 11 to 93 %, depending on the variety (Golino et al. 2007).

16.3.1.6 Thermotherapy

The meristematic dome of the shoot tips is not always free of viruses. Some of the viruses that invade the meristematic region are *carnation mottle virus*, *TMV*, *potato virus X*, *cucumber mosaic virus* and *odontoglossum ringspot virus* (*ORSV*). In such cases, meristem-tip culture alone is not effective in virus elimination. A combination of meristem-tip culture and thermotherapy has been helpful in raising virus-free plants in many such cases. Shoot tips or nodal explants from infected plants are cultured in vitro after proper sterilization and subjected to heat therapy at 30–40^{o} C in a thermal chamber for varying periods. The new shoots that appear are then used for isolating the meristem-tip and cultured on appropriate media for growth and development. Alternatively, meristem-tips are excised from mother plants and subjected to thermotherapy. Apple cultivar 'Idared' could be made free of *ACLSV* and *ASPV*, following the in vitro thermotherapy technique (Paperstein et al. 2008). This technique is regularly followed for virus elimination in strawberry plants (Kondakova et al. 2005). Thermotherapy of the mother plants prior to meristem-tip culture has an added advantage. It allows initiating cultures with comparatively larger meristem-tip explants, which greatly increases the proportion of explants surviving and developing into virus-free plants.

Potato Virus S (*PVS*) and *Potato Virus X* (*PVX*), which are not readily eliminated by meristem-tip culture or thermotherapy alone, could be eradicated by taking meristem-tips from heat-treated mother plants (Stace-Smith and Mellor 1968; MacDonald 1973). The frequency of *PVX*-free plants of potato cv. White Rose increased from 50 % after thermotherapy for 8 weeks to almost 100 % after heat treatment for 18 weeks. Eradication of *Potato Spindle Tuber Virus* (*PSTV*) required two consecutive cycles of thermotherapy. After the first cycle, 95 % of the regenerants were severely infected with virus. The 5 % of the regenerants, which carried mild infection, were again subjected to thermotherapy and meristem culture. Of the 248 plants regenerated, six were completely free of *PSTV* (Stace-Smith and Mellor 1970).

The duration of thermotherapy should be decided judiciously. If continuous exposure to high temperature required for virus elimination is harmful to the host plant, diurnal or daily cycles of high and low temperature can be tried. The efficiency of recovery of virus-free plants from infected individuals depends on the nature of the virus involved and the sensitivity of the mother plant to high temperature. Culturing the nodal cuttings of potato in the presence of 10^{-5} M acetyl salicylic acid for 4 weeks before thermotherapy improved not only the thermotolerance of the microplants but also increased the yield of *PVX*-free plants among the thermotherapy survivors (López-Delgado et al. 2004).

16.3.1.7 Chemotherapy

Chemical control of viral diseases under field conditions has not been successful so far. However, application of a range of chemicals, such as antibiotics, growth regulators, amino acids, purine and pyrimidine analogues, has met with some success in inactivation of viruses and inhibition of virus multiplication in tissue cultures.

So far, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; trade name 'Virazole'), a base analogue, has proved to be the most effective compound against plant viruses. This broad-range antiviral compound has been shown to be effective in eradicating a large number of plant viruses, such as *PVS*, *PVX* and *PVY* from potato (Griffith 1990; Nascimento 2003), *Chlorotic Leaf Spot Virus* (*CLSV;* Hansen and Lane 1985) and *ASGV* (James 2001; O'Herlihy et al. 2003) from apple, *PNRSV* from plum (Cieslinska 2007), *ORSV* and *Cymbidium Mosaic Virus* (*CyMV)* from *Cymbidium* (Lim et al. 2008) and *Sugarcane Mosaic Virus* (*SCMV)* from sugarcane (Balamuralikrishnan et al. 2002). In sugarcane, 50 mg l^{-1} ribavirin combined with meristem culture raised the frequency of virus-free plants from 95 to 62 % with meristem alone (Balamuralikrishnan et al. 2002). *Apple Stem Grooving Virus* (*ASGV*), which could not be removed by thermotherapy, was successfully eliminated by culturing shoots of *Malus domestica* on a medium containing 10 μM each of two viricidal compounds, ribavirin (a base analogue) and quercetin (a flavonoid) for 9–12 weeks (James 2001).

Actinomycin D (Renaudin and Bove 1977) and cycloheximide B (Alblas and Bol 1977) have been affective in reducing virus titre (Griffith et al. 1990), but their utility in virus elimination has not been established. Mayo (1983) found that actinomycin D and cordycepin were able to successfully protect tobacco protoplasts from being infected by *Tobacco Mosaic*, *Tobacco Rattle*, *Tobacco Ringspot* and *Potato Leafroll Viruses*. Inouye (1984) reported that pretreatment of isolated meristems of *Cymbidium* with the antiserum of *ORSV* for 2–24 h markedly improved the production of virus-free plants from shoot-tip cultures. Pardee et al. (2004) found that extracts from six marine algal species were able to inhibit *PVX* infectivity by 80 %.

16.3.1.8 Cryotherapy

Cryotherapy is a novel application of plant cryopreservation techniques to eradicate pathogens, such as viruses, phytoplasmas and bacteria, at a high frequency by a brief exposure of shoot tips to liquid nitrogen using cryopreservation protocols (Wang and Valkonen 2008; Wang et al. 2009). Since the first demonstration of *Plum Mosaic Virus* (*PMV*) elimination from an interspecific *Prunus* rootstock by cryotherapy of shoot tips (Brison et al. 1997), this technique has been successfully applied to eradicate *Cucumber Mosaic Virus* (*CMV*) and *Banana Streak Virus* (*BSV*) from *Musa* (Helliot et al. 2002), *Grapevine Virus A* (*GVA*) from grapes (Wang et al. 2003), *PLRV* and *PVY* from potato (Wang et al. 2006) and *Sweet Potato Chlorotic Stunt Virus* (*SPCSV*) and *Sweet Potato Feathery Mottle Virus* (*SPFMV*) from sweet potato (Wang and Valkonen 2008).

In cryotherapy, shoot tips (up to 2 mm long) are precultured, directly or after encapsulation in Ca-alginate, in increasing concentration of sucrose (0.25, 0.5 and 0.75 M for 1 day each) to impart desiccation tolerance, vitrified for dehydration (for details see Chap. 19) and plunged into liquid nitrogen, at −196 °C, for about 1 h. The shoot tips are retrieved from the super low temperature by rapid thawing in hot water (40 °C) for about 3 min and cultured on a suitable medium for shoot growth and rooting (Fig. 16.2). The plants thus obtained are indexed for viruses (Wang et al. 2006; Wang and Valkonen 2008).

Wang et al. (2006) compared the efficiency of cryotherapy with meristem-tip culture, thermotherapy and meristem culture after thermotherapy to free potato stocks from *PLRV* and *PVY*. Cryotherapy yielded plants free of *PLRV* and *PVY* with the frequencies of 84 and 93 %, respectively, which was considerably higher as compared to meristem-tip culture (56 and 62 %, respectively) or thermotherapy (50 and 65 %, respectively) but was comparable with meristem culture following thermotherapy (90 and 93 %, respectively). Since the frequency of surviving explants in meristem cultures following thermotherapy was almost half (40–50 %) of that following cryotherapy, the latter method proved to be most efficient to eradicate the two potato viruses.

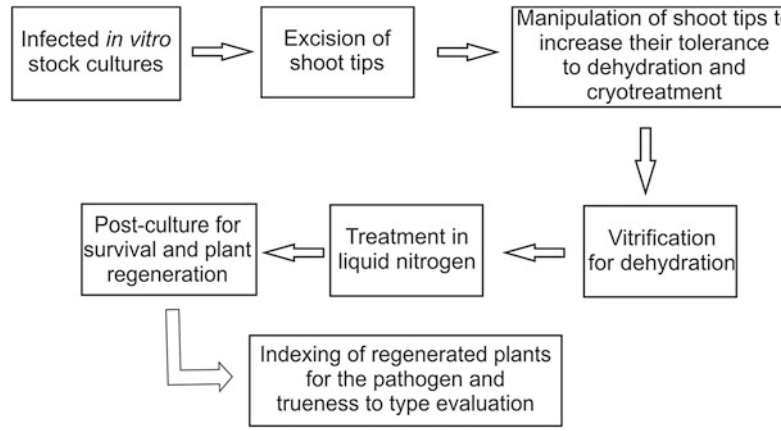

Fig. 16.2 Schematic representation of the main steps involved in cryotherapy of shoot tips for virus elimination (*after* Wang et al. 2009)

The major advantages of cryotherapy over more conventional methods of virus elimination are that in this technique, shoot tips measuring up to 2 mm are cultured, which have a higher survival rates than meristem-tips (0.3–0.5 mm). The explanation offered for the elimination of viruses in the cultures of larger shoot tips following cryotherapy is that exposure to −196 °C kills the older, highly vacuolated cells, which carry the infection, and the smaller, highly compact cells with high nucleus to cytoplasm ratio in the apical meristem, which are generally free of viruses, are able to grow and regenerate healthy plants. However, where viruses are able to migrate to the meristematic cells, as *Raspberry Bushy Dwarf Virus* (*RBDV*) in some genotypes of raspberry, neither meristem-tip culture nor cryotherapy alone is effective, a combination of cryotherapy with thermotherapy has proved to be effective (Fig. 16.3; Wang et al. 2008). The other merits of cryotherapy are that larger shoot tips facilitate the treatment of large numbers of samples at one time. Cryotherapy is especially suitable where viruses are heat resistant and the host tissues are sensitive to thermotherapy. A basic requirement for the application of this technique for pathogen elimination is a prior knowledge of the tissue culture and cryopreservation protocol of the species of interest. The response to cryopreservation technique is genotype specific. The development of this technique has the potential to replace more traditional methods like meristem-tip culture (Wang and Valkonen 2009).

16.3.2 In Vitro Shoot-Tip Grafting

Morel and Martin (1952), in their pioneering work to raise virus-free plants of *Dahlia* by meristem-tip culture, had to graft the meristem-tip-derived shoots on healthy rootstock of *Dahlia* to establish full disease-free plants because the meristem-tip-derived shoots could not be rooted. Since then, micrografting, which involves in vitro grafting of meristem-tip on young rootstock seedling, has been used to raise virus-free plants of some woody species where meristem-tip culture has not been possible.

All efforts to raise full plants of *Citrus* by meristem-tip culture have been unsuccessful. Murashige et al. (1972) could raise a few citrus plants by grafting shoot tips from diseased plants on young rootstock seedlings growing in vitro. Some of these plants were free of exocortis disease. This technique was improved by Navarro et al. (1975), and a routine protocol that allowed 40–60 % successful grafts was developed (Fig. 16.4). The technique has been used to cure citrus plants of as many as 16 diseases caused by viruses, viroids, mycoplasmas and fastidious bacteria (Navarro 1992). Micrografting of shoot tips has been successfully applied to fruit trees such as peach (Juarez et al. 1988),

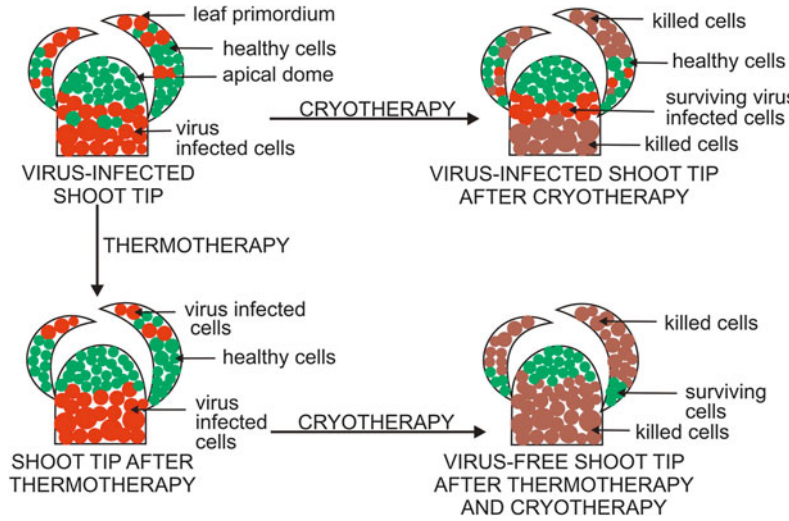

Fig. 16.3 Combination of thermotherapy and cryotherapy for the elimination of viruses that invade the shoot meristem cells. Cryotherapy alone does not eradicate the viruses that invade the meristematic tissue. However, cryotherapy after thermotherapy induces additional suppression of the viruses and propensity of the infected cells to be killed by cryotreatment. Thermotherapy causes stress and also accelerates the degradation of viral RNA (Wang et al. 2008) (*after* Wang et al. 2009)

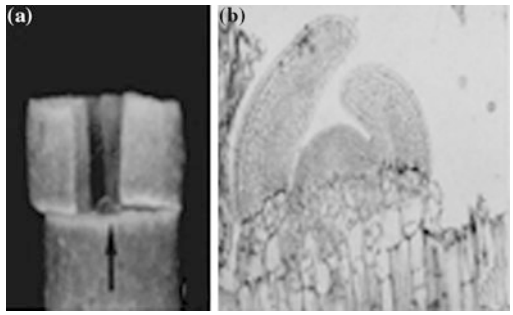

Fig. 16.4 In vitro micrografting of shoot tips of *Citrus sinensis* on a 2-week-old Troyer citrange rootstock. **a** Fresh graft. **b** Longitudinal section 5 days after grafting, showing callus formation at the graft union (*courtesy* L. Navarro 1992)

apple (Huth 1978), almond (Juarez et al. 1991), Kinnow (Sharma et al. 2008) and sweet pepper (Katoh et al. 2004).

16.3.3 Electrotherapy

Electrotherapy has been used for elimination of *PVX* from potato (Lozoya-Saldafia et al. 1996), *CMV* from banana (Helliot, FAO 2004) and *Tomato Yellow Leaf Curl virus* (*TYLCV*) from tomato (Falah et al. 2009). The technique of electrotherapy involves exposure of shoots to electrical impulses (5–15 mA) for 5–10 min. The shoots are then surface sterilized and inoculated in a suitable medium for regeneration. The regenerated plants are indexed for viruses. This method requires more studies and understanding before it can gain popularity as a routine technique for use.

16.3.4 Virus Elimination Through Other In Vitro Methods

Callus cultures obtained from infected plants have often been found to be free of viruses and so were the plants regenerated from such calli. The distribution of virus in the cells of the calli is not uniform. In the callus obtained from infected tobacco plants, about 40 % of the cells were actually free of viruses and could regenerate *TMV*-free plants (Hansen and Hildebrandt 1966). It is well known that virus titre in cultured cells becomes very low, and often, the virus may be lost in prolonged cultures (Wang and Hu 1980). Of the five infected grapevines

cultured to raise callus, only two were found to be still infected with closteroviruses (Salati et al. 1993). In the calli raised from infected grapevines, viruses could be detected at the end of 4 months but none at the end of 8 months (Gambino et al. 2006). The viruses that move via the vasculature are not transmitted easily in the callus cultures because of the obvious lack of a vascular system (Goussard et al. 1991). Though viruses are also translocated via plasmodesmatal connections, this process is very slow. In actively growing calli, the velocity of virus movement is not able to keep pace with the rapidly dividing callus cells. The rate of multiplication of virus and their rapid movement is dependent on the type of callus. The uneven distribution of viruses in the callus could be attributed to the possible inhibition of virus replication especially in the presence of cytokinin. Several authors have successfully eliminated *Grapevine Leafroll Virus* or *Grapevine Flock Virus* from grape cultivars through somatic embryogenesis alone or in combination with thermotherapy (Goussard and Wild 1994; Gambino et al. 2006, 2008; Popesen et al. 2003; Borroto-Fernandez et al. 2009). All the 46 plants regenerated via somatic embryogenesis, from anther callus of *Vitis vinifera*, carrying *Arabis Mosaic Virus*, were free of the virus and were true to type and diploid. This method has also been successfully applied to eliminate *Citrus Psorosis Virus* from three *Citrus* species (D'Onghia et al. 2001).

16.3.5 Practical Method of Virus Elimination

Currently, many techniques are available that are effective in elimination of a good number of viruses. However, no single technique is able to remove all the viruses. A large number of viruses are very reticent and require a combination of more than one technique for their eradication. In this regard, meristem-tip culture is the most widely used method in combination with any other suitable technique(s). A combination of thermotherapy and/or chemotherapy with meristem culture is often practised for complete removal of viruses. Thermotherapy combined with meristem-tip culture has been used to eradicate *SPFMV* from sweet potato (El Far and Ashoub 2009), *ACLSV* and *ASPV* from apple (Paprstein et al. 2008), *Onion Yellow Dwarf Virus* (*OYDV*) from garlic (Robert et al. 1998), *PPV* and *PNRSV* from *Prunus* (Manganaris et al. 2003) and four viruses from *Humulus* (Postman et al. 2005) to name a few. Meristem-tip culture was combined with chemotherapy to eliminate viruses from sugarcane (Balamuralikrishnan et al. 2002) and *Ornithogalum* (Vcelar et al. 1992).

16.4 Maintenance of Virus-Free Stocks

The plants freed of viruses have no additional resistance to diseases and may quickly become reinfected by the same or different viruses. It is, therefore, necessary that virus-freed plants are maintained in sterilized soil in a greenhouse or insect-proof cage. Alternatively, the virus-free plants could be cultivated in secluded regions where specific viruses have not been reported hitherto, and large-scale multiplication can be carried under strict observations. A large number of meristem-derived virus-tested plant species can be maintained and multiplied much more easily and economically in tissue cultures. If a method other than meristem-tip culture has been used to generate virus-free plants, then the micropropagation protocol should be developed for the maintenance of virus-free germplasm stock in vitro.

Meristem-tip-derived virus-free plants are expected to show very little or no genetic variation with respect to the parent plant, but it is a good practice to check their trueness-to-type.

16.5 Virus Indexing and Certification

Not all plants produced through meristem-tip culture alone or in combination with thermotherapy and/or chemotherapy or by any other

method of virus eradication programme are virus-free. Therefore, it becomes imperative to check each and every plant produced through any of the virus eradication programmes for the presence of viruses before a plant is labelled as free of specific virus and released for commercial use. In cultures, many viruses have a delayed resurgence. Hence, the plants should be indexed several times during the first 18 months, and only those plants which give consistently negative results should be treated as free of specific virus (Walkey 1978). Since virus-free plants can get reinfected after transfer to the field, it is necessary to repeat virus indexing at regular intervals.

The objective of certification schemes is to identify healthy, disease-free plant sources for large-scale multiplication through the use of well-established indexing procedures (Rowhani et al. 2005). Characterization and detection of plant viruses require expensive equipment and chemicals/reagents, besides a well-trained technical personnel, especially for the molecular techniques of virus indexing. It is, therefore, desirable to have national/state facilities for virus indexing (Rowhani et al. 2005; Hughes et al. 2001).

The Department of Biotechnology, Government of India, has established nine centres that have the know-how and facilities for virus indexing and to certify healthy nuclear stock for various crops, viz: (i) National Research Centre for Banana, Tiruchirapalli, Tamil Nadu; (ii) Central Potato Research Institute, Shimla; (iii) Indian Institute of Spices Research, Calicut; (iv) Indian Institute of Horticultural Research, Bangalore; (v) Institute of Himalayan Bioresource Technology, Palampur; (vi) University of Agricultural Sciences, Bangalore; (vii) Sugarcane Breeding Institute, Coimbatore; (viii) Agharkar Research Institute and Indian Agricultural Research Institute, Regional Station, Pune; and (ix) Central Research Institute for Jute and Allied Fibres (CRIJAF), Kolkata.

Some of the methods used for virus indexing are as follows: (i) biological indexing, (ii) electron microscopy, and (ii) molecular indexing. All these methods have their own advantages and limitations. It is recommended that more than one method be used to index any system reliably. These methods are briefly described below.

16.5.1 Biological Indexing

Observation for visible symptoms characteristic for a virus in the plants is the simplest test for knowing the presence or absence of viruses. However, symptoms take a long time to develop on the host plant. Therefore, more sensitive herbaceous plants, such as *Chenopodium, Cucumis, Lycopersicon*, *Nicotiana* and *Scopollia* species (indicator plants), are used for indexing sap-transmitted viruses in insect-proof glasshouse or nethouse. The succulent tissues, such as young leaves and shoot tips, from test plant are triturated in 10 mM phosphate buffer pH 7.5 containing 2 % nicotine (1:10, weight of tissue: ml buffer) and rub inoculated into the indicator plant, using 600-grade carborundum. Nicotine neutralizes the inhibitory effects of polyphenolic compounds and inhibitors from host cells on virus infectivity and also facilitates virus transmission (Mathews 1993; Rowhani et al. 2005). The inoculated plants are maintained in an insect-proof greenhouse separated from each other and other plants. Successful virus transmission is indicated by the development of primary symptoms such as local lesions, ring spots within a few days of incubation. The systemic infections of mosaic, vein clearing, leaf deformation, tissue necrosis, etc. take a longer time to appear (Brunt et al. 1996).

For woody plants, in addition to the herbaceous indicator plants, woody indicator plants of the same genera as the accession under test are used, especially for other graft-transmissible viruses and phloem-limited phytoplasmas, which are refractory to sap transmission. Their detection is accomplished by bud-chip grafts on a panel of woody indicators. These plants require to be incubated for 2–3 years before they exhibit visible symptoms specific for the virus (Rowhani et al. 2005).

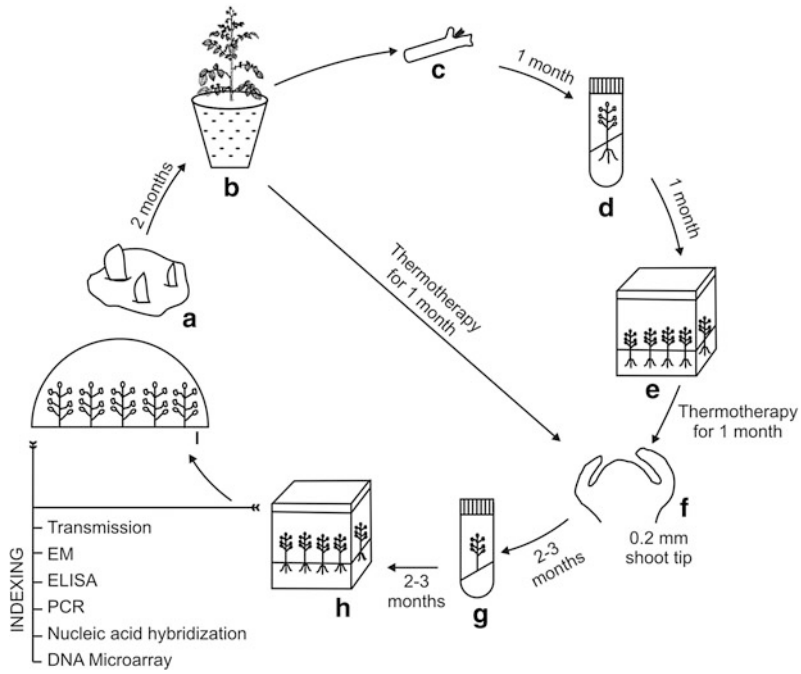

Fig. 16.5 Schematic representation of virus eradication process in potato. Aseptic plantlets are established from nodal segments (**c**) or tuber pieces (**a**) and micropropagated (**d**, **e**). The parent plant (**b**) or the in vitro–raised plants (**d**, **e**) are subjected to thermotherapy, and 0.2-mm meristem-tips (**f**) are excised from them and cultured. The plants derived from these meristems before (**g, h**) and after (**i**) transfer to greenhouse are indexed for viruses and viroids (*after* CIP 1993)

The sap transmission method is widely used to detect viruses and viroids. Though slow, it is a sure method. However, it is not suitable to detect latent viruses that do not show visible symptoms on the host plant and where different strains of a virus produce markedly different symptoms on the same host (Lawson 1986). Molecular assays have been developed, which are much more sensitive and rapid and can handle thousands of samples in a short time. These molecular assays are performed in addition to the biological indexing (Fig. 16.5).

16.5.2 Molecular Assays

(i) *Serology*. Serology is a rapid plant pathogen detection and identification method and was the first laboratory method to be developed. This method is based on the recognition of the antigen with antibodies produced against them. The demonstration that glutaraldehyde cross-linked enzyme-antibody conjugates retained both the specificity of the IgG molecule and the catalytic properties of the enzyme (Avrameas 1969) revolutionized the use of the enzyme-linked immunosorbent assay (ELISA) as a rapid and highly sensitive serological method for qualitative and quantitative analysis of virus infection (Clark and Adams 1977; Converse and Martin 1990). Most of the economically important and widespread viruses can now be characterized by ELISA.

There are two types of ELISA protocols used for virus detection. One is the direct method, called double-antibody sandwich (DAS)-ELISA. It involves enzyme attachment to the antibody probe (Barbara et al. 1978; Koenig 1978; Bar-Joseph and Salomon 1980). The second method is the indirect method, called double-antibody sandwich indirect (DASI)-

ELISA, in which the antibody probe remains unlabelled and the enzyme is attached to a second antibody (direct antigen coating or DAC; Mowat 1985) or to protein A that is reactive to the probe antibody (protein A coating or PAC; Edwards and Cooper 1985). Because of its greater sensitivity, broader reactivity and convenience, DASI-ELISA is preferred over DAS-ELISA. Moreover, in DASI-ELISA, only a single-enzyme conjugate is required for the assays of different viruses, and usually, a suitable conjugate is available commercially. However, neither of the ELISA is applicable to viroids and protein coat-free viruses. Moreover, several luteoviruses, such as *PLRV*, do not react with the antiserum developed for the main virus (Mellor and Stace-Smith 1987).

(ii) *Electron microscopy*. Serological methods can also be combined with electron microscopy (immunosorbent electron microscopy; ISEM).

(iii) *Nucleic acid-based assay*.

(a) *Polymerase chain reaction (PCR)*: PCR identifies the pathogen through their DNA. PCR assays are fast, reliable, highly sensitive and very versatile. To detect plant viruses which have RNA, the reverse transcriptase-PCR (RT-PCR) has been developed (Hanson and French 1993; Candresse et al. 1998). Preparation of the plant material and isolation of DNA/RNA are very critical because of the high levels of polysaccharides and phenolic compounds that mask the activities of the enzymes used (Borja and Ponz 1992). This problem can be partly overcome by using special resin columns that bind to RNA or by the use of inhibitors and absorbents of contaminants. Alternatively, the viruses can be trapped specifically and separated from the rest of the extract. The development of automated RNA extraction instruments and protocols has eased the situation (Wells and Harren 1998).

To detect very low amounts of viruses, several variations of RT-PCR have been developed, such as the nested-, one-step-, duplex-, multiplex- and real-time RT-PCR (Foissac et al. 2001; Dovas and Katis 2003; Viswanathan et al. 2008). Another efficient and sensitive diagnostic method, based on detection of the coat protein gene, is the loop-mediated isothermal amplification (LAMP) (Fukuta et al. 2003). It explicitly and specifically detects viral RNA.

(b) *Nucleic acid hybridization*: This technique employs complementary RNA (cRNA) as a probe that forms duplexes of greater stability with the target RNA than do DNA probes with the same RNA target (Hull 1993; Davis et al. 1994; James et al. 1999). Specific hybridization between nuclei acids has been used in viroid detection (Rowhani et al. 2005).

(c) *DNA microarray technology*: This technology has been suggested for simultaneous detection of a large number of plant pathogens. The unique pattern of hybridization for each of the pathogen will be the distinguishing feature of this technique. New pathogens could be identified through the use of microarray sequences derived from highly conserved regions in a given pathogen group. Microarray technique is the only assay that can detect a myriad of viruses, viroids and phytoplasmas infecting the plant and can still discriminate between the pathogens detected. The microarray technique can reduce the overall labour involved in virus detection, increase the sensitivity and process a large number of samples for simultaneous detection (Hadidi et al. 2004).

16.6 Importance of Virus Elimination

Virus infection affects the plants both qualitatively and quantitatively. This fact is realized once the crops are rescued from viruses. In clonally propagated plants, virus elimination has dramatically improved the crop economics. Yield losses due to virus infection may be up to 100 %, leading to complete crop failure (Haidi et al. 1998). *Peanut Mottle Potyvirus* and *Peanut Stripe Potyvirus*, individually and in combination, caused up to 20 % yield reduction in peanut (Moris et al. 1997). Selective elimination of *Grape Leafroll-associated Virus* (*GLRaV*)-1, *GLRaV-2*, *GLRaV-3* and *GVB* from *Vitis vinifera* increased vigour and yield. Of these,

GLRaV-2 elimination had the greatest beneficial effect. It improved growth by 21 %, fresh fruit yield by 22 % and sugar content of fruit juice by 9 % (Komar et al. 2007). Ornamentals have also benefited from the elimination of viruses in terms of improved flower colour and increased yield of bulbs and their size.

Virus elimination is a recurring process. The plants freed of virus are liable to be reinfected by the same or more devastating viruses or other pathogens. In a number of vegetatively propagated fruit crops, a nuclear stock of virus-free and indexed plants is maintained in proper greenhouses/nethouse. This nuclear stock serves as the source for virus-free plants both as rootstocks and as healthy scions or complete plants.

Certified pathogen-free germplasm of crop plants facilitates its free movement across nations for breeding purposes. Thus, germplasm conservation at repositories, gene banks and seed banks needs to be certified and virus indexed.

16.7 Concluding Remarks

The field grown crops, especially the perennial crops, harbour a variety of pathogens, such as fungi, bacteria, phytoplasma, viruses and viroids, which cause considerable economic losses to the agriculture, horticulture and floriculture industries. Infection by the pathogens also restricts free movement of germplasm from one country to the other for breeding purposes. Whereas fungal and bacterial diseases can be controlled by the application of fungicides and bactericidal compounds, viruses, viroids and phytoplasmas generally do not respond to chemical treatment.

