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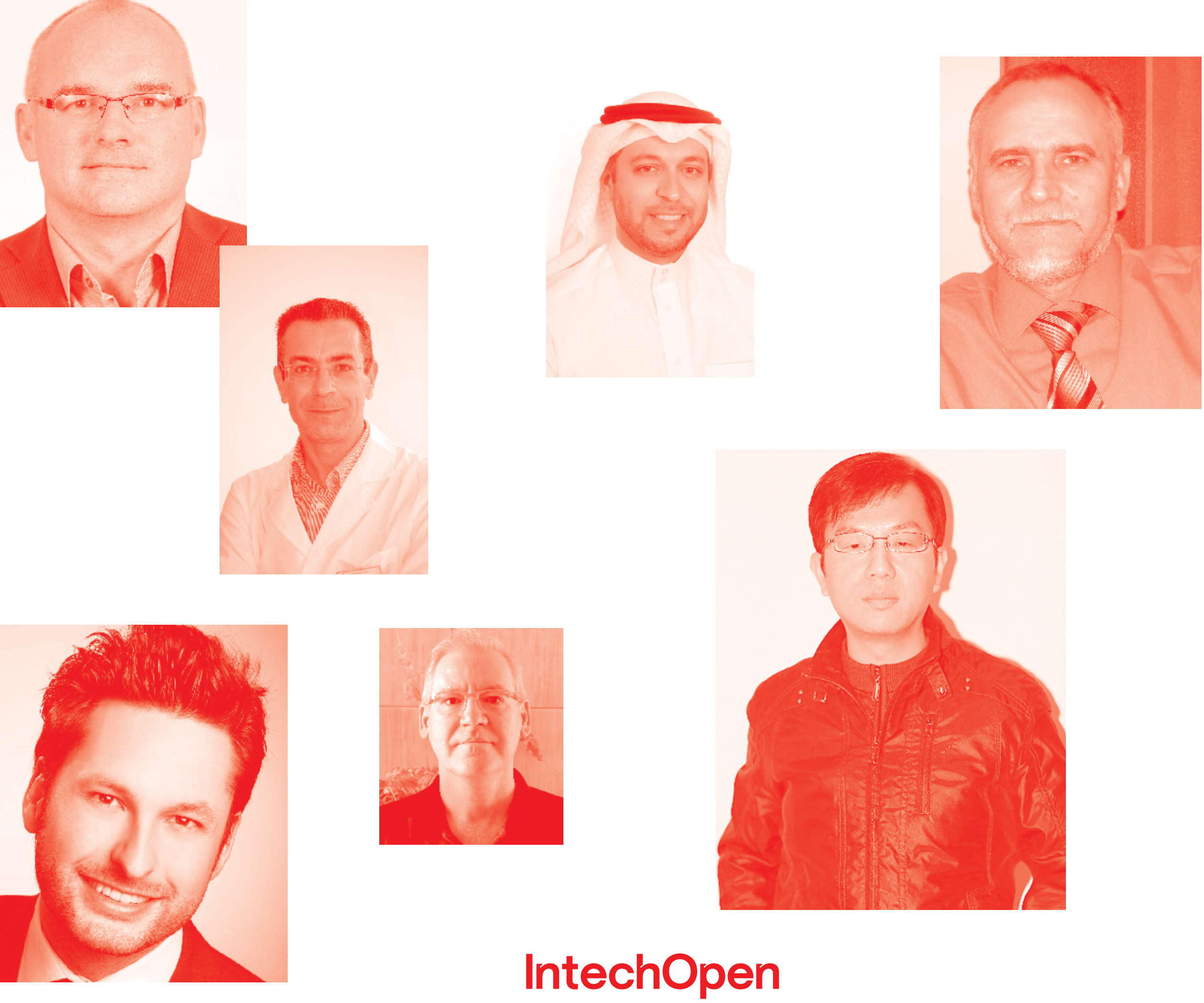

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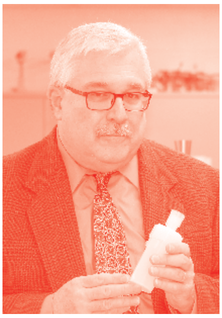

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Contributors
Usman Khalid Chaudhry, Zahide Neslihan Öztürk Gökçe, Ali Fuat Gökçe, Casper Nyaradzai Kamutando, William Makaza, Smita Srivastava, Abhisek Shrestha, Bharti Thapa, Mohammad Hasanuzzaman, Jasmine M. Shah, Joyous T. Joseph, Istvan Jablonkai, Samir Medhioub, Ali Ellouze, Slah Bouraoui, Hassen Sabeur, Pralhad Rambhaji Jaybhaye, Sajal Roy, Nasrin Chowdhury, Amit Chawla, Nikita Rathore, Dinesh Thakur, Nang Elennie Hopak, Josphert N. Kimatu

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Meet the editor

Prof. Josphert Ngui Kimatu is an associate professor in the School of Sciences and Computing, Department of Life Science, South Eastern Kenya University. He has a Ph.D. in Plant Molecular Epigenetics from Northeast Normal University (NENU), Changchun City, China, and an MPhil in Forestry Pathology and a bachelor's degree in Botany and Zoology, both from Moi University, Kenya. He also has a Post Graduate Diploma in Education (PGDE) from Maseno University, Kenya. Dr. Kimatu was trained in post-harvest management in maize, legumes, and rice via a four-module certificate course at Stellenbosch University, South Africa; Sydney University, Australia; and Kwame Nkrumah University of Science and Technology, Ghana. He also has a certificate in molecular diagnostics from the University of Nairobi, Kenya. Dr. Kimatu obtained a training certificate in integrated pest management and pesticide safety (IPM) from Soybean Innovation Lab in 2020 and a training certificate on COVID-19 from the London School of Hygiene and Tropical Medicine.

Contents

Preface

Studies involving *Arabidopsis thaliana* and other crop plants have continued to advance our knowledge of the mechanisms used by plants to survive in dynamic biotic and abiotic environments. Plants have adapted to diverse ecologies for ages; however, recent human migrations, agricultural activities, and climate change-induced phenomenon have forced plants to increasingly adapt to new environments. Furthermore, agricultural production is becoming progressively more vulnerable to climate variability and change characterized by altered frequency, timing, and magnitude of precipitation, temperature, and wind.

This book focuses on current morphological, anatomical, physiological, molecular, and genomic advances in plant defenses. It discusses advances in plant stress signaling mechanisms and molecular defense responses involving recently discovered biomolecules. It also discusses advances in gene expression changes, which can be inherited as described in epigenetic mechanisms and linked to observed phenotypic plant plasticity. The book also looks at emerging areas like plant growth-promoting microorganisms (PGPMs), which consist in a vast group of microorganisms found in the rhizosphere, on the root surface or associated to it. It examines the mechanisms of action of PGPMs, including their ability to produce different plant growth regulators or phytohormones.

The studies in this book indicate that researchers have found intriguing interactions among plants and their physical, microbial, and biotic environments. These interactions have been highlighted and linked to novel mechanisms, increasing our understanding of how sessile plants adapt and thrive in challenging environments. These studies consider the resilience and sustainability of plant genomes and epigenomes and the role they will play in the next generation of food systems.

Some of the other topics addressed in this book include the effects of hailstorms, control of secondary damages due to insect pests and diseases, the removal of fallen fruit to reduce the spread of disease and pests during their decay, analysis of wounds on branches and fruit thinning, and the effects of bud-breaking chemicals, bioregulators, and fertilizers on vegetative growth. In general, the major and chronic threats to ecosystem stability and sustainable agriculture are phytopathogenic microorganisms. The unlimited and regular use of chemical pesticides and fungicides can destroy soil ecology, degrade soil fertility, and disrupt the environment, causing harmful effects on human health and contaminating groundwater. However, our studies found that PGPMs are environmentally friendly and offer a promising way of obtaining sustainable soil fertility and plant growth, although indirectly. Nevertheless, antibiotic production is considered one of the most powerful and studied biocontrol mechanisms of PGPMs against phytopathogens.

Josphert Ngui Kimatu, BSc., Mphil., PGDE, Ph.D.
Professor,
South Eastern Kenya University,
School of Sciences and Computing,
Department of Life Sciences,
Kitui County, Kenya

Chapter 1

Introductory Chapter: Inevitable Cytogenetic, Genetic, and Epigenetic Changes Contributing to Phenotypic Plasticity for Plant Defence Mechanisms in Dynamic Environmental Conditions

Josphert Ngui Kimatu

1. Introduction

Plants are currently encountering many changes in the environment, which are being brought about by human activities due to increasing population demands and land fragmentations. Studies show that over 50% of the regions on the earth are expected to suffer from water scarcity by 2050 [1, 2]. These activities include pollutions, increase in temperature, lack of pollinators and dispersal mechanisms. The observed morphological changes in plants are due to changes in gene expressions [3]. These gene expression changes do not involve permanent changes in the DNA sequences; otherwise, the species would either become extinct or modified to be another. However, these changes have been identified in the functional genomics and names as mainly epigenetic. Epigenetic variations can be used to indicate the degree of plant responses due to environmental stresses. Plant adaptations in stress conditions can be induced by long-term or short-term stress exposures [4]. In stressful conditions, plants use three main strategies to survive. They either tolerate, resist, or escape but also employ stress recovery mechanisms after the stress challenging environmental conditions [5–7].

Climate change coupled with other environmental pressures is making the rate of formation of new plant gene combinations to seem quite slow compared with the occurrence of the environmental pressures [8]. Plants employ processes such as stress avoidance via regulating characteristics such as leaf structure, root growth, flowering patterns, seed development, so as to optimize prevailing morphological and physiological processes.

2. The relationship between environmental stress and epigenetic variability

Plants have to endure adverse environmental conditions at all times. This is because plants are sessile and do not physically escape stresses by changing locations. Plants defend themselves by employing epigenetic mechanisms [9].

Experiments have been done using plants of similar genetic compositions, which have grown in varied environmental conditions. For example, dandelion plants were grown in conditions of high salinity, low nutrients, and pathogenic induced by jasmonic acid or salicylic acid showed DNA methylation polymorphisms [10]. To confirm the results, the experimental control plants showed less epigenetic variations. Thus, epigenetic changes are important abiotic plant defense biomarkers in plant defense. Plants employ various mechanisms to sense environmental changes and then initiate epigenetic gene expression responses to enable adjustments in such situations [11]. More research should be done because, to date, only one transgenic maize cultivar has been commercialized among so many crop plants [6, 12].

3. The relationship between plant metabolism and epigenetic variability

Metabolism is defined as all chemical reactions in a cell that occur to maintain life. The level of acceptable rate of metabolism is determined by the number of environmental and cellular resources available for the plant. For example, water is a major plant resource that if there is water stress, much metabolism activity is shut down. This is done mainly via cytosine DNA hypermethylation. The mechanism that most plants use to respond to water stress is the expression of the abscisic acid (ABA) genes. For example, studies using repeated dehydration steps upregulated several ABA-induced genes in the model plant *Arabidopsis thaliana* [13, 14], while review studies by [4], suggested that changes in DNA methylation served as regulatory mechanisms affecting gene expression responses to drought stress. Previously, transposon mobility, activation of methyltransferase, and siRNA-mediated methylation have been implicated in phenotype variation in stress conditions [15].

4. The relationship between plant microbes, pests, abiotic stresses, and epigenetic variability

Plants establish biotic relationships, which are either beneficial or harmful. The beneficial relationships include those with the bacteria *Rhizobia*, *mycorrhiza*, with insect pollinators and seed dispersers. The harmful relationships involve viral, fungal, protozoan, bacterial pathogens and other competitors [16]. Thus, studies show that infection of plants by RNA viruses triggers epigenetic changes. For example, plants have been observed to recognize inserted viral double-strand RNA molecules and then inducing DCL2 and DCL4 for their degradation into siRNAs [17]. Other complex mechanisms to deal with single-stranded RNA (ssRNA) have also been observed. In this process, the genomes of ssRNA viruses are first converted into dsRNA molecules by RNA-dependent RNA polymerases and then the DCL family endoribonucleases act on the dsRNA,

Studies in *Arabidopsis* showed that an infection by a bacterial pathogen such as *Pseudomonas syringae* pv. *tomato* (Pst) elicited a defense response in plants that was suppressed by bacterial virulence factors. The data showed that cytosine DNA methylation pattern changes of some genes are associated with plant defense mechanisms. For example, *de novo* methylation can occur in a process where previously unmethylated DNA cytosine residues are methylated. This leads to new DNA methylation patterns being formed [18]. Other DNA expression related modifications include acetylation, phosphorylation, biotinylation, sumoylation, and ubiquitination at specific amino acid residues [19]. Furthermore, epigenetic changes were related to transcriptional changes of defense-related root genes [20–22].

Plants trigger gene expressions to survive all kinds of biotic and abiotic stresses. However, if the stress occurs in a short term, the plant can trigger rapid epigenetic changes to survive. Cassava plants have been observed to rapidly produce hydrogen cyanide (HCN) in case of biotic environmental disturbance and also could mistake a long-term abiotic stress and continually produce HCN to defend itself [23, 24]. Plants such as *Arabidopsis* when subjected to several cycles of water stress were found to spring back to normal metabolism faster than plants that experienced the water stress for the first time [25]. This is because even the mechanisms employed by plants to overcome various kinds of stresses are metabolically expensive and can drain plant resources. Plants transmit the epigenetic changes to the next generation in a phenomenon called transgenerational inheritable epigenetic changes or epimutations [25–27]. Extremes of most essential substances for plant growth and development can trigger extensive DNA methylations. For example, water stress can interfere with almost all metabolic processes, and if not checked, the plant can die. Furthermore, studies have shown essential trace elements such as Cu^{2+} and Cr^{3+} caused gross changes in DNA methylation only at high concentrations. Studies on *Laguncularia racemosa* showed that same plant species can be in different environmental conditions and be genetically similar but be varied epigenetically [28]. The mechanisms underlying these epigenetic changes remain largely unknown.

Other questions that linger are, for example, if plant epigenetic plasticity could be the cause invasive plants to be established in new habitats or it is new habitats that caused epigenetic variability? [4, 29]. More information is still needed in deciphering the significance of epigenetic mechanisms in influencing activities of specific plant growth regulators (PGRs) in the regulation of plant drought resistance and plant-microbiome interactions [6]. Studies have already been done on various tolerance-enhancing PGRs such as agonists, polyamines, antioxidants, and osmoprotectants [30–32] while further studies on the model grass *Brachypodium distachyon* gave more light on the genetic, epigenetic, and cytogenetic polymorphism mechanisms in plants. This is because the plant has a small genome and has fully been sequenced. It has a fast growth and is also almost cosmopolitan. This makes it to portray a great deal of observable phenotypic plasticity [33]. Statistical modeling can be used to establish the relationship between genetic distance and epigenetic variability or phenotypic variance and additive genetic effects [4].

5. Conclusions and prospects

Recent studies have confirmed the perceived relationship between epigenetic variations and environmental adaptations. The phenomenon of phenotypic plasticity as guided by varied epigenetic expressions enables plants to survive in changing environments while maintaining DNA sequence integrity. An understanding of the correction between genetic distances, epigenetic variations, heritability, and stability in the face of climate change in future might be used as a measure of selectable features in environmentally induced adaptations (**Figure 1**). Plant stress driven changes can be either temporal or permanent and stable. These are carried to the next generations and make the plant adaptable to future higher levels of stresses [34].