Of the various methods developed to eradicate viruses, shoot meristem-tip culture is the most popular method. In some cases, especially where the viruses invade the cells of the meristematic dome, meristem-tip culture is combined with in vitro thermotherapy or/and chemotherapy. Cryotherapy of the shoot tip is emerging as a powerful technique to eliminate viruses. Recently, electrotherapy (35 mAmp current for 5–10 min) has been successfully used to eradicate *Potato Virus A* and *Potato Virus Y* from potato cultivars (Meybodi et al. 2011). Meristem-tip culture also eradicates viroids and phytoplasmas, which are restricted to the phloem cells.

It must be emphasized here that meristem-tip culture does not allow selective elimination of a virus. It eradicates all the viruses in the plant. Sometimes elimination of resident viruses makes the plant susceptible to more severe viruses (Fulton 1986). Moreover, the plants freed of viruses are not resistant to viruses. They can get reinfected with viruses and other pathogens when grown in the field. Therefore, it is essential to replace, periodically, the planting material by pathogen-free nuclear stock.

Of late, the development of virus-resistant transgenic plants is gaining grounds with the promise of producing more stable virus-free plants. A plant may, however, be infected at any given time by more than one virus. In such a situation, a single virus-resistant gene introduced by genetic engineering might not be effective. Further, the allergenicity to humans of the introduced viral gene that will be producing the coat protein requires to be studied critically. Moreover, the viruses being evolutionary marvels, their newer and more infectious strains may develop in course of time.

16.8 Appendix

1. Virus eradication from potato plants by meristem culture following thermotherapy (*after* International Centre for Potato 1993; Fig. 16.5).
   (i) Expose 2-month-old potted plants or 1-month-old in vitro plants to high temperature (36 °C for 16 h and 30 °C for 8 h daily for 30 days) under continuous light (5000 lux).
   (ii) Excise single-node segments with intact axillary bud. Remove the leaves carefully and disinfect them with a wide-spectrum acaricide

(Morestan–Bayer 0.5 % for 10 min). Rinse the explants in 70 % ethanol for 30 s, followed by treatment with calcium hypochlorite (2.5 %) for 15 min. Wash the nodal segments four times with sterilized distilled water (5 min each time) to remove all traces of hypochlorite.

(iii) Under a stereoscope, remove the leaflets surrounding the apical meristem until the meristematic dome and only 1–2 youngest leaf primordia are left.

(iv) Excise the meristem-tip by a clean cut with a sharp scalpel and transfer it to the culture medium (MS salts + 2 mg L^{-1} glycine + 0.5 mg L^{-1} nicotinic acid + 0.5 mg L^{-1} pyridoxine HCl + 0.4 mg L^{-1} thiamine HCl + 0.1 mg L^{-1} GA_3 + 0.04 mg L^{-1} kinetin + 2.5 % sucrose and gelled with 0.6 % agar). Make weekly transfers of the explant to fresh medium. After 6–8 weeks, plantlets would develop.

(v) Index the plants grown from meristem for the suspected viruses by nucleic acid hybridization (NASH), ELISA test, and indicator host plant tests. All these tests can be carried out in 2 months. Micropropagate the virus-free plants.

2. Meristem-tip culture to eradicate viruses and other pathogens from grapevines. (Sim 2006).

(i) Collect 2 cm long shoot tips from actively growing shoots and bring them to the laboratory. Rapidly growing shoots in the spring and early summer provide the best plant material.

(ii) Wash the shoot tips under running tap water for 1 h with the addition of a drop of dishwashing liquid every 20 min. Surface sterilize the explant by immersion in 10 % commercial bleach plus one drop (~0.1 ml) of liquid detergent for 10 min.

(iii) Remove the tissue under aseptic conditions and wash three times with sterile distilled water.

(iv) Excise the meristem-tip aseptically in a transfer hood with the aid of a stereoscope. Remove individual leaf scales one by one to expose the meristematic dome. After each cut, flame sterilize and cool the forceps and scalpel to prevent contaminating younger, inner tissues with virus particles from older tissues, which might be transferred by the blade.

(v) When the meristematic dome becomes visible, give a final cut below the 2nd or 3rd youngest leaf primordium to excise the meristem-tip (0.4–0.5 mm long, with 1–3 leaf primordia) and gently place it on the surface of the culture medium (MS salts and vitamins + 1.0 mg L^{-1} BA +3 % sucrose, and 0.6. % agar, pH 5.8).

(vi) Incubate the cultures in a growth chamber at 25 °C, 70 % relative humidity and 16 h photoperiod, under cool white fluorescent and incandescent bulbs. Transfer the meristem-tips to fresh medium every 3 weeks.

(vii) When the explants develop into a 2 cm long shoot, with 4–5 well developed leaves (6–8 weeks after excision), transfer them to rooting medium (half-strength MS salts and vitamins +1.0 mg L^{-1} *IAA* + 1.5 % sucrose +0.6 % agar, pH 5.8).

(viii) When well-developed roots are formed and the shoot has attained a height of the tube (after 3–9 weeks), transfer the plants to sterilized potting mix in pots and maintain them in greenhouse for hardening. Before transplantation, rinse the roots to remove the medium and trim them if necessary. Place the pots inside a clear plastic magenta box with the lid on.

(ix) Over the next 2 weeks, the plants are gradually acclimatized to ambient humidity by leaving the box lid slightly loose and later removing it. Finally, transfer the plants to 4" pots and place in the greenhouse. Normally, 10–30 %

of the meristem-tips survive tissue culture and form well-rooted plants.

(x) Index the surviving plants for suspected viruses. Usually, 70–100 % of the surviving plants are virus-free, depending on the virus type and the source plant.

(xi) The whole process from excision of a <0.5-mm meristem-tip to a plant in a 4 inch pot may take 4 months to over a year depending on the variety.

3. To produce virus-free plants from virus-infected raspberry by cryotherapy (Wang et al. 2008)

Maintenance of the shoot cultures

(i) Initiate shoot cultures on MS + 100 mg L^{-1} myo-inositol + 3 % sucrose + 0.5 mg L^{-1} BAP + 0.05 mg L^{-1} IBA + 0.3 % Bacto agar (Difco Laboratories, Madison, WI) + 0.12 % Gelrite (Merck & Co. Inc., Rahway, NJ), pH 5.0. Incubate the stock cultures at a temperature of 22 ± 2 °C under 16 h photoperiod with a light intensity of 45 μE m^{-2} s^{-1} provided by cool white fluorescent tubes. Maintain the shoot cultures on the same medium by 4 weekly subcultures.

Thermotherapy

(ii) Excise individual shoots (>2 cm long) from 4-week-old stock cultures and transfer to 15 × 2.5 cm culture tubes containing 20 ml of the same medium (1 shoot/tube). Incubate the cultures under the conditions as in Step 1.

(iii) After 3 days, seal the tubes with Parafilm to maintain high humidity and transfer them to thermotherapy chamber, programmed for a 16 h photoperiod at 38 °C, followed by an 8 h dark period at 26 °C, for 21–42 days.

Cryotherapy

(iv) Excise 1 mm shoot tips from the heat-treated stock shoots and stabilize them by culturing for 2 days on the shoot culture medium supplemented with 0.25 % activated charcoal.

(v) Encapsulate the stabilized shoot tips in Ca-alginate. Suspend the shoot tips in 2.5 % (w/v) Na-alginate solution containing 2 M glycerol and 0.4 M sucrose and drop it by plastic pipette in 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose to form beads. The orifice of the pipette should be such that each bead contains only one shoot tip.

(vi) Preculture the encapsulated shoot tips step-wise on the shoot culture medium with increasing sucrose concentration (0.25–0.75 M) for 3 days.

(vii) Treat the precultured beads for 90 min with a loading solution, containing 2 M glycerol and 0.8 M sucrose, followed by dehydration with PVS2 at 24 °C for 180 min. The PVS2 solution contains 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) dimethylsulphoxide (DMSO) and 0.4 M sucrose in MS medium with pH set at 5.8.

(viii) Quickly surface dry the dehydrated beads by blotting on cellulose tissue and transfer them into a 2 ml cryotube (10 beads/tube; Nunc CryoTube, Roskilde, Denmark).

(ix) Directly immerse the cryotubes in liquid nitrogen for 1 h, and then, rapidly thaw in a water bath at 40 °C for 3 min.

(x) Wash the beads with MS medium containing 1 M sucrose for 20 min before post-culture in a Petri dish (9 cm diameter) containing 35 ml of solidified shoot culture medium supplemented with 50 mg L^{-1} Fe-EDTA.

(xi) Incubate the cultures in dark at 22±2 °C and, after 3 days, transfer them for survival and regrowth under the conditions described above.

Plant regeneration

(xii) In all experiments, survival is defined as percentage of shoot tips or meristem-tips showing green colour 2 weeks after post-culture. Regrowth

is determined as percentage of shoot tips or meristem-tips that develop shoots 6 weeks after post-culture.

(xiii) Elongated shoots (>3 mm) are used for further in vitro multiplication.

(xiv) For in vitro rooting, grow shoots longer than 5 mm in solid shoot culture medium without a hormone for 4 weeks.

(xv) Index the plants for the presence or absence of virus.

Suggested Further Reading

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Micropropagation 17

17.1 Introduction

Clonal propagation of plants, which refers to multiplication of genetically identical individuals by asexual methods of regeneration from somatic tissues or organs, is a common practice in horticulture and forestry to preserve the desirable characters of selected genotypes or varieties. Traditionally, it is achieved by cuttings, layering, splitting, grafting and so on. However, for many plants, especially the orchids and tree species, these methods are either very difficult or painfully slow. In early 1960s, when totipotency of plant cells was established and regeneration of large number of plants starting from small pieces of somatic tissues could be achieved, tissue culture was projected as a potential alternative method for rapid clonal propagation of plants (Murashige 1974). With the passage of time the regeneration of plants from cultured tissues and organized structures was variously modified based on the innate properties of the plant species, the rate of multiplication achieved and the quality of regenerated plants. In vitro clonal propagation of plants, popularly called micropropagation because of the miniaturization of the process, has now become an industrial technology and is being widely used the world over to multiply orchids, other ornamentals, and fruit and forest tree species (Debergh and Zimmerman 1991; Pierik 1991; George et al. 2008; Yam and Arditti 2009). The history of micropropagation is closely linked to the development of techniques for in vitro multiplication of orchids (Yam and Arditti 2009).

The main advantages of micropropagation over the conventional methods of clonal propagation are: (a) in a relatively short time and space a large number of plants can be produced starting from a single individual, (b) very small pieces of plant tissues are required to initiate aseptic cultures, (c) the rate of multiplication in vitro is often much faster than the in vivo methods of vegetative propagation because in cultures it is possible to manipulate the nutrient and growth regulator levels, temperature, and light more effectively, (d) it is applicable to many such genotypes for which in vivo vegetative propagation is difficult or impossible, (e) it goes on round the year, (f) if the plants are derived from virus-free stock they remain protected from re-infection, and the micropropagated plants can be exported with little quarantine problem, (g) the plants are relatively free of microorganism infestation, (h) in vitro production can be better planned by storing the cultures at low temperature in the season of low market demand, and (i) micropropagated plants may acquire new desirable traits, such as bushy habit of ornamental plants and increased number of runners in strawberry.

Since the pattern of in vitro growth and multiplication of orchids is very distinct from other categories of plants, the micropropagation of orchids is described before dealing with the general techniques of micropropagation.

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_17, © Springer India 2013

17.2 Micropropagation of Orchids

Orchids are a fascinating group of plants belonging to the largest family (Orchidaceae) of angiosperms, with about 750 genera, 25,000 species, and over 100,000 bi- or plurigeneric hybrids registered the world over (Paek et al. 2011). They constitute an important category of ornamental plants, with a wide variety of flowers with respect to their shape, size, color, and fragrance. There are several unique biological features associated with orchids. They produce a large number of dust particles-like seeds enclosing an unorganized embryo and lacking the endosperm tissue. Due to the absence of nutritive tissue, the orchid seeds require association with specific type of fungi for their germination. Since the survival of seedlings in nature is poor, the discovery and development of asymbiotic germination of orchid seeds in vitro on rather simple mineral and sugar-containing medium by Knudson (1922, 1956) laid the foundation of orchid tissue culture.

Commercially, important orchids are complex hybrids of two or more genera. Sympodial orchids grow and multiply by the formation of lateral buds which form pseudobulbils. These orchids are traditionally propagated by separation of the oldest pseudobulbils to force the development of dormant buds, a method referred to as 'back-bulb propagation'. This is a very slow process and can, at best, double the number of plants in a year. On the other hand, the only in vivo method for the propagation of monopodial orchids, which do not produce lateral bulbs, is through seed germination that results in highly heterogeneous population, and the plants take almost 3–5 years to flower.

Roton (1949) is credited for initiating the work on orchid micropropagation. He demonstrated that 74 % of the single node segments from floral stalks of *Phalaenopsis* plant cultured on Knudson C medium developed leaves after 14–60 days, which later formed roots and produced a complete new plant. However, his work, which was published in a magazine of hobby growers, did not catch the imagination of scientists or orchid growers. Clonal propagation of highly heterozygous varieties of orchids on large scale started only after the work of Morel (1960, 1964). He demonstrated that in vitro cultured shoot tips of *Cymbidium* and other orchids, instead of developing into a shoot, formed protocorm-like bodies (PLBs; small spherule tuber-like bodies with rhizoids at the base as formed by germinating orchid seeds). In cultures, the protocorms originate from epidermal or subepidermal cells of the leaves (Champagnat et al. 1966). Each protocorm proliferated to form a cluster of protocorms. If these protocorms were left in the medium undisturbed, they developed into a complete plant with normal shoot and root (Fig. 17.1). However, if the cluster of protocorms was cut into 3–6 pieces and planted on fresh medium each piece produced additional protocorms (Fig. 17.2). The new protocorms are formed by the activity of superficial cells as the internal tissues do not have the regeneration potential. Each piece produced about 3–5 protocorms in a month, which could be further cut and made to proliferate on a fresh medium. At this rate of multiplication, many millions of plants could be produced in a year starting from a single shoot tip measuring <1 mm (Morel 1960, 1972).

Culture of somatic tissues of orchids, such as shoot tip, leaf (Tanaka 1977), root tip (Park et al. 2003), or floral stalk (Tanaka 1978), generally result in PLBs formation directly or through an intervening callus phase (Ichihashui 1999).

The technique developed by Morel was rapidly adopted by commercial orchid growers with remarkable success. Today micropropagation is the only commercially viable method of clonal propagation of orchids. Commercial growers initiate cultures from 5 to 10 mm shoot tips as too small tips take much longer to form protocorms (Kunisaki et al. 1972; Murashige 1974).

Monopodial orchids such as *Phaelonopsis* and *Vanda* do not form laterals and grow by the apical shoot tip. Therefore, initiation of cultures from shoot tips in these cases is not advisable for the fear of loss of precious germplasm. These orchids are multiplied in vitro through segments

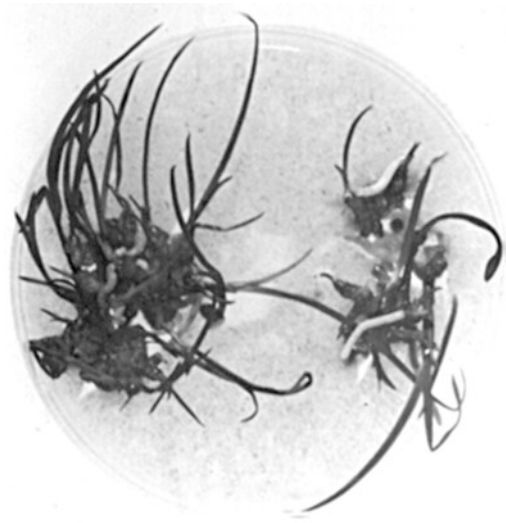

Fig. 17.1 Regeneration of shoots and roots from in vitro multiplied protocorms of *Cymbidium*

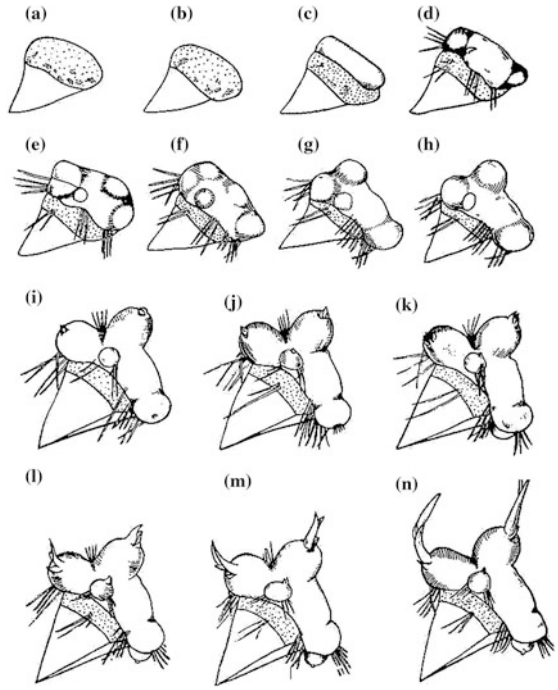

Fig. 17.2 Diagrammatized stages in the regeneration of protocorms (**b**–**h**) and shoots (**i**–**n**) from a piece of protocorm (**a**). (*after* Morel 1972)

of floral stalk with vegetative buds as the initiating explants (Intuwong and Sagawa 1973; Paek et al. 2011). Even sympodial orchid cultures could be initiated with aerial explants as this procedure is not only easy but also minimises the contamination problem.

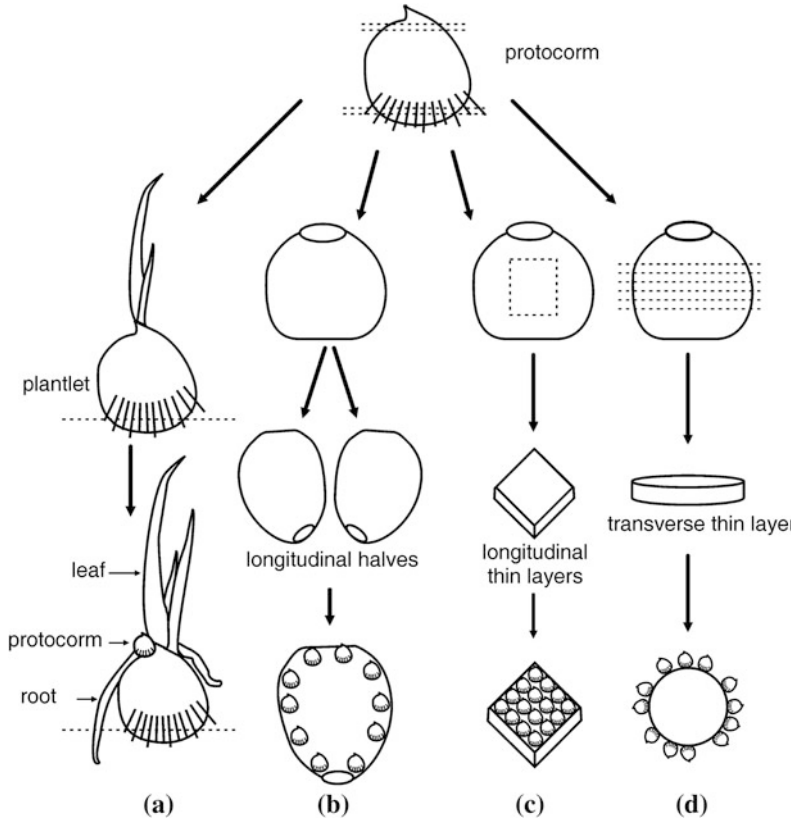

Fig. 17.3 Diagrammatized representation of four methods to multiply *Cymbidium* hybrids. **a** A complete Protocorm-like-body (PLB) from shoot tip culture on transfer to fresh medium develops a shoot and roots and a PLB. **b** The PLB is trimmed at the top to exclude shoot apical meristem and at the bottom to remove brown portion, if required. The trimmed PLB is cut into two longitudinal halves and cultured with the cut surface in contact with the medium. Within 60 days the parent explant gradually dies after producing ideal sized new PLBs from the peripheral tissue. **c** From the primary PLB 0.5–1 mm thick 3–5 × 3–5 mm square longitudinal TCLs (two per PLB) are prepared and immediately planted on the medium with the cut surface in contact with the medium. It develops new PLBs all over the surface of the explants. **d** The primary PLB is cut into five transverse TCLs (0.5–1 mm thick) and planted on fresh medium. All the TCL peels differentiate multiple PLBs from the peripheral tissue as in **b**. (*after* Teixeira da Silva and Tanaka 2010)

Teixeira da Silva and Tanaka (2010) have described a Thin Cell Layer (TCL) culture method for rapid clonal multiplication of the *Cymbidium* hybrid Twilight Moon 'Day Light', which are difficult to propagate by the traditional methods of clonal propagation. They compared the rate of multiplication by four methods as depicted in Fig. 17.3. Shoot tips (5–10 mm) from 3-years-old mature plants of the hybrid were cultured on highly organic substrate (1/2 strength MS salts + 0.2 % activated charcoal + 8 % (w/v) homogenate of ripe banana). Once the shoot began to elongate, but before the appearance of roots, it was transferred to another medium (1/2 MS salts + 2 % ripe banana homogenate + 10 % coconut water) gelled with 0.5 % Gelrite. After 6 months, the shoot developed roots and a Protocorm Like Body (PLB) at the base of the leaf sheath (Fig. 17.3a). If this primary PLBs were excised and transferred to another medium (VW + micronutrients of Nitsch and Nitsch medium + 2 mg L^{-1} tryptone + 0.1 mg L^{-1} each of NAA and kinetin + 2 % sucrose), with 10 PLBs per 100 ml flasks containing 40 ml

of the medium, and incubated at 25 °C under 16 h photoperiod with photon flux density of 30–40 μmol m^{-2} s^{-1} they produced more PLBs. This process of PLB multiplication could be repeated by two monthly transfers of individual PLBs to fresh medium of the same composition. By this method 5,353-fold multiplication in 10 months could be achieved, which is very slow.

The rate of multiplication of *Cymbidium* hybrid could be considerably enhanced by splicing the PLBs into two longitudinal halves and planting them on the multiplication medium with the cut surface in contact with the medium (Fig. 17.3b). After 45–65 days, the parent PLB tissue gradually died but before that it differentiated many PLBs from the outer epidermis. These PLBs could be used for further multiplication by three monthly subcultures. Through this method an estimated number of 36,350 PLBs of uniform size, shape, and developmental stage could be produced in 12 months.

In the third method of multiplication the primary and secondary PLBs were used to prepare longitudinal TCL (L-TCL; 0.5–1.0 mm thick and 3–5 × 3–5 mm square). Two peels per PLB were prepared and cultured on the same medium as above (Fig. 17.3c). Through three monthly subcultures an estimated number of 351,700 PLBs can be obtained in 9 months. Similarly, transverse TCL (0.5–1.0 mm thick 5 T-TCL per PLB) can yield 28,100 PLBs in 9 months (Fig. 17.3d). Thus, L-TCL culture system being the fastest of the four methods of multiplication and is recommended for micropropagation of this hybrid.

A protocol for the clonal propagation of *Phalaenopsis* by root and leaf segment culture is described in Appendix 1.

The simple media used for the germination of seeds, such as Vacin and Went (1949), Knudson (1946) and Hyponex (Kano 1965), are generally employed for clonal propagation of orchids (*for composition of these media* see Table 17.1). Some genera require more complex media like the MS medium. The three simple media mentioned above, which lack amino acids or vitamins, are often supplemented with undefined natural complex addenda, such as coconut water, and homogenate of ripe banana and potatoes, as supplementary substances. Low levels of auxin and cytokinin are sometimes added at the initial stage of protocorm multiplication but not in the media used for shoot and root development from the protocorms. The requirement for these growth regulators and their type and concentration may vary with the species and genotype. Sucrose is added to the medium for the initiation of cultures but often the protocorm formation improves in its absence.

Transfer of cultures to sucrose-free medium is essential in some genera to promote greening and plantlet differentiation, such as *Phalaenopsis*. Activated charcoal improves the growth of *Paphiopedilum* (Ernst 1974) and *Phalaenopsis* (Ernst 1975). The charcoal probably helps improving the aeration and adsorbs phenolic inhibitors.

17.3 General Micropropagation Technique

Micropropagation is a well-defined multistep process. It comprises of five steps, each with its specific requirements and problems (Bhojwani and Razdan 1996): *Stage 0*: It is the preparatory stage to provide quality explants; *Stage 1*: Initiation of aseptic cultures; *Stage 2*: Multiplication; *Stage 3*: Rooting of in vitro formed shoots; and *Stage 4*: Transfer of plants to greenhouse or field conditions (transplantation).

17.3.1 Stage 0: Preparatory Stage

This step was initially introduced to check the contamination problem, especially of fungi, but now it is considered to be important for the success of the establishment and eventual multiplication at Stages 1 and 2, respectively. Any measure(s) taken to improve the quality of the parent plant, whether involving its hygiene or physiological status, are included in this Stage.

Contamination is one of the serious problems at Stage 1, and for successful establishment of

Table 17.1 Composition of modified MS, VW (Vacin and Went) and Knudson C media used for orchid tissue cultures. (*after* Paek et al. 2011)

Constituents	Amounts (mg L^{-1})		
	Modified MS	VW	Knudson C
NH_4NO_3	825	–	–
$(NH_4)_2SO_4$	–	500	500
$Ca_3(PO_4)_2$	–	200	–
$Ca(NO_3)_2.4H_2O$	–	–	1,000
$CaCl_2.H_2O$	220	–	–
$MgSO_4.7H_2O$	185	250	250
KNO_3	950	525	–
KH_2PO_4	85	250	250
Na_2EDTA	18.65	–	–
$FeSO_4.7H_2O$	13.9	–	25
$Fe_2(C_4H_4O_6)_3.H_2O$	–	28	–
H_3BO_3	3.1	–	–
$CoCl_2.6H_2O$	0.0125	–	–
$CuSO_4.5H_2O$	0.0125	–	–
$MnSO_4.4H_2O$	11.15	7.5	7.5
KI	0.415	–	–
$Na_2MoO_4.2H_2O$	0.125	–	–
$ZnSO_4.4H_2O$	4.3	–	–
Glycine	2	–	–
Myo-insitol	100	–	100
Nicotinic acid	0.5	–	1
Pyridoxin.HCl	0.5	–	–
Thiamine.HCl	0.1	–	1
Adenine sulphate	10	–	–

aseptic cultures, the hygiene of the mother plants is very important. Generally, the explants derived from the plants maintained in greenhouse yield better results. The donor plants should be given basal irrigation to minimize the contamination problem during the initiation of cultures. It would reduce the requirement of strong sterilization measures and increase the number of healthy cultures. Before the introduction of Stage 0, infection-free *Cordyline* cultures could be obtained only from apical buds and not from the axillary buds. Maintaining mother plants under hygienic conditions of glasshouse made it possible to use all the axillary buds and apical bud to establish aseptic cultures (Debergh and Maene 1981). *Theobroma cocoa* is another classic example where aseptic cultures could be obtained only from greenhouse grown mother plants (Senawi 1985).

Stage 0 may also include manipulation of such parameters as light and temperature regimes under which the mother plants are maintained and the application of growth regulators, which could influence the response of explants at the later stages. *Petunia* stock plants grown under red light branched profusely, and the leaf explants obtained from such plants produced three times more shoots than the leaves obtained from untreated plants (Read et al. 1978). Some plants require temperature pre-treatment for better in vitro response. Ko (1986) found that storage of 'Carnegie' hyacinth bulbs at 5 °C influenced the number and quality of bulbs produced in vitro.

17.3.2 Stage 1: Initiation of Cultures

The aim of this stage is to establish aseptic cultures of the plant to be micropropagated using suitable explants. Although 100 % infection-free cultures are difficult to obtain but reproducibly high percentage of aseptic cultures are desirable for a satisfactory micropropagation protocol. The success at this stage is dependent on the choice of the right explant, proper sterilization procedure, and preventing any hypersensitivity reactions of the explants.

(i) *Explant.* The choice of explant is mainly dependent upon the mode of regeneration and multiplication desired and aim of the study. The explant most commonly used for micropropagation is either apical bud or nodal segments with at least one axillary bud. If the aim is virus elimination one goes for meristem tip culture (Chap.16). Otherwise, meristem tip culture should be avoided because of its poor survival and complex culture requirements. Moreover, some characters of horticultural interest controlled by the presence of virus, such as the clear-vein character of the geranium cv. Crocodile, may be lost in the plants derived from meristem tips (Cassells et al. 1980). For the micropropagation of plants such as cauliflower and the Boston fern chopped pieces of the curd and rhizome, respectively, has been used to initiate the cultures. Propagation from apical buds and axillary buds has the advantage of true-to-type progeny.

The choice of explants increases when the aim is *de novo* regeneration of shoots or somatic embryogenesis. Direct or indirect adventitious bud formation can be obtained from root, stem, leaf, or nucellus explants. Young immature zygotic embryos have been used for obtaining somatic embryogenesis in cereals, legumes, and tree species. Nucellus is the explant of choice for obtaining somatic embryos in mango and citrus. The only tissue from the adult plants of cashew that could be used for micropropagation is the nucellus (Cardoza and D'souza 2000). In monocots, the intercalary meristem at the base of young leaves or bulb scales has been successfully exploited for regenerating adventitious buds.

(ii) *Sterilization*. Commonly followed sterilization procedures are described in Chap. 2. Quite often field material is required to be micropropagated. In such cases, it is advisable to establish the donor plants in the greenhouse from cuttings in order to avoid wind borne contaminants. Aseptic shoot tips can be dissected out, inside a sterile hood, by carefully removing the outer leaves or scales from the buds and bulbs, respectively, surface sterilized by wiping with 70 % ethanol.

(iii) *Browning*. The explants from many plants, especially tree species and some horticultural crops, release phenols, which upon oxidation form quinones and turns the medium black that could be toxic to the tissue. This problem and the measures to offset it are discussed in Sect. 17.5.3.

17.3.3 Stage 2: Multiplication

The success of micropropagation protocol is largely dependent on the efficiency of this stage. Shoot multiplication is achieved through: (i) regeneration from callus, (ii) direct adventitious bud formation from the explant, and (iii) forced axillary branching. Each of these methods has its own advantages and disadvantages.

(i) *Regeneration from Callus*. Plant cells from almost all parts of a plant are capable of forming callus under suitable culture conditions, and the callus can be induced to regenerate plants via organogenesis or somatic embryogenesis. Both these methods have the potential for producing a large number of plants, the latter being much more efficient. Regeneration of shoots and somatic embryogenesis in cell and callus cultures has been described in Chaps. 6 and 7, respectively.

Somatic embryogenesis is a highly efficient process capable of producing large numbers of embryos that can germinate as zygotic

embryos to form plants with a primary root system. This is in contrast to a separate rooting step required for the *de novo* formed shoots. Moreover, once the protocol for somatic embryogenesis is standardized, the process is amenable to greater degree of control and scaling up for mass production in bioreactors and automation (Das Gupta and Ibaraki 2006; Preil 1991; Etienne et al. 2006). The embryos can be converted into synthetic seeds for possible mechanical transplantation on a large scale.

However, despite the many advantages, an efficient and reproducible protocol for somatic embryogenesis is not available for many species. Synchronization of embryogenic cultures is not easy to achieve and, often the conversion of embryos into plants has been very poor due to morphological and physiological abnormalities (Soh et al. 2003). The major disadvantage of somatic embryogenesis is an intervening callus phase which can induce variability. Therefore, regeneration from callus is not the preferred method for mass clonal multiplication. Yet, in vitro clonal propagation of palms, such as coconut, oil palm, and date palm, has been possible only through adventive embryony in callus cultures derived from inflorescence segments or shoot tips from offshoots (Bhojwani et al. 2003). The micropropagation protocols for *Citrus*, mango, and cashew are based on embryogenesis in the cultures of nucellar tissue. Whereas in polyembryonate *Citrus* and mango, the embryos arise from the pre-existing adventive embryo initials or immature nucellar embryos, in cashew the nucellar cells are induced to form embryos after callusing.

(ii) *Adventitious bud formation*. The shoot buds formed directly from any part of the plant other than apical or axillary bud is termed adventitious bud. Strictly speaking, *de novo* regenerated shoot buds from callus are also adventitious in nature. Many horticultural plants exhibit natural capacity to form adventitious shoots from leaf pieces (*Begonia, Saintpaulia*) and root cuttings (blackberry, raspberry), which have been exploited for clonal propagation of these plants. The advantages of in vitro propagation by adventitious shoot formation are: (i) the number of shoots per propagule can be considerably enhanced, (ii) very small pieces of tissues that do not survive in vivo can form shoot buds in cultures, and (iii) many plants that do not exhibit adventitious shoot formation in nature do so in vitro.

Adventitious bud formation from leaf scales has been most exploited in the Liliaceae and Iridaceae members (Ascough et al. 2009). Using small segments of outer bulb scales almost 100 bulblets could be obtained from a single scale of lily (Hackett 1969; Gupta et al. 1978). This tremendous regeneration potentiality in lily has raised the technique to mass production on a commercial scale in bioreactors (Takahashi et al. 1992). Apical corm tissues of *Ixia flexuosa*, an Iridaceae member, regenerated adventitious buds when cultured on a BA containing medium (Meyer and van Staden 1998).

Begonia x *heimalis* is a classical example of the efficiency of adventitious bud formation by plant tissues. In nature leaf pieces of *Begonia* form shoot buds only along the cut ends but in a medium containing BAP the entire surface of the explant gets covered with shoot buds (Reuther and Bhandari 1981). In cultures almost 10^{14} plants could be obtained in a year from a 7 × 7 mm segment of leaf (Takayama and Misawa 1982). It could also be rapidly multiplied in liquid shake cultures (Simmonds and Werry 1987). *Ficus lyrata* can be very effectively multiplied through adventitious bud formation from leaf segments (Debergh and De Wael 1977; Jona and Gribaudo 1988).

The excised shoot tips of grape fragmented into 20 pieces retained the potential to regenerate plants directly, and using this approach 8,000 plants could be produced from one shoot tip in 3–4 months (Barlass and Skene 1978, 1980). All the plants so produced were normal diploid. Regeneration of shoots from fragmented shoot tips has also been reported in *Asclepias rotundifolia*

(Tideman and Hawker 1982). Tissue pieces from homogenated plants of the ferns *Davallia* and *Platycerium* produced numerous plants (Cooke 1979).

Genetic fidelity of plants obtained through direct adventitious shoot bud formation is definitely greater as compared to those regenerated from callus. However, examples are known where adventitious bud-derived plants showed off types (potato—Cassells et al. 1983; *Brassica*—Bhojwani and Arora 1992). Multiplication of genetic chimeras through this method may not be possible because of the risk of splitting of the chimeras and regeneration of pure types (Skirvin 1978; Johnson 1980). Apple plants regenerated from internodes behaved differently with respect to growth habits and fruit characteristics as compared to the parent plant (Dayton 1970).

(iii) *Forced axillary branching*. Normally, the buds present in the axil of leaves remain suppressed due to the well-known phenomenon of apical dominance. Removal of the apical meristem stimulates the axillary bud to grow out into a shoot. In the well-established horticultural practice of clonal propagation by stem cuttings, the new plant develops from the axillary bud at the node. However, this process of clonal multiplication is very slow and is limited by the number of cutting (10″–12″ long with 2–3 nodes) that can be obtained from the mother plant. Exogenous application of growth regulators, particularly a cytokinin, can force the axillary buds to grow even in the presence of terminal bud and increase the number of useable flushes. However, the effect of exogenous growth regulators does not last long.

Forced axillary branching has been successfully exploited for in vitro clonal propagation of plants (Figs. 17.4). In fact, it is the most popular method for commercial micropropagation of the desired plants. Continuous multiplication through axillary branching can be obtained in vitro by culturing nodal explants in a medium containing an appropriate cytokinin at a suitable concentration, with or without an auxin. Due to the continuous presence of cytokinin in the medium the shoot developed from the axillary bud, present on the nodal cuttings at the time of culture, develops axillary buds which grow directly into shoots. This process may be repeated several times and the initial explant is transformed into a clump of branches (Fig. 17.5a). However, due to gradual depletion of the nutrients and the growth regulator/s in the culture medium, there is limit to which shoot proliferation can occur in a single multiplication cycle. Therefore, after some time (3–4 weeks) further multiplication of shoot stops. At this stage the shoot clump is cut into pieces, each with at least one well-formed shoot and transferred to fresh medium of the same composition for another cycle of shoot multiplication. This process can be repeated several times over till sizeable number of shoots is achieved. It has been recommended that multiplication cycles should be limited in order to avoid any risk of introducing abnormalities due to the culture environment. For banana, maximum number of multiplication cycles recommended is seven (Singh et al. 2011). This method of shoot multiplication has been used for a large number of species, which include monocots (*Alstomeria*, *Asparagus officinalis*, orchids such as *Dendrobium*, *Phalaenopsis*), herbaceous dicots (*Angelonia*, *Glycirrhiza*), and woody dicots (*Juglans regia*, *Eucalyptus grandis*, *Tectona*).

In some plants, such as Neem (*Azadirachta indica*), potato, *Leucaena* and *Feijoa*, the apical dominance is so strong that even in the continuous presence of cytokinin the axillary bud grows into a single long unbranched shoot. In these cases, at the end of a passage the solitary shoot is cut into single node segments and transferred to fresh medium. The rate of multiplication would depend on the number of nodal cuttings that can be prepared from the solitary shoot at the end of a passage. Even by this

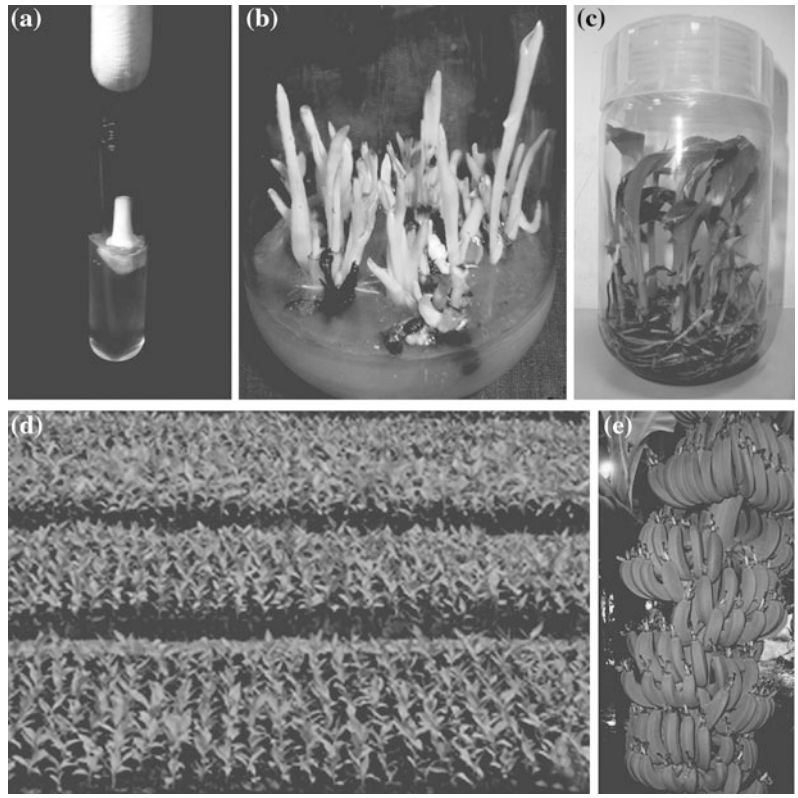

Fig. 17.4 Micropropagation of banana by forced axillary branching. **a** A fresh culture of shoot tip. **b** Shoot proliferation. **c** Rooting of the shoots. **d** Hardened micropropagated plants in a net house. **e** A fruiting micropropagated plant 11 months after transplantation. (*courtesy* Dr Sanjay Saxena, TERI, New Delhi)

method 3 to 4-fold shoot multiplication every 4–6 weeks can be achieved. In neem, the solitary axillary shoot attained a length of 8–9 cm (Fig. 17.6) and allowed 5 to 7-fold multiplication every 5 weeks by axillary branching (Chaturvedi et al. 2003).

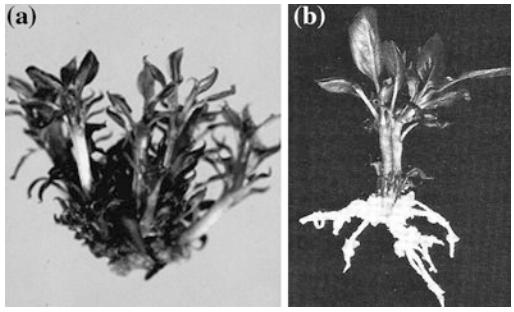

Fig. 17.5 Micropropagation of *Pyrus serotina*. **a** A shoot cluster developed from a nodal cutting by forced axillary branching. **b** In vitro rooted shoot from a cluster as in **a**

The forced axillary branching method may be initially slow but with each passage the number

Fig. 17.6 Nodal segments of *Azadirachta indica* (Neem), 8 weeks after culture on MS medium containing 1 μM BAP and 0.025 % casein hydrolysate. Both explants have developed a long multinodal, unbranched shoot. (*after* Chaturvedi et al. 2003)

of shoots increases logarithmically, and within a year very large number of shoots are produced starting from a single nodal cutting.

This method of shoot multiplication is most reliable in terms of the genetic uniformity of the micropropagated plants, and therefore is the most preferred method of in vitro clonal propagation of plants. Moreover, clonal multiplication of chimerical plants can be successfully achieved through forced axillary branching.

Excess cytokinins or a wrong cytokinin used may cause epigenetic changes in the plants obtained through forced axillary branching. Bushiness in the micropropagated *Gerbera* plants, which is accompanied by excessive leaves, decrease in flower number, and short peduncle, has been attributed to the use of excess BAP. Similarly, long exposures to culture conditions adversely affected the size and shape of fruits in strawberry.

17.3.4 Stage 3: Shoot Elongation and Rooting

Somatic embryos are bipolar structures with root and shoot primordia and can, therefore germinate to form complete plants. However, the shoots formed through regeneration from callus, direct adventitious bud formation or forced axillary branching require an additional step of rooting for complete plant formation. In some cases, the shoots formed in vitro by any of the three methods described above, being continually exposed to cytokinin, may remain short and require an intermediate elongation step before transfer to rooting medium. The elongation medium may be liquid medium of the same composition as for shoot multiplication (*Gladiolus*, Dantu and Bhojwani 1987, 1995) or with reduced level of cytokinin. It is advisable to transfer shoots in clusters to the chosen elongation medium, which will not only result in uniform elongation of all the shoots but also reduce the cost of handling.

Cytokinins present in the medium also prevent the formation of roots, and therefore it is essential to transfer the shoots to a suitable medium for rooting. Rooting is generally achieved by transferring individual shoots (about 2 cm long) to a medium supplemented with a suitable auxin (Fig. 17.5b). It will, of course, be economical if shoot clusters can be used as a single unit for this step as in the case of onion and garlic. The rooting stage is labor intensive, accounting for about 70 % of the cost of micropropagated plants. Therefore, the rooting percentage should be high (>95 %).

To cut down the cost of rooting of micropropagated shoots, many commercial companies resort to in vivo rooting. For this, the micropropagated shoots are treated as microcuttings and planted directly in potting mix after treating the cut basal end with a commercial rooting mix or an auxin solution. In vivo rooting has many advantages: (i) the roots formed in vitro die after transplantation and the new roots are formed which sustain the plant, (ii) the vascular connection between the in vitro formed roots and the shoot may not be well developed, (iii) the in vitro formed roots lack root hairs that makes them less effective when transplanted, (iv) transplantation is generally done by unskilled labor and the plants to be transferred being very large, quite often the in vitro roots get damaged during this process, and (v) callusing at the junction of roots and shoot is often a problem faced with in vitro rooting. In vivo rooting not only cuts down the cost but also circumvents the above problems associated with in vitro rooting.

17.3.5 Stage 4: Transplantation and Acclimatization

The ultimate success of micropropagation depends on the establishment of the plants in soil or potting mix. The plants grown in vitro are exposed to the artificial environment of the culture vial, which is characterized by the culture medium rich in inorganic and organic nutrients, sucrose and growth regulators, high humidity, low light, and poor gaseous exchange. Under these unnatural conditions, the plants are

able to grow well but suffer from many morphological, anatomical, cytological, and physiological abnormalities, which necessitate their careful acclimatization to the in vivo conditions. The two main abnormalities of these plants are heterotrophic mode of nutrition due to the culture medium being rich in organic nutrients and poor control of water loss. Under high humidly of the culture vials the leaves show poor development of cuticle, scanty deposition of wax, abnormally large stomata, which do not close even in response to ABA, high CO_2 or dark treatment, poor differentiation of the mesophyll tissue, which mainly comprised spongy parenchyma, and poorly developed chloroplasts with low chlorophyll content and disorganized grana (Preece and Sutter 1991; Bhojwani and Razdan 1996). The lack of cuticle and impaired structure and movements of the stomata cause excessive water loss on transplantation, reducing their chances of survival. Therefore, the in vitro plants should be carefully hardened (acclimatization) before transfer to field conditions.

17.3.5.1 *Acclimatization*

The principle governing acclimatization of the in vitro plants, habituated to grow under high humidity and low light heterotrophic conditions is to make them grow under low humidity and high light autotrophic conditions. It takes about 4–6 weeks for hardening the in vitro plants, so that they can survive under normal conditions. The individual micropropagated plants are first taken out of the agar medium, the roots are washed to remove the agar and individually planted in pots containing appropriate potting mix (peat, vermiculite, perlite, polystyrene beads, or coco compost). The plants are irrigated with a mild nutritive solution such as Knop's solution or one-fourth strength MS salt solution.

The simplest method to maintain high humidity around the transplanted in vitro plants, often used in research and teaching laboratories, is to cover them with plastic bags perforated with small holes for air circulation. The plants are maintained in shade or low light for about 15–20 days, and slowly acclimatized to low humidity conditions by removing the polybags for few hours every day in the beginning and slowly increasing the time of exposure until the plants are able to withstand complete removal of the cover. The plants during this phase are hardened to survive on inorganic nutrition and their photosynthetic machinery is reactivated to become autotrophic. The plants are able to survive under field conditions only when new roots and leaves are formed.

In a commercial laboratory, where a large number of plants have to be handled, a completely different approach is followed. The plants are transplanted into protrays having 96 holes, which are easy to handle for irrigation and transfer purpose and drastically cut down on the labor for handling the plants. High humidity is maintained by fogging or misting, of which fogging, with extremely small droplets (ca 20 μm), is preferred. In tropical countries high humidity, cooler temperatures and low light conditions are maintained in large polyhouses fitted with heavy duty exhaust fans at one end and pads, constantly drenched with water, at the other end (fan and pad system). Within the polyhouse, the plants in protrays are shifted from high humidity near the pad end to low humidity near the fan end over a period of about 4 weeks. During this period, the plants are irrigated with high phosphorous containing nutritive solution to promote rooting, and as the protrays are moved toward the fan the fertilization is changed to solution containing equal NPK. Frequent sprays of fungicides and insecticides are necessary as these conditions are conducive to the growth of pests and pathogens.

Alternately, the rooted plants could be partially hardened in vitro by reducing the humidity inside the culture vessel by gradually unscrewing the lid of the culture jar over a period of 3–4 weeks. The humidity inside the culture vial could also be reduced by using desiccants, use of culture vials with microporous closure to allow gaseous exchange and cooling the bottom of the culture vessels (Bhojwani and Razdan 1996). The photosynthetic ability of the plants in cultures could be enhanced by increasing the irradiance to 80 μmol m^{-2} s^{-1}. Inclusion of growth

retardants, such as paclobutrazole or ancymidol, in the rooting or shoot proliferation medium is reported to increase desiccation tolerance, and thus survival ability of plants on transplantation (Novello et al. 1992; Roberts et al. 1992; Ziv and Ariel 1991; Ziv 1992).

17.3.5.2 *In Vitro Formation of Storage Organs*

Plant species that produce storage organs (tuber, corm, bulb, rhizome) in nature can be promoted to do so in cultures to facilitate transplantation with high survival rates. This may also eliminate the rooting step altogether. The storage organs can be easily stored or shipped and planted manually or by machines. In vitro formation of storage organs has been achieved in *Gladiolus* (Dantu and Bhojwani 1987, 1995), *Crocus* (Plessner et al. 1990), *Dioscorea* spp. (Forsyth and van Staden 1984), *Freesia* (Hirata et al. 1995), potato (Akita and Takayama 1994; Donnelly et al. 2003; Kämäräinen-Karppinen et al. 2010), *Dierama luteoalbidum* (Madubanya et al. 2006) by increasing sucrose concentration, and altering the growth regulator and light and temperature treatments.

Potato is a classical example where in vitro formation of tubers has been extensively studied (Fig. 17.7). The precise conditions that promote in vitro tuberization for various cultivars of potato have been established and the whole process has been raised to the level of commercial production and is also a subject for automation (Sect. 17.6). Generally, shoot multiplication (Fig. 17.7a) occurs under long day conditions on a medium with 2–3 % sucrose, and in vitro microtuberization (Fig. 17.7b) is promoted by short day conditions or complete darkness and elevated sucrose level (8–9 %) in the culture medium. Microtubers are harvested and sown in plastic trays to produce minitubers (Fig. 17.7c), which can be used as seed tubers for crop production.

The requirement of growth regulator(s) and/or growth retardants for microtuber formation is variety specific and dependent on the propagator (Donnelly et al. 2003). Some varieties produce better microtubers in the absence of any growth regulator or retardant while quite a number of varieties require the presence of either or both. However, since in vitro microtuber formation in the presence of growth regulator (BAP) and growth retardant (CCC, alar, ancymidol) is associated with tuber anomalies, such as abnormal shapes (round instead of elongated), less number of eyes, bigger lenticels, and less well organized, thinner periderm (Nasiruddin and Blake 1994), many propagators prefer to induce microtuberization solely by varying environmental conditions, such as reduced photoperiod or complete darkness.

The number of microtubers formed per original node, their size, and fresh weight are some measures to compare efficiency of environment

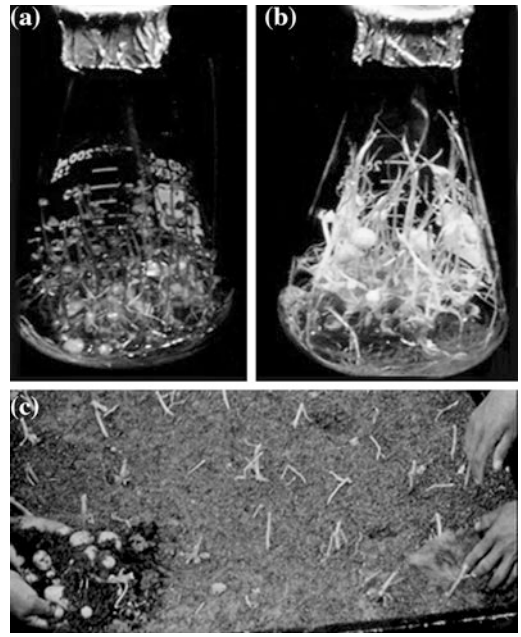

Fig. 17.7 Micropropagation of potato. **a** Shoot multiplication in light on a medium with 3 % sucrose. **b** Microtuber formation on the stem on a medium containing 9 % sucrose in dark. **c** Microtubers planted in trays have developed minitubers, which can be used as vegetative seeds for growing potato crop

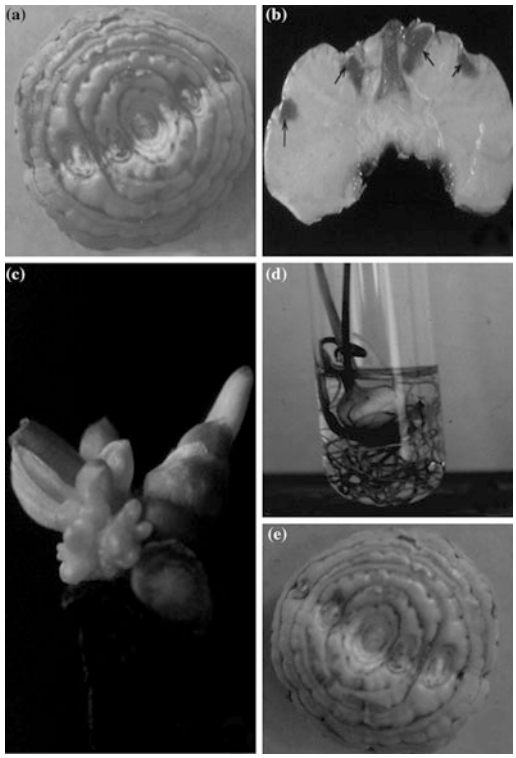

Fig. 17.8 In vitro clonal propagation of *Gladiolus*. **a**, **b** Corms (complete and a longitudinal half, respectively), showing positions of axillary buds (arrow marked in **b**). **c** Formation of multiple shoot buds on MS + 0.5 mg L^{-1} BAP in a culture of axillary bud. **d** In vitro corm formation by a micropropagated shoot in MS containing 6 % sucrose. **e** An in vitro formed corm. Morphologically, It is identical to the in vivo formed corm as in **a** (*after* Dantu and Bhojwani 1987)

and media conditions adopted for microtuber formation. A yield of 30–50 microtubers of 200–300 mg per 100 shoots in 4 months in stationary containers is considered to be quite efficient (Wang and Hu 1982). However, such a system is not suitable for microtuber production on commercial scale, for which different types of bioreactors have been developed (see Sect. 17.6).

Dantu and Bhojwani (1987) reported corm formation by micropropagated shoots of *Gladiolus* in static liquid cultures with increased concentration of sucrose (6 or 10 % depending on the cultivar; Fig. 17.8). Corm formation in this crop could also be induced in liquid shake cultures by treatment with GA_3 biosynthesis inhibitors, such as paclobutrazol (Steinitz et al. 1991; Ziv 1992). Temperature requirement for corm formation differs for winter and summer flowering *Gladiolus* species (De Bruyn and Ferreira 1992). The summer flowering *G. dalenni* formed corms at 24 °C while the winter flowering *G. tristis* formed corms at 15 °C. Light intensity also influences corm formation in some cultivars of *Gladiolus* (Thun et al. 2008). In *Dierama luteoalbidum*, another member of the family Irridaceae, corm formation with 6–8 % sucrose occurred in 6 months, which could be reduced to 3 months by adding 17.1–34.2 μM paclobutrazol to the culture medium (Madubanya et al. 2006).

17.4 Factors Affecting Micropropagation

17.4.1 Initiation of Cultures and Shoot Multiplication

The most widely used basal medium for, both, culture initiation and shoot multiplication is MS (Murashige and Skoog 1962). It has been variously modified for different systems. For a number of plants, the full strength of MS is very toxic and requires to be reduced to half strength or even less. Bamboos, such as *Dendrocalamus,* showed better shoot proliferation on one-half strength than on full strength MS basal medium. For multiplication of *Pingicularia* even one-half strength MS medium was toxic, and the concentration of basal medium required to be reduced to one-fifth. The promoting effect of reduced salt concentration of MS could be because of reduced strength of nitrogen, especially the ammonium salt. In *Gladiolus*, reducing ammonium nitrate to half strength and supplementing the MS medium with 300 mg L^{-1} of NaH_2PO_4 improved the rate of multiplication (Dantu and Bhojwani 1992).

The medium requires to be supplemented with suitable growth regulator/s for culture initiation and later shoot multiplication. Whether a cytokinin alone is sufficient or an auxin is also required is determined by setting up suitable experiments. More often than not a cytokinin alone is sufficient to elicit responses, both, at initiation level and shoot multiplication stage. Of the various cytokinins available commercially (kinetin, BAP, 2iP, zeatin, TDZ) BAP has been most widely used. However, for some plants other cytokinins have proved to be more beneficial. For example, in the case of *Rhododendron* (Anderson 1975), *Ficus benjamina* cv. Starlite (Dantu unpublished), garlic (Bhojwani 1980) and blueberry (Cohen 1980) 2iP proved superior to BAP. Zeatin being expensive is not recommended for commercial micropropagation. Thidiazuron (TDZ), a urea derived cytokinin, has proved useful for hard to regenerate species.

Topolins, the latest group of aromatic cytokinins introduced in tissue culture studies, have given promising results with many systems (Bhim et al. 2008). *m*T (*meta*-Topolin) improved acclimatization and ex vitro survival rate in *Spathiphylum* spp (Werbrouck et al. 1996), multiplication and control of hyperhydricity in *Aloe polyphylla* (Bairu et al. 2006) and the multiplication of plantains (Escalona et al. 2003). *m*TR (*meta*-Topoline riboside) improved the survival rate of potato cultures (Baroja-Fernandez et al. 2002), and *m*T derivatives improved histogenic stability and anti-senescence affects in *Petunia* and rose cultivars, respectively (Bogaert et al. 2006). *m*TR proved better than BAP in terms of rate of multiplication and the quality of shoots in *Baleria greenii*, an endangered perennial ornamental shrub (Amoo et al. 2011). Bairu et al. (2008) compared the effect of three topolins with BAP for shoot multiplication and somaclonal variation levels in banana and noted that in 6 weekly subcultures 22.2 μM *m*TR (5.3 fold) or *m*T (4.8 fold) promoted the rate of shoot multiplication over equimolar concentration of BAP (3.0 fold). However, in the presence of *m*TR rooting during shoot multiplication was completely suppressed, whereas with BAP profuse rooting occurred (10.4 roots per culture).

The most effective concentration of cytokinins has been between 1 and 2 mg L^{-1}, although much higher concentrations have also been used. TDZ is effective at very low concentrations of 0.002–0.10 mg L^{-1}. Higher concentrations of this cytokinin can induce callusing and may bring about morphological abnormalities, such as hyperhydration.

To maximize regeneration in some plants, the cytokinin requires to be complemented with a suitable auxin. The most commonly used auxins are NAA and IBA in the range of 0.1–1 mg L^{-1}. 2,4-D is preferred for inducing somatic embryogenesis but is avoided where shoot multiplication is desired. In some species, as *Gardenia* GA_3 improved the rate of multiplication and the quality of shoots formed.

Generally, media gelled with agar or gelrite are used for initiation of cultures and shoot multiplication. However, multiplication and even survival of a large number of plant systems is better in liquid cultures. In some orchids, such as *Cattleya*, cultures could be established only in liquid medium. The pH of culture medium is generally set at 5.7–5.8. However, some plants, such as *Magnolia* and *Dianthus*, prefer a highly acidic pH of 3.5 and 4.5, respectively, for improved rates of shoot multiplication.

For shoot multiplication the cultures are mostly maintained in light. Amoo et al. (2011) observed that culturing the shoots of *Baleria greenii* under 16 h photoperiod resulted in high production of shoots with length >10 mm as compared to culturing under continuous light.

17.4.2 Rooting

Most of the micropropagated plants are able to root in vitro on a full strength MS medium supplemented with an appropriate auxin. However, some plants could not root in full strength

MS medium. *Narcissus* and *Rhododendron* preferred half strength MS medium for increased percentage of in vitro rooting. As in the case of shoot multiplication, ammonium ions were detrimental to in vitro rooting. Shoots of some cultivars of apple rooted only when ammonium nitrate were reduced to half strength or completely deleted from the medium (Sriskandarajah et al. 1990; Hyndman et al. 1982).

Presence of an auxin is essential for most of the herbaceous monocots and dicots and woody trees. IBA is preferred over IAA or NAA. IAA has a short life in the culture medium because of photooxidation. NAA, on the other hand, does induce rooting but has a tendency to promote callusing at the base. However, it is the preferred auxin for rooting of woody plants. IAA-induced rooting in some tree species, specially rosaceous fruit trees, is promoted in the presence of bi- or polyphenolic substances, such as phloroglucinol and ferulic acid. These phenolic compounds protect IAA from oxidative decarboxylation (DeKlerk et al. 2011).

The period of exposure to auxin varies with plant species. Herbaceous plants and soft wood species require a short exposure while woody species require long or continuous exposure to the auxin. Long auxin exposure may induce callusing at the base or cause chlorosis of leaves and make acclimatization difficult. Auxins have been used at a concentration of 0.1–1.0 mg L^{-1} for rooting though woody plants require a higher concentration. A root length of 5–10 mm is sufficient as any longer root may get damaged at the time of transplantation. This root length is generally attained within 10–15 days.