Epigenetic changes can be observed in same species in different habitats and can provide raw materials for natural selection in climate change induced stresses. For example, changes in DNA methylation patterns were correlated to morphological modifications abundance of trichomes and spines in plants [35], modified leaf

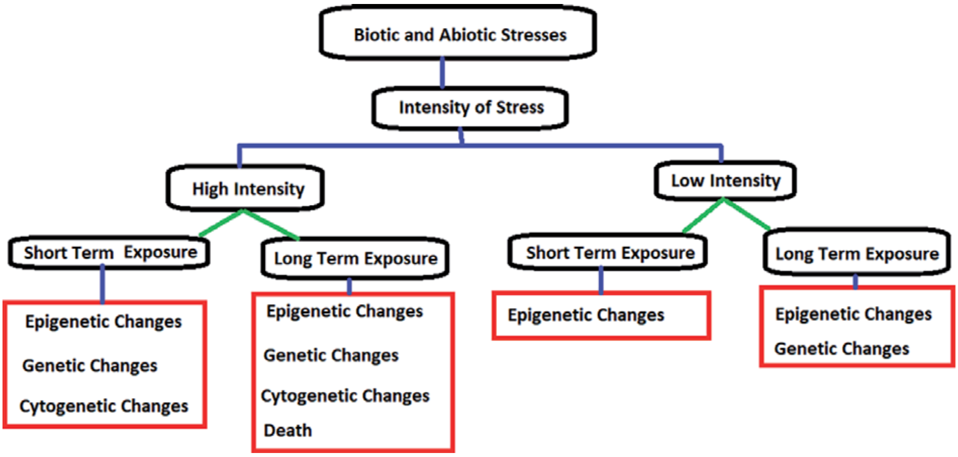

Figure 1.
The impact on the plant cells, genetic and epigenetic constitution by various intensity levels and durations of biotic and abiotic environmental stresses. The short-term effects are mainly for survival strategies while the long-term effects drive a plant into dimensions of adaptive speciation. Most epigenetic changes occur more in short durations and are highly reversible unlike long-term genetic and mutational changes. However, epigenetic changes can be assimilated into phenotypes in long-term evolutionally durations.

palatability [10], and later long-term differential vegetation browsing was observed [36]. This is because environmental stresses have been known to alter growth and productivity of even agricultural crop plants [15]. These epigenetic variations occur rapidly and can be used to predict the long-term effect of similar environmental stresses or hazards including heavy metals, air pollution, electromagnetic radiations, and high temperatures.

Epigenetic changes can affect genetic processes including DNA replication, DNA repair, transcription, transposon stability, and even cell differentiation [37]. These predictions can be simulated and studied in controlled laboratory environments, for example, recent analysis of plant mutations showed that epigenome-associated mutation bias could contribute to environmental effects on mutation [38, 39]. Studies in cosmopolitan plants can give an idea of the particular key mechanisms, which make them to survive and thrive in diverse environmental conditions. Species such as grasses can survive extreme temperature by shutting down entire metabolism and seem as if they are dead. They, however, rejuvenate rapidly at the onset of favorable conditions. The seeds of such plants can serve as study materials for such studies. They actually make all their processes to be in dormant state in adverse conditions.

Recent studies on plant microbiome show interactions that have symbiotic relationship, which reduce plant stress signaling [40]. Another epigenetic plant molecule of interest in abiotic stress that has been given recent attention is melatonin. The exogenous application of melatonin influences both physiological and molecular activities in a plant [41–44]. Furthermore, chitosan has been found to have multifaceted effects in various plant crops such as maize, sun flower, and potato in adjusting in abiotic stresses and improving on crop productivity. Studies in the correlations between epigenetics and biotic interactions also are addressing morphological plasticity to identify epigenome markers to improve crop productivity [45]. The molecular basis of such effects is still yet to be fully understood [46, 47], although recent bioengineering predicts possibilities of more precision genome editing using the CRISPRCas9 system application in the generation of alleles to improve plant yields under various abiotic stresses [2]. This is future, which was predicted by [48].

Author details

Josphert Ngui Kimatu
Department of Life Sciences, School of Sciences and Computing, South Eastern Kenya University, Kitui, Kenya

*Address all correspondence to: jkimatu@seku.ac.ke

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Chapter 2

Protein Metabolism in Plants to Survive against Abiotic Stress

Bharti Thapa and Abhisek Shrestha

Abstract

Plants are frequently subjected to several abiotic environmental stresses under natural conditions causing profound impacts on agricultural yield and quality. Plants can themselves develop a wide variety of efficient mechanisms to respond environmental challenges. Tolerance and acclimation of plants are always related to significant changes in protein, cellular localization, posttranscription, and posttranslational modifications. Protein response pathways as well as pathways unique to a given stress condition shared by plants under different stressed environment are discussed in this chapter. The various signaling of protein such as fluctuation, overexpression, and silencing of the protein gene are observed to be modulated in drought-tolerant plants. Similarly, gene expression, RNA processing, and metabolic process take place to cope with drought conditions. For adaption in water-submerged conditions, plants undergo reactive oxygen species (ROS), cell wall modification, proteolysis, and post-recovery protein metabolism. Heat shock protein and protein and lipid contents vary and play pivotal role in resisting low and high temperatures. In a nutshell, this paper provides an overview of several modification, synthesis, degradation, and metabolism of protein in plants to cope with and revive again to normal growing conditions against abiotic stress, emphasizing drought, submerged, extreme cold, and heat temperatures.

Keywords: protein, abiotic stress, tolerance, acclimation, yield

1. Introduction

Plants have developed a wide variety of highly sophisticated and efficient mechanisms to sense, respond, and acclimatize to a wide range of environmental changes. They have responded by activating tolerance mechanisms at multiple levels of organization (molecular, tissue, anatomical, and morphological), through the adjustment of membrane systems and cell wall architecture. This includes altering the cell cycle and rate of cell division and also by metabolic tuning [1]. Many molecular genes are induced and repressed by abiotic stresses at molecular level involving a precise regulation of extensive stress-gene networks [2, 3], and their products may function in stress response and tolerance at cellular level. Proteins involved in multiple protein functions, such as biosynthesis of osmoprotectant compounds, detoxification enzyme systems, proteases, transporters and chaperones, act as a first line of direct protection from stress. Moreover, regulatory proteins, for instance, transcription factors, protein phosphatases and kinases, and signaling molecules activation are essential in regulation of signal transduction and stress-responsive gene expression [4, 5].

Generally, observed tolerance responses toward abiotic stress in plants are composed of stress-specific response mechanisms and adaptive responses that confer strategic advantages in adverse conditions. In energy maintenance, general response mechanisms related to central pathway are involved, including calcium signal cascades [6], reactive oxygen species (ROS) signaling elements [7, 8], and energy deprivation signaling (energy sensor protein kinase, SnRK1) [9]; and induction of these central pathways is observed during plant acclimation toward different stress. Protein kinase SnRK1, despite being central metabolic regulator of the expression of genes related to energy-depleting conditions, also get activates when plants face different sorts of abiotic stresses such as drought, salt, flooding, or nutrient depravation [10, 11]. SnRk1 kinases alter over 1000 stress-responsive genes expression allowing the re-establishment of homeostasis by repressing energy consuming processes, thus promoting stress tolerance [10, 12]. Optimization of cellular energy resources during stress for plant acclimation has been found to be imperative; and partially arrested energetically expensive process, such as reproductive activities, translation, and some biosynthetic pathways [13]. For instance, in maize, during salt stress and potassium deficiency stress, nitrogen and carbon assimilations are impaired; also, the synthesis of free amino acids, chlorophyll, and protein is also affected [14, 15]. After cessation of energy-expensive process, energy resources can be redirected to activate protective mechanisms [16].

1.1 Plant stress tolerance and resistance

Plants are sessile organisms, which are continuously being confronted with several detrimental factors rising from ever-changing environment, and to cope with these problems, they have developed sophisticated and delicate defense mechanisms. In fact, diverse defense signal including the production of reactive oxygen species (ROS), change in redox potential or cellular level of Ca^{2+} ion, disruption of ion homeostasis, and membrane fluidity adjustments are activated [17, 18]. Once external stress is sensed via specific receptors, foreign signal is induced into intracellular downstream signaling pathways including the activation of protein kinase or phosphatase, stimulation of downstream target proteins, and biosynthesis of phytohormones for the control of plant growth/development [19, 20].

1.2 Role of amino acid during stress

Gene expression can be adversely affected by salinity, drought, and temperature stress, and many genes coding for enzymes involved in cellular metabolism are differentially expressed upon stress, thus modeling some stress-related transcription factors to induce changes in stress-associated metabolite levels [4].

For the synthesis of secondary metabolites and signaling molecules, several amino acids can act as precursors, for instance, polyamines are derived from Arg [21], Met synthesized the plant hormone ethylene [22], and conversion of Lys to N-hydroxy pipecoline is necessary for immune signaling [17, 23]. Moreover, several aromatic amino acids, such as Phe, Tyr, and Trp, or intermediates of their synthesis pathways produce a broad spectrum of secondary metabolites possessing multiple biological functions and health-promoting properties [24]. Usually, plants exposed to different abiotic stresses tend to accumulate free amino acids [25, 26], as exemplified to this response, [27] reported extensive accumulation of amino acid in response to drought stress in maize, cotton, tomato, and the resurrection plant. Also, recent studies conducted by [26, 28–30] suggest increment in free amino acids as a result of autophagy and abscisic acid. Similarly, plants surviving in stressed environment can use amino acids as an alternative for mitochondrial respiration substrates during inadequate carbohydrate supply due to a decrease in photosynthesis rates [29, 31]. Although ambiguity still remains for the specific role of catabolic pathways, the degradation pathways for Lys and the branched-chain amino acids Val, Leu, and Ile have already been identified as crucial factors for dehydration tolerance for *Arabidopsis* [32]. And, soon after reviving of plants to favorable growth conditions, reprogramming their metabolism to switch back for survival and active growth is necessary.

Members of the AP2/EREBP (Apetala2/ethylene-responsive element binding protein) family of transcription factors, CBF/DREB1 proteins (C-repeat binding factor or dehydration responsive element binding proteins), such as CBF1/DREB1B, CBF2/DREB1C, and CBF3/DREB1A play an important role in the transcriptional response to osmotic stress [33–37] and stated improved tolerance of *Arabidopsis* to freezing, drought, and/or salt stress via overexpression of these transcription factors, and [38, 39] supported above researchers confronting plants overexpression of CBF/DREB1 accumulated higher levels of proline and soluble sugars (glucose, fructose, sucrose, and raffinose) when grown under normal growth conditions and during cold acclimation. Conclusion made from [39, 40] suggested overall metabolic profile of CBF3/DREB1A overexpressers grown at normal growth temperatures resembled that of cold-exposed plants.

2. Proteomic overview on abiotic responses in plants

The biological research of abiotic stress in plants can be studied in broad range of transcriptomic and proteomic-based, provides the comprehensive information, during and following stress condition, on alteration of gene expression and proteome profile, the study about 30 min to 1 day after induction, and time lapse between transcriptomic and proteomic suggest more than 50% of genes responsive to flood, heat, and other stress were found to encode transcription regulators [41].

2.1 Protein metabolism in plant roots and shoots due to drought stress

Prolonged water deficit in the soil causes drought, which vastly affects the metabolism and physiological function in growing plant especially roots and responsive for water supply from soils to leaves and photosynthesis, respectively. Most of the proteomic evidence has been noticed due to drought condition, six steps prominently occur in the responsive drought stress. Signaling and sensing receptor, yet not specially but drought-responsive photoreceptor, phytochrome C1, found in maize, phytochrome gene (i.e., *PHYA*, *PHYB*, and *PHYE*) in *Arabidopsis*, believed to regulated the transcription of light responsive genes by modulating the activity of several transcription factors and involved in suppressing drought tolerance. Other signaling cascades, G protein subunits (alpha and beta), small G protein (e.g., Ras-related protein Rab7 and Ras-related nuclear protein Ran), and Ran-binding protein 1 (play important role in cell cycle and DNA synthesis) regulate positive role in drought stress [42], involved in vesicle trafficking, intercellular signaling, polar growth, plant hormone signal cross talk, and stress response [43]. The *PgRab7* gene was upregulated by dehydration in *Pennisetum glaucum* [44], while overexpression of the peanut *AhRabG3f* exhibited an enhanced tolerance to drought stress in transgenic peanut (*Arachis hypogaea* L.) [45] but negative role in Arabidopsis. Calcium-binding proteins (CaBs), such as calmodulin (CaM), calcium-sensing receptor (CaSR), calreticulin (CRT), and calcium-dependent protein kinase (CDPK), enhanced the survival of *Triticum aestivum* [46], and several protein kinases (e.g., serine/threonine-protein kinase, germinal center kinase (GCK)-like kinase

MIK, receptor-like protein kinase HERK 1-like, phototropin family protein kinase, and salt-inducible protein kinase), imply their role in drought response signaling pathway, in addition to phosphorylation level of protein phosphate 2C (PP2C), acts as negative regulator for plant drought tolerance in the abscisic acid (ABA) signaling pathway, which can inhibit the activity of SnRK, leading to a decrease of the phosphorylation of its substrates in the signaling cascade [47]. Similarly, 14-3-3 protein availability fluctuation shows the drought condition and reported that drought stress can directly alter the abundance of 14-3-3 proteins [48]. In addition, overexpression or silencing of the 14-3-3 protein genes can modulate drought tolerance of transgenic plants (e.g., *Gossypium hirsutum* and *Arabidopsis*) [49, 50].

Phytohormones play important role in signal transduction pathways such as drought-increased ethylene-responsive transcription factor (ERF) in *Gossypium herbaceum* [2] (and some members of drought-responsive auxin-binding protein (ABP) family in *Quercus robur* [51] *Zea mays* (41) and polar clones [52]. Under drought stress, *ERF* gene was induced in *G. herbaceum* [53, 54], and its overexpression in various plants, such as sugarcane *SodERF3* overexpression in tobacco, tomato *TERF1* in rice, and *Brassica rapa BrERF4* in *Arabidopsis*, can improve plant drought tolerance. *BpERF11* was found to negatively regulate osmotic tolerance in *Betula platyphylla* [42] ABP members (i.e., ABP2, ABP20, and ABP19a) are in response to drought stress, additionally TRIP-1 was phosphorylated by the brassinosteroid (BR)-insensitive I (BRI-1) protein, drought-increased TGF-β-receptor interacting protein 1 (TRIP1) was found in *Sporobolus stapfianus* and triggered the BR signaling pathways in water deficit condition.

Gene expression plays important role in the transcriptional regulatory networks, requires chromatin structure modification, i.e., histone, major protein of chromatin, and regulates the expression and high mobility group protein (HMG), involved in cell cycle progression. Among several histones, histone H1 was decreased in a drought-sensitive *Z. mays* cultivar, but increased in a drought-tolerant one [55], and H2B histone H1 was decreased in a drought-sensitive *Z. mays* cultivar, but increased in a drought-tolerant one [55]. Similarly, the phosphorylation level of HMG was significantly decreased in a drought-tolerant wheat cultivar, but increased in a drought-sensitive one [42] that reduced its binding to DNA, inhibiting replication and transcription.

Several RNA processing-related proteins changed over the stress condition, represent the critical for plants to cope with. Five glycine-rich RNA-binding proteins (GR-RBPs) increased with drought and three GR-RBPs decreased with drought, which bind to RNA molecules for transcriptional gene regulation and suspected to function in the regulation of specific gene expression. For instances, transgenic rice consists of GR-RBPs gene showing higher yield and drought recovery rate as compared with wild rice [56], besides overexpressed in *Camelina sativa,* reduced the drought tolerant. Similarly, S-like ribonucleases (RNases) specialized function as stress regulation, defense against microorganisms, phosphate scavenging, and even nitrogen storage, increased in rice under drought [57]. Additionally, an intron splicing-related protein, and maturase K (MatK) and multiple organelle RNA editing factor 9, involved in RNA editing in mitochondria and plastids, was found fluctuated in *Brassica napus* with the extension of drought stress [58] indicating the transcriptional regulation.