17.5 Problems Inherent with Micropropagation

17.5.1 Hyperhydration

Often the shoots regenerated under highly humid environment and heterotrophic conditions inside the culture vials exhibit morphological, anatomical, physiological, and metabolic abnormalities (Ziv 1999; Kharrazi et al. 2011). Such plants, referred to as vitrified or hyperhydrated, appear water soaked, glassy, translucent, succulent or fleshy with rigid, thick, and brittle leaves and stem. The other abnormalities associated with these plants are poor differentiation of mesophyll tissue (palisade tissue is either absent or highly reduced), chlorophyll deficiency, poor lignification, poor internal differentiation of chloroplasts, faulty deposition of wax on the leaves, and the stomata remain open even in dark and do not respond to water stress, ABA, high CO_2 or hypertonic solutions of mannitol or sucrose. Hyperhydration of shoots is one of the most serious problems in commercial micropropagation because these abnormal shoots exhibit poor rooting response and transplantation survival.

After the initial report of hyperhydration in in vitro regenerated plants of carnation by Ziv et al. (1991) it has been observed in several plant species by different scientists but it is still poorly understood. The degree of hyperhydration depends on the species or even cultivar, physical nature of the medium, type of closure device of the culture vial, type and quantity of the gelling agent, concentration and type of cytokinin (Debergh 1987). Improving aeration to reduce humidity in the head space of the culture vial by using suitable closure, using an agar of high gelling strength, low NH_4^+ and cytokinin concentration, and low salt concentration are known to reduce the hyperhydration response of the in vitro plants. The major problems in the micropropagation of *Macadamia tetraphylla* have been shoot tip necrosis, hyperhydration of the shoots, and callus formation at all stages (Bhalla and Mulwa 2003; Mulwa and Bhalla 2006; Gitong et al. 2008). Cha-um et al. (2011) observed that the micropropagated shoots of *Macadamia* on a semi-solid medium containing 88 mM sucrose in a closed, poorly ventilated culture vial showed only 30 % rooting. Poor rooting was associated with shoot and leaf necrosis (>80 %). However, in the culture vessels vented by making a hole in the plastic cap and covering it with a microporous membrane (0.20 μm pore) and with vermiculite as the supporting substrate 100 % shoots rooted, and

the plants were healthy, free of shoot tip necrosis, hyperhydricity, and callus formation.

17.5.2 Contamination

Contamination is a serious problem for any tissue culture laboratory, especially for a commercial laboratory, as it can cause huge economic losses not only through direct loss of cultures but also throwing the production schedules in disarray. The loss because of contamination may be as high as 20 % in some commercial laboratories (Ilan and Khayat 1997). In well-established commercial labs, the problem of contamination is accentuated by the fact that the type of contamination changes with time. Initially, the bacterial contaminations are due to the ones associated with plants but with the passage of time the bacteria associated with humans may appear (Cassells 1991). The problem of contamination is aggravated by latent, slow growing bacteria which can be carried inside the tissue without detection and make their appearance at a very late stage when everything is apparently clean. Latent infection can also result in variable growth, tissue necrosis, reduced shoot proliferation, and rooting (Kane 2002; Sluis 2006; Rani and Dantu 2011). Therefore, culture indexing, whereby plantlets and tissues are assayed for the presence of internal or nonobvious bacteria using standard media such as nutrient broth and potato dextrose agar, which promote bacterial growth, is a common practice in commercial laboratories.

Movement of humans into the clean area cannot be prevented, although it may introduce fungal spores and bacteria carried on the body hairs and clothing into the sterile area. Therefore, entry into the clean area should be restricted, and those entering should have a head gear and pass through an air curtain. Frequent fumigation with formaldehyde or thiobendazole should be carried out to reduce the incidence of contamination (Debergh et al. 1990). Another serious problem is the movement of thrips in the culture rooms. These tiny insects multiply very rapidly in the conductive atmosphere of culture storage facility and cause havoc, destroying large number of cultures in a short time. Thrips can be controlled by daily spray of Baygon^{TM} or two treatments with 10 % dichlorovos at 5-day interval (Debergh et al. 1990). With the application of 5 mg L^{-1} acephate (*O,S*-dimethyl acetylphosphoramidothioate) and imidacloprid (1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine) the western flower thrips could be completely eliminated from the infested shoot cultures of apple (*Malus* x *dometica*) (Bhagwat and Lane 2003).

Mites are another pest that can multiply very rapidly in culture vessels (Pype et al. 1997). These cannot be identified through unaided eye and their presence becomes evident only after microbial infection, mostly fungal (Leifert and Cassells 2001). The fungal infection spreads so fast that discarding cultures becomes the only way of control. Healthy shoots of *Hibiscus moscheutos* from cultures heavily infested with mites and fungi could be retrieved by encapsulating them in sodium alginate capsules containing acephate and benomyl. Acephate killed mites while benomyl was effective in controlling fungal infection (West and Preece 2006). Regular checking for contaminated cultures should be done and those found infected must be removed immediately.

The most obvious consequence of cell culture contamination is the loss of time, money, and efforts spent in developing cultures and setting experiment. It may also adversely affect the cultures leading to inaccurate and erroneous experimental results and loss of valuable product.

17.5.3 Oxidative Browning

Many woody plants and some herbaceous species, when cultured in vitro cause browning of the medium. Sometimes the browning is so intense that the explants also turn brown/black, become necrotic, and eventually die (Ko et al. 2009). The explants release phenols which get

oxidized and cause browning of the medium. The oxidation products of the phenols could be phytotoxic. However, a blackened tissue should not be discarded without careful investigation. In mango, a large number of healthy embryos differentiated from absolutely black tissue (Ara et al. 1999, 2000).

The degree of browning depends on the species or genotype of the donor plants, age of the tissue (older tissues show more browning), season of initiation of cultures (more in winters and autumn), and composition of the medium. A simple method to protect the explant is initial frequent transfers (2–3 days) of the tissue within the same vessel or to a new vessel. Photo-oxidation of phenols can be reduced by keeping the cultures in dark initially. Elements such as Mn^{2+} ad Cu^{2+}, which are cofactors for the enzymes peroxidase and phenolase, respectively, should be reduced or eliminated to inactivate these enzymes and prevent browning (Debergh and Read 1991). Reduction in MS salt concentration and low light intensity (3–4 μmol cm^{-2} s^{-1}) reduced callus browning in *Actinidia arguta* (Han et al. 2010). Sometimes addition of adjuvants such as ascorbic acid (AA), cysteine-HCl or citric acid (antioxidants) to the culture medium helps to check browning of the explants and medium. In shoot proliferation cultures of the somaclonal banana cv. Formosana browning of the plants caused up to 100 % loss of cultures. Addition of 0.01 % AA not only prevented lethal browning of the shoots but also greatly increased the number of plantlets formed (Ko et al. 2009). However, AA was effective only if it was added to the surface of the medium; it was ineffective if included in the medium before autoclaving. The most effective treatment to combat the problem of explant browning in leaf segment cultures of *Sideritis trojana* was a combination of 100 mg L^{-1} of AA and 50 mg L^{-1} of citric acid (Cördük and Aki 2011). Other supplements to the medium that have helped preventing browning are polyvinylpyrolidone (PVP) and activated charcoal which adsorb phenols. These adsorbents may also adsorb useful compounds from the medium and, therefore their concentration should be selected judiciously. Initial culture in liquid medium helped initiating the cultures of *Rubus* (Zimmerman 1978) and *Eucalyptus citriodora* (Gupta et al. 1981) which are prone to browning.

17.5.4 Recalcitrance of Some Plants

Many plant species are not amenable to tissue culture procedure as they do not regenerate in vitro or show any axillary bud break. This recalcitrance is more pronounced in the tree species and can be overcome by: (i) using the most juvenile tissues of tree, (ii) repeated treatment with cytokinins can rejuvenate the donor plants, and (iii) coppicing of the tree may induce the development of new flush of shoots which could then be used to initiate cultures. In many plants such as *Prunus*, *Eucalyptus*, *Pinus* and *Sequoia,* repeated transfer to cytokinin medium reactivated their meristem (Thorpe et al. 1991).

17.5.5 Off-Types

A major problem faced by the commercial laboratories interested in clonal multiplication of desired plant varieties is the production of off-types in cultures. The appearance of abnormalities is more pronounced in regeneration schemes involving a callus phase. Adventitious shoot formation directly from explants is also not always free from abnormal off-types. It is important that such methods of regeneration should be avoided as far as possible. Where no alternative is available, the first generation of shoots can be obtained through callus or direct adventitious bud formation and these shoots should be used for subsequent production through forced axillary branching. The abnormalities developed in vitro are influenced by the choice of explant, media composition, growth regulator/s used, exposure periods to culture conditions, and the genotype of the plant.

The off-types can be distinguished only after transplantation to the field conditions when the plants are ready to flower. Date palm, banana,

and cardamom are three case points where abnormalities of in vitro produced plants devastated the commercial laboratories causing huge economic losses to the propagators and the farmers. Some of the tissue culture companies were closed because of these reasons. Several companies micropropagate banana on a very large scale for plantations. Almost 3–25 % of the tissue culture produced banana are affected by the development of the dwarf phenotype, which is characterized by plants that are short in height, produce small fruits with closer packing of the hands on the bunches, chocking of the branches when temperatures are below 15 °C, and the pseudostem fails to emerge out (Bhojwani and Razdan 1996).

A serious problem experienced by commercial growers with the micropropagated plants of the Ericaceae family (*Rhododendron, Kalmia* and *Pieris*) in mid-1980s was the formation of nonpathogenic tumors at or near the crown or base of the plants. Although the exact cause of this morphological abnormality is yet not clear, a probable reason suggested is cytokinin habituation causing tissue proliferation and adventitious shoot formation (Brand 2011). Some of the recommendations made in this regard, which have helped minimizing the problems, are: (i) regular initiation of fresh cultures from nontissue cultured plants, (ii) use of minimal concentration of cytokinin, (iii) correct identification of stock plants, (iv) avoidance of adventitious shoot production, and (v) rooting only distal tip shoot to avoid the base with clustered nodes (see Brand 2011). Another abnormality associated with the kind and concentration of cytokinin is the shoot tip necrosis (STN). Excessive accumulation of cytokinin in the tissues was associated with STN in *Harpagophytum procumbens* (Bairu et al. 2011). Some tissue cultured plants develop widened and flattened shoots (fasciated shoots) due to increased shoot tip meristem size and active cell division (Iliev and Kitin 2011).

It is necessary to check the genetic fidelity of in vitro produced plants at regular intervals even when the shoots are in cultures or at an early stage after transplantation. The trueness to type could be checked through cytological tests and/or use of molecular methods such as random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), DNA amplification fingerprinting (DAF), inter-simple sequence repeats (ISSR), sequence-tagged sites (STSs), and so on (*see* Chap. 12). The results obtained through molecular analysis of the in vitro shoots at an early stage of multiplication will help in discarding the off-types and modifying the protocol to obtain genetically uniform plants, thus increasing the confidence of the commercial laboratories and the farmers (Rani and Raina 2000). A National Certification System for Tissue Culture Plants was established in India by the Department of Biotechnology, Government of India. It has identified the Indian Agriculture Research Institute, New Delhi for virus diagnosis in the plants and Centre for DNA Fingerprinting and Diagnostic, Hyderabad for genetic fidelity.

17.5.6 High Cost

A large number of commercially important plants, including important vegetatively propagated vegetables, flowers, ornamentals, fruit trees, forest trees, and medicinal plants are propagated by tissue culture. Over a billion plants per year are produced by micropropagation (Towler et al. 2006). However, the micropropagated plants are generally more expensive than the conventionally propagated plants, because the micropropagation operation is capital intensive.

Cost calculations for a research laboratory, where the objective is to develop protocols, are neither feasible nor desirable. However, for a commercial set up it is of utmost importance in order to remain competitive in the market. The major portion of the TC plant cost is contributed by the manpower and electricity charges (Fig. 17.9). In developed countries the labor cost is the major contributor (∼70 %). Therefore,

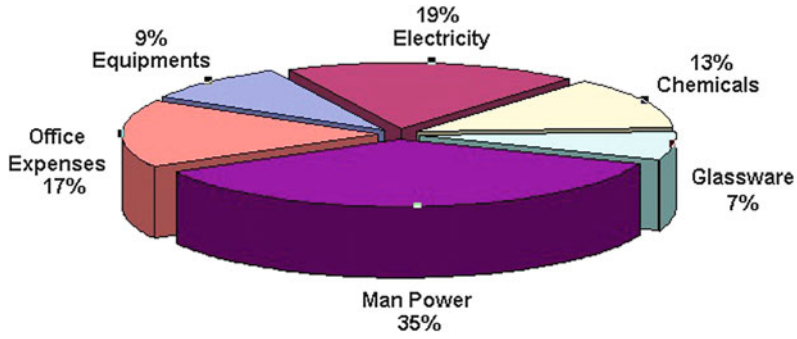

Fig. 17.9 Pie chart indicating contribution percentage share of different factors in tissue culture plants (*after* Tomar et al. 2008)

several tissue culture units have been established in developing countries, including India, where the labor is fairly low (35 %) (Tomar et al. 2008). These units are involved, mainly in contractual micropropagation for the growers in developed countries. Reducing in vitro stages, such as resorting to in vivo rooting of the shoots and introducing automation can contribute considerably toward cost reduction of tissue culture plants. To some extent, automation has been introduced in media preparation and during the hardening phase of micropropagation. Automation has also been attempted at the multiplication stage by use of robot and bioreactors, of which the latter holds greater potential.

17.6 Bioreactors

Bioreactors were initially developed for plant cell suspension culture (see Chap. 4) and secondary metabolite production (see Chap. 18), which were later modified and improved for somatic embryogenesis (see Chap. 7; Etienne et al. 2006) and hairy root culture (see Chap. 18) in many species.

Application of bioreactor technology for plant propagation was first reported by Takayama and Misawa (1981) in the case of *Begonia*. Since then the technology has been applied for large-scale propagation of lilies, strawberries, potato, *Spathiphyllum, Stevia*, and so on. (Takayama and Akita 2006). Somatic embryogenesis in liquid shake cultures or bioreactors has also been reported in alfalfa, caraway, carrot, coffee, celery, poinsettia, fa, *Eschcholtzia californica*, *Nerine*, *Octea catharinensis*, rubber and sweet potato (Ziv 2000; Etienne et al. 2006). The efficiency of propagation is quite high in bioreactors as compared to solid or shake cultures, resulting in the saving on equipment and labor costs (Table 17.2).

Due to shear sensitivity of plant systems, particularly shoot system, standard stirred tank bioreactors are not suitable for large-scale propagation of plants. Therefore, most frequently used bioreactors for the purpose are unstirred bubble column and air-lift bioreactors. The major advantage of air-lift bioreactors is their relative simple construction, the lack of region of high shear, reasonably high mass, and heat transfer and relatively high yields at low input rates (Ziv 2000).

The highly humid and the gaseous environments inside the bioreactor, comprised mainly nitrogen (78), oxygen (21), and CO_2 (0.036 %) and minor quantities of ethylene, ethanol, acetaldehyde etc., cause hyperhydricity of the shoots. One approach adopted to offset this problem is to add to the medium inhibitors of gibberellin synthesis to allow only limited elongation of leaves, resulting in the multiplication of shoots as clusters of meristems or buds (mass of highly reduced shoots). This system has been used for banana, ferns, gladiolus, *Nerine*, *Philodendron* and potato (Ziv 2000).

Akita et al. (1994) developed a 500 L separated impeller type bioreactor (1000 mm × 760 mm height) fitted with a porous disk type sparger (300 mm diameter) at the bottom and a cylindrical mesh in the center (Fig. 17.10) for the propagation of *Stevia rebaudiana* known for

Table 17.2 Comparison of the specifications for *Spathiphyllum* propagation in bioreactors and agar cultures. (*after* Takayama and Akita 2006)

Items	Bioreactor	Agar culture
Equipment		
Vessel volume	20 L	500 ml
Medium volume (L/vessel)	16.6 L (liquid)	100 ml (gelled)
Number of vessels	6	1000
Number of inocula used for subculture	96 test tubes	150 test tubes
Culture period	90 days	60 days
Culture space	0.5 m^3	36 m^3
Number of fluorescent lamps (40 W)	6	30
Labour		
Operational time	200 min	2500 min
[a]Medium preparation (100 L)	(60 min)	(450 min)
Autoclaving	(10 min)	(140 min)
Inoculation	(45 min)	(1250 min)
Transfer to culture room	(10 min)	(60 min)
Removing cultures	(45 min)	(300 min)
Vessel washing	(30 min)	(300 min)
Transplanting	1800 min	1800 min

[a] The volume of culture medium was 100 L in both bioreactor culture and agar culture

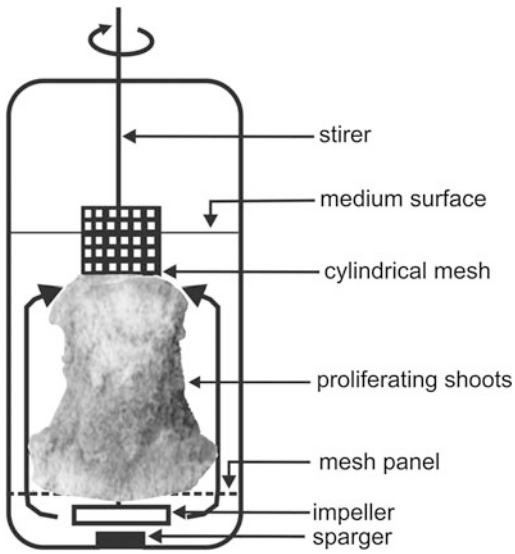

Fig. 17.10 Diagram of a separated impeller-type 500 L bioreactor used for mass propagation of *Stevia rebaudiana*. Shoot cultures in the bioreactor are illuminated with fluorescent lamps. The proliferating shoots could be seen adhering to the cylindrical mesh which can be taken out for harvesting propagules. (*based on* Takayama and Akita 2006)

the synthesis of noncaloric, thermostable intense sweetener stevioside, and rebaudioside-A (300–400 time sweeter than sucrose). In this bioreactor fitted with four fluorescent lamps, a multiplication rate of 140 times in a month was achieved. Cluster of shoots from shake cultures with modified MS medium (KNO_3, NH_4NO_3 and $CaCl_2.2H_2O$ reduced to ½ strength) supplemented with 0.1 mg L^{-1} NAA, 1 mg L^{-1} BAP and 3 % sucrose, served as the inoculum. The bioreactor containing 300 L of MS medium with 1 % sucrose was inoculated with 460 mg (FW) of the shoot primordia and aerated with 15 $Lmin^{-1}$ of compressed air was maintained at 25 °C under 16 h photoperiod. After 3 weeks of culture, 20 L of the medium was withdrawn and 50 L of fresh medium containing 6,300 g of sucrose was added to replenish the consumed nutrients and sucrose. Within a month the shoots multiplied to fill up the culture vessel and produced a biomass of 64.6 kg. The shoots lacked roots and could be easily transplanted with 90 % survival.

Permanent immersion of shoots in liquid medium is often deleterious for shoot growth and general health. Therefore, different types of temporary immersion bioreactors (Piao et al. 2003) such as ebb and flow in glass fermentor (Akita and Takayama 1994), Rita™ System (Teisson and Alvard 1999), twin-flask system (Jiménez et al. 1999), nutrient mist bioreactor (Hao et al. 1988), plastic bag cultivation (Brigoriadou and Leventakis 2003), and Liquid-Lab™ Rocker System (Kämäräinen-Karppinen et al. 2010) have been tried for the production of microtubers of potato.

Kämäräinen-Karppinen et al. (2010) used thin film Liquid-Lab™ Rocker (L-L-Rocker) System comprised of autoclavable polycarbonate plastic container (27 × 10 × 10 cm) with microporous patches attached to each side of the vial to allow gas exchange and eliminate ethylene and other gases built up in the head space of the vial. Liquid MS medium (125 ml) containing 2 % sucrose was used for shoot elongation and MS with 8 % sucrose for microtuber induction and growth phase. The L–L rocker was adjusted to move liquid from one side to another once per hour in the stem elongation phase and once every 2 h in tuberization phase. Shoot elongation was carried out under 16 h photoperiod and 22–27 μmol m^{-2} s^{-1} irradiance, and for microtuberization short day conditions (10 h photoperiod) was applied. The vegetative growth phase lasted 4–5 weeks and the tuberization phase 8 weeks (short culture time) or 10–11 weeks (long culture time). The mean number of microtubers formed per jar inoculated with 50 single node cuttings from 10 node shoots multiplied in stationary agar medium, varied from 30 to 75 depending on the cultivar. The majority (63 %) of the tubers were larger than 0.2 g which makes them suitable for minituber production.

One major problem encountered in large-scale liquid cultures in bioreactors is of contamination by microorganisms introduced with the plant material and during the operation process. In scaled-up liquid cultures the losses are even greater.

17.7 Photoautotrophic Micropropagation

With respect to the source of carbon and energy for plant growth, there are two types of micropropagation systems: (i) Heterotrophic system, in which the plant is entirely dependent on sugar in the culture medium (nonchlorophyllous cell and embryogenic cultures), and (ii) Photomixotrophic systems, in which the plant growth is dependent on photosynthesis and sugar in the medium (green shoot cultures). Considerable efforts are being made to develop Photoautotrophic micropropagation systems, in which the plants can grow in sugar-free medium.

The major disadvantages of the first two systems, in which the plants are grown on sugar-containing medium, are that with low CO_2 concentration in the vessel and low light intensity the plants are unable to do photosynthesis, as a result of which the plants exhibit a range of morphological, physiological, and phenotypical abnormalities. In contrast, under photoautotrophic conditions, in which the culture vessel is ventilated with CO_2 through a microporous filter and the cultures are maintained under high light intensity ($\sim$150 μmol m^{-2} s^{-1}), growth, and quality of the plants is considerably improved, and the rooting of the plants and their acclimatization ex vitro are also better. Moreover, in the absence of sugar in the medium contamination of the cultures is minimized. Photoautotrophic in vitro multiplication has been successfully applied to as many as 13 plant species that include *Acacia mangium*, *Brassica oleracea*, *Chrysanthemum morifolium*, *Citrus macrophylla*, *Coffea arabusta*, *Eucalyptus camaldulensis*, *Vitis vinifera*, *Solanum tuberosum*, *Rubus idaeus*, *Saccharum* spp, *Ipomoea batatas*, *Nicotiana* tabacum, and *Lycopersicon esculentum* (Kauota 2002).

A comparative study of micropropagation of calla lily (*Zantedeschia elliottiana*) and China fir (*Cunninghamia lanceolata*) under photoautotrophic (PAM) and photomixotrophic (PMM) conditions revealed that PAM reduced the period of in vitro multiplication as well as rooting to half

(from 30 to 15 days), improved the quality of the shoots, lowered the losses due to infection (from 5 % to 0), improved the quality of rooting in the absence of an auxin, resulted in higher survival ex vitro, and lowered the cost of production (Kozai and Xiao 2006). CO_2-enriched (1000 μmol mol^{-1}) photoautotrophic conditions, without sucrose and with vermiculite as the substrate in vented culture vessels significantly improved the rooting response (number and length of roots) of in vitro multiplied shoots of macadamia (*Macadamia teraphylla*) and the plants thus formed appeared healthy and exhibited increased survival after transplantation to soil in greenhouse (Chaum et al. 2011). However, PAM system suffers from certain key disadvantages which require to be addressed before its mass acceptability. The technique and knowledge required to control the in vitro environment are relatively complex and the expenses of maintaining light intensity and CO_2 concentration are very high.

17.8 The Indian Scenario of Micropropagation

In India, tissue culture research began nearly five decades ago primarily due to the efforts of the late Prof. P. Maheshwari and his colleagues at the Department of Botany, University of Delhi, and they were indeed the pioneers in this field in India. However, the importance of this technique dawned very late in India, many years after the Western world not only demonstrated but established many large-scale micropropagation companies. Even today, contribution of India is a miniscule percentage of the global production of plants through micropropagation.

Realizing the importance of tissue culture and other biotechnologies, the Government of India established the Department of Biotechnology (DBT) in 1986 to promote research in these areas. DBT soon recognized the potential of plant tissue culture technology in revolutionizing the commercial agriculture sector by enabling mass propagation of elite, high yielding, and disease free plants throughout the year, and identified it as a priority area and initiated a number of programs aimed at development and commercialization of the technology in an integrated manner. A number of research and development projects in various research institutes and universities have been supported for perfecting protocols of important plant species namely forest trees, horticulture crops, plantation crops, medicinal plants, and a large number of recalcitrant plants.

To demonstrate the feasibility of tissue culture technology for large-scale multiplication of elite species DBT set up two Tissue Culture Pilot Plant Facilities (TCPP) in 1989: (i) Tata Energy Research Institute (TERI), New Delhi, and (ii) National Chemical Laboratory (NCL), Pune. These two facilities refined and scaled up the protocols developed at other research centers for large-scale multiplication. The two TCPPs were later converted into Micropropagation Technology Parks (MTPs).

The major bottleneck of large-scale multiplication through tissue culture is the hardening procedure. The DBT also set up, on a priority basis, six regional hardening units catering to the requirements specific to different agro-climatic regions. These hardening units were established at: (i) Jai Narayan Vyas University, Jodhpur, (ii) Regional Plant Resource Centre, Bhubaneswar, (iii) G. B. Pant Institute of Himalayan Environment and Development, Almora, (iv) West Bengal Council of Science and Technology, Kolkota, (v) Regional Research Laboratory, Jammu, and (vi) Tata Energy Research Institute, Guwahati.

17.9 Applications of Micropropagation

A large number of commercially important plants are clonally propagated by tissue culture worldwide and over a billion plants are produced annually (Towler et al. 2006). Multiplication of plants in vitro from small parts has changed the concept of plant handling on a large scale and has been put to a varied number of applications. Plants grown or growing in vitro could be used to mass propagate selected cultivars or clones round

the year, transport pathogen-free plants safely, early release of a variety, and long-term storage. The major advantage of micropropagation is the possibility of establishing cultures from very small parts and to scale-up multiplication of a large number of plants in a short time and in limited space. One of the commercial labs, GrowMore Biotech (P) Ltd., propagates and supplies over 6 million plants in a year from a lab space of 1,300 sq. m (14,000 sq. feet) using 330,000 kw/h^{-1} of electricity/year and with 100 technical staff. The cost of a plant produced in the lab is between Rs 3 and Rs 7. The low cost of production is one of the indications for efficiency of this laboratory at the given level of power cost and salary (www.growmorebiotech.com).

It takes almost 10–15 years for the development of a new cultivar by a breeder and for scaling it up for release to farmers. Micropropagation can reduce this time by almost half. Actively growing plants in vitro being free of pathogens such as bacteria and fungi can pass quarantine easily and pave the way for quick introduction of new crops/plants. Because of over exploitation and habitat destruction several important medicinal plants have become endangered. In vitro multiplication is the strategy of choice for conservation of such endangered plants (Chap. 19). Plants cultured in vitro can be stored for long times by maintaining at low temperature (4 °C) and scaled up as and when required. Besides, by changing media composition, these cultures can be maintained at culture room conditions without subculture for almost 6 months or longer sometimes. Such conservation of plants in vitro is routinely carried out at National Bureau of Plant Genetic Resources (NBPGR), New Delhi.

Maintenance of a more commercially important sex in a dioecious species could be achieved only through tissue culture. The male plants of *Asparagus officinalis* are important for their spears. However, the seed population segregates into 1:1 male and female plants. The desired male plants can be scaled up through in vitro propagation for commercial release (Desjardins 1992). Similarly, papaya is a dioecious plant and the female plants that bear fruits can be increased in number using in vitro propagation (Jordan 1992; Mishra et al. 2007).

17.10 Concluding Remarks

Micropropagation is one of the in vitro techniques that has become an industrial technology, and is being used the world over for rapid clonal propagation of important horticultural, medicinal, and silvicultural plants. Indeed, tissue culture is the only commercially feasible method for clonal propagation of orchids. Micropropagation is particularly useful for the species which are difficult to propagate by the conventional methods of cloning or are of high value. It allows rapid clonal multiplication of plants under disease-free controlled environmental conditions round the year. It helps to maintain high health status of virus-free plants. Historically, commercial micropropagation is based on enhanced axillary branching by overcoming the natural apical dominance with cytokinin and other factors to encourage lateral buds to grow into shoots. The alternative systems being used for micropropagation are adventitious bud differentiation and somatic embryogenesis.

For routine clonal propagation this in vitro technique is still an expensive approach as it is labor intensive and requires a set up with high initial capital investment. Efforts are being made to make it cost-effective by scaling up the production through introduction of automation at different stages, such as the use of bioreactors for scaling-up multiplication, mechanization of subdividing the shoot clusters and transplantation at subcultures, and introduction of photoautotrophic micropropagation system (Sluis 2006; Kozai and Xiao 2006). However, so far a combination of human eyes, hands and brain are the practical approach to micropropagation in terms of accuracy of manipulation and economics.

17.11 Appendix

1. Protocol for micropropagation of the orchid—*Phalaenopsis* (*after* Paek et al. 2011)
   (i) Excise 3–4 cm segments from floral stalk at the 3–5 open flowers stage, each with single lateral bud. Surface sterilize the segments with 3 % sodium hypochlorite solution containing one drop of Tween 20 for 10 min. Rinse the segments three times in sterile water, trim-off the bleached ends of the sections and plant them on VW medium (Table 17.1) containing 2 % sucrose, 20 % coconut water (CW), 3 mg L^{-1} BAP or 0.1 mg L^{-1} TDZ, and 1 % agar.[1]
   (ii) Incubate the cultures at 26–28 °C under 16 h photoperiod and 30 µmol m^{-2} s^{-1} irradiance.
   (iii) After 1–2 months, when 2–3 leaves have developed, excise the youngest leaf and cut 5–7 one mm thick sections from its proximal end. Soak the segments in half strength MS liquid medium for 2 h.
   (iv) Plant the sections on half strength MS medium + 2 mg L^{-1} TDZ or 10 mg L^{-1} BAP + 10 mg L^{-1} adenine sulphate + 20 % CW +2 % sucrose + 0.23 % Gelrite, and pH adjusted to 5.7.
   (v) After incubation in dark at 27 °C for 1 week, transfer the cultures to 25 °C under 16 h photoperiod and 20 µmol m^{-2} s^{-1} irradiance for 6 weeks.
   (vi) Excise the PLBs from leaf segments, trim their apical portion to remove the small shoot and leaf and transfer them (20 pieces per 10 cm Petri plate containing 25 ml of medium) to protocorm multiplication medium (1 g L^{-1} each of Hyponex- N:P:K- 6.5:6:19 and Hyponex- N:P:K 20:20:20 + 2 g L^{-1} Peptone + 10 % CW + 30 g L^{-1} homogenate of unsprouted potatoes + 0.05 % activated charcoal (AC) + 0.8 % Agar, with pH adjusted to 5.5).
   (vii) Subculture the protocorms every 4 weeks as above. The protocorms can be cut into 2–4 longitudinal sections to enhance the rate of multiplication (10–20 protocorms per piece after 4 weeks). Since the basal tissue of protocorms shows genetic instability it should be discarded. Also discard the small, yellow protocorms at the time of subculture to avoid formation of abnormal plants. Do not extend the multiplication of PLBs from one explant beyond a year.
   (viii) Maintain the cultures at 25 °C under 16 h photoperiod and 30 µmol m^{-2} s^{-1} irradiance for 4 weeks.
   (ix) Once the PLBs develop the first leaf transfer them to 1st TP medium (1 g L^{-1} each of Hyponex- N:P:K- 6.5:6:19 and Hyponex- N:P:K 20:20:20 + 2 g L^{-1} Peptone + 10 % CW + 0.05 % AC + 0.8 % Agar, with pH adjusted to 5.5) as for symbiotic seed germination and subsequently to grow.
2. Micropropagation protocol for *Musa acc-uminata* (*after* Singh et al. 2011)
   (i) Remove sword suckers from healthy disease-free stock plants. Cut it to expose the shoot tip of 10 cm^3 and trim further to about 3 cm diameter and 5 cm length without damaging the meristem.
   (ii) Wash the shoot tip thoroughly in tap water, and surface sterilize with 0.1 % mercuric chloride for 10 min. Wash thoroughly under running tap water.
   (iii) Carefully remove the outer 1–2 leaves and trim the corm base with sharp sterilized scalpel. Wash it with sterile distilled water three times under aseptic conditions, and disinfect with

[1] Root tips (<5 mm long) from in vitro plants derived from floral stalk cuttings can also be used for clonal multiplication of *Phalaenopsis*. The root tip is planted with the cut end down. For PLBs formation from root tip TDZ is more effective than BAP (MS + 1 mg L^{-1} TDZ + 20 % CW + 10 mg L^{-1} Adenine sulphate + 0.23 % Gelrite).

Table 17.3 Culture media used at different stages of Banana micropropagation at the National Research Centre of Banana, India (*after* Singh et al. 2011)

Ingredients	Amount (mg L^{-1})		
	Initiation	Shoot multiplication	Rooting
KNO_3	1,900	1,900	1,900
NH_4NO_3	1,650	1,650	1,650
$CaCl_2.2H_2O$	440	440	440
$MgSO_4.7H_2O$	370	370	370
KH_2PO_4	170	170	170
Fe-EDTA	36.7	36.7	36.7
$MnSO_4.2H_2O$	22.3	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CoCl_2.6H_2O$	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025	0.025
L-ascorbic acid	10	10	–
Glycine	2	2	2
Nicotinic acid	0.5	0.5	0.5
Pyridoxine. HCl	0.5	0.5	0.5
Activated charcoal	–	–	2.5
BAP	4	4	–
IAA	1	1	–
IBA	–	–	1
NAA	–	–	2
Sucrose	3 %	3 %	3 %
Agar	–	0.75 %	0.75 %
Phytagel	0.2 %	–	–
pH	5.8	5.8	5.8

5 % sodium hypochlorite followed by 0.1 % mercuric chloride, each for 15 min.

(iv) Wash three times in sterile distilled water and trim to remove 1–2 mm of peripheral tissue of the corm.

(v) Transfer the shoot tip to culture initiation medium (Table 17.3; 30 ml medium in 250 ml glass jar container), and incubate the cultures at 24–26 °C, under 16 h photoperiod and light intensity of 1500–3000 lux.

(vi) As the explant turns green after 20–25 days remove the shoot tip from the jar, trim to remove the outer dead tissue and peel off 1–2 leaves to the base until fresh meristem tip gets exposed. Give two gentle cross incisions to the meristem tip and transfer it to multiplication medium (Table 17.3).

(vii) In 20–15 days the meristem would have produced a cluster of proliferating buds, and 1–3 axillary buds would have regenerated from the basal part of the explant around the central apical meristem.

(viii) In subsequent 3–4 weekly subcultures trim to remove the dead basal tissue and cut the shoot cluster into 3–4 pieces, each with up to four shoot buds and transfer to fresh multiplication medium.

(ix) After 5–6 cycles of shoot multiplication transfer the proliferating shoots to (Table 17.3) rooting medium. Proliferating shoots can also be rooted in polybags under greenhouse conditions to reduce the cost and better establishment of the plants ex vitro.

(x) For hardening, transfer the plants from culture vessels and leave exposed in a room at ambient temperature for 4–6 days.

(xi) Wash the plants (8 cm shoots with 3–4 ramified roots) to remove the agar medium and plant individually in micropots in a protray. Keep them under high humidity (90–95 %) and diffuse light for 6–8 days. Reduce the humidity and raise the light intensity gradually.

(xii) After 5–6 days remove the plants from micropots, dip in fungicide solution (0.1 % Bavastin) and plant in polybags and maintain under 70 % RH, which is gradually reduced to 40 %.

3. Micropropagation protocol for *Gladiolus* (*after* Dantu and Bhojwani 1992)

(i) Dehusk the corm and scoop out individual axillary buds along with corm tissue. Trim the corm tissue to 5 mm^3.

(ii) Rinse the excised buds in 90 % ethanol, air dry for 15 min, dip in a 0.5 % solution of Cetavlon or any other surfactant for 5 min and wash thoroughly under running water for 60 min. Surface sterilize the buds in a 2.5 % (w/v) solution of sodium hypochlorite for 15 min and give three quick rinses in sterile distilled water under aseptic conditions.

(iii) Trim the peripheries of the corm tissue and plant the bud on MS + 0.5 mg L^{-1} BAP (BAP concentration might vary with cultivar) and incubate the cultures at 25 °C in continuous light of 10 W m^{-2} s^{-1} irradiance.

(iv) After two passages of 4 weeks each on the same medium as in step (iii), when multiple shoot buds have developed, cut the bud cluster into pieces, each bearing at least five buds and transfer them individually to shoot multiplication medium (Table 17.4).

(v) At the end of 4 weeks again cut the shoot clusters into small propagules each bearing about 10 buds and transfer them individually to fresh shoot multiplication medium (Table 17.4). At this stage the cultures are incubated at 25 °C in complete dark.

(vi) For shoot elongation, cut the shoot clusters into small clumps each bearing about 10 shoots and transfer them to liquid MS medium containing 0.5 mg L^{-1} BAP. Incubate the cultures as in step (iii).

Table 17.4 *Gladiolus* shoot multiplication medium (*after* Dantu and Bhojwani 1992)

Constituents	Amount (mg L^{-1})
Macronutrients	
NH_4NO_3	825
KNO_3	1,900
$CaCl_2.2H_2O$	400
$MgSO_4.7H_2O$	370
NaH_2PO_4	300
Micronutrients	
H_3BO_3	6.2
$MnSO_4.2H_2O$	22.3
$ZnSO\text{-}_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.125
$CuSO_4.5H_2O$	0.0125
$CoCl_2.6H_2O$	0.0125
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
Organic nutrients	
Myo-Inositol	100
Nicotinic acid	0.5
Pyridoxine.HCl	0.5
Thiamine.HCl	0.1
Glycine	2.0
Growth regulator	
BAP	0.5
Sugar cubes	30,000

Table 17.5 Culture media for adventitious shoot differentiation from leaf discs (shoot multiplication) and rooting of the shoots (rooting) of *Ficus lyrata* (*after* Jona and Gribaudo 1987)

Constituents	Amounts (μM)	
	Shoot Multiplication	Rooting
Macronutrients		
NH_4NO_3	9,000	–
KNO_3	9,400	–
$CaCl_2$ $2H_2O$	1,500	–
$MgSO_4 \cdot 7H_2O$	750	500
KH_2PO_4	500	1,000
$Ca(NO_3)_2.4$ H_2O	–	2,250
KCl	–	900
Micronutrients		
H_3BO_3	161	161
$MnSO_4 \cdot 4H_2O$	112	112
$ZnSO_4 \cdot 7H_2O$	35	35
$Na_2MoO_4 \cdot 2H_2O$	1	1
$CuSO_4 \cdot 5H_2O$	0.1	0.1
Iron		
$FeSO_4 \cdot 7H_2O$	100	50
$Na_2EDTA.2H_2O$	100	50
Sucrose	2 %	1 %
Other growth factors		
Myo-Inositol	550	550
Nicotinic acid	40	40
Pyridoxine·HCl	2.5	2.5
Thiamine. HCl	15	15
Folic acid	1	1
Biotin	0.2	0.2
Amino acids		
Glycine	25	25
Growth regulators		
BA	24	–
2iP	24	–
Kinetin	24	–
IBA	12.5	–
NAA	–	5
Agar	0.7 %	0.7 %

(vii) After 4 weeks separate individual shoots (ca 10 cm long) transfer them individually to MS liquid medium with 6–10 % sucrose for corm formation. Incubate the cultures in continuous light. Corms should be ready for harvest in 10–12 weeks.

(viii) Remove the corms, wipe off the medium, and store them at 5 °C for 6–8 weeks.

(ix) Sow the corms directly in the field following the standard horticultural practice. The corm should sprout within a fortnight.

4. Micropropagation protocol for *Ficus lyrata* (*after* Jona and Gribaudo 1988)
   (i) Select fully opened but not completely expanded leaf that is still very soft as the source of explants. Bring the leaves to the laboratory for further preparation. Care should be taken that the mother plant is growing in bright conditions and is healthy.
   (ii) Wipe the whole leaves with 70 % ethanol, and allow it to dry. Dip the leaves in water containing 2–3 drops of Savlon for about 5 min followed by thorough washing under running tap water for about 15 min. Place the leaf on a Petri plate and remove the margins on both side of the mid rib by cutting with a sharp blade leaving about 1–1.5 cm of margin on both sides of the mid rib. Cut the mid rib portion of the leaf into 1 cm long pieces resulting in 1 × 1.5 cm ready to use leaf explants.
   (iii) Sterilize the leaf pieces in 1 % sodium hypochlorite solution for about 12 min. Remove all traces of hypochlorite by washing 3–4 times with sterile distilled water in a laminar air flow hood.
   (iv) Inoculate the leaf pieces on the initiation medium, consisting of Nitsch and Nitsch (1969) basal medium + 24 μM BAP + 24 μM Kn + 24 μM 2iP + 12.5 μM IBA in culture tubes (Table 17.5). Incubate the cultures at 24 ± 1 °C and continuous light. Leave the cultures undisturbed for almost 3 months. If medium in the culture tubes is sufficient there is no need of transfer to fresh medium.
   (v) At the end of 3 months tiny adventitious shoots could be seen arising all along the margins. In another fortnight these shoots grow slightly big. At this stage, the leaf explants along with the young shoots are transferred to fresh medium to allow the shoots to grow.
   (vi) The cluster of shoots is cut into small clumps and inoculated into fresh medium of the same composition for shoot multiplication.
   (vii) Individual shoots, about 5–6 cm long, are transferred to the rooting medium (Table 17.5).
   (viii) Rooted shoots are acclimatized in a net house.

Suggested Further Reading

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Production of Industrial Phytochemicals

18

18.1 Introduction

The higher plants synthesize an overwhelming range of small organic molecules that are not directly involved in primary metabolic processes of growth and development of the plant but serve it in a variety of other ways, such as chemical defence against microorganisms, insects and higher predators, and as attractant of pollinators and seed dispersing agents. These secondary metabolites include over 25,000 terpenoids, 12,000 alkaloids, and 8,000 phenolic compounds (Sato 2003). Many of these natural phytochemicals have been used by man to produce a large number of commercial products, such as industrial chemicals, pharmaceuticals, antimicrobial agents, insecticides, cosmetics, dyes, fragrance, and flavoring agents (Lila 2005). The plants are renewable natural resource of these valuable compounds produced at normal temperature and pressure without emitting toxic effluents or gasses.

With upsurge in preference for natural, ecofriendly, and renewable resources; the demand for natural plant products, including traditional medicines, is increasing. The developed countries market of herbal drugs, which was about US$ 30 billion in 1998, doubled to US$ 60 billion in 2003 (Kamboj 2005). Moreover, some of these compounds are difficult to synthesize due to their structural complexities and several chiral centers (Michael et al. 2005), and novel compounds are still being discovered in plant extracts as more and more hitherto unsurveyed plants are being analyzed. The natural plant products also serve as model compounds for chemical synthesis of more potent analogs (Michael et al. 2005; Schmidt et al. 2007).

Destruction of natural habitats of wild plants and environmental and geographic instabilities is making it difficult to acquire enough plant-derived chemicals from whole plants. Plant cell, tissue, and organ cultures under controlled physical and chemical conditions provide an attractive alternative to whole plant for the production of high value industrial phytochemicals (Dantu and Bhojwani 2005). Some of the advantages of this technology over the conventional approach of extraction of industrial phytochemicals from whole plants are:

1. In cultures, factory-type production of natural compounds can be carried out throughout the year, unaffected by the season, pests and diseases, climate, and geographical constraints.
2. The risk of crop failure due to natural hazards and the danger of extinction of some species due to their mass extraction from the natural populations are eliminated.
3. A more consistent product quality could be assured with cultivation of selected cell lines under controlled conditions.
4. The intensive nature of production of compounds in cultures might release land for the production of food and cash crops.
5. Biotic and abiotic stresses (elicitors), which are known to enhance the accumulation of

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_18, © Springer India 2013

valuable secondary metabolites, qualitatively and quantitatively, can be introduced into the medium to improve the productivity of the cultures.

6. Due to technological advancements, several cell lines capable of synthesizing the natural compounds in quantities higher than the whole plant have been isolated (Table 18.1). Some of the systems have been commercialized (Table 18.2).
7. Cell cultures not only provide means for de novo synthesis of natural products but also serve as factories for bioconversion of low value compounds into high value products.
8. Novel compounds not known in intact plants may be produced by cell cultures. At least 85 novel compounds, including 23 alkaloids, 30 quinones, and 11 phenol compounds have been isolated from 30 different plant cell culture systems (Phillipson 1990).
9. Genetic manipulation of single cells to improve yields and quality of the product is easy.
10. Extraction of products from in vitro tissues is much simpler than the complex in vivo tissues because of the absence of significant amount of pigments, making efficient downstream recovery, and product yield.

In the last half-century many technological advances have been made in this field. This chapter deals with the strategies used to improve metabolite production in cell, tissue, and organ cultures.

Table 18.1 Some examples where cell cultures produced secondary metabolites at higher levels than the whole plant

Plant species	Compound	Yield (% Dry Weight)	
		Culture	Plant
Lithospermum erythrorhizon	Shikonin	20	1.5
Panax ginseng	Ginsenoside	27	4.5
Morinda citrifolia	Anthraquinones	18	0.3
Coleus blumeii	Rosmarinic acid	13	3
Thalictrum minor	Berberine	10	0.01

Table 18.2 Some examples of commercial production of secondary metabolites in vitro

Compound	Culture system	Plant
Ginseng	Hairy root cultures	*Panax ginseng*
Shikonin	Cell cultures	*L. erythrorhizon*
Berberin	Cell cultures	*Coptis japonica*
Taxol	Cell cultures	*Taxus baccata*

18.2 Strategies to Optimize Phytochemical Production in Vitro

18.2.1 Culture Conditions

The productivity of cell lines is greatly influenced by culture conditions, of which culture medium is the most important. In general, growth and production of secondary metabolites are inversely related, both, in whole plants and in tissue cultures. Consequently, in tissue cultures on media defined for optimum growth, production of secondary metabolites occurs in the late stationary phase when the medium gets depleted of some of the important constituents. Growth inhibition is often associated with cytodifferentiation and induction of enzymes for secondary metabolism. In such cases, a 'dual culture system' is preferred. It involves biomass production in a medium optimum for cell proliferation ('growth medium') followed by transfer of healthy cells to a different medium ('production medium'), which does not support good growth of the cells but is favorable for product yield. Zenk et al. (1977) were the first to use this strategy for the production of alkaloids by *Catharanthus roseus* cells. This strategy was also applied for commercial production of Shikonin by cell cultures of *Lithospermum erythrorhizon* (Fujita et al. 1982).

The most useful modifications made in the growth medium to promote secondary metabolite production are: (a) reduction or elimination of 2,4-D or other phytohormones, (b) reduction

in phosphate level, and (c) increase in the sucrose level or alteration of carbohydrate (C)/nitrogen (N) ratio.

Fujita et al. (1981) observed that the growth of *L. erythrorhizon* cell cultures was best on LS medium (Linsmaier and Skoog 1965), but shikonin derivatives were produced only on White's medium (White 1954). This is because of the type of nitrogen in the two media. Stable production occurred when nitrogen was supplied solely as nitrate, as in White's medium. Even part substitution of nitrate with ammonium strongly inhibited shikonin production. Therefore, Fujita et al. devised a two-stage method. The first stage involved biomass production in LS medium followed by the second stage of shikonin production in White's medium. By further modification of LS and White's media, they developed MG-5 and MG-9 media (Table 18.3), respectively, which enhanced shikonin production 13-fold over that obtained in earlier media (Fujita 1990). The effect of nitrogen on alkaloid production is dependent on the carbon available to the cells, which makes the C/N ratio an important factor to be taken into account (Moreno et al. 1995).

Optimum media for the production of different alkaloids by a cell culture system may also vary. In cell cultures of *C. roseus* the optimum production of ajmalicine occurred on a medium containing nitrate and high glucose concentration and lacking phosphate and ammonium, whereas for the production of tryptamine, low glucose, negligible amount of nitrate, and presence of high levels of phosphate and ammonium were required. Increasing sucrose level to 6 % favored both growth and alkaloid production in the same medium, eliminating the need for two-stage culture system.

Plant growth regulators effect growth and differentiation and thus effect secondary metabolite production by cell cultures. Auxins are generally included in the medium to increase biomass of cell cultures and are omitted for secondary metabolite production. The physical environment, such as pH of the medium, light, and gasses in the enclosures also play important role in the production of secondary metabolites in cell cultures.

Table 18.3 Composition of MG-5 and M-9 media for biomass and shikonin production, respectively, in cell cultures of *L. erythrorhizon* (*after* Fujita 1988)

Constituent	Amount (mg L^{-1})	
	MG-5	MG-9
NH_4NO_3	500	–
KNO_3	1,900	80
$NaNO_3$	2,480	–
$Ca(NO)_3.4H_2O$	–	694
KH_2PO_4	170	–
$NaH_2PO_4.2H_2O$	–	19
KCl	–	65
$CaCl_2.2H_2O$	150	–
$MgSO_4.7H_2O$	120	750
$MgCl_2.6H_2O$	203	–
Na_2SO_4	–	1,480
$FeSO_4.7H_2O$	27.8	–
$Na_2.EDTA.2H_2O$	37.3	–
$Na_2.EDTA.3H_2O$	–	1.8
$MnSO_4.4H_2O$	22.3	–
$ZnSO_4.7H_2O$	8.6	3
H_3BO_3	1.9	4.5
$Na_2MoO_4.2H_2O$	0.25	–
$CuSO_4.5H_2O$	0.025	0.3
Sucrose	30,000	30,000
Inositol	100	–
Thiamine.HCl	0.4	–
Indole-3-acetic acid	–	1.75

18.2.2 Genetic Enhancement

Plant cell cultures are heterogenous with regard to the metabolic productivity of their constituent cells, and in cultures the pool of variation further increases. Selection and cloning of high yielding cells from this spontaneous variability is an effective method to improve in vitro production of secondary metabolites. For colored compounds, such as berberine, shikonin, and betaline, it is very convenient to visually identify and isolate the cells of interest. Cells with highly fluorescent compounds may be detected under UV light using fluorescence microscope. Flow cytometry enables rapid analysis and subsequent sorting of a large number of single cells based on their fluorescence (Aiken and Yeomen 1986;

Adams 1990). For colorless compounds specific reactions of squashed cells or immunological tests of their extracts may help to identify best clones.

It is important that the selected lines are stable over several subcultures. Cell lines of *C. roseus* showing 10-fold increased production of ajmalicine and serpentine were highly unstable and often reverted back to the level of unselected lines. However, cell lines of *Euphorbia millii* (Yamamoto et al. 1982) and sweet potato (Nozue et al. 1987) with stable production of anthocyanin have been established. Similarly, stable high shikonin producing cell lines of *L. erythrorhizon* were obtained based on protoplast cloning (Fujita et al. 1985). It has been possible to isolate high menthol yielding somaclones of *Mentha arvensis* by adding menthol in the medium as a selection system (Shasany et al. 2000; Dhawan et al. 2003).

A new approach to genetic enhancement of cell cultures for the production of phytochemicals is biochemical engineering of enzymes of their biosynthetic pathway. The first example of successful modification of a medicinal plant for its secondary metabolite is the introduction of cDNA encoding hyoscyamine-6β-hydroxylase (*h6h*) from *Hyoscyamus niger* into *Atropa belladonna* which normally produced hyoscyamine as the main alkaloid and very little of scopolamine (Yun et al. 1992). The hairy root cultures of the transgenic *A. belladonna* exhibited increased hyoscyamine hydroxylase activity and produced five times more scopolamine than the wild-type hairy roots (Hashimoto et al. 1993). Root cultures of *H. muticus* carrying the transgene *h6h* produced over 100 times more scopolamine than the wild-type control (Jouhikainen et al. 1999). Hairy root cultures of *Datura metel* overexpressing the SAM N-methyltransferase gene, that encodes for putrescine, accumulated higher amounts of tropane alkaloids (hyoscyamine and scopolamine) than the control hairy roots (Moyano et al. 2003). Overexpression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR) gene (*hmgr*) from *Catheranthus roseus* in *Artemesia annua* enhanced the production of artemisinin by the transgenic plants up to 39 % higher than the nontransgenic plants of *A. annua* (Nafis et al. 2011).

Resveratrol, a phytoalexin, found in grapes, red wine, and other foods, exhibits cancer chemopreventive activity. It acts as antioxidant and antimutagen and induces Phase II drug mobilizing enzyme. Chia and Ng (2003) isolated cDNA coding for resveratrol from *Vitis vinifera* and introduced it into lettuce, which produced resveratrol at an amount higher than that in red wine.

A rice variety, called "Golden Rice", has been developed by genetic engineering of carotenoid pathway by combining a daffodil gene and a bacterial gene involved in terpenoid pathway, as a result of which β-carotene, a vitamin A precursor (Provitamin A), accumulates in the grain imparting it golden color (Ye et al. 2000; see Sect. 15.5.5).

It is now possible to create transgenic plants with multiple gene transfers for producing completely alien metabolites involving multiple steps. Transgenic plants of *Arabidopsis* with stable expression of the 3-step pathway for dhurrin biosynthesis have been developed. Similarly, *Arabidopsis* plants have been created that accumulate cyanogenic glucoside up to 4 % D.W. by inserting a gene from *Sorghum bicolor* (Tattersall et al. 2001). RNAi technology (see Chap. 15) has been used to develop genetically modified poppies that accumulate reticuline, a benzylisoquinoline alkaloid, instead of the usual narcotic morphine (Allen et al. 2004). Such genetically modified plants may yield tissue cultures capable of producing the desired compounds in higher quantities.

18.2.3 Elicitation

Exposure of cell or hairy root cultures to a variety of biotic [enzymes, cell wall fragments of microorganisms, polysaccharides from microorganisms (chitin, glucan), glycoproteins, and phytochemicals produced by plants in response to physical damage, fungi or bacteria attack, polysaccharides from plant cell wall (pectin, cellulose), chitosan, glucans, salicylic acid, methyl jasmonate that are formed by the

action of plants on microbial cell wall] and abiotic (inorganic salts, heavy metals, UV irradiation, high salinity, high and low osmolarity, extreme temperatures, and high pressure) factors have been shown to improve the yield of secondary metabolites (Cai et al. 2012; Wink et al. 2005; Table 18.4). An advantage of using these elicitors is that the products are often released into the medium (Brodelius 1990).

Addition of conidia of the fungus *Verticillium dahliae* to cell cultures of *Gossypium arboreum* raised the yield of gossypol from 5–10 to 500 mg L^{-1} within 5 days (Heinstein 1985). Similarly, solubilized chitin could elicit sanguinarine production by cell cultures of *Papaver somniferum* (Kurtz et al. 1990). Quite often fungal elicitors have been used in combination with other factors, such as chemicals (*Verticillium* with oxalate for gossypol production; Asada and Schuler 1989). Simple inorganic molecules, such as NaCl, have been shown to increase catharanthine production in *C. roseus* cultures by 90 % over the control (Smith et al. 1987). Vanadyl sulfate added to the cell cultures of *C. roseus* promoted the production of catharanthine, serpentine, and tryptamine (Tellevi and DiCosmo 1988).

Addition of methyl jasmonate to the culture medium improved the accumulation of ginsenosides in cell suspension (Wang and Zhong 2002) and hairy root (Yu et al. 2002) cultures of ginseng and taxol production (17–19 %) by *Taxus* cell cultures (Wang et al. 2001). Three days exposure of hairy root cultures of *Plumbago indica* to chitosan (200 mg L^{-1}) and methyl jasmonate (80 μM) significantly improved total plumbagin production (13.6 mg g^{-1} D.W. as compared to 5.29 mg g^{-1} D.W. in the untreated controls) (Gangopadhyay et al. 2011). The elicitor treatment also induced secretion of 34 % of the metabolite produced. There was no leaching in the untreated cultures.

Most of the culture systems respond to the elicitors when applied during the growth phase (Eilert et al. 1985; Kurtz et al. 1990).

18.2.4 Biotransformation

The chemical conversion of exogenously supplied substances by living cell cultures, permeabilized cells, or entrapped enzymes derived from cell cultures into high value products is referred to as biotransformation. The substances used for biotransformation may be natural or synthetic, and the product could be novel or compounds already known to occur in plants.

The cost of production of arbutin by biotransformation of hydroquinone using cell cultures of *C. roseus* is comparable to the chemical process. A semisynthetic method has been developed for the production of vinblastine, an anticancerous drug, from *C. roseus* cell cultures through biotransformation (Misawa et al. 1988). The dimeric alkaloid 3,4-anhydrovinblastin has been produced by coupling of the monomeric alkaloids catharanthine produced by cell cultures and commercially available vindoline with the aid of cell-free enzyme extracted from suspension cultures of *C. roseus*. Similarly, cell

Table 18.4 Effect of some elicitors on secondary metabolite production

Cell culture	Elicitor	Product	Increase over the control (%)	Reference
Catharanthus roseus	*Pythium*	Ajmalicine	400	Asada and Shuler (1989)
Morinda citrifolia	Chitin	Anthraquinones	2.3	Dornenburg and Knorr (1997)
Rubia tinctorum	Chitosan	Anthraquinones	2.2	Vasconsuelo et al. (2004)
Papaver bracteatum	Dendryphion	Sanguinarine	9	Dicosma and Misawa (1995)
Vitis vinifera	Jasmonic acid	Anthocyanins	2.2	Curtin et al. (2003)

cultures of *Digitalis lanata* and *D. purpurea* were able to rapidly transform exogenously supplied progesterone to pregnane (Graves and Smith 1967). Leaf and root cultures of *D. lanata* and shoot forming cultures of *D. purpurea* accumulated an increased amount of digoxin and/or digitoxin when progesterone was added to the cultures (Hagimori et al. 1980; Lui and Staba 1981).

Paclitaxel yields in the cell cultures of *Taxus cuspidata* were improved up to six times by feeding phenylalanine and other potential paclitaxel side-chain precursors (Benzoic acid, N-benzoylglycinen, serine; Fett-Neto and Di-Cosmo 1996). Addition of phenylalanine, one of the biosynthetic precursors of rosmaric acid, to *Salvia officinalis* suspension cultures stimulated the production of rosmaric acid and shortened the production time as well (Smetanska 2008).

A step-wise feeding of 50 mg L^{-1} of cholesterol (in four instalments), a precursor of conessine alkaloid biosynthesis, to cell suspension cultures of *Holarrhena antidysenterica*, during growth phase enhanced the production of the alkaloid from 63 mg L^{-1} to 106 mg L^{-1} in 6 L stirred tank bioreactor (Panda et al. 1992).

18.2.5 Immobilization of Cells

One of the major problems in commercialization of cell culture-based processes for secondary metabolite production is its high cost due to slow growth of plant cells, low product yield, genetic instability of selected lines, low shear resistance of cells, and intracellularization of the product. Some of these problems can be overcome by immobilization of cell cultures by entrapping them in gels (calcium alginate, agar, agarose, carrageenen), polyurethane foam or within membrane reactors. Immobilization of cells on the surface of an inert support such as fibreglass mats and unwoven short fiber polyester has also been examined for in vitro production of secondary metabolites.

Immobilization of cells is applicable to only such systems where the production process involves two steps. In the first step, conditions are optimized for biomass production by suspension cultures and in the second stage the conditions are optimized for product formation by immobilized cells.

Immobilized cells of *Capsicum frutescens* produced almost 50 times more capsaicin than in suspension cultures (Lindsey et al. 1983). Similarly, diosgenin production was enhanced by almost 40 % over the suspension cultures by, basically, entrapping the cells of *Dioscorea deltoidea* into polyurethane foam cubes and growing them in a medium with 3 % sucrose (Ishida 1988). However, commercial production of secondary metabolites by immobilized cells is yet to be developed.