Most fundamental metabolic process to cope with drought stress, a plant can attribute to protein synthesis and turnover. Several proteins are involved in protein biosynthesis, such as ribosomal protein (RP), elongation factor (EF), translation initiation factor (TIF), tRNA synthase (TRS), and ribosome recycling factor (RRF), beneficial to protein synthesis, besides protein folding and processing varies cultivars and species. Instances, peptidyl-prolyl *cis-trans* isomerases (PPIases) were significantly increased in *Oryza sativa* [6] and *Q. robur* [51], but decreased in a drought-sensitive cultivar of *Phaseolus vulgaris* [59]. Protein disulfide isomerases (PDIs) were increased in barley and *B. napus*, but decreased in *Agrostis stolonifera*, *Q. robur*, and poplar. Additionally, ER-luminal binding protein (BiP), trigger factor-like protein (TIG), most heat shock proteins (HSPs), and other molecular chaperones (i.e., calnexin, endoplasmin) were increased, but T-complex protein and HSP70-HSP90 organizing protein were decreased in drought-treated leaves. This protein helps in maintaining the normal protein folding, repairing, and renaturation of the stress-damaged protein, whereas HSP must popular function in protein folding in Arabidopsis and yeast to improve the drought tolerance.

Protein degradation, process of removing the abnormal, damaged protein, and maintenance of certain level of regulatory proteins during drought, includes the components such as ubiquitin/26S proteasomes, small ubiquitin-like modifier (E3 SUMO) ligase, and proteases/peptidases (ATP-dependent Clp proteas, cysteine proteinase, zinc metalloprotease, aspartic proteinase, serine carboxypeptidase, and aminopeptidases (APs)). These components show positive response in *P. vulgaris* [59] *Hordeum vulgare* [60], *B. napus* [58], and *Medicago sativa* [61] under drought condition, involved in ubiquitination, exhibited significantly increased values in drought tolerant and decreased in drought-sensitive leaves.

Due to drought condition, it interrupts the normal cellular mechanism, results to produce the ROS. Plants evolve diverse mechanism to keep ROS homeostasis in cells, including antioxidative enzymes, e.g., SOD (first defense mechanism by converting O_2 into H_2O_2) and CAT (convert H_2O_2 into H_2O and O_2) and chemical antioxidant (e.g., glutathione and ascorbate). Diverse abundance of SODs in cystolic, peroxisomeas as well as in chloroplast helps in the drought tolerance and avoidance. For instance, increment of cystolic Cu-Zn SODs drought avoidance CT9993 and drought tolerance IR62266), while e chloroplast Cu-Zn SODs were increased in CT9993, but decreased in IR62266 [57], additionally in cultivar of *Malus domestica*, Cu-Zn SOD decreased and Fe SOD increased [54]. Similarly, in Ascorbate-Glutathione (AsA-GSH) pathways, the ascorbate peroxidase (APX) reduces H_2O_2 to H_2O using ascorbate (AsA) as an electron donor, then the oxidized AsA is restored by monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [62]. GR catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form GSH and work out for the drought tolerance. In addition, some proteins that involved in glutathione-mediated ROS scavenging: glyoxalse (GLO), phospholipid hydroperoxide glutathione peroxidase, glutamate-cysteine ligase (GCL), glutaredoxin (Grx), and monothiol. GLo catalyzes the detoxification of methylglyoxyl, whereas GCL, first enzymes in GSH biosynthesis pathways found in drought stressed *B. napus* [58]. Additionally, Prx/Trx also catalyzes the reduction of H_2O_2, whose abundance response to drought. Trx-linked enzyme, methionine sulfoxide reductase (MSR), involved in conversion of methionine sulfoxide to methionine, protects cells and tissues from H_2O_2-induced stress. Besides, Glutathione Peroxidase/Glutathione S-Transferase pathways, GPX catalyzes the reduction of H_2O_2 using Trx [63], and GST catalyzes conjugation reactions between GSH and a number of xenobiotics, playing a crucial role in the degradation of toxic substances. To cope with drought, GPXs found increased in *Boea hygrometrica* [64], *E. elongatum* [65], and *B. napus* [58].

There will be occurrence of pathogen when left plant for water deficit condition, but some pathogenesis-related protein, namely chitinase, disease resistance protein (DRP), polyphenol oxidase (PPO), oryzacystain, pathogen defense-related protein 10 (PR10), and disease resistance gene analog PIC15 increased in the response of drought condition. These proteins act as the pathogen by acting on insect exoskeleton and fungi cell walls, catalyzing the oxygen-dependent oxidation of phenols to quinines' during plant defense, acting as cysteine proteinase inhibitor in the phytocystatin family of proteinase inhibitors. For example, overexpression of oryzacystain gene in Tobacco displayed an increase of drought tolerance by improving total SOD and guaiacol POD activities.

Osmotic regulation will be hindered due to exposed to water deficit, but important osmotic homeostasis-related protein, namely embryogenesis abundant (LEA) protein, dehydrin (DHN), and betaine aldehyde dehydrogenase (BADH), which function as cellular protectants to stabilize cellular components, protein structure through detergents and chaperone like properties, act as calcium buffer. LEA proteins were also increased in *Z. mays* [55] and *B. napus* [58] under certain drought conditions.

Cell division and cell wall formation decreased due to decrease of phosphorylation of several protein (cell division cycle protein, division protein ftsZ1, and cyclin A2) when exposed to drought, which implies the suppression of cell growth. Cytoskeleton and cell wall component require for cell division, morphogenesis, and signal transduction, while cytoskeleton protein, namely actin, kinesin motor protein, tubulin, profilin, actin depolymerizing factor, and fibrillin to check the cell growth during stress. Additionally, the translationally controlled tumor protein homolog (TCTP) is a Ca^{2+}-binding protein, which protect against stress and apoptosis, cell growth, and microtubule organization, which was significantly drought increased in *H. vulgare* [60], *T. aestivum* [66], and *B. napus* [58], which would facilitate plant adaptation to drought stress.

Cell wall extensibility was directly affected by water loss, while cell wall polysaccharide synthesis/hydrolysis, lignin biosynthesis, and cell wall loosening in leaves were drought-responsive enzymes. Two enzymes, glycosylated polypeptide and pectinacetylesterase, involved for polysaccharides synthesis, another two enzymes xylanase inhibitor and polygalacturonase inhibitor, involved in polysaccharide hydrolysis inhibition. Three lignin biosynthesis-related proteins, phenylalanine ammonia-lyase (PAL), caffeic acid 3-*O*-methyltransferase, and caffeoyl-CoA *O*-methyl-transferase, catalyze the transformation of phenylalanine to cinnamylate of lignin biosynthesis, while two drought-increased cell wall structural proteins (i.e., glycine-rich protein and fasciclin-like arabinogalactan protein) enhance cell wall synthesis in response to drought by providing the UDP-glucose directly to the cellulose synthases and/or callose synthases [67], hence, improve the mechanical strength for minimizing water loss and cell dehydration. Another important activity of cell wall loosening/expansion, important aspect in the adaption to drought, which was related enzymes, polygalacturonase/pectin depolymerase (PG) in *O. sativa* [6] and xyloglucan endotransglycosylase (XTH), where PG degrade pectin, while XTH can cleave and reform the bonds between xyloglucan chains to regulate cell wall rigidity.

Membrane trafficking localized in mitochondrion, plasma, and vacuole. Two mitochondrion protein carriers (dicarboxylate/tricarboxylate carrier (DTC) and 2-oxoglutarate/malate carrier protein (OMC)), catalyze the transport of various metabolites (e.g., dicarboxylates, tricarboxylates, amino acids, and keto acids), play important role in gluconeogenesis, nitrogen metabolism, as well as biotic stress [68]. Another, Remorin, aquaporin and PEG, plant-specific plasma membrane protein have importance in plant-microbe interaction and signal transduction [69]. In addition, vacuolar H^+-pyrophosphatase (V-PPase), vacuolar-ATPase (V-ATPase), and ABC transporter ATPase, important for or translocating H^+ into the vacuoles to generate a gradient of H^+, which provide a driving force for the accumulation of ions and other solutes in the vacuole and function for abiotic stress.

Photosynthesis inhibition is the primary detrimental effect due to drought stress, and related protein decrease. To cope with this situation, drought increased protein involved in the photoreaction and Calvin cycle in leaves. Light-harvesting chlorophyll a/b-binding proteins (LHCB), involved in ABA signaling partially by modulating ROS homeostasis, besides, abundance of sedoheptulose-1,7-bisphosphatase (SBPase) and carbonic anhydrase (CA), catalyzes the reversible hydration of CO_2, and influence in internal conductance and abundance of protein involved in photorespiration, significantly increases and decreases glycolate oxidase, glycine dehydrogenase, serine glyoxylate aminotransferase, and serine transhydroxymethyl transferase, aminomethyl transferase (AMT), and glycine dehydrogenase to adapt the drought stress. The mechanism in photorespiration can protect the photosynthesis from photoinhibition and prevent ROS accumulation in green tissues.

Involvement in carbohydrate and energy metabolism is important step to cope with drought condition. Phosphoglucomutases (PGluMs), fructose-bisphosphate aldolase (FBPA) in glycolysis and aconitate hydratases in TCA cycle increased in drought condition, which inhibit the accumulation of sugars as osmolyte or energy source for recovery, while the increase of glycolysis and TCA may act as a strategy for providing energy during the activation of stress defenses, especially when the photosynthesis was inhibited. The change in mitochondrial electron transport chain and ATP synthesis related protein implies ability to enhance energy production to maintain physiological activity and inhibit stress damage.

Due to the drought condition, nitrogen assimilation decreased in the reduction of NR, GS, and GOGAT, which was main reason for yield reduction. Similarly, the decline of aspartate aminotransferase (AST) and alanine aminotransferase (ALAT) indicates that drought stress inhibits the amino-acid metabolism and synthesis of other metabolites. At the same time, *S*-adenosyl-L-methionine (SAM) cycle was generally increased in leaves, including drought-increased. 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MetE), *S*-adenosyl-L-homocysteine hydrolase (SAHase), *S*-adenosylmethionine synthase (SAMS), and methionine synthase (MS), which implies that it enhances the methionine and osmo-regulant metabolism for plants to cope with drought stress.

Acetyl-coenzyme A carboxylase carboxyl transferase, acyl carrier protein, enoyl-acyl carrier protein reductase, and lipoxygenase 6 involved in fatty acid biosynthesis, and enzymes thiolase I, thiolase II, and acyl-CoA dehydrogenase used for fatty acid degradation. Greater composition of unsaturated fatty acid in membrane lipids contribute to superior leaf dehydration tolerance and maintain membrane integrity and preserve cell compartmentation under water stress, in addition, two flavonoid biosynthesis related proteins (i.e., chalcone isomerase (CHI) and dihydroflavonol-4-reductase) involved in secondary metabolism were also changed in response to drought.

2.2 Protein metabolism under flooding and submerged stress condition

Deprivation of the soil oxygen due to consequence of flooding and forced the plant to shift from aerobic to anerobic respiration [70], which regenerate NAD^+, through ethanol fermentation by selectively synthesizing flooding-inducible proteins involved in sucrose breakdown, glycolysis, and fermentation [13]. Several glycolysis-related proteins, including fructose-bisphosphate aldolase, phosphoglycerate kinase [64], glyceraldehyde-3-phosphate dehydrogenase [71], enolase [72], sugar isomerase, phosphofructo-kinase [73], and pyruvate kinase [72] are increased in soybean under flooding stress, indicate the glycolysis and fermentation pathways activation, initiating for plants protecting plant from flooding induced damage, whereas decrease of fructose-1,6-bisphosphate aldolase and sucrose-fructan 6-fructosyl transferase in wheat show response to flooding stress. Othersides, fermentation under anaerobic condition, influence the accumulation of fermented related proteins such as alcohol dehydrogenase (ADH) and pyruvate carboxylase, and indicates that activation of the alcohol fermentation pathways, to cope the hypoxic condition. The conversion of acetaldehyde to ethanol by ADH with concurrent reoxidation of NAD^+ for the continuation of glycolysis. The fermentation related enzyme pyruvate decarboxylase, and aldehyde dehydrogenase increase to accelerate the energy production via nonoxidative pathways, even growth is suppressed.

In other sides, flooding stress induces impairment of the electron transport chain in plants. Protein related to complexes II, IV, and V of the electron transport chain decreased in abundance and while, succinate-semialdehyde dehydrogenase, 2-oxoglutarate dehydrogenase, and gamma-amino butyrate are significantly increased, which are required for energy production via non-oxidative pathways [72]. Oxaloacetate produced in TCA cycle stimulates phosphoenol pyruvate synthesis and provides the indirect simulation for the continuation of glycolysis. Reduction of energy metabolism-related proteins, including citrate synthase, glutamate dehydrogenase, and adenosine kinase, in wheat roots under waterlogging stress [74]. In addition, energy-related proteins such as beta-amylase, malate dehydrogenase, fructose-1,6-bisphosphatase, and phosphoenol pyruvate carboxykinase are decreased in response to flooding stress, indicating that gluconeogenesis is suppressed in wheat under these conditions [10]. RuBisCo sub unit binding protein alpha sub unit and RuBisCO activate degraded and senescence in high ROS condition and decreased the chlorophyll content, results to decrease in net energy production.

ROS recognized as toxic byproduct of aerobic metabolism and controlled by anti-oxidants and anti-oxidative enzymes. The plant development of well-organized scavenging mechanism to overcome ROS toxicity likely to led to the use of reactive molecules as signal transducers in plant cells. ROS production in cellular organelles, such as plastids, mitochondria, and peroxisomes, involved in signaling cascades controlled by production and scavenging of ROS intermediates [27]. ROS scavengers, such as peroxidase, APX, cytosolic APX, and superoxide dismutase (SOD), linked to bio photon emissions and decreased photosynthesis and beneficial for normal metabolism and cell signaling.

Cell wall modification related proteins, namely polygalactouronase inhibitor-like and expansion-like B1-like proteins and cell wall synthesis related protein such as cinnamyl-alcohol dehydrogenase and cellulose synthase-interactive protein-like protein abundance response under water logged condition. Flooding stress induces the assimilation of methionine and promotes cell wall hydrolysis, thereby restricting growth so, under the waterlogged stress, cell wall synthesis related proteins decrease, cell wall loosening related protein increase and cell wall lignification is suppressed.

Proteolysis, protein folding and storage plays important role in the removing the flooding damage induced non-active proteins [40]. Heat shock proteins act as molecular chaperones in preventing protein aggregation, translocation of nascent chains across membranes, assembly or disassembly of multimeric protein complexes, and targeting proteins for lysosomal or proteasomal degradation [40]. The ubiquitin/proteasome-mediated proteolysis of enzymes involved in glycolysis and fermentation pathways may be negatively controlled under the hypoxic condition caused by flooding stress [40].

Post recovery protein metabolism is less studied but studied by [75] Gro-EL-like chaperone ATPase, 26 S proteasome regulatory subunit 7, 26 S regulatory subunit S 10B, and cyclophilin were decreased in seedlings recovering from flooding stress, whereas globulin-like protein, Kunitz trypsin protease inhibitor, and peptidyl-prolyl cis-trans isomerase 1 were increased, and soybean root recovers from flooding by altering cell structure, strengthening cell wall lignification, and scavenging toxic ROS.

2.3 Cold stress

One of the major abiotic stresses is cold or low temperatures (LTs) that severely affect plant growth and survival. Chilling or freezing with temperatures <20°C and < 0°C respectively can induce ice formation in plant tissues which causes cellular dehydration [39]. To be able to withstand in this adverse condition, plants adopt several strategies, such as production of more energy via activation of primary metabolisms, leveling up of antioxidants content and chaperones, and maintenance of osmotic balance by altering membrane structure [76].