18.2.6 Permeabilization

Generally, the secondary metabolites synthesized by plant cells accumulate intracellularly making their efficient and continuous production very difficult. Although these products can be extracted from the cells using destructive methods, it would be more desirable if the metabolites can be sequestered from the cells without undue loss of their viability and synthetic ability, so that each culture can be used for repeated cycles of secondary metabolite production and harvest. It would be helpful in reducing the cost of in vitro production of industrial chemicals, which is currently very high.

Permeabilization of cells to allow controlled release of products into the medium has been attempted by various techniques, such as dimethylsulfoxide (DMSO) application (Parr et al. 1986), low pH of the medium, sonication with continuous ultrasound and electric treatments (Hunter and Kilby 1990). The essential feature of such permeabilization is that it could be reversible (Parr et al. 1986).

18.3 Removal of Secreted Products

The secondary metabolites released into the medium naturally or through permeabilization of cells may suffer enzymatic or nonenzymatic degradation. Therefore, it is important that the secreted metabolites are removed from the medium. It would also eliminate any feedback inhibition regulating the biosynthetic pathway. A number of experiments have been carried out, using 'two phase cultures' to trap excreted products into the 'second phase' which may be liquid or solid.

Amberlite XAD-7 was used to adsorb indole alkaloids (ajmalicine and serpentine; Payne et al. 1988) and anthraquinones (Robins and Rhodes 1986) from the culture medium of *C. roseus* and *Cinchona ledgeriana*, respectively. Activated charcoal (Knoop and Beiderbeck 1983) and reverse phase silica (Becker and Herold 1983) are solid phases employed to increase the production of secondary metabolites in cultures.

18.4 Hairy Root Cultures

Valuable secondary metabolites can be produced in callus and suspension cultures of a wide range of plants. However, in the plants where secondary metabolite production is organ specific, in vitro production of a metabolite occurs only when callus or cell culture is induced to undergo organogenesis or embryogenesis (Conastabel and Kurz 1999). A prominent example is ginseng (*Panax ginseng*), which produces saponins and other valuable metabolites in the roots. In this plant, root cultures are required to produce the phytochemicals. Similarly, undifferentiated calli of *Atropa belladonna* do not produce the alkaloid hyoscyamine which, however, was produced on differentiation of roots (Raj Bhandary et al. 1969). The roots are capable of accumulating a large range of secondary metabolites, reflecting their biosynthetic capacity.

The major problem in phytochemical production by in vitro culture of normal roots is their slow growth rate. In contrast, *Agrobacterium rhizogenes* (a gram negative, soil bacterium) induced hairy roots are characterized by extensive branching, high growth rate under hormone-free condition, low doubling time, ease of maintenance, and genetic stability (Srivastava and Srivastava 2007). The ability of the hairy roots to produce secondary metabolites at levels comparable to or greater than that of intact plants, like the normal roots, changed the prospects for metabolite production by root culture dramatically (Ciau-Uitz et al. 1994; Seven and Oksman-Caldentey 2002; Lila 2005; Srivastava and Srivastava 2007). Manipulation of culture medium (Vozumi et al. 1993), physical factors (Yu et al. 2005), O_2 starvation (Giri and Narasu 2000), metabolic engineering (Hu and Du 2006) etc., have been tried to improve the yield of target products by hairy root cultures. Growth rates as high as 2,500–5,000-fold increase of biomass in 3 weeks could be obtained in the hairy root cultures of *Hyoscyamus muticus* (Flores et al. 1987) which is faster than the fastest growing suspension cultures. In some hairy root cultures certain metabolites are secreted into the medium. For example, in *Glycyrrhiza uralensis* hairy root cultures up to 98 and 94 % of licochaleone A and flavonoids, respectively, were secreted into the culture medium (Zhang et al. 2011). Similarly, in *Arachis hypogaea* hairy root cultures over 90 % of the resveratrol arachidin-1 and arachidin-3 were accumulated in the medium (Condori et al. 2010).

Production of secondary metabolites by hairy root cultures equal to or greater than normal roots has been reported in *Beta vulgaris*, *Valeriana officinalis* and *Lupinus*. The yields of camptothecin have been low in suspension cultures of *Camptotheca acuminata* or *Nothapodytes foetida* (0.0003–0.01 %) but a good production (0.1–0.3 % dry weight) of this compound occurs in root and hairy root cultures of *Ophiorrhiza pumila, O. mungos and C. acuminata* (Wink et al. 2005). Hairy root cultures of *Trigonella foenum-graecum* produced twice the amount of diosgenin than the normal roots (Merkli et al. 1997). *Atropa baetica* hairy roots

synthesized and accumulated a conspicuously high amount of alkaloid (Zárate 1999).

The feasibility of exploiting hairy root cultures as a commercial source of bioactive compounds depends on the development of suitable bioreactor (Fig. 18.2). While designing bioreactors suitable for hairy root culture, several physical and chemical parameters (nutrient availability, nutrient uptake, oxygen depletion in the medium, mixing, and shear sensitivity) must be taken into consideration (Srivastava and Srivastava 2007).

Hairy roots provide an efficient way of biomass production due to fast growth and displays high biosynthetic capabilities comparable to those of normal roots in nature. Hairy root cultures have been established for ginseng but there are reservations for utilizing hairy roots for healthy food (Choi et al. 2006). Therefore, hairy-like adventitious root (HLAR) culture system has been developed by culturing normal root tips of ginseng in the presence of auxin (Son et al. 1999, 2000). HLARs grown in 20 L balloon type bubble bioreactor showed 30 times increase in biomass after 42 days of culture (Choi et al. 2006).

The hairy root cultures of *Catharanthus* with modified strictosidine and the transgene for bacterial halogenases could synthesize chlorinated tryptophan and other downstream products such as monoterpene indole alkaloids, including 10-chlorocatharanthine and 13-chlorotabersonine (Runguphan et al. 2010).

In hairy root cultures of *P. ginseng* biomass accumulation and ginsenoside production were optimal under 20 °C/13 °C and 16 h/8 h day/night cycle. Biomass production was higher in dark and red light, whereas ginsenoside accumulation was optimal under fluorescent light (Yu et al. 2005).

18.5 Bioreactors

Cell suspension cultures have more immediate potential for production of industrial phytochemicals than callus and organ cultures, because suspension cultures can be grown on large scale in bioreactors with automated replenishment of nutrients, aeration, temperature, and light control, and pH maintenance (see Chap. 4). Although the basic equipment and process related requirements for suspension culture of plant cells are similar to those of submerged microbial cultures, some of the features of bioreactors used for microbial cultures are not suitable for plant cells because of striking differences in the nature and growth pattern of the two types of cells: the plant cells, (i) being larger are shear sensitive, (ii) grow as cell aggregates rather than single cells, (iii) exhibit slower growth rate, (iv) have lower oxygen requirement, (v) plant cell suspensions are viscous at high cell density and the viscosity is further enhanced by the excretion of polysaccharides at the later stage of cell growth, and (vi) product synthesis is nongrowth associated. Aggregation of cells, which is desirable for the biosynthesis of many secondary metabolites by plant cells, results in sedimentation, insufficient mixing, and diffusion-limited biochemical reaction. Proper mixing promotes better growth by enhancing the availability of nutrients from liquid and gaseous phase to cells and dispersion of air bubbles for effective oxygenation.

Proper mixing promotes better growth by enhancing the availability of nutrients from liquid and gaseous phase to cells and dispersion of air bubbles for effective oxygenation.

Different types of bioreactors have been used for mass cultivation of plant cells for the production of phytochemicals (Chattopadhyay et al. 2004; Fig. 4.4). Stainless steel Stirred Tank Bioreactors have been most extensively used despite the fact that it exerts hydrodynamic stress on plant cells. Low agitation speed and modified impellers have been used to reduce shear stress. The first commercial application of large-scale cultivation of plant cells was carried out in stirred tank bioreactors of 200 and 750 L capacities to produce shikonin by cell cultures of *L. erythrorhizon* (Payne et al. 1987). Cells of *C. roseus, Digitalis lanata, Panax notoginseng, Taxus baccata and Pododphyllum hexandrum* have been cultured in stirred tank bioreactors with suitable modifications for the production of

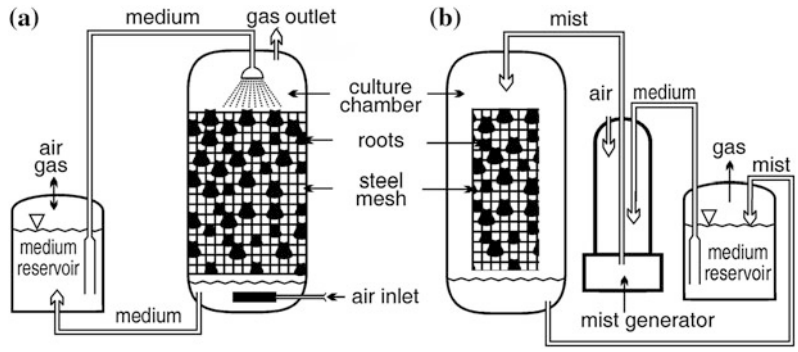

Fig. 18.1 Bioreactors for hairy root culture. **a** Spray reactor. **b** Mist reactor. The roots anchored to steel mesh in the culture chamber are exposed to humidified air or gas mixture, and the nutrients are delivered by spray nozzles (**a**) or ultrasonic transducers (**b**), respectively

phytochemicals (Chattopadhyay et al. 2004). Phyton Biotech (Germany) used 70,000 L stirred tank bioreactor, with low shear force, like those of ship impeller type, to culture *Taxus* cell suspensions (Wink et al. 2005). Cells of *C. roseus, D. lanata, Curdania tricuspidata, L. erythrorhizon* and *Taxus chinensis* have been successfully cultivated in airlift bioreactor for the production of secondary metabolites. Rotary drum bioreactor has been shown to be superior to all other bioreactors for the cell cultures of *Vinca rosea* (Goldstein et al. 1980) and *L. erythrorhizon* (Tanaka 1987). A modification of bubble column bioreactor (balloon type bubble reactor) was adopted for the production of taxol by *Taxus cuspidata* suspension cultures (Son et al. 2000).

The standard stirred tank bioreactor is not suitable for mass cultivation of hairy roots, because the contact of impeller not only damages the root tips but also induces callus formation. Therefore, these bioreactors are modified by isolation of roots from the impeller and gentle mixing to lower the shear stress. However, gentle mixing results in poor gas exchange and insufficient nutrient transfer to the roots. The growth pattern of the hairy roots in bioreactors is very different from cell cultures. The hairy roots exhibit profuse branching leading to the formation of a packed root mass of interlocked matrix that causes resistance to uniform nutrient flow and creates O_2 gradients within the root matrix, resulting in growth limiting effect. Therefore, several different types of bioreactors have been designed and used for hairy root culture. The merits and demerits of these bioreactors have been discussed by Srivastava and Srivastava (2007).

Of the various reactors used, trickle bed reactors (Fig. 18.1) are considered as most suitable for hairy root culture. In this, the roots are planted on the top of a support, such as glass beads and the medium trickles over the roots. The medium is drained from the bottom of the reactor to a reservoir and re-circulated at a specific rate. For better dispersion of the medium it is mixed with humidified air. Of late, a special gas-phase bioreactors such as spray (droplet) reactors (Fig. 18.1a) and mist reactors (Fig. 18.1b), have been designed in which the roots, anchored to a support, such as horizontal or cylindrical stainless steel mesh, are exposed to humidified air or gas mixture, and liquid nutrient medium is delivered as droplets by spray nozzles or mist on the top of root bed. The medium is drained from the bottom of the reactor and is recirculated.

Son et al. (1999) designed a balloon type bubble bioreactor (BTBB; Fig. 18.2) which proved superior for biomass growth than the bubble column reactor, stirred tank reactor for cell cultures of *Taxus cuspidata* (Son et al. 2000), hairy root culture of *B. vulgaris* (Shin et al. 2002) and ginseng (Yu et al. 2000) and hairy-like adventitious root (HLAR) culture of ginseng (Yu et al. 2002). The gain of biomass of HLAR in 20 L BTBB was three times greater

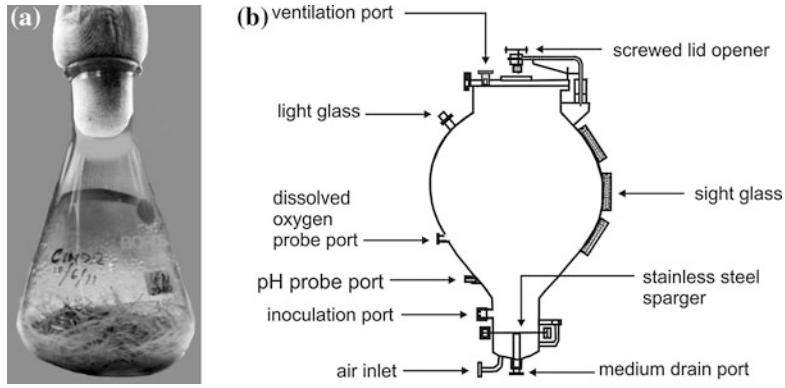

Fig. 18.2 **a** A hairy root culture of *Azadirachta indica*. **b** Schematic diagramme of pilot scale culture vessel (1,000 Litres) for hairy root culture developed by Choi et al. (2006). (**a,** *courtesy* Professor AK Srivastava, IITD, New Delhi)

than the stirred tank reactor (Son et al. 1999). The maximum biomass production of 2.2 kg FW in 20 L bioreactor was obtained after 42 days of inoculation of 240 g root (Choi et al. 2000). The biomass growth of HLARs of mountain ginseng line in BTBB reached 30 fold of the inoculum after 42 days (Choi et al. 2006).

18.6 Commercialization

Shikonin was the first in vitro produced phytochemical to be commercialized. It is of medicinal importance and a valuable vegetable dye which assumes a wide spectrum of colors from red and purple to blue. Traditionally, shikonin is obtained from the roots of 3 to 4years old plants of *L. erythrorhizon*. Extensive exploitation of this plant has resulted in its extinction in Japan and now similar fate awaits it in China and Korea. Scientists at Mitsui Petrochemical Co., Japan developed a commercial tissue culture process for the production of Shikonin using cell cultures of *L. erythrorhizon* established by Tabata et al. (1974). High yielding lines (15 % as compared to 1–2 % in the parent plant) have been selected and are cultured in huge bioreactors for the production of shikonin on a commercial scale (Fujita 1990). In 1984, the first product (lipstick) made with plant tissue culture produced shikonin was marketed. However, the production had to be discontinued due to limited market demand (Sato 2003).

The Japanese company Nitto Denko scaled up the cultivation of ginseng cells and marketed the biomass as a food additive. The product has been used as additive to wine, tonic drinks, soups, herbal liquors, and others (Misawa 1994). In South Korea, several laboratories have established continuously growing root cultures of ginseng in bioreactors and the harvest is being marketed. Three companies produce ginseng roots (normal) commercially using pilot-scale 10,000–20,000 L BTBB. The root materials are processed into various types of health foods and food ingredients (Choi et al. 2006).

Taxol (paclitaxel), a diterpine amide, is an anticancerous drug, manufactured by extraction from the bark of *Taxus brevifolia*. Catalytic Inc. and ESCA Genetics, USA, announced in 1992, that they had an in vitro process to manufacture taxol (Misawa 1994). Samyang Genex, in South Korea, commercially produced "Genexol" (Peditaxel) by cell cultures of *T. chinensis* grown in 35,000 L bioreactors (personal visit to the lab). Phyton Biotech, Germany, is also reported to be producing taxol on a commercial scale (Wink et al. 2005). Lee et al. (2010) were able to enhance taxol production in 3 and 20 L airlift bioreactors by establishing suspension cultures from cambial meristematic cells (CMC) instead of dedifferentiated plant cells (DDC) of *T.*

cuspidata. The CMCs secreted 98 mg kg^{-1} of paclitaxel as compared to 11–13 mg kg^{-1} by the DDCs. The perfusion cultures of CMC produced as high as 264 mg paclitaxel per kg of cells while the DDCs were largely necrotic.

Two other metabolites produced commercially through cell or root culture are the polysaccharides and the saikosaponins. Both these compounds are useful in skin protection and are used as cosmetics. The polysaccharides are produced in cell cultures of *Polyanthes tuberose* (Honda et al. 1996) and the Saiko extracts from the cultured roots of *Bupleurum falcatum* (Kausakari et al. 2000).

18.7 Concluding Remarks

Numerous studies to-date have demonstrated the ability of cultured cells, tissues, and organs to synthesize phytochemicals of industrial interest sometimes at levels higher than the parent plants. Therefore, tissue cultures are regarded as a suitable alternative to whole plant cultivation for the production of desired natural plant products. In vitro production of phytochemicals can go on round the year like a factory, independent of climatic and soil conditions, free of microbes and insects. The in vitro systems provide ample opportunity to enhance the yield of natural compounds and induce the cells to synthesize novel compounds by manipulating the growth conditions. Some compounds have been produced on commercial scale by cell cultures but the degree of success so far is below the expectations.

Production of recombinant proteins by plant cells indicates continued interest in this in vitro technology. Dow Agrosciences, USA received world's first regulatory approval from USDA for a plant cell made vaccine in January 2006, (*see* Ducos et al. 2008). Israeli Biotech Company Protali reported the production of glucocerebrosidase by plant cells in disposable plastic bioreactors (Shaaltiel et al. 2007).

The first requirement for a commercially viable in vitro system for phytochemical production is to be able to culture the cells/organs on large scale for which a suitable bioreactor is required. In many cases, two bioreactors are required for optimum production of the desired compound. In the first reactor, cell suspensions or hairy roots are cultured under conditions optimum for biomass production, whereas the second reactor is set with conditions optimum for product formation.

Vincristine and vinblastine (dimeric indole alkaloids), very important for chemotherapy, are produced by *C. roseus*. All efforts to produce these compounds in cell suspension or hairy root cultures have failed so far. Field cultivation and production of these alkaloids is still the method of choice (Wink et al. 2005).

The creation of new pathways in plants through suppression of the existing gene (Hagel and Facchini 2010; Liscombe and O'Connor 2011) and introducing new ones (Runguphan et al. 2010) will be the most potent method for biosynthesis of novel molecules (De Luca et al. 2012).

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Conservation of Phytodiversity 19

19.1 Introduction

Plants form the key element in maintaining the biodiversity on this Earth by providing suitable habitat, food, and oxygen to all other living beings. Endowed with the unique capacity to convert solar energy into chemical energy, plants synthesise thousands of compounds of importance to our lives, at normal temperature and pressure, without polluting the atmosphere. Our survival on this Earth depends on the survival of plants. India, with a wide spectrum of phytogeographic and agro-climatic zones, is blessed with a rich diversity of plants (Rao 2000). With about 45,000 species of flora and 80,000 species of fauna, India ranks among 12 mega-biodiversity centers of the world. Unfortunately, this valuable natural resource is depleting at an alarming pace.

Extinction of the existing species and their replacement by new species by natural selection imposed by environmental shifts is a natural phenomenon of evolution. However, change in human attitude by fulfilling his needs to satisfying his greed is causing extinction at a much faster rate than the appearance of new species. Urbanization, industrialization, hydroelectric projects, and clearing forests to cut roads and for agriculture fields, orchards, tourism, and grazing are some of the anthropogenic activities endangering the biodiversity. In addition, a large number of wild species of human utility are being ruthlessly collected for commercial exploitation in a destructive and unsustainable manner. Over 8,000 species of angiosperms are being used in some 10,000 herbal formulations, and nearly 25 % of the prescriptions made in developing and developed countries include the curative factor from higher plants (Rao 2000). For this industry, 90 % of the plant material are obtained, most destructively, from tropical forests. The value of yet undiscovered pharmaceuticals in tropical rainforests is estimated at US$ 147 billion (Mendelsohn and Balick 1995). If suitable strategies for the conservation of germplasm are not developed well in time, we may lose this valuable natural resource and many wild relatives and old cultivars of crop plants, which harbor valuable genes for future breeding programs.

At the Earth Summit at Rio de Janeiro, Brazil, in June 1992, the World Conservation Union (IUCN) brought into sharp focus, the concern raised by conservationists during the last few decades over the rapid loss of biological diversities. Following this, many countries, including India, have developed strategies to conserve their plant genetic resources.

19.2 In Situ Conservation

The most desirable plant conservation strategy is to protect and nurture the plants in their natural ecosystems. However, there are numerous problems with in situ conservation of biodiversity. The Government of India has banned the collection of some orchids and several medicinal

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9_19, © Springer India 2013

plants whose number has fallen below a critical level but this has not worked and their collection from nature, for commercial purpose, continues unabated. In situ conservation of the identified hot spots of biodiversity (areas with rich and unique native biodiversity but facing anthropogenic disturbances) faces similar problems. Moreover, many of the endangered species are highly habitat specific, which confine them to unapproachable habitats, often at an altitude of over 3,000 m where their conservation management is cost prohibitive.

19.3 Ex Situ Conservation

Alternatively, the biodiversity can be conserved away from its natural habitat. Seed preservation is the most convenient method of ex situ conservation of germplasm, and many countries have established their own seed banks. The National Gene Bank at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, has 390,000 accessions of seeds under long-term storage. However, preservation of germplasm in the form of seeds is not possible for the plants with recalcitrant seeds or species that are propagated vegetatively. Seed storage is also not applicable where clonal material of popular cultivars is to be preserved. In such cases, in-field gene banks are maintained. In 1992, the Department of Biotechnology, Government of India, established three gene banks in the country to conserve medicinal and aromatic plants: NBPGR, New Delhi, Central Institute for Medicinal and Aromatic Plants (CIMAP), Lucknow, and Tropical Botanical Garden and Research Institute (TBGRI), Thiruvenanthapuram. The fourth gene bank at the Regional Research Laboratory (RRL), Jammu was started in 1998 (Natesh 2000).

Maintaining species in Botanical Gardens and Arboreta has traditionally provided a valuable safeguard against loss of many rare species (Laliberté 1997). Botanical gardens have played a commendable role in conserving a large number of rare and exquisite tropical plants, such as *Victoria amazonica*, *Nymphaea gigantea*, tree ferns, palms, bromeliads, insectivorous plants, and many colorful orchids. Indeed, some of the endangered species exist only in botanical gardens today. However, in-field gene banks are not only very expensive but also make the germplasm vulnerable to loss due to diseases, pests, and adverse climatic conditions.

19.4 In Vitro Conservation

Tissue culture offers tremendous advantages over the other ex situ methods of plant conservation. For endangered species, where the available plant material is very limited and in vivo conservation runs the risk of its being lost due to natural or human factors, tissue culture is a safer method. For in vitro storage, very small amounts of tissues are required, and numerous plants can be regenerated in a short time for re-planting in the natural habitat, as reported for *Cypripedium calculus* (Ramsay and Stewart 1998) and *Nepenthes khasiana* (Rathore et al. 1991). The other advantages of in vitro storage of germplasm are:

(i) *Economical.* Relatively little space is required for the storage of a large number of clonally multiplied plants. Eight hundred cultivars of grapes, each replicated six times, can be maintained in 2 m^2 of laboratory space, instead of 1 hectare of land required for their field maintenance (Morel 1975). Similarly, nearly 6,000 accessions of cassava can be stored in a $5 \times 6 \times 3$ m room (Roca et al. 1984). Moreover, under special in vitro storage conditions the plants do not require frequent pruning and splitting.

(ii) *Safe.* The plants are maintained free from pests, pathogens, virus, and other natural hazards.

(iii) *Special situation.* For plants, such as cocoa, coconut, and mango, which produce large recalcitrant seeds, lacking dormancy, and are incapable of withstanding desiccation or low temperature exposure, embryo culture has proved useful.

(iv) *International exchange of germplasm.* Being free from known viruses and pathogens, the clonal material could be sent from country to country, thus minimizing the obstructions imposed by quarantine system on the movement of live plants across national boundaries.

In cultures plants can be maintained for virtually unlimited period by serial subcultures every 4–8 weeks (short-term storage). Chaturvedi (1999) has recommended root cultures as a safe method for long-term germplasm conservation. The roots of *Solanum khasiana* regenerated true-to-type plants even after 20 years of culture. Similarly, the root cultures of *Rauwolfia serpentina* retained their regeneration potential up to 16 years (Chaturvedi 1991). Some important in vitro gene banks and their holdings are listed in Table 19.1.

However, frequent subculture risks the loss of plant material by microbial contamination and is also uneconomical. A basic requirement for the practical feasibility of a plant tissue culture method in the preservation of germplasm, therefore, is to reduce the frequency of subcultures to a bare minimum. There are two options for in vitro storage of plant material: (1) medium-term storage, by maintaining the cultures under growth limiting conditions, and (2) long-term storage, at the super-low temperature of liquid nitrogen (−196 °C; Cryopreservation).

19.4.1 Medium-Term Storage

It involves maintaining the cultures under growth limiting conditions to prolong the intervals between subcultures. The plant species which have inherently slow growth can be maintained for several months under standard culture conditions. For example, *Coffea arabica* plants could be maintained for 12 months at 27 °C without the need for a subculture (Bertrand-Desbrunais and Charrier 1990). However, medium-term storage mostly requires alteration of environmental and/or media conditions to slowdown the growth of the cultures.

The most successful strategy for medium-term storage of germplasm is to store the cultures at low temperature. Modification of the culture medium by adding osmotically active compounds (e.g. mannitol) or reduction of growth regulators or nutrient status, lowering of oxygen level, and desiccation of tissues are some other methods used for medium-term storage of germplasm.

One of the earliest programs to use in vitro propagation method for rare and endangered species was the establishment of micropropagation unit at the Royal Botanic Garden, Kew, in 1974 (Benson 1999). Since then numerous countries have initiated their own programs for the micropropagation and conservation of endangered species (Table 19.1; Pence 1999; González-Benito et al. 1999). In 1986, a National Facility for Plant Tissue Culture Repository (NFPTCR) was established at NBPGR, New Delhi, with the objective to store on short-, medium- and long-term basis in vitro cultures, pollen and seeds of economic plants, including endangered species. The NFPTCR has over 2,000 accessions in in vitro gene bank maintained for almost 25 years (NBPGR Report 2010).

(i) *Cold Storage*. Germplasm storage at non-freezing low temperature has been very successful. At lower temperatures, the aging of the plant tissues is slowed down. Consequently, subculture of plant material is required albeit not very frequently. The optimum storage temperature depends on the sensitivity of the species. Whereas for temperate species it ranges from 5–9 °C, for tropical species it is often slightly higher.

Mullin and Schlegel (1976) maintained virus-free strawberry plants in vitro for 6 years at 4 °C. A few drops of liquid medium were added to the cultures every 3 months. Preil and Hoffmann (1985) stored about 700 breeding lines of chrysanthemum at 2–3 °C in diffuse light (10–15 lux). Some of the lines survived 5 years at the growth limiting conditions. Aeration of the cultures was essential to prevent vitrification of the shoots during storage. Cold storage in dark is also known to induce vitrification of

Table 19.1 Some important in vitro genebanks and their holdings[a]

S. no.	Gene bank	Number of accessions	Crops
1.	International Potato Centre	13,866	Potato, sweet potato, Andean roots, and tubers
2.	Centro International de Agricultura tropical (CIAT), Colombia	5,714	*Manihot*
3.	Gatersleben Genebank, Germany	3,122	Potato, *Allium* spp
4.	National Centre for Genetic Resources and Biotechnology (CENARGEN), Brazil	2,221	Cassava, sweet potato, potato, yam, *Asparagus*, stevia, strawberry, and banana
5.	International Institute of Tropical Agriculture, Nigeria	1,500	*Dioscorea* spp
6.	National Bureau of Plant Genetic Resources (NBPGR), New Delhi	1,327	*Musa, Fragaria, Allium* spp, C*olocasia, Dioscorea, Ipomoea Curcuma, Zingiber, Simmondsia*, medicinal, and aromatic plants
7.	National Clonal Germplasm Repository (NCGR), USA	2,000	*Corylus, Fragaria, Humulus*, M*entha, Pyrus, Ribes, Rubus,* and *Vacinium*
8.	International Network for the Improvement of Banana and Plantain (INIBAP), France	1,051	*Musa*
9.	Institute of Plant Breeding, University of Philippines, Philippines	866	Cassava, sweet potato, potato, yam, banana, manila hemp, shallot, and garlic
10.	Sweet Potato Repository, USA.	800	Sweet potato
11.	Braunschweig Genetic Resources Centre (BGRC), Germany	778	Potato

[a] after Benson (1999), Engelmann (1999) and NBPGR (2010)

shoots (Williams and Taji 1987). Shoots of *Beta vulgaris* could be kept alive for 12 months at 5 °C in diffuse light. The rooted shoots showed better survival, as also reported for *Coffea arabica* (Kartha et al. 1981). Germplasm of potato can be cold stored in the form of microtubers instead of shoot cultures (Kwiatkowski et al. 1988).

Shoot tip cultures of kiwifruit could be maintained at 8 °C for about a year with 100 % survival (Monette 1986, 1987). The shoots retrieved after 52 weeks of storage exhibited

normal growth and proliferation. Similarly, the cultures of *Colocasia esculenta*, another tropical species, tolerated storage at 9 °C for 3 years (Zandvoort and Staritsky 1986). The plantlets and somatic embryos of oil palm are unable to tolerate even a short exposure to a temperature below 18 °C (Corbineau et al. 1990). Similarly, banana cultivars stored below 5 °C suffered damage and/or died within 3 months. At 15 °C some of the genotypes survived for up to 17 months with viability of 92 % but in others the viability was reduced to 50 % after 3 months (Withers and Williams 1986). According to Ko et al. (1991), the best condition for the storage of banana shoots is to place them on cotton saturated with a 3 % solution of ribose, wrapped in cheesecloth and incubated at 17 °C. Cavendish banana stored under these conditions for 24 months showed 64 % survival, and most of the plants regenerated from cold-stored material appeared normal. The cassava plants need to be stored at temperatures higher than 20 °C (Roca et al. 1984).

Cold storage could be very useful in plant propagation industry employing the micropropagation technique. The nucleus stock of cultures can be maintained in dormant state during the low demand season without the need for frequent subcultures. Similarly, the in vitro research material can be stored if not required for some time.

One of the problems with cold storage of the germplasm may be the gradual habituation of some material to slow growth conditions.