2.3.1 Protein metabolism in cold stress

Several articles and reviews deals with the metabolic responses of plants at low temperature, where some attempted correlating metabolic and biochemical responses with cold tolerance. Solaw [77] noted correlative studies of biochemical changes does not enable understanding of cold acclimation (CA) leading to increased freezing tolerance and till date no any new approaches in molecular biology and genetics have been extensively enlisted on study of cold-tolerance and injury mechanisms. However, few studies of CA started focusing on some of the more rapid plant physiological and molecular responses subjected to LTs, which revealed that within the hours of LT exposure, plant and algal cells can rapidly initiate to alter membrane lipid composition [78], RNA [79], and protein content. These findings of rapid biochemical alterations in response to L T convince the rapid induction of freezing tolerance at inductive temperatures and by desiccation and ABA at non inductive temperatures [80] and ABA [10]. This suggests a possible molecular basis, at minimum, for the adjustment of metabolism to low nonfreezing temperature, and perhaps for freezing tolerance. Also, upon exposure to LT, it consists of repeated observations that a number of enzymes show shifts in isozymic composition, whereas both quantitative and qualitative differences in the protein content is shown by numerous electrophoretic studies between non-acclimated and cold acclimated tissues.

2.3.2 Enzyme variation

Compared with plants maintained at warm temperature, [20] reported changes in activity, freeze stability, and isozymic variation in plants subjected to LTs. He mentioned increased peroxidase activity in hardened stems of four widely unrelated woody species when electrophoretic techniques to separate enzymes from non-hardened and hardened tissues. Here, peroxidase isozymes present in hardened tissues were not found in other three non-hardened tissues. Similarly, during deacclimation, no change in peroxidases, glucose-6-phosphate, 6-phosphogluconate, and malate dehydrogenases was observed in willow stem [81], however, differences were observed in lactate dehydrogenase where the activity increased during deacclimation. Similarity as above findings was illustrated by [82] in which invertase enzyme in wheat leaves undergoes a shift from a lower-molecular weight form to a higher-molecular-weight form at LT. Different kinetic properties is exhibited in larger form functionally replacing small form in cold-hardened plants [83]. Also, Krasnuk and colleagues [6, 84] observed increased activity of a number of dehydrogenases associated with respiratory pathways, including glucose-6-phosphate dehydrogenase, lactate, and isocitrate dehydrogenase [6] during a comprehensive studies with alfalfa. Thus [85] suggests higher amounts of enzyme may increase in activity and soluble protein content indicating increased soluble protein content and enzyme activity could be part of the adjustment of metabolism to the kinetic constraints imposed by LTs.

A more recent study of glutathione reductase from spinach carried out by [26] demonstrated not only additional isozymic forms in cold-acclimated tissue but also increased activity, freezing stability, and altered kinetic behavior and the activity of this particular enzyme was decreased by freezing/thawing both in vitro and in vivo. However, the enzyme found in cold-acclimated plants was less sensitive than its counterpart from non-acclimated plants to freezing from nonacclimated plants. In contrary, kinetic parameters and freeze/thaw inactivation was observed identical in ferredoxin NADP reductase from nonacclimated and cold-acclimated wheat [86], whereas activity was increased during CA. Therefore, [85] illustrated the potential for alterations in enzymes in response to low temperature exposure and the apparent selective basis where such changes can occur.

Ribulose bisphosphate or oxygenase from winter rye is the best-characterized enzyme relative to non-acclimated and cold-acclimated plants. Early in vitro studies studied by [87] noted purified enzyme from both non- and cold-acclimated plants demonstrated an increased stability of catalytic activity to denaturants and storage at −25°C of the enzyme from cold-acclimated plants. Moreover, [87] presented evidence of a stability in vivo conformational change during low-temperature adaptation that was not altered by purification of the enzyme. Also, osmotic concentration of the purified enzyme caused a greater degree of aggregation through intermolecular disulfide bond formation of the large subunit from non-acclimated plants [4] also claimed, similar to rye, the enzyme purified from freezing sensitive and -tolerant potato species demonstrated structural differences that paralleled variation in freezing tolerance much in the same way. However, the study still remains in ambiguity for the stable change in conformation, kinetic properties, and differential cryostabilities of this enzyme from cold-acclimated leaves or cold tolerant potato species. Given that a single chloroplastic gene encodes for large subunit and not possess even a minute chance for the synthesis of an alternative cryostable large subunit from another gene. Also, in many plants, always, a small gene family codes the small subunit, a change in the small subunit may possess subtle effect on the cryostability and other properties of the holoenzyme. Equally possibility occurs in LT -specific posttranslational processing, although there is no evidence to support this concept. In addition to isozymic and conformational differences of enzymes in response to L T exposure, Griffith, stated supramolecular interactions can also be affected.

2.3.3 Protein content

According to [88], accumulation of soluble protein in cold-acclimated cortical bark cells of black locust trees was first correlated with freezing tolerance about 40 years ago. These study may not be explained as simply as stated in past, [88] suggests there are many reasons why some plants might accumulate soluble proteins during CA; but with the exception of a protoplasmic augmentation hypothesis without clear mechanistic rationale for conferring greater freezing tolerance for this hardening response. In temperate deciduous perennials like black locust could provide the nitrogen source for the accumulation of proteins in the cortical cells of the living bark, in expense of nitrogenous materials during senescence. Parsell and Lindquist [89] supports a possible functional role of the increased soluble protein in cold tolerance was the fact that an evergreen, red pine, also accumulated soluble proteins during the winter, similarly, cortical bark cells need not to act as vegetative storage in evergreens nevertheless, it cannot be refused that one or more minor components of the total protein content could function in freezing-tolerance mechanisms.

Most of the studies have confirmed the presence of new protein species in cold-acclimated and freezing-tolerant plants. When these plants are compared to non-acclimated plants, subtle shift in protein content in cold-acclimated tissues involving mostly the appearance and disappearance of minor bands in gel can be observed [85]. Existing evidence at present includes several studies of purified plasma membranes from non-acclimated and cold-acclimated tissue. Tzin and Galili [90] emphasized on declination of more than 20 proteins in cold-acclimated leaf plasma membranes, whereas 11 had increased their concentration, while 26 proteins were new and unique to membranes from hardened tissue, yet increased levels of high-molecular-weight glycoproteins were other alterations included during CA.

2.4 Heat stress

Some defense mechanisms can be triggered in response to several stresses, such as expression of obvious genes which were not expressed under normal situations, resulting increased synthesis of protein groups [60]. These groups in cases of heat are called as Heat Shock Proteins (HSPs), "Stress-induced proteins" or "Stress proteins" [49].

According to [14] declination in normal protein synthesis occurs when exposed to high temperatures, thereby increasing selective translation of mRNAs for characteristic sets of HSPs. Heat responding phenomena in plants generally observed with concomitant reduction in protein synthesis of new or constitutive HSPs. Jin et al. [35] observed reduction in total protein synthesis at of 40°C and above in soybean. The HSPs, in plants, consists an abundant group of low mo1 wt polypeptides with higher molecular weight families [91], where some of them found to function as chaperones minimizing high-temperature stress damage partially denaturing proteins and preventing breakdown or aggregation. Response toward heat includes increase in binding of ubiquitin to conglomerated high molecular weight protein [11] which both increase and decrease ubiquitin transcripts expression [17].

2.4.1 Role of heat shock proteins

Levitt et al. [92] reported formation and folding patterns of any protein in three dimensional structure determines their function and [93] favored above statement and confronted with the findings illustrating 50% of principle amino acids sequence is necessary for formation of three dimensional structure which signifies the importance of HSPs in folding of other proteins. As plants were induced in heat stress, HSPs protects cells from injury and facilitates their recovery and survival to normal growth conditions [49]. Also, [94] specified the role of HSps as molecular chaperones during heat stressed condition, apart from ensuring maintenance of correct protein structure, which is basically different than in non-thermal stress where proteins unfolding is not the primary effect and protection could occur in any ways.

Seo et al. [95] also focused the general role of HSPs as molecular chaperones, regulating folding and accumulation of proteins as well as localization and degradation in all plant and animal species, thus preventing the irreversible aggregation of other proteins and participate in refolding proteins during heat stress conditions [96].

Different HSPs with their unique role are described below:

2.4.1.1 Small heat shock proteins (sHSPs)

These contains a common alpha-crystallin domain containing 80–100 amino acid residues contained in the C-terminal region [97]. The characteristics features of this proteins is degradation of protein having unsuitable folding [11]. Another attributes that make it indifferent from other class is independency of its activity from ATP [98]. A recent review from [99] concluded the presence of some indications that sHSPs play crucial role in membrane quality control, thereby potentially contributing the maintenance of membrane integrity under stress conditions.

2.4.1.2 HSP60

This class, called as chaperonins, is known to be important in assisting plastid proteins such as Rubisco [100]. Some studies like [101] pointed out that they might participate in folding and aggregation of proteins that were transported to chloroplasts and mitochondria. These proteins are different from other proteins, after transcription and before folding, to prevent their aggregation [42].

2.4.1.3 HSP70

These HSP70 functions as chaperones, in almost all organisms, for newly formed proteins to check their accumulations as aggregates and folds in a proper

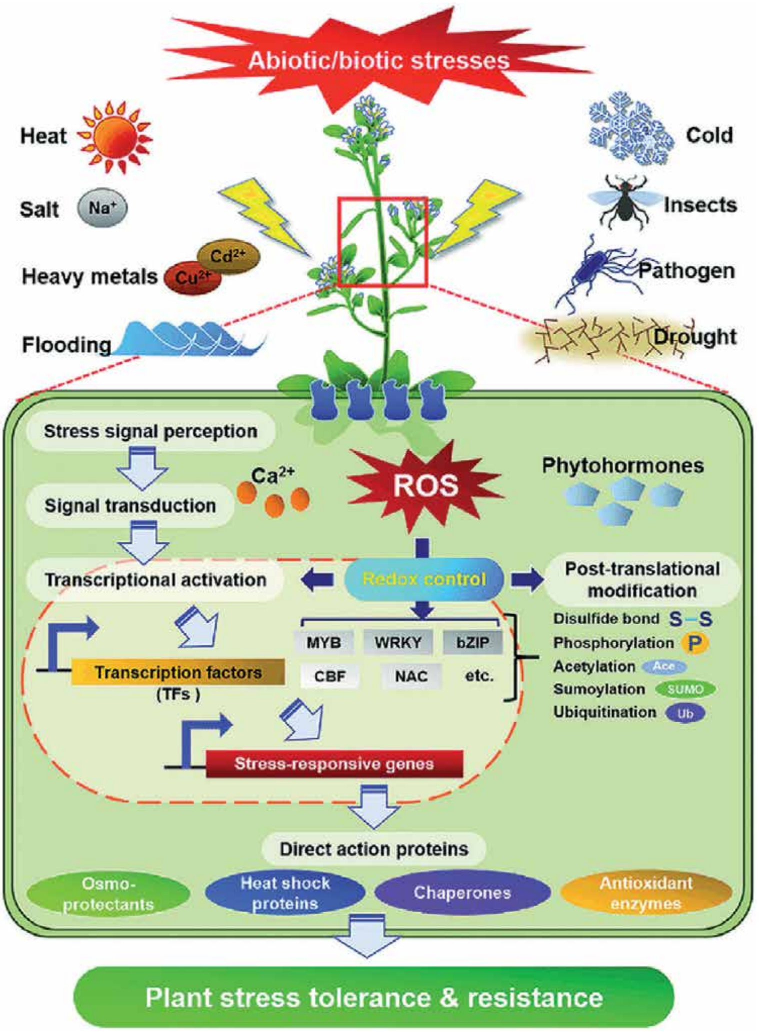

Figure 1.
Plant stress tolerance and resistance [100].

way during their transfer to final location [102]. Furthermore, cooperation in the activity of HSp70 and sHSPs primarily function as molecular chaperone and play an important role in protecting plant cell from detrimental effects of heat stress [83] stressed on crucial role played by HSp70 and sHSP17.6 in the development of cross adaptation to temperature stress in grape vines induced by heat acclimation (HA) and cold acclimation (CA).

2.4.1.4 HSP90

The protein from HSP90 class shares the role in many chaperone complexes and has important role in signaling protein function and trafficking [89]. Furthermore, other important attributes retained by these class includes regulation of cellular signals, such as the regulation of glucocorticoid receptor (GR) activity [103].

2.4.1.5 HSP100

What makes it unique from other class is the reactivation of aggregated proteins [42] by re-solubilizing nonfunctional protein aggregates and also by degrading irreversibly damaged polypeptides [104, 105]. Members of this class are not restricted only to acclimation to high temperatures but also housekeeping functions necessary in chloroplast development are also provided by specific member (**Figures 1** and **2**) [106].

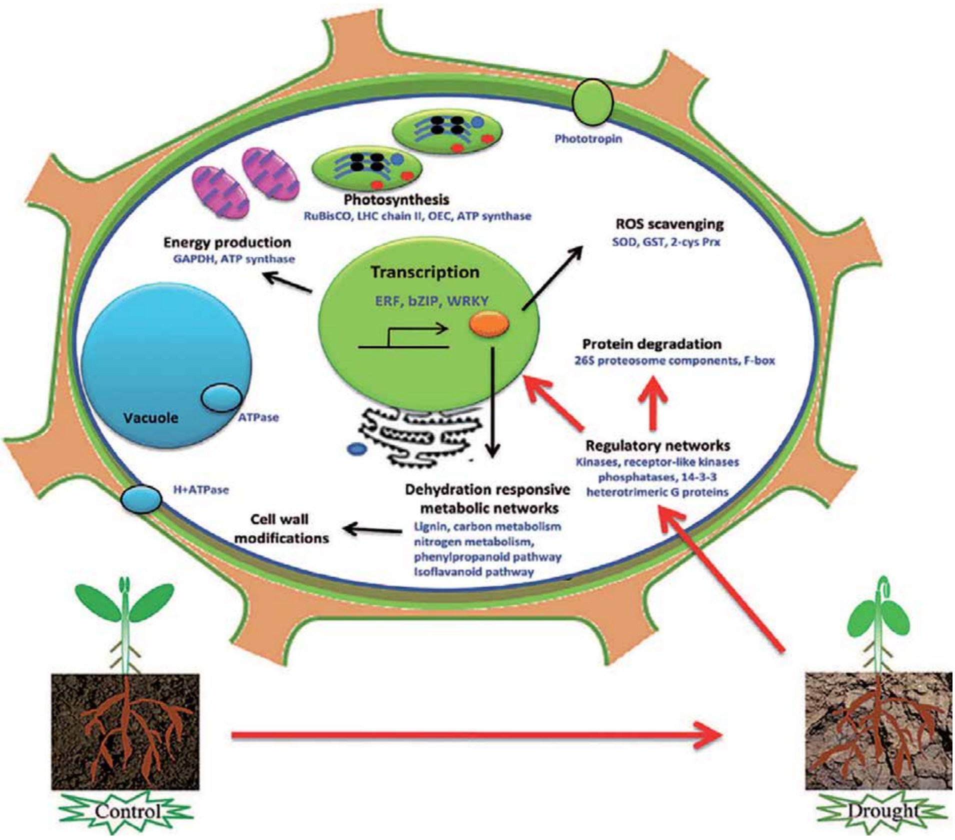

Figure 2.
Transcription [100].

3. Conclusion and future prospective

Abiotic stresses are major limiting factors for plant growth and yields and with various acclimation responses at morphological, physiological, metabolic, and molecular level coordinated by complicated regulatory networks comprising genes, phytohormones, ROS, and other signaling components. The abundance of ion channels protein and trans-membrane water found indicated the change in ions/osmotic balances, but the phenomenon was not observed in flooding conditions. In addition, the preventive measure against the oxidative damage caused due to ROS levels under abiotic stress, higher abundance of ROS scavengers plays a great role in this matter, whereas the abundance of ROS scavengers was low in the flooding condition. On the other hand, protein folding due to molecular chaperone and disease, defense-related proteins such as proteolytic enzymes and proteosomal factors under stress, indicating the refolding of denatured proteins and proteolytic elimination of damaged proteins. This review paper showed the different protein metabolism occurs during the metabolic stages, and secondary metabolism-associated proteins escape and tolerate mechanism under different abiotic stress.

At the recovery stages, increased lignin biosynthesis results in enhanced mechanical strength by hardening of cell wall. Changes in abundance for cyto-skeletons associated proteins can be overlooked upon compensation against the reduced cell size as well as repairing injuries caused by drought and flood stress. Moreover, the levels of proteins related to *de novo* proteins synthesis, *growth-related* signaling and secondary metabolism are enhanced during flood replenishment of the stress induced effects. These stress-induced effects can be recovered by compensatory mechanism.

Only after proteomic studies could make us aware about the mechanism involved in abiotic stress condition. Analyzing of the plant response and abundance of protein and stress tolerant crops will lead to better understanding of the mechanism of plant to overcome the stress and recover. Moreover, some proteins showed the dynamic changes depending on plant species and stress intensity, which gives a clear interpretation of the mechanism in stress response. The integration of those finding from physiological, gene expression, and other large scale “omics” will help us to establish molecular networks of stress response and tolerance.

Author details

Bharti Thapa[1] and Abhisek Shrestha[2]*

1 Centre for Biotechnology, AFU, Rampur, Chitwan, Nepal

2 National Sugarcane Research Program, NARC, Jitpur, Bara, Nepal

*Address all correspondence to: shrestha.avi1425@gmail.com

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Chapter 3

Salt Stress Tolerance in Rice and Wheat: Physiological and Molecular Mechanism

Mohammad Hasanuzzaman

Abstract

Salinity is a major obstacle to global grain crop production, especially rice and wheat. The identification and improvement of salt-tolerant rice and wheat depending upon the genetic diversity and salt stress response could be a promising solution to deal with soil salinity and the increasing food demands. Plant responses to salt stress occur at the organismic, cellular, and molecular levels and the salt stress tolerance in those crop plant involving (1) regulation of ionic homeostasis, (2) maintenance of osmotic potential, (3) ROS scavenging and antioxidant enzymes activity, and (4) plant hormonal regulation. In this chapter, we summarize the recent research progress on these four aspects of plant morpho-physiological and molecular response, with particular attention to ionic, osmolytic, enzymatic, hormonal and gene expression regulation in rice and wheat plants. Moreover, epigenetic diversity could emerge as novel of phenotypic variations to enhance plant adaptation to an adverse environmental conditions and develop stable stress-resilient crops. The information summarized here will be useful for accelerating the breeding of salt-tolerant rice. This information may help in studies to reveal the mechanism of plant salt tolerance, screen high efficiency and quality salt tolerance in crops.

Keywords: antioxidant enzyme, Na^+/K^+ ratio, osmolytes, salt Tol gene, salinity, rice and wheat

1. Introduction

Salinity is an abiotic stress factor that has severe negative impacts on agricultural crop growth and yield potentiality [1]. The seed germination, traits of growth and yield-related components of crop plants such as rice and wheat are seriously damaged under salinity conditions [1, 2]. Currently, 230 million hectares of world's areas are used for cultivation in which 20% lands are affected by varying degree of salinity and amount of saline affected area is increasing continuously due to the effect of sea level rise, coastal subsidence, increased tidal effect and continuous reduction of river flow, particularly during dry periods [3–6]. Therefore, soil salinity is the major obstruction to crop cultivation. When the crop plants are exposed to salt-affected soil, the plants primarily develop osmotic stress and instantly inhibits the normal plant growth and development [7]. Later, ion toxicity is created in crop plants due to excess salinity level and causes imbalance in mineral homeostasis [3]. Osmotic stress and ion toxicity are major salt-induced stresses which create secondary oxidative stress in plants and overall growth inhibition. Salinity stress also interferes with the photosynthetic process by severely damaging photosynthetic pigments [8] and ultimately results in huge loss of crop yield globally [9]. To feed up the huge global population and ensure the food and nutritional security of the rising population, it is urgent to increase the crop production at least 70% by 2050 [10].

Rice and wheat are the first two ranked cereal crops that jointly feed approximately 80% of the world's population, and are sensitive to salt stress [11, 12]. Developments of salt tolerant rice and wheat varieties is a prerequisite for salt stress management. To improve rice and wheat production, a thorough understanding of physiological response, biochemical activity, metabolism, epigenetic response, and gene expression under salt stress conditions is required. In this chapter, we discuss the salt-induced oxidative stress, osmotic stress and mineral toxicity to rice and wheat plants. Moreover, we systematically summarize the current understanding of salt-stress response and morpho-physiological and molecular mechanisms, and epigenetic response underlying salt tolerance in rice and wheat. This theoretical basis could be further useful for developing salt-tolerant and high yielding rice and wheat varieties in future.

2. Salt stress response of rice and wheat plants

Plant showed several morphological and anatomical changes under high salinity stress conditions e.g. shorter shoot, root system impairment, total biomass reduction, shoot tip rolling, leaf chlorosis, fewer tillers per plant, lower grain weight, and fewer spikelets per panicle ultimately leading to decrease in harvest index and grain yield [13–16]. Salt stress significantly affects plant physiology and biochemical activity at germination, seedling and reproductive stages [17–19]. Excess salt causes osmotic stress and ionic toxicity on rice plants leading to overall oxidative stress and nutrient depletion [20, 21]. Plants show various morpho-physiological or biochemical changes and salt injury symptoms and even may die under high salinity stress conditions (**Figure 1**). Higher amounts of sodium ion (Na^+) directly cause cellular damage in plants and excess Na+ in root areas inhibits K^+ uptake because of their antagonistic effect which hampered photosynthetic activity [22]. Ionic or mineral stress causes excess accumulation of sodium (Na^+) and chlorine (Cl^-) in plant cells which finally results in premature leaf senescence and often plants die by salt antagonistic effects [23, 24]. Excess presence of Na^+ in plant cells cytoplasm has an extreme inhibitory effect on enzyme activities which disrupted many cellular metabolism (e.g. protein synthesis), bio-molecular function, and photosynthesis [25–29]. Furthermore, abundance of Na^+ in the cytoplasm hampers the normal uptake and transport of potassium (K^+) and other macro- and micronutrients, for example nitrogen (N), phosphorus (P), calcium (Ca^{2+}), magnesium (mg^{2+}), zinc (Zn^{2+}), and iron (Fe^{2+}) [25, 30–32].

Salinity-induced continuous stress diminish the plant cell turgor pressure, which in turn decreases normal cell growth, and plants must enhance enzymatic activity to adjust osmotic potential and maintain cell expansion and growth [17, 25, 32–34]. Many investigations reported that Na^+ accumulation in plant shoots is directly correlated with the rice and wheat plants' survivability under saline condition [25, 26]. Therefore, lowering the cytosolic Na^+ level is playing a vital role to enhance salt tolerance mechanisms in rice and wheat [25]. Because of osmotic stress in plants, water uptake is obstructed and due to lower water potential

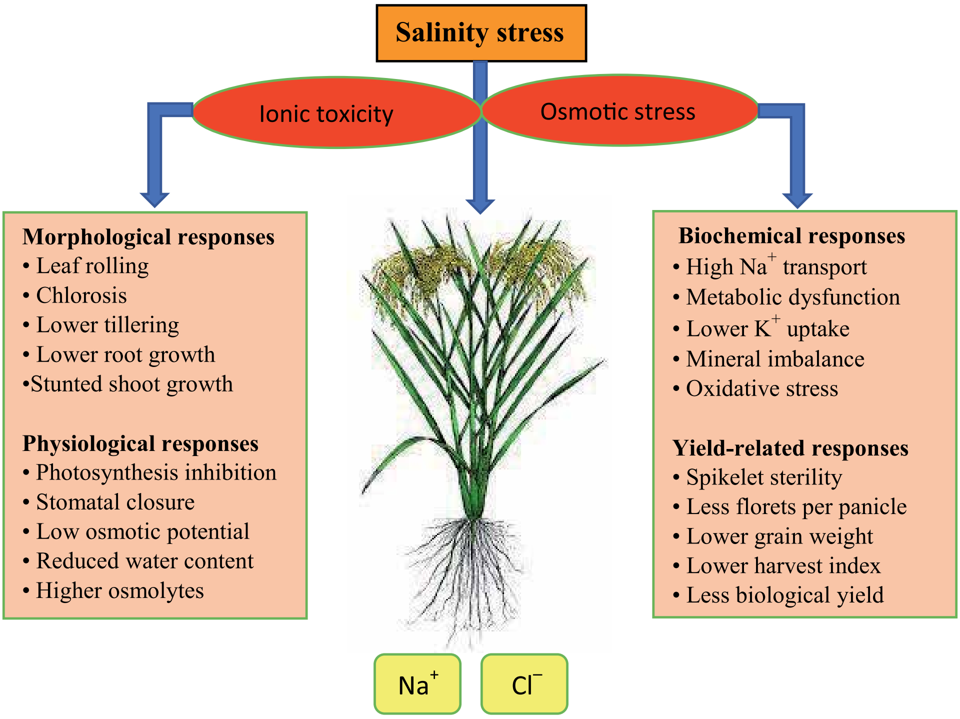

Figure 1.
Schematic diagram represents the morphological, physiological, biochemical and yield associated response of rice and wheat plants under salt stress conditions. High salinity stress severely damage the plants mineral homeostasis and osmotic maintenance, thereby retarded the plant growth and developments and finally reduce crop yield.

physiological drought is created in plants. Osmotic stress also results in interruption of nutrient uptake and rapid stomatal closure, which decline the CO_2 assimilation capability of plants and highly inhibits photosynthesis [35]. Oxidative stress due to osmotic and ionic imbalance, causes higher accumulation of reactive oxygen species (ROS), which may severely damage the cellular macromolecules (e.g. DNA, lipids and enzymes) and structural components [36, 37]. Under salinity conditions, rice and wheat plants must amplify their physio-biochemical and enzymatic activity involved in the regulation of ion and osmotic homeostasis, oxidative stress, and nutritional balance [1, 23, 38, 39].

3. Salinity tolerance mechanism in rice and wheat

3.1 Maintenance of ionic homeostasis

Soil salinity induced stress in crop plants is commonly caused by high concentrations of Na^+ and Cl^- [38, 39]. The ionic homeostasis must be regulated and re-established in saline stressed conditions [40]. The final determinants of salt tolerance mechanisms are different ion transporters that transport toxic ions at plant organ and cell levels [41]. Therefore, the most important fact of salinity tolerance studies is to find which transporters interfere with the entry of Na^+ into the cell [42]. Na^+ and K^+ are normally transported into the plant cell by the same transporters, where these two cations compete for space [43]. Excess Na^+ competes with K^+ for absorption across the plasma membranes of plant cells, and K^+ is required for the activities of several important catalytic enzymes [44].

Moreover, K^+ is essential for the preservation of cell turgor, protein synthesis, osmoregulation, normal photosynthesis [45, 46]. Therefore, the regulation and adaptation of cellular Na^+/K^+ ratio is a crucial indicator that discerns the plant's capability to survive in salinity stress conditions. Moreover, mechanisms to reduction in cytoplasmic Na^+ uptake, compartmentalization of Na + in the vacuole and increase of Na^+ efflux [1, 47]. It was reported that the removal of cytoplasms Na^+ into the apoplast is due to the salt-inducible enzyme Na^+/H^+ antiporter of cell plasma membrane [3].

In the rice plants, the plasma membrane Na^+/H^+ antiporter (OsSOS1) excludes Na^+ from the shoot and roots, adjusting the lower Na^+/K^+ ratio at cellular level and developing salt tolerance in rice [48, 49]. The vacuolar Na^+/H^+ antiporters (for instance OsNHX1, OsNHX2, OsNHX3, OsNHX4, OsNHX5 and OsARP/OsCTP) contribute a necessary roles in the vacuolar compartmentalization of Na^+ and K^+ which is accumulated by cells cytoplasm and so determine salt tolerance in rice [50–52]. In wheat, the accumulation of Na^+ is controlled by the genes Nax1 and Nax2, located at 2A and 5A chromosomes position, respectively [53, 54]. These are also used as molecular markers in a wheat breeding program for salt tolerance. Under salt stress, vascular Na^+/H^+ antiporter (TNHX1, TNHX2, and TVP1) are responsible for wheat seedling growth and development by generating the pH gradient and facilitating sodium sequestration into [31].

3.2 Adjustment of osmotic potential

Osmotic adjustment is critical for regulating cell turgor for the maintenance of plant metabolic activity, growth, and finally productivity [55]. Crop plants synthesize compatible osmolytes for example proline, polyamines, soluble sugars and proteins, betaine, glycine to provide osmotic balance at the cellular level [56–58]. Under salinity stress, those important osmolytes provide the plants osmotic adjustment under as they reduce osmotic potential, stabilizing proteins and finally maintain cellular structures [2, 59]. Proline, a dominant substance for osmotic adjustment, possesses high water solubility, low molecular weight and in a free state, thereby no net charge in the physiological pH range in crop plants. The plant cells tend to uptake soluble osmotic adjustment substances to mitigate salt-mediated osmotic stress caused, importantly the biosynthesis of proline is clearly activated [60]. Therefore, proline level may be used as a physiological indicator of plant stress tolerance, especially salinity [61]. Wheat Ta-UnP, can significantly enhance the salt tolerance of transgenic Arabidopsis and rice. Liang et al. [2] described that proline content in transgenic Arabidopsis thaliana was significantly increased for maintaining the osmotic potential and protecting plant cells from salinity stress. The proline synthesis genes OsP5CS1 and OsP5CR enhances the proline production to improve the rice salinity stress tolerance [62]. A monosaccharide transporter, encoded by OsGMST1 increases monosaccharide accumulation and develops the salt tolerance in crop plants [63]. Glycine and betaine in rice, which is synthesized by the choline monooxygenase OsCMO and the betaine aldehyde dehydrogenase OsBADH1, enhance salt tolerance by promoting glycine betaine accumulation [64, 65]. In wheat plants, some investigation proved that betaine can inhibit the transport speed and quantity of Naþ and Cl^- from roots to the aerial parts and promote Kþ transport to enhance tolerance capability against salinity stress [33]. The salt-regulated gene OsSALP1 encodes a small plant-specific membrane protein that develops salinity tolerance mechanism by enhancing the expression of OsP5CS and free proline content under salt stress [66].

3.3 ROS scavenging and antioxidant enzymes

Oxygen is one of the essential biomolecules which involves in cellular metabolism, mitochondrial respiration, and oxidative phosphorylation for energy production in plants. However, oxygen is converted into reactive oxygen species (ROS) during plants metabolic process. Plants under salt stress conditions can up-regulate the excess production of ROS e.g. $O2^-$ (superoxide radical), H_2O_2 (hydrogen peroxide), and OH^- (hydroxyl radical) [67, 68]. Although lower presence of ROS can act as a signal to salt stress responses, excess uptake of ROS causes cytoplasmic membrane damage, DNA mutation, peroxidation of carbohydrates, lipids and protein degradation, irreversible metabolic dysfunction, and finally cell death [68, 69]. In other crop plants, several important antioxidant enzymes and non-enzymes are activated during salinity stress to alleviate ROS stress in rice and wheat [69–71]. Various Enzymatic scavengers includes catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutaredoxin (GRX), glutathione peroxidase (GR), glutathione S-transferase (GST), and glutathione peroxidases (GPXs) [1, 72–78]. Nonenzymatic scavengers include ascorbic acid (ASH), alkaloids, carotenoids, flavonoids, glutathione (GSH), phenolic compounds, and tocopherol [45, 70, 79].

Catalase (CAT) and glyoxylic acid-circulating is found in plant peroxisomes and bodies, respectively, which is one of the main enzymes that remove H_2O_2 and APX. Studies revealed that CAT is a main enzyme for scavenging H_2O_2 and is essential for H_2O_2 tolerance of crop plants [45]. The increment of ROS in the crop plant leads to lipid peroxidation in the cell membrane during higher and continuous salinity stress. Superoxide dismutase (SOD), the first line of defense of the plant antioxidant enzyme system, can eliminate the excess superoxide anions in the cells. SOD can disproportionate O_2 to form H_2O_2 and diminish the toxicity of the superoxide anion. Ascorbate peroxidase (APX) is one of the main enzymes that remove excess H_2O_2 in cells Chloroplast APX mainly removes the H_2O_2 produced by the Miller reaction. Malondialdehyde (MDA) is the key product of membrane lipid peroxidation when plants are under salt stress, and its content represents the degree of cell membrane damage [80]. Therefore, MDA content can indicate plant salt stress and salt tolerance. OsAPX2, OsAPX7, OsAPx8, OsAPXa, and OsAPXb increase APX activity, lower H_2O_2 and malondialdehyde (MDA) levels, decrease oxidative stress damage, and enhance rice tolerance to salt stress [81–84]. Glutathione responsive rice glyoxalase II (OsGLYII-2) functions in salinity adaptation by maintaining better photosynthetic efficiency and increasing the antioxidant pool [85]. All these studies suggest that enhancing ROS-scavenging ability can efficiently increase the salt tolerance of rice and wheat.