(ii) *Storage under low oxygen environment*. Low temperature storage is a costly option in the tropical countries. Power/equipment breakdown, especially in summer, endangers the loss of stored germplasm. In this context, storage of plant material under normal culture room temperature (25 °C) in a growth limiting medium or gaseous environment is an attractive alternative to medium-term germplasm storage.

Remarkable success has been achieved to store plant materials at ambient temperature by reducing the available oxygen level. The simplest way to reduce the concentration of available oxygen is to cover the tissue with mineral oil. This technique was first used by Caplin (1959) with carrot callus. None of the embryogenic calli of grape retrieved after 360 days of storage at 10–15 °C survived. However, if the calli were immersed in silicon during cold storage they showed high survival (95 %) and regenerated plants (Moriguchi et al. 1988). Somatic embryos of oil palm stored for 4 months in an atmosphere of 1 % oxygen exhibited rapid proliferation (Engelmann 1990). The control embryos, cultivated under standard conditions, were severely damaged. Silicon has almost eight times higher oxygen solubility than water (Moriguchi et al. 1988).

A major problem in incubation of cultures under low O_2 level is the vitrification of shoots. The cell lines of *Panax ginseng* and *Catharanthus roseus* preserved under mineral oil retained their growth potential as good as the cryopreserved cells, but lost their biosynthetic capacity like the lines maintained at normal temperature by regular subcultures. In contrast, the cryopreserved cells retained their capacity to synthesise catharanthine (*C. roseus*) and ginsenosides (*P. ginseng*) (Mannonen et al. 1990).

(iii) *Modification of medium.* Growth of the cultured shoots can be retarded at normal temperature by adding osmotically active substances (such as mannitol, growth retardants), reducing the concentration of specific minerals (such as nitrogen, magnesium), or raising sucrose concentration. Modification of culture medium by addition of 10 % sucrose or raising agar concentration to 2 % or reducing salt concentration to one quarter was effective in obtaining high survival rates (73–80 %) of pear shoots stored for 2 years at 5 °C (Oka and Niino 1996). Yam collections at the Institut de Recherche pour le Developpement, France are maintained in medium with low minerals and sucrose concentration (Malaurie, 2001). Most of the potato accessions at the International Potato Centre (Lima, Peru) could be maintained for up to 4 years, without a subculture, by combining

osmotic stressants (4 % sorbitol and 8 % agar), low temperature (6 °C) and low light intensity (100 lux) (Golmirzaie and Toledo 1999).

(iv) *Desiccation.* Partial desiccation of callus and somatic embryos has also been tried for medium-term storage of germplasm. ABA treatment, often in combination with high concentration of sucrose (9 % or higher), enhances the desiccation tolerance of the cells. Carrot callus dried in the air system of laminar air flow cabinet overnight, after pre-treatment with ABA and a high concentration of sucrose, could be stored at −80, −20, or –15 °C for 1 year in a viable state (Nitsche 1980). Calli of several varieties of japonica and indica rice preserved in desiccated form for 3 months by treating them with 10^{-5} M ABA and 9 % sucrose followed by slow drying in a medium maintained their totipotent nature (Shin et al. 1991). Plant regeneration from dried somatic embryos has also been reported for several species, including orchard grass (Gray 1987) and alfalfa (Senaratna et al. 1989). Somatic embryos of alfalfa could be dehydrated down to 15 % water content and stored for 8 months at room temperature. Desiccation tolerance of axillary buds of asparagus was considerably enhanced by pre-culture in a medium containing 0.7 M sucrose (Uragami et al. 1990).

19.4.2 Long-Term Storage

Long-term preservation of germplasm is through cryopreservation, in which the material is frozen at the temperature of liquid nitrogen (–196 °C). At this super-low temperature, all metabolic activities of the cells are suspended. Therefore, in theory, the cryopreserved cells can be stored for unlimited period without a subculture. However, adequate care is required to protect the cells from cryogenic injury during freezing, storage, and retrieval. During the past three decades, considerable work has been done to develop effective protocols for cryopreservation of plant cells, tissues, and organized structures (shoot tips, embryos etc.), and the technique is being increasingly used for conservation of germplasm (Ashmore 1997; Benson 1999; Engelmann 2004, 2011; Gonzalez-Arnao et al. 2008). The number of plant species for which cryopreservation protocols have been established is over 200 (Engelmann 2004). The in vivo buds of silver birch cryopreserved for 5 years were as viable as the nonfrozen buds (Ryynänen 1999). Some of the centers where germplasms have been maintained in liquid nitrogen are (Panis et al. 2001): International Potato Centre, Peru, Laboratory of Tropical Crop Improvement, Belgium, National Seed Storage Lab, USA, ORSTOM, France, and Tissue Culture Research Inc., Canada (5000 accessions of conifer species). In India, NBPGR (New Delhi) and TBGRI (Thiruvenanthapuram) are working on cryopreservation of germplasm

The main consideration in cryopreservation is to protect the cells by preventing or minimizing ice crystal formation during cooling. This has led to two main approaches to cryopreservation of plant materials: (1) Slow or Step-wise freezing method (also known as the traditional method) (Fig. 19.1), and (2) Ultra-rapid freezing method, which is comparatively new and is becoming increasingly popular. In both the methods, cells are sufficiently dehydrated during or before freezing, so that the protoplasm becomes concentrated and ice crystal formation is prevented. A pre-culture step is generally helpful in enhancing the desiccation tolerance of the cells. There are four main steps in cryopreservation of plant materials: (1) Pre-culture, (2) Freezing, (3) Thawing, and (4) Re-culture. The choice of the tissue also contributes toward the success of a cryopreservation protocol.

19.4.2.1 Plant Material

Small, highly cytoplasmic cells, especially soon after mitosis, from suspension cultures in the early growth phase are most tolerant to freezing. In contrast, cells with large vacuoles are extremely sensitive to cryoprotectant treatment and would not survive freezing and thawing (Seitz 2000). Similarly, shoot tips smaller than 1 mm are very susceptible to the toxic effect of highly

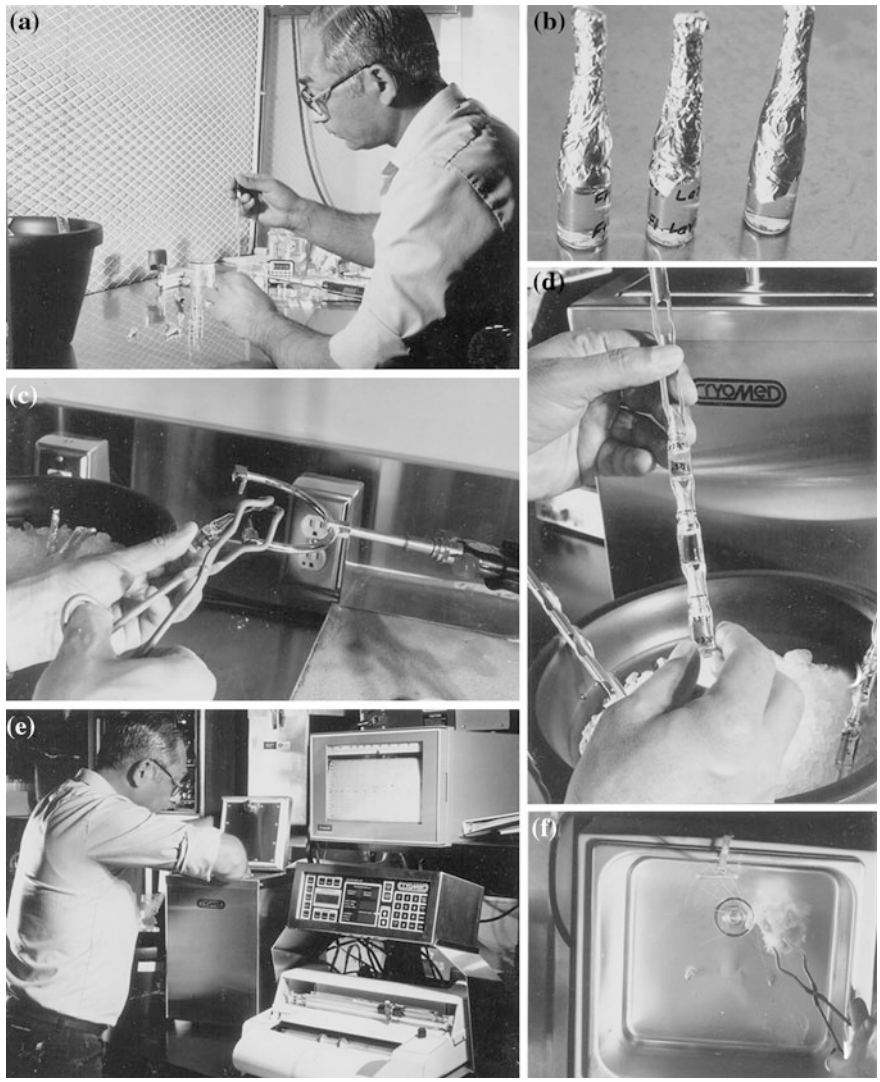

Fig. 19.1 Stages in slow freezing method of cryopreservation. The tissue of choice is excised and treated with cryoprotectant under aseptic conditions **(a)** and transferred to cryovials **(b)** The vials are flame sealed **(c)** loaded on canisters **(d)** and transferred to freezing chamber of a programmable LN_2 Freezer **(e)** The frozen material is thawed rapidly by plunging the vials into water bath at 37–40 °C (**f**)

concentrated vitrification solution, leading to poor survival after cryopreservation. A reasonable size of excised shoot tip for successful cryopreservation is 1–1.5 mm (Sakai 1997). The nature of the tissue may also effect survival after cryopreservation. Embryogenic cultures of olive showed higher survival (38 %) than shoot tips (15 %) when directly immersed in liquid nitrogen after vitrification in PSV2 solution for 60 min (Benelli et al. 2001).

19.4.2.2 Preculture

A key to successful cryopreservation lies in the induction of dehydration and or cold tolerance of the cells to which they are exposed during the freezing regime. This is achieved by 2–4 days of culture of the tissue in a medium modified with the additives such as sugars (sucrose), sugar alcohols (sorbitol, mannitol), and amino acids (proline). Adaptation of the cells to high osmotic stress is also achieved by low temperature

treatment, which induces accumulation of heat shock proteins, cold regulating proteins, and dehydrins. The cellular protein pattern changes by high sucrose concentration, which protects freeze labile enzymes. Pre-culture of plants at 4 °C for 4 days before excising shoot apices for freeze-preservation raised the survival rate from 30 to 60 % (Seibert and Wetherbee 1977). Similarly, the survival of the cryopreserved shoot tips of white clover significantly increased by pre-culture at 4 °C for 2 days in B_5 medium supplemented with 5 % each of DMSO and glucose (Yamada et al. 1991).

19.4.2.3 Freezing

This is one of the most critical steps in cryopreservation of plant materials. The first successful cryopreservation of shoot tips and somatic embryos using the conventional methods of freeze dehydration was achieved with pea (Haskins and Kartha, 1980) and carrot (Lecouteux et al. 1991), respectively. Conventional methods have been successfully applied to undifferentiated culture system such as cell suspensions and calli as well as to shoot tips of cold tolerant species (Engelmann 2004). However, when this technique is applied to shoot tips, particularly of tropical plants, large zones of the apical domes are destroyed, and plants regenerate indirectly via callus formation. The somatic embryos of *Citrus* cryopreserved by this method regenerated plants via adventive embryony instead of direct germination. The removal of intracellular water by slow cooling is not sufficient to avoid ice formation. Cassava is an exception in this regard. This led to the development of a more effective ultra-rapid freezing methods of cryopreservation. In both the freezing methods, (slow and ultra-rapid) the cells are dehydrated before immersion in LN to minimize intracellular cryogenic injury by ice crystal formation.

(i) Slow freezing. In this classical method (Fig. 19.2), the plant sample is first cooled at a controlled rate of 0.5–2 °C min^{-1} down to –30 to –40 °C, and held at this terminal pre-freezing temperature for about 30 min before transferring them to liquid nitrogen. The controlled slow cooling is achieved using a computer programmable cooling system, manufactured, for example, by Planner Products Co., UK and Cryomed, USA.

During slow freezing the extracellular liquid (intercellular fluid and freezing mixture) freeze first, and this extracellular ice nucleation creates water vapor deficit between the inside and outside of the cell. As a result, water from inside the cell moves out causing dehydration of the cells and a concomitant decrease in the freezing point of the cell contents due to its increased viscosity. The protective dehydration of the cells during slow cooling effectively prevents or reduces detrimental intracellular ice crystal formation in the cytoplasm or vacuoles when transferred to liquid nitrogen.

Dehydration during slow cooling may cause osmotic injury due to lethal changes in cell

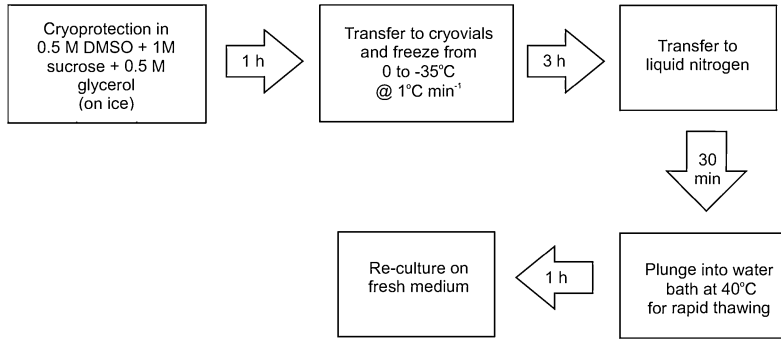

Fig. 19.2 Slow freezing method for cryopreservation of cell cultures. (*based on* Withers and King 1980)

volume and toxic concentration of cell solutes (Benson 1999). To offset this danger, certain cryoprotecting agents, such as DMSO and glycerol, are used. Generally, they are used in combination. The penetrating cryoprotectants act as a stabilizing factor. For cells from suspension cultures, a mixture of 0.5 % DMSO, 0.5 % glycerol and 1 M sucrose is widely applicable. Sometimes only sucrose has been used at low concentration (0.3 M) in the preculture medium to induce desiccation tolerance and at high concentration (1 M) as cryoprotectant (Seitz 1997). The plant cells are generally incubated in cryoprotectant solution, on ice, for about 1 hour prior to freezing. Cryoprotectants are especially important for cells with large vacuoles. The cryoprotectants also act as antioxidant and membrane and protein stabilizers.

(ii) Ultra-rapid freezing. In this method, also called vitrification based method, the plant material is first desiccated, and then directly immersed in liquid nitrogen. During desiccation, the solute concentration of the protoplasm increases and becomes very viscous. When such a material is directly plunged into liquid nitrogen the water undergoes a phase change from liquid to glassy state. This physical process is called vitrification (Fahy et al. 1984). Vitrification based cryopreservation protocols are simple and more effective for complex organs, such as embryos and shoot tips, and do not require programmable freezer (Urgami et al. 1989; Sakai et al. 1990; Lambardi et al. 2001).

This method has allowed significant progress in cryopreservation of germplasm, especially in the form of shoot tips and somatic embryos that could not be effectively cryopreserved following the conventional method of slow freezing (Panis 1995; Gonzalez-Arnao et al. 2003, Gonzalez-Arnao et al. 2008). When a vitrification-based protocol is applied under optimum conditions, the whole or most of the meristematic structures remain intact which guarantees direct regeneration of plant after cryostorage without the transitory callus phase, avoiding genetic instability associated with the callusing phase (Matsumoto et al. 1994; Hirai and Sakai 1999).

The pre-freezing desiccation of cells is achieved by exposing them to sterile air in a laminar airflow cabinet or, more precisely, by using stream of sterile compressed air or by drying over silica gel. However, this simple method of desiccation is applicable only to desiccation-insensitive materials. Therefore, protocols of wider applicability have been developed in which cells are dehydrated with an osmotically active substance, such as sucrose, before desiccation treatment.

A Plant Vitrification Solution (PVS2), comprising high concentrations of cryoprotective agents (0.4 M sucrose, 30 % glycerol, 15 % ethylene glycol, and 15 % DMSO in MS medium), developed by Sakai and his co-workers in Japan, has been widely used. The time and temperature of treatment with this highly concentrated cocktail are critical factors. The ultra-rapid cooling method, using PVS2 solution, has been successfully applied to in vitro growing shoot tips of several cultivars of temperate fruit crops (see Sakai, 1997), *Populus* sp. (Lambardi et al. 2001), and mulberry (Niino et al. 1992). The general protocol involves: (i) cold/desiccation hardening at 4 °C in a medium containing an osmoticum for 2–4 days, (ii) incubation in vitrification solution for 30–90 min on ice, (iii) transfer to a suitable sterile cryovial containing the freezing mixture (ca 0.75 ml in a 2 ml vial) and seal it, and (iv) transfer to liquid nitrogen Dewar for storage (Lambardi et al. 2001). Addition of 10^{-4} M acetylsalicylic acid to the PVS2 solution improved the survival frequency of cryopreserved shoot tips of *Glehnia littoralis* from 43 to 87 % (Otokita et al. 2009). Many different vitrification based methods for cryopreservation of plant materials have been developed:

- *Pregrowth.* The samples are cultivated in the presence of cryoprotectants before freezing them rapidly by direct immersion in LN. This technique has been developed for *Musa* meristem cultures (Panis et al., 2002).
- *Dehydration.* This is the simplest vitrification based procedure. The samples are dehydrated by exposure to air current of a laminar airflow cabinet or a stream of sterile, dry compressed air (Flash drying; Berjak et al. 1989) or using Silica

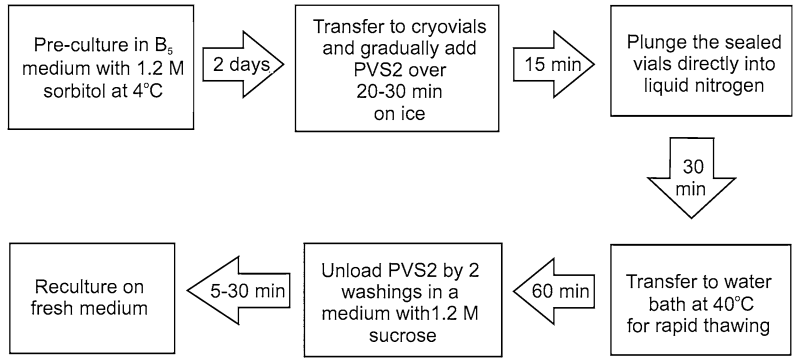

Fig. 19.3 *Vitrification* (in PVS2) method for cryopreservation of 1-1.5 mm shoot apices from 2–3 weeks old aseptic seedlings of *Trifolium repens* (Ymada et al. 1991)

gel before plunging them into LN. Optimum survival to direct exposure to LN is achieved when water content of the samples is reduced to 10–20 % (FW basis). This method is applied mainly to zygotic embryos and embryonal axis of large number of recalcitrant species.

- *Pregrowth dehydration.* This is a combination of above two methods. It involves cultivating the explants in the presence of cryoprotectants followed by dehydration before immersion in LN. This method has been applied to *Asparagus* stem segments, oil palm polyembryonic cultures and coconut zygotic embryos.
- *Encapsulation and dehydration.* The samples are encapsulated in Ca-alginate like synthetic seeds (see Sect 7.10) and precultured in liquid medium with high sucrose concentration before dehydration. It allows drastic dehydration process prior to freezing, which would otherwise be highly damaging or lethal for nonencapsulated samples (Gonzalez-Arnao and Engelmann, 2006). This method is very effective for freezing apices of different plant species from temperate and tropical regions (Gonzales-Arnao and Engelmann, 2006).
- *Vitrification.* In this method (Fig. 19.3), the sample is first treated with a cryoprotectant at low concentration or an osmoticum in a liquid medium for 2–4 days to impart dehydration tolerance and to mitigate mechanical stress caused by subsequent treatment with highly concentrated vitrification mixture for 2–4 days. A mixture of 2 M glycerol and 0.4 M sucrose in liquid medium (Loading solution) applied for 20 min at room temperature is very effective to enhance osmotolerance (Sakai 2004). The most frequently used vitrification mixture is PVS2 (Sakai 1992, 2004).
- *Encapsulation and vitrification*. In this method (Fig. 19.4) of rapid freezing the encapsulated samples are subjected to vitrification treatment before immersion in LN. This method has been used for cryopreservation of shoot apices of increasing number of plant species, such as yam, pineapple, sweet potato, and cassava (Gonzalez-Arnao et al. 2008).
- *Droplet vitrification*. The explants are treated with loading and vitrification solutions as in vitrification protocol and frozen in minute droplets of PVS2 placed on aluminum foil strips, which are plunged directly into LN. This method is being successfully applied to an increasing number of species (Sakai and Engelmann 2007).

19.4.2.4 Storage

The frozen material is stored in Dewars containing liquid nitrogen. It is important that adequate level of LN in the container is maintained. While storing a large number of samples, it is vital to follow an efficient inventory system. This would not only facilitate checking what has been stored and for how long but also reduce the

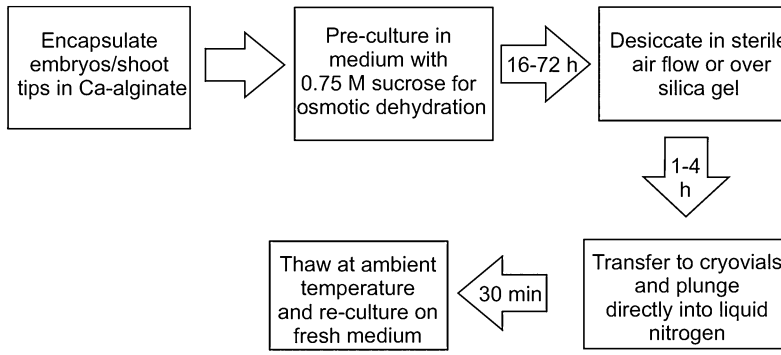

Fig. 19.4 Encapsulation-dehydration-desiccation method for cryopreservation of shoot tips and embryos (*after* Fabre and Dereuddre 1990)

time the other samples are exposed to the ambient temperature while trying to remove a particular sample.

19.4.2.5 Thawing

Rapid thawing is generally beneficial. For this, the ampoule containing the frozen material is plunged into water at 37–40 °C (Fig. 19.1f). After 90 min or so it is transferred to ice bath or culture medium. Removal of cryoprotectants from the retrieved material is unnecessary or, at times, deleterious. Therefore, the thawed material is plated on agar medium where the cryoprotectant is diluted gradually. Similarly, the vitrified and excessively desiccated material may require sequential lowering of the medium osmotica during thawing and reculture (Benson 1994). The tissues frozen by encapsulation and desiccation are frequently thawed at ambient temperature.

19.4.2.6 Reculture

The culture media for the cryopreserved material is generally the same as for unfrozen material. However, some systems may require slight modifications. For example, shoot tips from frozen seedlings of tomato directly developed into plantlet only if GA_3 was added to the medium. In its absence, regeneration occurred indirectly via callusing. Unfrozen shoot tips did not require GA_3 for direct regeneration (Grout et al. 1978). GA_3 also improved the survival rate of cryopreserved zygotic embryos of coffee (Abdelnour-Esquilre et al. 1992).

19.4.2.7 Stability Assessment

It is important to check the viability and stability of the germplasm retrieved from cryopreservation. The best test of viability is of course the regrowth of the tissue/cells/organized structures. However, there are several histochemical tests to check cell viability rapidly. The genetic stability of the material can be checked by modern molecular techniques, such as RAPD.

19.5 Concluding Remarks

In vitro storage of plant cells, tissues, and organized structures is a promising approach to conservation of biodiversity and the valuable germplasm of crop plants. Maintenance of cultures under growth limiting conditions of nutrients and environmental conditions provides a simple method for short-time (1–3 years) conservation of biodiversity and is being used at many germplasm centers. Concurrently, considerable progress has been made in developing efficient cryopreservation methods for long-term storage of plants. The conventional methods of cryopreservation by slow programmed freezing from −30 to −40 °C before immersing the tissue in LN has been successfully applied to a

large number of plant species but it has some limitations: (i) Desiccation of cells by slow freezing method does not remove sufficient intracellular water due to which the survival after exposure to LN is poor, (ii) It is not very effective for the cryopreservation of organized structures such as shoot tips and embryos which comprise different types of cells. Large patches of cells in the explants are damaged during storage in LN and regeneration occurs through callusing or adventive embryony, and (iii) The technique has not been very successful with tropical plants. Since 1990, the introduction of vitrification-based cryopreservation methods has considerably improved the efficiency and scope of cryopreservation of plant materials. Several protocols have been tried for ultra-rapid freezing, of which vitrification and vitrification after encapsulation or in small drops are most successful. These techniques have been applied to different tissues of over 100 species of temperate and tropical origin (Sakai and Engelmann 2007). The survival of somatic embryos of *Citrus* cryostored by the conventional method and encapsulation-dehydration method was 3.7–30.5 % and 75–97 %, respectively (Gonzalez-Arnao et al. 2001). The first international symposium on Cryopreservation in Horticultural Species, held in Belgium in April 2009, is an indication of active research interest in the basic and applied aspects of cryopreservation of phytodiversity.

It has been suggested that concomitant to cryopreservation a strong research focus should be directed towards improved techniques for seed storage. Attention should also be paid to the economics of cryopreservation as compared to other methods of germplasm storage. Recently, a detailed study has demonstrated the cost efficiency of long-term cryopreservation of coffee genetic resources (Dullo et al. 2009).

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Dr. Bhojwani has over 40 years of experience of research and teaching Plant Biotechnology to undergraduate and postgraduate students. After 33 years of service at the Department of Botany, University of Delhi, in 2002 Professor Bhojwani moved to Agra as the Director of the Dayalbagh Educational Institute (Deemed University). He continues as Emeritus Professor of Botany with the DEI. Prof. Bhojwani has published over 90 original research papers in reputed international journals and guided 17 doctoral and 11 M.Phil. thesis and authored/edited seven books on Plant Tissue Culture and the Embryology of Angiosperms, some of which have been translated into Japanese and Korean languages. Prof. Bhojwani has been a Member of Organizing Committee, Session Chairman, Organizer of Workshop, and Invited Speaker for several National and International Conferences held in India and overseas. He has been the recipient of many international Fellowships for advanced research in Canada, Japan, New Zealand, Germany, South Korea, and U.K. He has been on the editorial boards of the journals *Scientia Horticulurae, Plant Biotechnology Reports and Plant Tissue Culture*.

Dr. Dantu has 20 years of research and teaching experience in the field of Plant Biotechnology. After completing Ph.D. in 1992, he joined IARI and worked on genetic modification of *Lathyrus sativus* to produce OX-DAPRO-free lines. He was instrumental in setting up a commercial plant tissue culture laboratory with a production capacity of half-a-million horticultural species. In 1997, Dr. Dantu returned to academics and after a brief stint at University of Delhi, in 2004 he joined the Department of Botany, DEI as Associate Professor and was promoted to Professor in 2012. Prof. Dantu is currently working on various biotechnological aspects of medicinal plants. He has guided five doctoral and four M.Phil. theses and 10 M.Sc. dissertations. He has published 20 research papers and contributed six book chapters. He participated in several national and international conferences and was invited as Resource person to the International conference on "Biodiversity Conservation and Education for Sustainable Development: Learning to Conserve Biodiversity in a Rapidly Developing World" held during CBD COP-11 in Hyderabad.