3.4 Regulation of RGRs under salinity stress

Phytohormone levels fluctuate downstream of the early salt signaling phase, and the salt-induced signaling cascade eventually leads to adaptive responses [14]. Phytohormones are important endogenous chemical signals that regulate plant growth and development in both ideal and challenging environments [14]. Multiple phytohormones must be integrated and coordinated in order to respond and adapt to salt stress, such as abscisic acid (ABA), indole acetic acid (IAA), cytokinins (CK), ethylene (ETH), jasmonic acid (JA), gibberellic acid (GA), cytokinin (CK), and salicylic acid (SA), brassinosteroids (BR), triazoles (TR) which regulate normal growth and mediate responses to abiotic stress [14, 38, 86–88].

4. Salt stress tolerant gene in rice and wheat

Seed germination, seedling growth and development, vegetative and flowering stage growth, fruit setting, and root system structural development are all harmed by high saline levels. Crop yields will eventually suffer as a result of this phenomenon [89]. Modern agricultural research includes the use of biotechnological technologies and genetic engineering to study crop tolerance mechanisms, explore tolerant genes, and cultivated varieties of tolerant crops using molecular biology, molecular genetics, and other methods. The goal of plant tolerance research is to develop tolerant cultivars; nevertheless, the mechanism of plant stress tolerance is a difficult problem to solve. We can offer strategies to avoid salt damage and increase salt tolerance of plants based on basic research and a full understanding of the mechanisms of salt stress and salt tolerance of plants. A number of genes involved in salt stress have been investigated in order to better understand the salt tolerance process. It is hypothesized that overexpression of a single gene can increase transgenic plants' salt tolerance. *A. thaliana* plasma membrane Naþ/ Hþ antiporter gene SOS1 and vacuolar Naþ/Hþ antiporter gene AtNHX1 can considerably improve the salt tolerance capacity of the transgenic plants [90]. Researchers found that AtSAT32 transgenic plants have higher salt tolerance and activity of vacuolar H transport pyrophosphatase in high salt environments after overexpressing AtSAT32 in *A. thaliana*. The AtSAT32 mutant, on the other hand, is extremely sensitive to salt [91]. Furthermore, through rice ABA-dependent regulatory mechanisms, the rice OsbZIP71 gene can considerably improve transgenic plant tolerance to high salt and drought [92]. Plant tolerance can also be improved by increasing the activity of antioxidant enzymes and increasing the degree of antioxidant metabolism in plants. The use of genetic engineering to create highly effective transgenic plants makes it possible to investigate the role of antioxidant enzymes in the scavenging of active oxygen. In numerous transgenic plants, the necessity of scavenging active oxygen has been demonstrated, and overexpression of SOD, APX, GR, and GAT improves plant tolerance to oxidative stress. The SOD isoenzyme has been the subject of the most extensive investigation among the numerous antioxidant enzymes [93]. Wheat salt tolerance gene research could lead to the utilization of saline soil and the expansion of wheat planting areas, resulting in higher wheat yields. Wheat salt tolerance genes have been studied extensively by researchers, with some successes. Wheat genes Ta-UnP, TaZNF, TaSST, TaDUF1, and TaSP have been shown in lab research to improve the salt tolerance of transgenic plants [1, 94–97]. Furthermore, ectopic expression of the wheat TaCIPK14 gene increases transgenic tobacco's salt tolerance [98]. Bread wheat with TaHKT1; 5-D has a high salt tolerance [99]. The ability to tolerate salt is a multi-gene trait. Hundreds of thousands of genes and dozens of physiological systems are usually involved in its regulation. -As a result, research into the huge number of salt-tolerant genes is required. The study of salt-related gene function provides a theoretical foundation for increasing the stress signal network and improving rice and wheat plant tolerance to stress.

4.1 Epigenetic response to abiotic stress in rice and wheat

Histone post-transcriptional modifications, histone variations, DNA methylation, and non-coding RNAs are examples of chromatin state modification which can arrange diverse chromatin states that epigenetically define specific transcriptional outputs. The identification of epigenetic markers and their effect on plant response to abiotic stressors has been made easier because to recent advances in the field of -omics of important crops.

Recent research using –omics technologies have found a link between changed DNA methylation patterns and varied gene expression across the genome in three rice cultivars with variable susceptibilities to increasing salt and drought stress [100]. DNA methylation alterations were seen in both salt-tolerant and salt-susceptible rice varieties upon exposure into high salinity [101]. DNA immunoprecipitation with the 5-methylcytosine antibody and high throughput sequencing (MeDIP-seq) were used to determine the genome-wide methylation status of a salt resistant rice variety under increased salinity in a recent study [102]. These research identified new epigenetic variables and target genes linked to rice's response to salt stress, which could be used to improve resistance under salt stress conditions. The role of miRNAs in rice response to abiotic stress has been the subject of a lot of research over the last few years [103–106]. Rice tolerance to salt stress was boosted by overexpression of a *miR156*, while the expression of transcription factor target genes like *SQUAMOSA PROMOTER BINDING ROTEIN LIKE-9 (SPL9)* and *DIHYDROFLAVONOL-4-REDUCTASE (DFR)* which mostly associated with developmental processes was reduced [107].

Based on 850 RNA-seq data collected from 32 tissues sampled at different growth stages and/or under different stress treatments, an analysis of the wheat whole genome expression pattern (e.g. "expression atlas") found higher average methylation status in low expressed genes [108]. Gardiner et al. [109] discovered that methylation patterns in wheat are not evenly distributed across the A, B, and D subgenomes, indicating that methylation patterns in progenitor species are different. In recent years, several research on epigenetic modifications in wheat in response to abiotic stress have been published. Wheat drought stress response has been linked to heterochromatic small interfering RNA (hc-siRNA) and micro RNAs (miRNA), both tiny regulatory RNAs [110]. For example, 2055 putative sites for 113 conserved durum miRNAs and 131 targets for four novel durum miRNAs that may contribute to genotypic stress tolerance have been found [111]. Differential epigenetic modifications in certain genes such as HKTs (high-affinity potassium transporters) were discovered in the shoots and roots of wheat genotypes with variable levels of susceptibility to salt stress [112].

5. Conclusion

Salinity stress is the one of the main abiotic factors that limits crop growth, development and yield. In this chapter, we briefly summarize how crop plants, especially rice and wheat respond to salinity induced osmotic, ion, and oxidative stresses and collect a significant number of studies and progress on the effects of salt stress on plants. Much research was undertaken to clarify physiological and biochemical and molecular mechanisms of plants salt tolerance applying molecular biology and biotechnological approach which will further explain the plant salt-tolerance mechanism and provide sufficient theoretical guidance for the future cultivation of salt stress tolerance rice and wheat. Moreover, by understanding the role of epigenetics in gene expression under abiotic stress could provide guidance in the development of climate-smart crops. Further enhancing plant salt tolerance and rich salt tolerant crop plants still need further study.

Conflict of interest

No

Notes/thanks/other declarations

No

Author details

Mohammad Hasanuzzaman[1,2]

1 Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, Bangladesh

2 Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh

*Address all correspondence to: hasanbge50450@bau.edu.bd

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Chapter 4

Involvement of Epigenetic Regulation in Plant Defence during Biotic Stress

Jasmine M. Shah and Joyous T. Joseph

Abstract

Plants being organisms that lack locomotion and vocabulary, they are not privileged to escape and communicate during unfavourable conditions of biotic/abiotic stresses, like their animal counterparts. Therefore, plants have evolved with higher adaptive skills that tune them during unfavourable conditions. In this context, regulation of gene expression plays a crucial role in controlling the cellular pathways required for survival during unfavourable conditions. This chapter is about the epigenetic regulation of plant defence during biotic stress. Researchers have taken various approaches to understand the epigenetic regulation of plant defences and these approaches are described here. Epigenetic regulation also has the potential to be inherited and this phenomenon has aided plants for better adaption. Such reports on transgenerational memory during biotic stress in plants are also compiled. A deeper understanding of epigenetic regulation of defence pathways during biotic stress, and identification of epigenetic marks on the genomes, can aid the development of crop improvement strategies. With the recent advancement in epigenome editing, it should become possible to develop epigenetically improvised plants, devoid of genetic modification.

Keywords: epigenetic regulation, epigenetic modification, biotic stress, plant defence, heritable epigenetic changes, methylation

1. Introduction

Regulation of gene expression is the ultimate criteria that decide the role of each player in all cellular pathways. Gene regulation occurs in the nucleus and cytoplasm at multiple levels such as chromatin conformation, transcription regulation, post-transcription regulation, regulation of translation, protein modification, and protein degradation. Regulation at transcription is one of the prominent mechanisms as it involves the so called 'switching-on and switching-off' of genes. Gene regulation is required for organisms not only for their routine growth and maintenance but also for survival during unfavourable conditions. In this context, epigenetic regulation is of much significance because it not only offers adaptive skills to the organisms under stress but also has the potential to be heritable, thereby contributing to transgenerational memory. Owing to lack of locomotion and vocabulary in plants, like their animal counter parts, it is imperative for them to survive in unfavourable conditions such as biotic and abiotic stress. Therefore, plants have evolved with an elaborate mechanism of gene regulation, especially epigenetic regulation. Researchers have deduced gene functions by adopting various approaches and the key findings in epigenetic regulation and transgenerational memory in plants during biotic stress are described in this chapter.

2. Promoters, the switches for perceiving stress-induced communications

Promoters serve the function of a switch as they are the site for the binding of RNA polymerase and other transcription regulators. Promoters harbour *cis* regulatory sequences that can perceive information from regulatory factors in *trans*. This mechanism serves as an apt platform for perceiving signals of growth, development, and survival during unfavourable conditions of stress. In this context, promoters of plants play a significant role as they can support the organism to thrive unfavourable conditions, which they cannot escape owing to their sessile nature. Plants have developed an elaborate defence mechanism to survive during conditions of biotic stresses such as herbivory and disease. The defence mechanism comprise of pathways such as systemic acquired resistance (SAR) and induced systemic resistance (ISR), apart from structural adaptations and certain constitutively expressing defence proteins [1]. The secret for survival during vivid conditions of biotic stresses, which the plants are forced to undergo, lies in the regulation of gene expression involved in defence. Regulation at the switches, the gene promoters, mainly involves response to transcription factors (TFs) and epigenetic modifications, which tune the transcription by RNA polymerase. Special TFs belonging to the families such as bZIP (basic leucine zipper), MYB (myeloblastosis), WRKY, and NAC [NAM (No apical meristem), ATAF (*Arabidopsis* 69 transcription activation factor), and CUC (cup-shaped cotyledon)] are required to perceive biotic stresses [2]. Epigenetic modulators of the entire genome, including the promoters, involve the DNA and histones modifiers, and small non-coding RNAs. Change in nuclear architecture was also found to be involved in the regulation of promoters [3]. Epigenetic changes associated with other genomic regions such as introns and not the promoter were also known to be involved in gene regulation as they could result in alternative splicing.

3. Epigenetic modifications

Epigenetic changes refer to all modifications or influences on the chromatin, except for changes in the DNA sequence [4, 5]. These changes if occurred in promoters or other regulatory regions can result in altered gene expression, thereby contributing to phenotype plasticity. Multiple factors can lead to epigenetic changes (**Figure 1**). Nucleotides in DNA, especially cytosine, undergo methylation/demethylation. In addition to methylation, histones can undergo other chemical modifications such as acetylation, phosphorylation, ubiquitylation, and sumoylation. Non-coding RNAs are involved in altering chromatin organisation and/or methylation of chromatin [6]. Replacement of histones by histone variants such as H3.3, H2A.X, and H2A.Z also influence chromatin organisation and gene expression [7, 8].

4. Different approaches taken to understand epigenetic regulation of plant defences

Discoveries in various aspects of epigenetic regulation in plants provided a deeper perception of various pathways, including defence. Different approaches

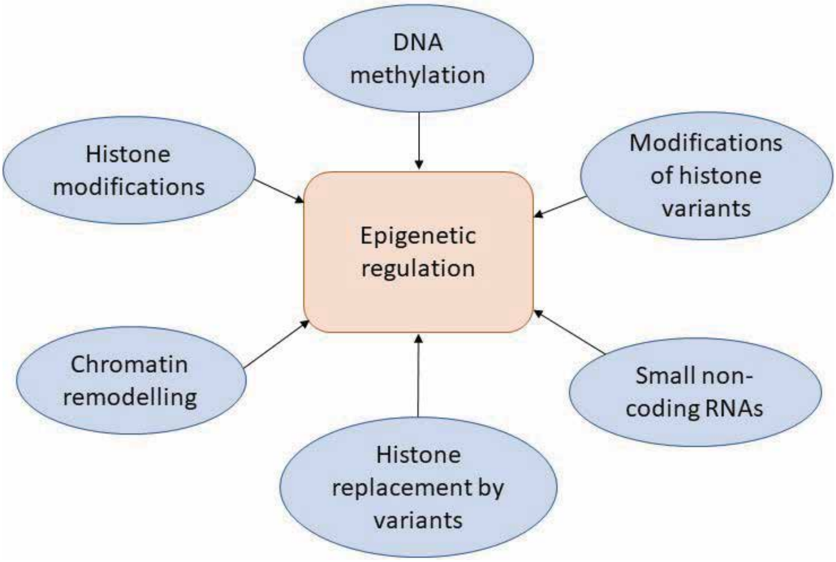

Figure 1.
Multiple causes of epigenetic regulation.

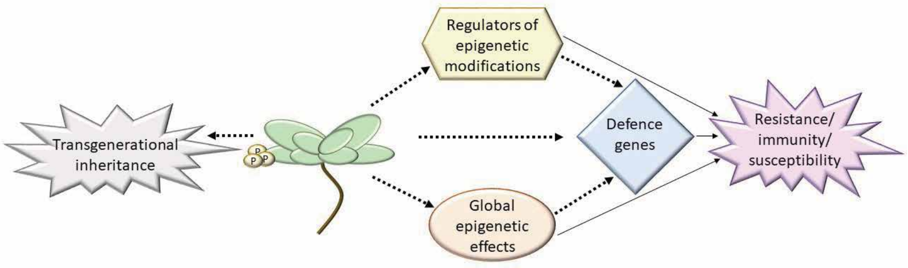

Figure 2.
Various paths chosen by researchers to understand the involvement of epigenetic regulation of plant defence, including the transgenerational memory of defence. The dotted arrows indicate the involvement of epigenetic modulations in that step.

taken by researchers include the study of epigenetic regulation of defence genes directly under pathogen stress, whole epigenome analysis, and understanding of the regulation of epigenetic regulators and their influence on disease (**Figure 2**). In this chapter, the research done in understanding different aspects of epigenetic regulation during plant-pathogen interaction and defence is described. Epigenetic changes have the potential to be retained after multiple cell divisions of both mitosis and meiosis. Therefore, heritable epigenetic changes lead to an interesting phenomenon of transgenerational memory. Epigenetic changes play a crucial role in plants as they not only lack locomotion, but also the vocabulary mode of communication. Biotic/abiotic stress-induced epigenetic changes have often generated plants and even their offsprings, with enhanced stress resistance. Such aspects are also elaborated in this chapter.

4.1 Biotic stress-induced epigenetic modulation of defence genes

Pathogen stress is well known to alter the expression of numerous genes, belonging to several pathways, including those involved in defence, in plants.

Interestingly, there are reports revealing the involvement of pathogen stress in altering the epigenetic status of various defence genes. For example, infection by *P. syringae* altered the histone methylation status of promoters of two key defence genes, *CPR5* and *PR1* [9]. Treatment with β-aminobutyric acid (BBA), which mimics infection, resulted in altered histone acetylation and methylation of promoters of few defence genes, *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*), *ARABIDOPSIS NON-RACE SPECIFIC DISEASE RESISTANCE GENE (NDR1)/HAIRPIN-INDUCED GENE (HIN1)-LIKE 10* (*NHL10*) and *CYTOCHROME P450, FAMILY 81* (*CYP81F2*) and *PR1* of *Arabidopsis* [10]. This treatment also resulted in the priming of *Arabidopsis* plants against the bacterial pathogens *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) [10] and *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 [11]. Similarly, priming of *Arabidopsis* plants using benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) resulted in altered acetylation and methylation of *AGO2* promoter, which in turn provided enhanced resistance against *Cucumber mosaic virus* [12].

Though regulation of defence genes was known to be modulated by many TFs as mentioned earlier in this chapter, it is interesting to note that epigenetic regulation is involved in modulating these modulators. For example, promoters of three WRKY TFs, WRKY29, WRKY6, and WRKY53 underwent histone methylation and acetylation under biotic stress conditions and some of these modifications facilitated gene expression in primed plants [13].

4.2 Epigenetic modulators under biotic stress

About 130 genes are known to be involved in epigenetic regulation in plants [14]. We selected 60 genes involved in DNA and histone methylation (**Table 1**) and looked whether there are any previous reports on their expressions in the eFP Browser (*Arabidopsis* eFP browser 2.0 – BAR) and this revealed that all the 60 genes invariably had altered expression under many biotic stresses such as infection due to *P. syringae*, *Botrytis cinerea*, *Erysiphe oronti* or *Phytophthora infestans* [30]. Similarly in rice, several genes involved in the RNA-directed DNA methylation (RdDM) pathway showed altered expression under many bacterial pathogens [31]. This indicates that epigenetic regulation in plants is closely associated with biotic stresses.

4.3 Regulators of epigenetic modifications that influence defence genes

Like any other pathway in organisms, epigenetic regulation is also maintained by many key players. There are reports that many such epigenetic regulators are directly associated with the expression of many defence genes as, observed in various loss-of-function mutant plants. JmjC DOMAIN-CONTAINING PROTEIN 27 (JMJ27), an *Arabidopsis* JmjC domain-containing histone demethylase 2 (JHDM2) family protein, was involved in suppressing the expression of three repressors of defence TFs, WRKY25, WRKY26, and WRKY33 in *Arabidopsis* [32]. Promoters of two of these TFs WRKY25 and WRKY33 and, *PR1* gene were found to be hypermethylated in *Arabidopsis* plants deficient for JMJ27 [32, 33]. Two sub-units of the elongator complex protein that interacted with RNA polymerase II, ELP2 [34] and ELP3 [35], also additionally functioned as epigenetic regulators of plant defence genes. ELP2 altered histone acetylation of the promoter of *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1* (*NPR1*), a key defence gene activator, and regulated the kinetics of the expression of many defence genes [34]. An *Arabidopsis* demethylase encoded by *REPRESSOR OF SILENCING 1* (*ROS1*) was shown to be involved in removing methyl groups from the promoter region of two genes *RECEPTOR-LIKE PROTEIN*

No.	Genes names and category		Function	References
1.	**DNA METHYLATION**	*CMT2*	Plant DNA methyltransferase	[15]
2.		*DRM2*	Maintenance of CHH methylation	
3.		*CMT3*	A chromomethylase involved in methylating cytosine at non-CG sites	
4.		*DDM1*	Gene silencing and maintenance of DNA methylation	
5.		*OTU5*	Phosphate (Pi) homeostasis during DNA and histone methylation	[16]
6.		*MT1*	Maintenance of CG and CHG methylation	[17]
7.		*ROS1*	Acts along with DME	
8.		*DME*	Catalyses the release of 5-methylcytosine (5-meC) from DNA by a glycosylase/lyase mechanism	
9.		*MET1*	Maintains DNA methylation in CHG context	[18]
10.		*NRPD1B*	Major trans acting locus affecting DNA methylation	
11.		*BRU1*	Link between responses to DNA damage and epigenetic gene silencing	[19]
12.		*MRE11*	DNA repair and meiotic recombination	
13.		*FLC*	High level delays flowering unless treated with prolonged cold	[20]
14.		*KYP*	Encodes a histone 3 lysine 9 specific methyltransferase involved in the maintenance of DNA methylation	
15.		*NRPD1*	Required for posttranscriptional gene silencing	[21]
16.		*NRPE1*	Normal RNA directed DNA methylation at non CG methylation sites and transgene silencing	
17.		*MOM1*	Prevents the transmission of stress-induced transcriptional changes to progeny of the stressed plants	
18.		*EDM2*	Prevents ectopic 3′ end processing of mRNA in atypically long introns containing T-DNA sequences	[22]
19.		*RDR2*	Encodes RNA-dependent RNA polymerase that is required for endogenous siRNA (but not miRNA) formation	
20.		*DCL3*	Encodes a ribonuclease III family protein that is required for endogenous RDR2-dependent siRNA formation	
21.		*AGO4*	SiRNA mediated gene silencing, CpNpG and CpHpH methylation	
22.		*NRPD2A*	encodes a shared subunit of RNA polymerase IV and V	
23.		*DRM2*	A putative DNA methyl transferase with rearranged catalytic domains	
24.		*AGO6*	Important for DNA methylation and transcriptional gene silencing	

No.	Genes names and category		Function	References
25.		*DCL2*	Encodes a Dicer-like protein that functions in the antiviral silencing response	
26.		*DCL4*	Encodes an RNaseIII-like enzyme that catalyses processing of trans-acting siRNA precursors	
27.		*LFY*	Encodes transcriptional regulator that promotes the transition to flowering, T DNA shows high methylation	
28.		*LHP1*	Required for epigenetic maintenance of the vernalized state	[23]
29.		*SUVH2*	Encodes a SET domain protein that is involved in epigenetic regulation	
30.		*MEA*	Has a SET domain for methyltransferase activity and is involved in the stable transcriptional silencing of target genes	
31.		*VIM1*	Involved in centromere heterochromatisation, CG methylation	[24]
32.	**HISTONE METHYLATION**	*ATX1*	Activates the expression of AtWRKY70 epigenetically by nucleosomal histone H3K4 trimethylations	[25]
33.		*WRKY70*	ATX1 leads to nucleosomal histone H3K4 trimethylations that activate AtWRKY70, which in turn activates *PR1* and *THI2.1* defence genes	
34.		*WRKY40*	Histone methylations at the AtWRKY40 promoter activate the SA-dependent pathway to control plant immunity	
35.		*FLD*	Epigenetically influences systemic-acquired-resistance induced expression of AtWRKY29 and AtWRKY6 through histone modifications at their promoters	
36.		*SUVH2*	Leads to H3K4me2 and H3K4me3 methylation that epigenetically regulates AtWRKY53 to mediate leaf senescence responses	[26]
37.		*SUVH5*	Histone methyl transferase activity and maintenance of H3 mK9	
38.		*SUVH6*	Binds to methylated cytosine of CG, CNN, CNG	
39.		*ATXR5*	Control of replication of transposable elements and repeated sequences	[27]
40.		*ATXR6*	Control of replication of transposable elements and repeated sequences	
41.		*EMF1*	Maintains vegetative development, encodes a putative transcriptional regulator	[28]
42.		*EMF2*	Maintains vegetative development, encodes a polycomb group	
43.		*PISTILLATA*	Maintains gene silencing via histone modification	
44.		*APETALA3*	Maintains gene silencing via histone modification	
45.		*APETALA2*	Epigenetic maintenance of reproductive development	[29]
46.		*PR1*	Gene involved in plant response to pathogen	[9]

No.	Genes names and category	Function	References
47.	*ASHH2*	Histone methyltransferase involved in di and tri-methylation of 'Lys-36' of histone H3 (H3K36me2 and H3K36me3)	
48.	*ASHR1*	Histone-lysine N-methyl transferase activity	
49.	*TOC1*	Contributes to plant fitness like biomass, carbon fixation by influencing circadian clock period.	[21]
50.	*PRR7*	Essential component of a temperature-sensitive circadian system	
51	*PRR9*	Essential component of a temperature-sensitive circadian system	
52	*HAC1*	Necessary component for bacterial resistance.	
53.	*SUVR4*	One of the four closely related *Arabidopsis* SUVR proteins that belong to the SU(VAR)3–9 subgroup of SET-domain proteins	[24]
54.	*CLF*	Putative role in cell fate determination. Involved in the control of leaf morphogenesis.	
55.	*MEA*	Has a SET domain for methyltransferase activity and is involved in the stable transcriptional silencing of target genes.	
56.	*SWN*	Encodes a polycomb group of protein	
57.	*SDG4*	Contains a SET domain which is known to be involved in modification of histone tails by methylation	
58.	*SDG26*	SET domain, histone methylation	
59.	*ATPRMT4A*	Encodes a type I protein arginine methyltransferase	
60.	*ATPRMT4B*	Encodes a type I protein arginine methyltransferase	

Table 1.
Various epigenetic modulators and their functions.

43 (RLP43) and *RESISTANCE METHYLATED GENE 1* (*RMG1*), which encode for receptors involved in pathogen recognition [36]. *ROS1* antagonised the action of RdDM and interestingly, removed methylation from the binding sites of WRKY TFs of *RLP43* promoter, thereby exposing the site for TF binding [36]. RNA polymerase V, an enzyme required for RdDM, also played a crucial role in differential histone modification of genes involved in SA pathway [37].

Using the *Arabidopsis-P. syringae* pathosystem, it was revealed that three *ASH1-RELATED* (*ABSENT, SMALL OR HOMEOTIC DISCS 1*) genes *ASHR1*, *ASHR3,* and *ASHH2* that were involved in histone methylation, also served the function of compromising resistance in plants [9]. There was decreased histone methylation of *PR1* gene promoter in plants with mutant *ashr1* and *ashh2* alleles [9]. While the avirulent *P. syringae* strain slightly increased the expression of *ASHR1*, *ASHR3,* and *ASHH2* genes in wild plants, an antagonistic response was induced by the virulent strain, indicating interference of pathogen in the expression of genes involved in writing the histone marks. Histone deacetylase 6 (HDA6) was found to bind to the promoters of *PR1* and *PR2* genes, thereby leading to their decreased transcription [38]. Expression of several defence genes and *R* genes was compromised and/or the histone methylation status of their promoters was altered under pathogen stress in

Arabidopsis loss-of-function mutant (*sdg8–1*) for the gene encoding a histone methyltransferase [*SET DOMAIN GROUP8* (*SDG8*)] [39, 40]. H2A.Z, a histone variant was involved in suppressing the expression of multiple SAR genes [8]. Infection of rice plants with the nematode *Meloidogyne graminicola* resulted in differential histone modifications at H3K9 that is associated with plant defence genes [41] MOS9, a protein that regulated two *R* genes, *CONSTITUTIVE 1* (*SNC1*) and *RECOGNITION OF PERONOSPORA PARASITICA 4* (*RPP4*), was found to be associated with a histone methyltransferase Trithorax-Related7 (ATXR7) and, MOS9 and ATXR7 together were involved in the regulation of the two *R* genes using histone methylation [42]. ATPase SPLAYED (SYD) was found to be involved in the chromatin remodelling of promoters of various genes involved in JA and ethylene (ET) signalling pathways [43]. A kind of cyclic interdependence of histone deacetylases (HDACs), SA, and nitric oxide (NO) was discovered in *Arabidopsis*. While NO increased histone acetylation and/or increased expression of many defence genes by adversely affecting HDACs, SA induced endogenous NO, inhibited HDAC activity, and increased histone acetylation [44].

4.4 Epigenetic regulators that provide immunity/resistance/susceptibility

There are several reports confirming that various epigenetic regulators are directly involved in either providing immunity to plants or, to render the plants susceptible to various stresses. For instance, studies on *Arabidopsis* mutants for genes encoding various subunits of RNA polymerases (Pol) IV and V, which are required for RdDM, revealed that Pol V and not IV was involved in host defence [37]. Interestingly, these mutants had enhanced and reduced expressions of genes involved in SA and JA pathways, respectively. As a consequence, the plants were resistant to the bacterial pathogen *P. syringae* and susceptible to the fungal pathogens, *B. cinerea* and *Plectosphaerella cucumerina*. Lack of *JMJ27* rendered *Arabidopsis* mutants more susceptible to virulent *P. syringae* and, the expression of various *PR* genes belonging to *PR1*, *PR3*, *PR4* and *PR5* was found to be compromised in these plants [32]. The *Arabidopsis REPRESSOR OF SILENCING 1* (*ROS1*) was shown to be involved in providing basal resistance towards *Pseudomonas syringae* and facilitated flagellin-triggered immunity [36]. Histone deacetylase 6 (HDA6) was identified as a negative regulator of defence as plants with mutated allele coding for HDA6 exhibited enhanced resistance against the pathogenic strain of *P. syringae* and, constitutively expressed *PR1* and *PR2* genes [38]. Null mutations of genes encoding H2A.Z histone variant in *Arabidopsis* also resulted in enhanced resistance to phytopathogenic *P. syringae* pv. *tomato* [8]. In contrast, loss-of-function *Arabidopsis* mutants of *HISTONE MONOUBIQUITINATION1* (*HUB1*) [45] and *SDG8* [39] exhibited increased susceptibility to two necrotrophic fungi *B. cinerea* and *Alternaria brassicicola*. HUB1 interacted with another protein MED21, which was required for defence against plant necrotrophic fungi [45]. *SDG8* regulated *LAZ5* that encoded an RPS4-like R-protein and as a consequence, the *Arabidopsis* mutants for *SDG8* were compromised for resistance against *P. syringae tomato* DC3000 as well [40]. However, the *syd* mutant *Arabidopsis* were susceptible to *B. cinerea* and not to *P. syringae*, a biotroph [43]. *Arabidopsis* mutants for *KYP* that coded for H3K9me2 methyltransferase exhibited reduced manifestation of the aphid *Myzus persicae* larvae and enhanced expression of aphid-resistance genes [46].

4.5 Biotic stress-induced epigenomic changes

Biotic stress such as microbes and herbivory have induced loci-specific as well global epigenetic changes, both at DNA and histone levels. Infection of *Arabidopsis* plants with both virulent and avirulent *P. syringae* strain resulted in hypomethylation and major decondensation of centromeric heterochromatin DNA [47]. Virulent and avirulent strains of *P. syringae* and SA treatment-induced differentially-methylated regions across the genome, many of which were associated with altered transcription [48]. This analysis also revealed that SA-induced transposon-associated differently methylated regions were often regulated by siRNA and influenced the transcription of proximal genes. Whole epigenome analysis of *Arabidopsis* plants infected with differentially pathogenic strains of *turnip mosaic potyvirus* (*TuMV*) though indicated no major differences in the induction of methylomes, they exhibited drastic changes in their transcriptomes [49].

Nematode-associated molecular patterns from different nematode species and bacterial pathogen-associated molecular pattern flg22 induced global DNA hypomethylation in rice and tomato plants [50]. Hypomethylation was more common at CHH and not CG or CHG nucleotides in these plants. While herbivory due to an insect *Heliocheilus albipunctella* de Joannis resulted in enhanced global methylation, SA resulted in hypomethylation of *Pennisetum glaucum* genome [51]. Also, the number of larvae significantly reduced in SA-treated plants, indicating an association between altered methylation and defence. In another example of herbivory due to the larvae of aphid *M. persicae* on *A. thaliana*, a global loss of methylation was seen, accompanied by enhanced expression of defence genes [46].