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9, © Springer India 2013

Subject and Plant Index

A
Abscisic acid, 32, 54, 55, 137
Acacia nilotica, 124, 125
Acer pseudoplatanus, 41
Acetosyringone, 204
Actinidia arguta, 262
Actinidia chinensis, 125
Actinidia deliciosa, 124, 125
Activated charcoal, 33, 80, 107, 108, 175, 242, 248, 249, 262, 269, 270, 281
Aegilops, 139
Agrobacterium mediated transformation, 201, 204, 205
  method for, 204
  protocol for, 224
Agrobacterium rhizogenes, 281
Agrobacterium tumefaciens, 7, 8, 9, 69, 201, 202, 224, 281
Albino plants, 107
Aloe polyphylla, 259
Alstromeria, 160
Amaranthus hypochondriacus, 215
Analysis of putative transformants, 208
Androgenesis
  applications of, 105–106
  early segmentation of microspores, 100
  effect of culture medium, 100
  effect of physiology of the donor plants, 98
  effect of pollen stage, 98, 100, 107, 110
  effect of pretreatments, 98
  factors effecting, 95
  genetic potential for, 95–98
  in *Brassica*, 97, 100, 103
  in *Brassica juncea*, 94, 95, 98, 103, 108
  in *Brassica* napus, 95, 98, 101
  induction of, 101–102
  in rice, 94, 97, 98, 106, 110
  in tobacco, 99
  media for, 100
  regeneration of plants, 103–104
  techniques, 95
Androgenic plants
  advantages of, 105
  albino plants, 106
  in *Brassica napus*, 101
  origin of, 100
  protocol for, 107–110
Aneusomaty, 144, 146
Anther culture
  of *Nicotiana tabacum*, 107
  of *Oryza sativa*, 109
Anthurium scherzerianum, 145
Antirrhinum majus, 155, 216
Apparatus required, 22
Applications of embryo culture
  haploid production, 97, 100, 137
  in basic studies, 135
  production of rare hybrids, 138–140
  propagation of rare plants, 137
  rapid seed viability, 137
  shortening of breeding cycle, 137
  transformation, 138, 168
Arabidopsis thaliana, 70, 71–74, 77, 85, 136, 180, 181, 187, 188, 204
Arabinogalactan, 59, 60, 81, 84
Arachis hypogaea, 46, 79, 281
Artemesia annua, 278
Artemesia sieversiana, 187
Asclepias rotundifolia, 252
Aseptic manipulation, 11, 12, 15, 21, 23
Asparagus, 2, 49, 58, 105, 268, 292, 295
Asparagus officinalis, 105, 106, 253, 268
Asymmetric hybridization, 187–189
Atropa baetica, 281
Atropa belladonna, 98, 189, 217, 278, 281
Autoclave
  horizontal, 18, 19
  vertical, 18, 19
Autoclaving, 11, 17, 19, 36, 88, 262, 265
Auxins, 3, 31, 32, 65, 79, 181, 259, 260, 277
Avena sativa, 185
Azadirachta indica, 125, 127, 254

S. S. Bhojwani and P. K. Dantu, *Plant Tissue Culture: An Introductory Text*,
DOI: 10.1007/978-81-322-1026-9, © Springer India 2013

B
B_5 basal medium, 43, 96, 109, 181, 294
Bacillus, 19
Bacillus amyloliquefaciens, 215
Bacillus circulans, 20
Bacillus thuringiensis, 199, 211
Baleria greenii, 259
Banana micropropagation, 254, 270
Barringtonia racemosa, 43
Begonia, 66, 69, 252, 264
Begonia x *heimalis*, 252
Beta vulgaris, 114, 116, 117, 182, 281, 283, 290
Biolistic gun, 7, 207–209
Bioreactors
  air-lift bioreactor, 42
  bioWave, 42, 45
  bubble column, 42, 45, 86, 264, 283
  for cell culture, 282–283
  for hairy root culture, 283
  for micropropagation, 263–264
  for plant cell culture, 42, 45
  for somatic embryogenesis, 86–90
  stirred tank, 42, 45
Biotechnology, 8, 288
Biotransformation, 279
Brachiara setigera, 121
Brassica campestris, 66, 67, 191
Brassica carinata, 67, 151, 184
Brassica juncea, 2, 65–68, 70, 94, 95, 98, 103
Brassica napus, 95, 98, 99, 102, 104–106, 151, 160, 188, 194
Brassica nigra, 67
Brassica oleracea, 66–68, 114, 157, 184, 191, 266
Brassica rapa, 98, 151
Brassinosteroids, 58, 59, 97, 99
Bupleurum falcatum, 285

C
Calliclones, 141
Callus, 121–124
  habituated, 40, 54, 256
  induction, 70–72
  of mulberry, 40
  of wheat, 40, 41, 66, 94
Callus culture
  applications of, 40
Calystegia sepium, 41, 46, 49
Capsella bursapastoris, 129
  embryo culture, 128
  embryo development, 134
  embryogenesis, 133
  embryo nutrition, 131
  isolation of embryo, 129, 130
Capsicum, 99
Capsicum annuum, 102
Camptotheca acuminata, 281
Capsicum frutescens, 280
Carica papaya, 125, 127
Catharanthus, 69, 276
Catharanthus roseus, 276–279, 281, 285, 291, 295
Cattleya, 259
Caulogenesis, 64, 66
Cell culture
  large scale, 45–46
Cell viability tests
  Evan's blue staining, 45
  fluorescein diacetate, 44, 53, 176, 195
  phase contrast microscopy, 44
  tetrazolium test, 44
Cellular totipotency, 1, 63, 79, 123
Centaurea cyanus, 56
Chemotherapy, 4, 228, 232, 234, 236, 238, 285
Cichorium, 69, 84
Cinchona ledgeriana, 281
Citrus sp., 75, 91, 124, 126, 173, 191–193, 236
Citrus clementina, 192
Citrus deliciosa, 192
C. grandis, 125, 127
C. nobilis, 192
Citrus sinensis, 125, 126, 192, 236
Citrus unshiu, 192
Clonal propagation, 4, 69, 89, 145, 245, 246, 249, 252, 258, 268, 269
Coccinia grandis, 127
Cochlearia danica, 129
Codiaeum variegatum, 125
Codonopsis ovata, 155
Coffea arabica
  medium-term storage, 289
Coffea arabusta, 267
Coffea canephora, 87, 90
  initiation of embryogenic callus, 87
  production of green cotyledonary stage embryos, 87
  production of torpedo stage embryos, 87
Coffea sp., 75
Cointegration binary vector
  salient features of, 205
Coix, 164, 168
Coleus, 53, 55, 56
Coleus blumeii, 276
Colocasia esculenta, 291
Conservation of germplasm, 6, 239, 267, 292
Conservation of phytodiversity
  in situ, 287–288
  ex situ, 288
Convolvulus arvensis, 70, 71
Cordyline, 249
Corynebacterium glutamicum, 215
Crambe abyssinica, 188, 192
Crepis capillaris, 144, 145
Crotolaria juncea, 67
Croton, 122, 123, 125
Croton bonplandianum, 122, 123
Cryopreservation
  reculture, 297
  slow freezing, 293, 294
  stability assessment, 297

storage of cells, 296
thawing, 296–297
ultra-rapid freezing, 292, 295, 298
Cryopreservation pre-treatments
dehydration, 293–296, 298
droplet vitrification, 296
encapsulation and dehydration, 296
encapsulation and vitrification, 296
pregrowth dehydration, 295
pregrowth, 295
vitrification, 296
Cryoprotectants, 294–296
Cryotherapy, 233–235, 240, 242
Cucumis, 123
Cucumis sativus, 138
Cucurbita pepo, 68, 114
Cucurbita sp., 199
Culture media, 3, 17, 27, 123
constituents, 27–34
for androgenesis, 94, 97
for banana micropropagation, 271
for *Capsella bursapastoris* embryo, 128, 134
for cell cultures of *Calystegia sepium*, 44
for *Ficus lyrata* micropropagation, 272, 273
for *Gladiolus* micropropagation, 269
for mesophyll cells culture, 44, 60
for orchid tissue culture, 250
for proembryo culture, 128, 132–133
for suspension cultures of tobacco, 43
inorganic nutrients, 29, 129
Kao90 medium, 129, 132, 134
pH, 34–35
preparation, 13, 35
sterilization, 17
Culture media constituents
amino acids, 29–30
carbon source, 31
gelling agents, 34
macronutrients, 28, 29
micronutrients, 28, 29
plant growth regulators, 31–33
undefined supplements, 33
vitamins, 29–30
Culture media preparation, 13, 35
Culture of in vitro zygotes, 162, 163
Culture room, 11, 16, 108, 158, 261, 265, 268, 291
Culture trolley, 16
Culture vials, 12, 14–17, 21, 22, 36, 67, 256, 260
Cunninghamia lanceolata, 68, 266
Curdania tricuspidata, 283
Cuscuta, 2
Cybridization, 5, 189, 190, 193
Cybrids, 5, 173, 184, 189, 190
Cymbidium
clonal multiplication, 141, 248, 263, 268, 269
Cymbidium hybrids, 69, 248
Cymbopogon, 144
Cypripedium calculus, 288
Cytodifferentiation, 51, 54–57, 276
Cytokinins, 3, 32, 79, 231, 255

D

Dahlia, 4, 8, 228–231, 233, 279
Datura, 2, 8, 9, 133
Datura innoxia, 6, 93, 98, 103
Datura metel, 278
Datura stramonium, 93, 113, 131, 132
Datura tatula, 132
Daucus carota, 75, 84, 91
Davallia, 252
Dedifferentiation, 60, 63, 80, 81, 143
Dendranthema x *grandiflorum*, 187
Dendrophthoe falcata, 123–125
Dianthus, 260
Dierama luteoalbidum, 254, 258
Digitalis lanata, 279, 280, 282
Digitalis purpurea, 279, 280
Dioscorea, 257
Diploidization, 97, 105, 195
Direct gene transfer, 205–207

E

Eichhornia crassipe, 1
Elaeis guineensis, 145
Electrofusion, 5, 160–162, 167, 178, 179, 192, 195
Electroporation, 182, 200, 202, 207
Electrotherapy, 235, 240
Endoreduplication, 144
Embryo culture
applications of, 135–140
culture medium for, 116, 131
culture requirements, 131
of *Capsella bursapastoris*, 128, 131, 133–135
precocious germination, 78, 80, 135
technique of, 129–130
Embryo development
applications of, 135
autotrophic phase, 133
heterotrophic phase, 133
role of suspensor in embryo development, 134–135
Embryo culture applications
haploid production, 98, 101, 138
in basic studies, 135
production of rare hybrids, 138–140
propagation of rare plants, 138
rapid seed viability, 138
shortening of breeding cycle, 138
transformation, 138, 167
Endosperm culture, 121, 123, 124
applications of, 127
callusing, 121–123
culture medium for, 123
effect of stage of endosperm, 121–123
of *Croton bonplandianum*, 122, 123

of *Exocarpus cupressiformis*, 6, 123–125
of maize endosperm, 121, 123, 160
of *Ricinus communis*, 122
plant regeneration, 123–124
Equipment suppliers, 23
Eruca sativa, 134
Ethylene, 33, 54, 55, 66, 67, 80, 220, 221, 264
Euphorbia millii, 278
Exocarpus cupressiformis, 6, 123–125

F
Feijoa, 253
Ficus benjamina, 259
Ficus lyrata, 252, 272, 273, 274, 275
Filter assembly, 19, 20
Filter sterilization, 13, 19, 23
Freesia, 257
Fusion of gametes, 162
Fusogen, 176, 178, 192

G
Gametoclonal variation, 105, 141
Gametoclones, 7
Gardenia, 259
Gelling agents
agar, 34, 35, 224, 231, 292, 296
agarose, 34, 180
gellan gum, 34
gelrite, 34, 171, 242, 248, 260, 270
isubgol, 34
phytagel, 34, 281
Gene banks
some examples, 290
General requirements, 11
General techniques, 11, 245
Genetically engineered plants, 7, 141, 199
Genetically modified crops, 149, 199, 218, 222, 223
for abiotic stress tolerance, 215
for biofuel, 222
for delayed fruit ripening, 223
for disease resistance, 212
for flower colour, 239
for herbicide resistance, 209, 210
for improvement of cotton oil, 221
for insect resistance, 209–211
for male fertility, 215, 216
for nutritive quality of food, 214
for parthenocarpy, 216
for plants as bioreactors, 216
for virus resistance, 213, 214, 218, 222, 239
golden rice, 200, 214, 215, 217, 278, 282
some examples of, 223
Genetic engineering, 73, 94, 119, 173, 200, 203, 209, 212, 222, 223, 239, 278
applications of, 73, 149, 191, 209, 211
Genetic transformation
Agrobacterium mediated, 202, 203, 203–205, 200, 201, 224
applications of, 119, 202
biolistic method, 205, 207
biosafety, 193, 222, 223, 214
direct gene transfer, 205–207
RNA interference based, 214, 218
Gene transfer, 7, 200, 201, 204–207, 209, 278
Gerbera, 114, 116–118, 256
Gibberellins, 19, 33, 53–55, 79, 83, 123, 135, 231
Gladiolus, 255, 257–259, 265, 272
Gladiolus tristis, 258
Glassware
washing, 1, 11–13, 17
Glehnia littoralis, 295
Glycine, 28–30, 44, 60, 66, 67, 133, 181, 241, 270
Glycyrrhiza uralensis, 281
GM crops. *See* Genetically modified crops
Golden rice, 200, 214, 215, 278
Gossypium arboreum, 139, 279
Gossypium hirsutum, 139
Greenhouse, 16, 180, 216, 224, 228, 230, 237, 238, 240, 242, 249–251, 267
Growth room, 11, 12, 14, 15, 21, 22
Gynogenic haploids, 6, 113, 114, 119

H
Habituated callus, 40, 54, 254
Hairy root culture, 217, 264, 278, 281–282, 284, 285–286, 288
Haplopappus gracilis, 41
Helianthus, 53, 55, 187
Helianthus annuus, 14, 16, 140, 144, 188, 189
Helianthus giganteus, 189
Helianthus maximiliani, 140, 188, 189
Helianthus tuberosus, 144
Helminthosporium maydis, 215
Heterokaryon, 173, 176, 177, 184, 197
High Efficiency Particulate Air (HEPA) filter, 21
History, 7, 193, 245
Holarrhena antidysenterica, 280
Hordeum, 131, 168
Hordeum vulgare, 102, 104, 113, 114, 116, 160
Hybrid embryo culture, 129, 140
Hyoscyamus niger, 104, 189, 217, 278
Hyperhydration, 88, 117, 259–261

I
IAPTC Newsletter, 9
Identification of transformed cells/plants, 207
Inorganic nutrients, 17, 29, 129, 254
Instruments, 12, 17, 20, 209, 239
International Association of Plant Biotechnology, 10
International Association of Plant Tissue Culture, 8, 9
International Association of Plant Tissue Culture and Biotechnology, 8

In vitro conservation of germplasm
advantages of, 292
long-term storage, 289
medium-term storage, 289
In vitro fertilization
applications of, 158
diagrammatic summary of, 160
embryo development, 78, 82, 115, 116
endosperm development, 159, 165, 167, 168
factors affecting, 49, 64, 113, 158, 183, 258
in maize, 33, 84, 105, 132, 138, 156–159, 160–163, 167, 217
in rice, 77, 99, 116, 117, 161, 167
preparation of explant, 68
protocols of, 267, 295
technique, 155, 156, 167, 168
In vitro formation of storage organs, 236, 257
In vitro ovular pollination, 156, 157
In vitro placental pollination, 156, 157
In vitro pollination, 155, 156
In vitro shoot-tip grafting, 234
In vitro stigmatic pollination, 155–157
In vitro therapy, 228, 229
In vivo thermotherapy, 232, 240
In vitro tuberization, 257
In vitro zygote culture, 135
medium for, 130, 131, 133, 136, 164
Isolation of protoplasts
enzyme treatment, 163, 175
osmoticum, 175
purification of, 175
viability of the protoplasts, 176
Intraovarian pollination, 157
Isolation of cells, 46
enzymatic method, 46, 50
mechanical method, 46
Isolation of central cell, 159, 163
Isolation of egg, 159, 163
Isolation of sperm cells, 161, 169
Isubgol, 34

J

Jatropha, 122, 123
Jatropha panduraefolia, 125
Journal of Plant Tissue Culture & Biotechnology, 8, 9
Juglans regia, 124, 125

K

Kalanchoe laciniata, 204
Kalopanax septemlobus, 86, 88
Kinetin, 64, 66, 123
discovery of, 3

L

Lactuca sativa, 65, 67
Laminar air flow cabinet, 107, 292
Larix eurolepsis, 34
Leptomeria acida, 124
Leucaena, 253
Lilium auratum, 139
Lilium longiflorum, 144
Lilium regale, 139
Lilium speciosum-album, 139
Lithospermum erythrorhizon, 6, 9, 276, 284
cell culture, 9
media for cell culture, 281
Linum austriacum, 1, 8, 127
Lolium perenne, 1, 8, 101, 127
Longterm storage of germplasm, 289
cryopreservation, 289
Lupinus, 2, 8, 184, 281
Ixia flexuosa, 252
Lycopersicon chilense, 139
Lycopersicon esculentum, 139, 183, 185, 266
Lycopersicon peruvianum, 139, 183, 187

M

Macadamia tetraphylla, 260
Macleaya cordata, 3, 9, 46, 75, 76, 82
single cell culture, 46, 49
single cell isolation, 46
somatic embryogenesis in, 3, 9, 75, 76, 82, 83
Magnolia, 259
Maintenance of virus-free stocks, 238
Mangifera indica, 76
Marker genes, 184
Matthiola incana, 184
Mature endosperm culture, 6, 124
importance of embryo association, 78
Mechanisms underlying somaclonal variation, 85
activation of transposable elements, 148
amplification of DNA, 147
changes in chromosome number and structure, 146, 187
gene mutations, 146, 147, 152
hypomethylation of DNA, 147
Media preparation, 11
Media room, 11–13, 19
Medicago sativa, 75
Medicago truncatula, 77, 84
Medium-term storage of germplasm, 289–292
by desiccation, 292
by modification of medium, 291
cold storage, 291
storage under low oxygen environment, 291
Melandrium album, 96, 115, 117
Mentha arvensis, 278
Meristem culture, 4, 8, 231, 233, 236, 243
Meristemoids, 66, 69, 70
Meristem-tip Culture, 228, 229, 231, 232, 234, 236, 237, 240, 241
chemotherapy, 236
culture medium for, 228, 229, 231
effect of genotype on, 67, 232

effect of physiological condition of explants, 231
effect of thermotherapy, 231, 232
meristem-tip isolation, 230, 231
storage conditions for, 231
Mesophyll protoplasts, 180
of *Trifolium repens*, 139, 156, 158, 174
Microelectrofusion, 178, 179, 197
Micropropagation, 56, 63, 81, 123, 181, 183, 184, 188
adventitious bud formation, 251, 252, 254
applications of, 268
factors affecting, 259
forced axillary branching, 253–255, 263
Indian scenario, 267
of banana, 254
of *Ficus lyrata*, 252, 272, 273
of gladiolus, 255, 271
of *Musa accuminata*, 269
of orchids, 246, 249, 251
of *Phalaenopsis*, 246
of potato, 257
of *Pyrus serotina*, 254
photoautotrophic, 266
Problems in *see* Problems in micropropagation
protocols for, 250, 251, 270, 272
regeneration from callus, 251
Micropropagation stages, 249, 258
acclimatization, 33, 253, 254, 259, 260
adventitious bud formation, 251, 252, 255, 263
forced axillary branching, 253–255, 263
initiation of cultures, 247, 250, 259, 260, 262
multiplication, 251
preparatory stage, 249
regeneration from callus, 146, 251, 252, 255
rooting, 255
shoot elongation, 255
transplantation, 255
Microprotoplasts, 188, 189
preparation of, 188
Microspore culture, 6, 94, 98, 100
Microspore embryogenesis, 112
in *Brassica napus*, 97
Microtubers of potato, 266
Molecular breeding, 199, 223
Morinda citrifolia, 277
Morus alba, 114, 116, 117, 125
Mosaicism, 146, 148
MS basal medium, 66, 109, 124, 259
composition of, 182
stock solutions, 13, 29, 35
Musa accuminata, 270
Myrothecium verrucaria, 5

N

Narcissus, 260
Nautilocalyx, 69
Neoplastic growth, 69
Nepenthes khasiana, 290
Nicotiana, 2, 41, 53, 94, 99, 104, 237
Nicotiana amplexicaulis, 171
Nicotiana glauca, 2, 5, 183, 184
Nicotiana glauca x *Nicotiana langsdorffii*, 9
Nicotiana langsdorffii, 2, 5, 9, 178
Nicotiana rustica, 155
Nicotiana tabacum, 41, 101, 102, 116, 118, 160, 168, 176, 192
Nitsch's medium, 248
Nothapodytes foetida, 281
Nymphaea gigantea, 288

O

Ontogeny of shoots, 69
Ophiorrhiza mungos, 281
Ophiorrhiza pumila, 281
Orchid micropropagation, 245
Organic nutrients, 29, 31, 131, 184, 257, 274
amino acids, 29, 30, 31
vitamins, 29, 30
Organogenesis, 3, 8, 33, 63, 64, 66–68, 70, 71, 82, 101, 185, 253, 285
in cotyledon cultures, 66, 65, 68, 70
Organogenic differentiation, 123
induction of, 70
Ornithogalum, 236
Oryza longistamminata, 212
Oryza meyeriana, 188, 192
Oryza sativa, 114, 117, 125, 170, 188
Osyris, 122

P

Paclitaxel production, 281, 285
Panax ginseng, 281, 282, 291
Panax notoginseng, 282
Papaver somniferum, 279
Paphiopedilum, 249
Parasexual hybridization, 173
applications of, 250
Petroselinum hortense, 125, 187
Petunia, 99, 191, 218, 250, 259
Petunia axillaris, 127, 157
Petunia hybrida, 177, 191
Petunia parodii, 177
Petunia violacea, 155
Phalaenopsis, 246, 249, 253
a protocol for clonal propagation, 249
Phaseolus acutifolium, 139
Phaseolus coccineus, 134–136
Phaseolus lunatus, 139
Phaseolus vulgaris, 55, 114, 139
Phloem differentiation, 5, 53
Phloroglucinol, 32, 33, 260
Photoautotrophic micropropagation, 266, 267
Phytosulfokine-α, 49, 58
Pinus gerardiana, 68
Pinus radiata, 65, 67–69, 70
Plant growth regulators, 31, 39, 59, 81, 277

abscisic acid, 32, 33, 54, 55, 135
auxins, 3, 30–32, 59, 65, 79, 184, 261, 262
cytokinins, 3, 30, 32, 33, 65, 68, 69, 79, 181, 231, 255
ethylene, 80, 295
gibberellins, 33
polyamines, 33, 83, 84, 116
TIBA, 32, 33, 66, 71, 132
topolins, 32, 259
Plantago ovata, 34
Plant material, 11, 14, 20, 21, 180, 239, 241, 266, 287–289, 292
Plant regeneration from endosperm
list of species showing, 90, 125
of *Exocarpus cupressiformis*, 123–125
of mulberry, 40, 126, 127
protocol for, 46, 50
Plasticware, 11, 14, 17
washing, 17
Platycerium, 253
Plumbago indica, 66
Plumbago zeylanica, 160
Pluripotent, 70, 71
Podophyllum hexandrum, 79
Polarity, 79, 81, 82, 176
Pollen culture
advantages of, 94
in *Brassica napus*, 95, 98
of *Brassica juncea*, 108
of *Nicotiana tabacum*, 107
of rice, 110
Pollen embryogenesis
nuclear fusion during, 104
Polyamines, 33, 83, 84, 116
Polyanthes tuberose, 285
Polysomaty, 144
Populus, 80, 295
Precision breeding, 223
Precocious germination, 78, 80, 127, 135
Problems with micropropagation
browning, 251, 261
high cost, 263
hyperhydration, 259–260
off-types, 262
recalcitrant plants, 267
Proembryo culture, 128, 132–133
Proembryogenic masses, 77, 78, 81
Protoclone, 141, 150
Protocorms, 69, 246, 247, 249, 269
Protoplast culture
8p medium, 181
agarose droplets or beads, 162, 180
agarose embedded cultures, 180
cell division and callus formation, 180–183
cell wall formation, 167, 180
culture medium for, 181–183
double layer method-protocol for, 180
in liquid medium, 180
microdroplet method, 181
nurse cell technique for the culture, 184
plant regeneration, 183–184
Protoplast fusion
by electrofusion, 178
by pH-high Ca^{2+}, 5, 176
by polyethylene glycol, 5, 159, 196
Protoplast isolation
enzyme treatment, 175, 178, 188
osmoticum, 175, 178, 182, 206, 295, 296
purification of, 175–176
viability of protoplasts, 176
Prunus persica, 125
Pseudomonas elodea, 34
Pulmonaria mollissima, 1
Putranjiva, 122, 125
Putranjiva roxburghii, 125
Pyrus malus, 125
Pyrus serotina, 254

R

Ranunculus sceleratus, 75–77
Raphanus caudatus, 127
Raphanus landra, 127
Raphanus sativus, 127, 191
Rauwolfia serpentine, 278, 279, 281
Recombinant DNA technology, 223, 285
Re-differentiation, 63
Regeneration of protocorms, 247
Regeneraion of transformed plants, 209
Requirements, 11–16
Rice spikelet
structure of, 130
Ricinus, 122
Ricinus communis, 122
Rhododendron, 259, 260
RNA interference (RNAi), 214–216, 218–221, 223
Root cultures, 2, 218, 278, 280, 281, 284, 289
Root specific genes, 72
Rosa sp., 41
Rubia tinctorum, 279

S

Saccharum, 6
Saccharum officinarum, 148
Saintpaulia, 252
Salvia officinalis, 280
Sambucus, 53
Santalum album, 124, 125
Sclerotinia sclerotorum, 106, 112
Scurrula pulverulenta, 65, 124, 125
secondary metabolite production
biotransformation, 279–280
commercialization, 209, 224, 267, 280, 284–285
elicitation, 278–279
genetic enhancement, 217, 277–278
immobilization of cells, 280
permeabilization, 280
strategies to optimize production, 276–280

Selection of transformed cells/plants, 211–212
Selective subculture, 79
Serratia marcescens, 215
Shoot apical meristem specific genes, 73, 103
Shoot bud differentiation, 64–68, 70, 71, 124
Shoot induction medium, 70–72
Shoot meristem, 71–73, 234, 237, 242
Shoot regeneration, 66, 71–73, 124
  differentiation, 68
  effect of culture medium, 64
  effect of electrical stimulation, 68
  effect of explant, 67
  effect of genotype, 67
  effect of sonication treatment, 68
  effect of ultrasound, 68
  factors affecting, 64–68
  the process of, 71
Shoot tip culture, 4, 146, 147, 238, 252, 294
Short interfering RNA (siRNA), 214, 219, 220
Sinapsis arvensis, 193
Single cell culture
  Bergmann's cell plating technique, 47
  factors affecting, 49
  filter paper raft-nurse technique, 46–47
  microchamber technique, 47
  microdrop method, 47
Single cell isolation, 46
Solanum, 69
Solanum lycopersicoides, 141
Solanum melongena, 80, 84, 189
Somaclonal variation
  advantages of, 155
  applications of, 150
  disadvantages of, 155
  mechanisms underlying, 148–150
  methods to assess, 143
  origin of, 144
  some examples of, 150
Somaclones, 143, 146–149, 151–153, 278
Somatic embryos
  large scale production, 86–89
  mass production, 88
  maturation and conversion of, 85–86
  single cell origin of, 82
  storage of dicot embryos, 87
  vs zygotic embryos, 86
Somatic embryogenesis
  development of, 81–82
  effect of auxin, 78
  effect of electrical stimulation, 79
  effect of explants, 77
  effect of genotype, 77
  effect of growth regulators, 78
  effect of medium, 78
  effect of *Nostoc* and *Anabaena*, 80
  effect of *Pseudomonas maltophilia*, 80
  factors affecting, 76–80
  in *Macleaya cordata*, 83
  in *Mangifera indica*, 76
  in *Ranunculus sceleratus*, 76
  induction and development of, 80
  induction of, 81
  molecular markers of, 84–85
  physiological and biochemical aspects of, 83–84
  synchronization of, 82
Somatic hybridization
  asymmetric hybridization, 190–194
  landmarks in the history of, 195–196
  symmetric hybridization, 189–192
Somatic hybrids
  characterization of, 189
  selection of, 188–189
  some examples of, 195
Sorghum bicolor, 282
Spathiphylum spp., 261
Stages in micropropagation. *See* Micropropagation stages
Sterile area, 11, 263
Sterilization, 11–13, 17–22
Sterilizing agents, 20
Steripot, 20
Stevia rebaudiana, 267, 268
Stigmatic pollination, 158, 159
Supermales, 107
Suspension cultures
  batch cultures, 41
  chemostat, 42
  close continuous cultures, 42
  continuous cultures, 41
  growth measurements, 41
  growth phases of, 43
  medium for, 43
  open continuous cultures, 42
  synchronous cultures, 43
  turbidostat, 42
  types of, 40–42
Symmetric hybridization, 188, 190–192
Syngamy, 121, 130, 157
Synthetic seeds, 4, 89, 90, 253

T

Taxillus cuneatus, 123, 125
Taxillus vestitus, 123, 125
Taxus, 279, 282
Taxus baccata, 276, 282
Taxus chinensis, 284
Taxus cuspidate, 280, 283
T-DNA binary vector, 202, 203
T-DNA vectors, 201–204
Teratoma, 69
Test tube fertilization, 5, 156
Thalictrum minor, 276
Theobroma cocoa, 250
Thermolabile compounds, 13, 19
Thermotherapy, 228, 229, 232, 234–236, 238, 241, 242
Thin cell layer culture, 68–69

TIBA, 32, 33, 71
Ti plasmid, 201, 202, 205
Topolins, 32, 259
Totipotency
of crown gall tumour cells, 69
Tracheary element, 51–59
Tracheary element differentiation
biochemical changes, 57
cytological changes, 56
effect of abscisic acid, 54–55
effect of auxin, 53–55
effect of calcium, 55
effect of cAMP, 54, 55
effect of cytokinin, 54
effect of ethylene, 54–55
effect of gibberellic acid, 54–55
effect of physical and physiological factors, 55
effect of sucrose, 55
factors effecting, 53–55
in *Helianthus tuberosus*, 146
in *Syringa*, 54, 55
in *Zinnia elegans*, 52, 53, 56, 57, 59
medium for, 52, 53, 56
molecular changes, 57
process of, 57
stages in, 57
Tradescantia, 1
Transdifferentiation, 52, 59, 60, 63
Transfer room, 14–15, 22
Transformation
Agrobacterium mediated, 200–205
biolistic method, 205, 207
biosafety, 222–223
direct gene transfer, 205–207
RNA interference based, 214, 218–219
Transgene, 141, 199, 200, 203, 208, 209, 212, 213, 214, 215, 217, 218, 222
Transgenic crops. *See* Genetically modified crops
Transgenic plants, 199, 201, 210, 211, 212, 216, 218, 222, 223
Trifolium, 157
Trifolium hybridum, 139
Trifolium repens, 139, 174, 296
Trigonella foenumgraecum, 281
Triple fusion, 121, 155, 166, 164
Triticum, 139, 168
Triticum aestivum, 101, 103, 114, 187
Tropaeolum, 2, 8
Tumour inducing principal (*Ti*P), 8

V
Valeriana officinalis, 281
Vanda, 246
Vascular tissue differentiation
factors effecting, 53–55
Vectors, 201–204, 221, 223
Verticillium dahliae, 279
Victoria amazonica, 288
Virus elimination
by chemotherapy, 232–233
by cryotherapy, 234–235
by electrotherapy, 235
by meristem-tip culture, 230
by other in vitro methods, 235–236
by thermotherapy, 232
by thermotherapy and cryotherapy, 232, 233
importance of, 240
in vitro shoot-tip grafting, 234–235
practical method of, 241
protocol for, 238, 241
Virus indexing and certification
biological indexing, 238–239
DNA microarray technology, 239
electron microscopy, 239
molecular assays, 238–239
nucleic acid-based assay, 239
nucleic acid hybridization, 239
serology, 238
Vitis vinifera, 236, 266, 278, 279
Vivipary, 137

W
Washing room, 12–13
White's medium, 65, 78, 91, 277

X
Xanthomonas oryzae, 212
Xylogen, 58–60
Xylogenesis
in *Zinnia elegans*, 52, 53, 55, 60

Z
Zea latifolia, 185
Zea mays, 75, 102, 114, 168
Zea mexicana, 168
Zinnia elegans
tracheary element differentiation, 56–58
Zygote culture, 123
of maize, 121
of rice, 130, 132
of wheat, 138, 141, 144
technique for, 129–130
Zygotic embryo culture, 123, 124
applications of, 135–140
Capsella bursa-pastoris, 128, 131, 133–135
culture medium, 130–132