Open chromatin is an indication of epigenetic changes such as histone acetylation, which loosen chromatin. About 10,129 open chromatin sites associated with about 3025 genes, most of which also had enhanced expression, were induced in *Arabidopsis* plants infected with *P. syringae* pv. *maculicola* [52].

A comparison of whole epigenomes in various *Arabidopsis* mutants for genes involved in methylation/demethylation indicated that both these antagonistic processes are required for plant defence [53]. This was understood based on the analysis on four different hypomethylated (*ddm1 F4*, *nrpe1*, *drd1*, and *cmt3*) and two hypermethylated (*ros3* and *ros1*) mutants of *Arabidopsis*, which were infected with the biotrophic fungal pathogen *Hyaloperonospora arabidopsidis*. All the hypomethylated mutants were more resistant compared to the uninfected control, and in contrast, the hypermethylated mutants were more sensitive. While infection with *H. arabidopsidis* lead to enhanced *PR1* expression in *nrpe1* mutant, lack of functional *ros1* lead to suppression of *PR1*. The two opposite mutants *nrpe1* and *ros1* differed in the expression of genes involved in SA and JA pathways. As expected due to SA-JA pathway antagonism, *nrpe1* and *ros1* mutants were more susceptible and resistant, respectively, to the necrotrophic fungi *Plectosphaerella cucumerina*. Transcription of about 49% of pathogen-responsive genes was affected in both *nrpe1* and *ros1*, as described in the same report [53].

The beneficial fungus *Trichoderma harzianum* T22 induced altered global genome methylation status, defence gene expression, and even post-transcriptional regulation in tomato plants [54]. Here, the DNA methylation first decreased and then increased at 24 h and 72 h post-inoculations, respectively.

4.6 Heritable epigenetic biotic stress-induced responses

There are several reports revealing the heritable nature of pathogen-induced epigenetic changes in plants [55, 56]. For example, *Tobacco mosaic virus* (*TMV*) induced global hypermethylation of genomes in *Nicotiana tabacum,* and this change was observed even in the progeny [57]. *Agrobacterium tumefaciens* induced loci-specific changes in *Arabidopsis,* which were retained even in the grand progeny [58]. Pathogen infection also induced heritable resistance in plants. For example, infection with *P. syringae tomato* DC3000 resulted in enhanced resistance against

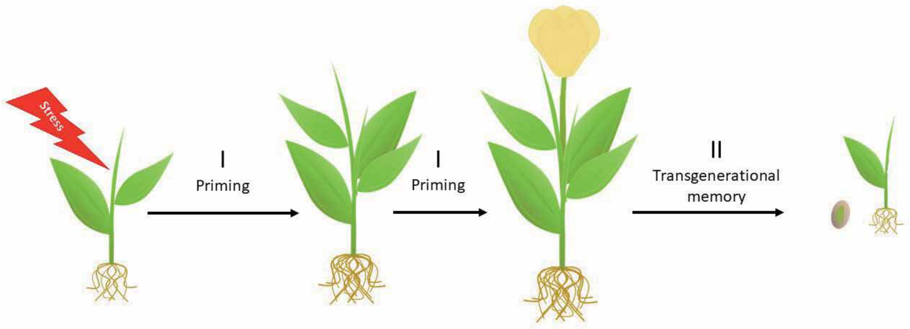

Figure 3.
Transfer of epigenetic memory in plants.

the subsequent infections by PstDC3000 and *H. arabidopsidis* in the progeny *Arabidopsis* plants [59]. Interestingly, altered histone modifications were associated with *PR1*, *WRKY6*, *WRKY53,* and *PLANT DEFENSIN1.2* (*PDF1.2*) of these plants and, the progeny of plants mutant for *NPR1* failed to exhibit this transgenerational defence [59]. Similarly, *TMV* caused hypomethylation of LRR region of the N-gene specific for resistance to *TMV* [57]. Thus, epigenetic memory in plants can be broadly categorised into two, one that retains within the affected generation, and two, that transfers to the progeny (**Figure 3**).

The epiRILs lines of *Arabidopsis* that differ in their DNA methylation patterns but not DNA sequence, have aided in confirming that SA and JA induced heritable phenotypic plasticity, indicating the involvement of heritable epigenetic regulation on plant defence system [60]. Infection of *Arabidopsis* hypo-methylated and hypermethylated mutants *nrpe1* and *ros1*, respectively, with a virulent *Pseudomonas* strain, indicated that NRPE1 and ROS1 could be involved in transgenerational memory [53]. While the progeny of the infected wild-type plants exhibited resistance against another pathogen, *H. arabidopsidis*, *nrpe1* mutants did not show any difference in their resistance level and, *ros1* mutants displayed enhanced susceptibility [53].

Similarly, to pathogens, herbivory also induced transgenerational responses. For example herbivory due to caterpillars in *Arabidopsis* and tomato plants induced reduction in subsequent caterpillar invasion and this priming persisted for two generations [61]. The same group also discovered that *Arabidopsis* mutants deficient in siRNA biogenesis did not inherit this resistance.

The close influence of pathogen stress on epigenetic modification of plant defence system and transgenerational memory offers an entire new array of promises for crop improvement. Approaches of whole epigenome studies under various conditions of biotic stress and resistance would unravel more aspects of the epigenetic regulation of host mechanisms. New avenues of epimutagenic studies that could serve as alternatives for methods that involve gene manipulations/mutations seem to be promising.

5. External application of chemicals or external factors that induce epigenetic changes

There are observations where application of certain chemicals such as SA, JA, methyl jasmonate, systemin, paraquat, abscisic acid, azelaic acid, and pipecolic acid on plants had resulted in enhanced resistance against pathogens, which could even be heritable [62, 63]. Even conditions of altered salinity, light, drought, and temperature had induced similar results [63]. Reports indicate that the action of some of these external factors involve epigenetic modifications in plants. For example, application of 1-isothiocyanato-4-methylsulfinylbutane on *Arabidopsis* induced chromatin modifications in the two defence genes, *WRKY6* and *PDF1.2*, and reduced the susceptibility to downy mildew disease caused by *H. arabidopsidis* [64]. Thus, such chemicals and other factors provide a non-mutagenic, or the 'epimutagenic' mode of inducing favourable changes in plants that can be used for crop improvement. But since epigenetic stress is highly dynamic and depends on other environmental factors and the stress-pressure [65] careful examination and repeated testing would be required to bring in a commercial-level application of epimutagens.

6. Conclusions

While understanding of the DNA sequence conveyed what information is there in the genome and expression analysis conveyed what information is disseminated, research on epigenetics conveyed how the information is disseminated. While the entire genome of an organism needs to be sequenced only once to get the sequence information, epigenome has to be sequenced and studied multiple times, with multiple approaches, based on the regulatory aspects of relevance. Owing to the dynamic and reversible nature of epigenetic regulation and, phenotypic plasticity, epigenetic regulation can play a crucial role in improvising traits of agronomic importance, including plant defence. Functions of more epigenetic modulators need to be analysed that can tune the plants towards a favourable trait. The function of more epialleles needs to be identified for their application in developing enhanced resistance in plants. With the recent development of non-transgenic method of epigenome editing, epialleles of agronomic importance can be generated and deployed.

Author details

Jasmine M. Shah* and Joyous T. Joseph
Central University of Kerala, Kasaragod, Kerala, India

*Address all correspondence to: jasmine@cukerala.ac.in

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Chapter 5

Molecular Defense Mechanisms in Plants to Tolerate Toxic Action of Heavy Metal Environmental Pollution

Istvan Jablonkai

Abstract

Toxic action of heavy metals on plants growing in contaminated soils intensified the research on detoxification and sequestering mechanisms existing in plants to understand and manipulate defense mechanisms that confer tolerance against metal ions. Increased biosynthesis of plant biomolecules to confer tolerance during toxic action of heavy metals is an intrinsic ability of plants. Induced formation of low-molecular weight amino acids, peptides or proteines as chelators such as proline (Pro), glutathione (GSH), phytochelatins (PCs) or metallothioneins (MTs) under heavy metal stress enhances metal binding and detoxification capability of plants. In addition, proline and GSH related enzymes such as GSH reductase, GSH peroxidases and glutathione S-transferases are also key components of the antioxidant defense system in the cells to scavenge reactive oxygen species (ROS). Protective action of oxidized fatty acids oxylipins at toxic levels of heavy metals is considered to activate detoxification processes as signaling molecules.

Keywords: heavy metals, stress, detoxification, glutathione, chelators

1. Introduction

Abiotic stress factors such as extreme temperatures, drought, salinity, heavy metals, xenobiotics have been considered as potential threats for agricultural productivity. To cope with abiotic stress, plants can initiate a number of molecular, cellular, and physiological changes to respond and adapt to such stresses. Stress-tolerance of plants involves the activation of cascades of molecular networks leading to expression of stress-related enzymes.

The accumulation of heavy metals in the plant is accompanied by damage to the structural components and an imbalance of various metabolic processes in the cells, which leads to disruption of plant growth and development. Plants have evolved a number of mechanisms to adapt to increasing concentrations of metal ions. These include the immobilization, exclusion, chelation, and compartmentalisation of metal ions.

The toxic action of heavy metals can produce excessive reactive oxygen species (ROS). Glutathione and GSH related enzymes such as GSH reductase, GSH peroxidases and glutathione S-transferases are fundamental parts of the antioxidant defense system in the cells to scavenge ROS and electrophilic organic molecules as well. The significance of plant thiols and glutathione *S*-transferases involved in plant response to almost all stress factors will be discussed with special attention to their overexpression, redox status and regulation that confer stress tolerance.

A number of metal-binding ligands have now been recognized in plants [1]. Polypeptide ligands include the metallothioneins (MTs), small, gene-encoded, cysteine-rich polypeptides, and the phytochelatins (PCs), which, in contrast, are enzymatically synthesized are effective metal binding ligands have been identified. Recent advances in understanding the role, biosynthesis and protective action of phytochelatins and metallothioneins as metal-binding ligands in heavy metal detoxification are reviewed.

Proteinogenic amino acid proline protects cell from environmental stress factors by several protective mechanisms such as osmoprotectant, acting as chemical chaperone by stabilization of proteins and antioxidant enzymes, chelation of metals, scavenging ROS, balancing the intracellular redox homeostasis ($NADP^+$/NADPH ratio, GSH pool) and participating in cellular metabolic signaling. Proline accumulation has been observed in response to environmental stress in a variety of living organism including plants.

Reactive electrophile species oxylipins exhibit protective action under toxic concentration of pollutants by activation of detoxification processes. The less studied oxylipin signaling in plant stress response will be detailed as an important factor in plant adaptation to stress by heavy metal pollutants.

2. Role of glutathione (GSH) and glutathione related enzymes in protection of plants against toxic action of heavy metals

The accumulation of heavy metals in plant organs results in the damage of the structural components and disruption of cell metabolic processes leading to plant growth retardation. The toxic action of heavy metals can produce excessive reactive oxygen species (ROS). Glutathione and GSH related enzymes such as GSH reductase, GSH peroxidases and glutathione S-transferases are fundamental parts of the antioxidant defense system in the cells to scavenge ROS and electrophilic organic molecules as well (**Figure 1**).

2.1 Glutathione (GSH) and GSH/GSSG redox system

The most abundant biological thiol the tripeptide glutathione (GSH, γ-Glu-Cys-Gly) is a low molecular weight, water soluble compound that is ubiquitous in most plant tissues and has a key function in stress management. Besides the ROS detoxification, GSH also participates in detoxication of methylglyoxal and electrophilic xenobiotics [2]. As a component of the glutathione-ascorbate (AsA-GSH) cycle GSH has a key role in H_2O_2 detoxication. GSH is used as co-factor by a) various peroxidases in detoxication of peroxides formed in the reaction of oxygen radical with biological molecules and b) by glutathione S-transferases (GST) to conjugate GSH with endogenous substances and xenobiotics. Interaction of GSH with thioredoxin systems fine-tune photosynthetic and respiratory metabolism by modifying the sensitive protein Cys residues [3]. GSH is a substrate of phytochelatin synthase and oligomeric GSH products phytochelatins (PCs) can effectively sequester heavy metals by complexation alleviating metal stress of plants [4]. Interaction of GSH with known signaling molecules such as salicilic acid, jasmonic acid and ethylene can be important in treatment of plant biotic stress [5].

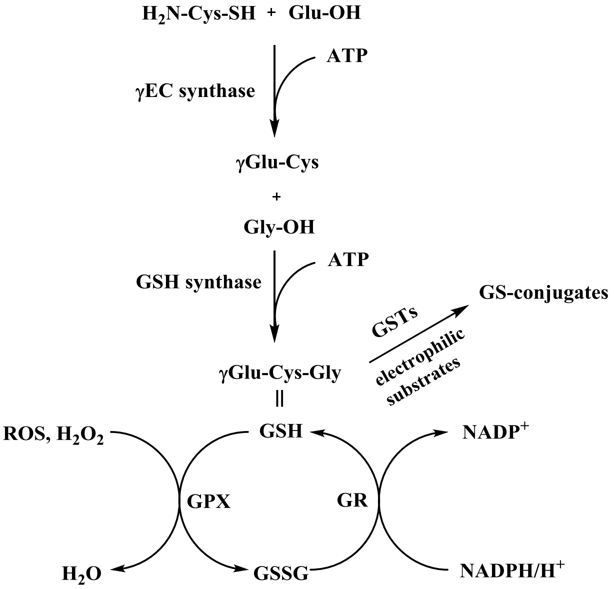


Figure 1.
Biosynthesis of GSH and GSH/GSSG redox system with related enzymes such as glutathione peroxidase (GPX) and glutathione reductase (GR) as well as glutathione S-transferase.

GSH biosynthesis is two-step pathway mediated by γ -glutamylcysteine synthase (γ-ECS) and glutathione synthase (GSHS) enzymes using 2 molecules of ATP (**Figure 1**). The first step occurs mainly in the chloroplast while the second step predominantly takes place in the cytosol [3, 6]. GSH produced in the cytosol can be transported directly to other cellular organelles by glutathione transporters [7].

The GSH redox state in plants, particularly in leaves is normally very stable but is extremely sensitive to oxidative stress. In the absence of stress, in leaf tissues measurable GSH: GSSG ratios typically around 20:1 [8]. Conversion of the reduced GSH into oxidized glutathione or glutathione disulfide (GSSG) can occur under stress conditions. Non-enzymatic reactions of GSH with ROS species such as $^1O_2$, $O^-{}_2$, OH, H_2O_2 and $O_2{}^{2-}$ convert the reduced GSH to the oxidized form (GSSG, glutathione disulfide) [9, 10]. Glutathione reductase (GR) and glutathione peroxidase (GPX) enzymes in conjuction with AsA-GSH cycle are responsible for the balanced state of GSH/GSSG and GSH homeostasis [11].

Plethora of information on alteration of plant GSH pool and GSH/GSSG redox system as a results of heavy metal stress are available. Elevated levels of GSH have been observed in various plant species with increasing Cd concentration. But depletion of GSH in roots of variety of species under cadmium and lead stress has also been reported [12]. In two genotypes of *Brassica juncea* treated with Cd increased levels of GSH and GSSG were detected. Higher increase in GSH content was found in Cd-tolerant genotype while in Cd-sensitive genotype the enhancement of level of the oxidized form, GSSH was more pronounced [13]. Remarkable decreases in $O_2{}^-$, H_2O_2, and MDA (malondialdehyde) accumulation were detected with exogenously applied GSH in rice seedlings treated with Cd as a result of modulation of the antioxidant system [14]. The oxidative stress induced by Cd in coontail, a free-floating freshwater macrophyte (*Ceratophyllum demersum)* was alleviated by exogenous Zn application. The application of Zn restored thiols and also inhibited oxidation of AsA and GSH, and sustained the redox state balance. Application of zinc enhanced the activities of AsA-GSH enzymes (APX, MDHAR, DHAR, and GR), GST, and GPX, conferring tolerance to Cd stress [15].

Toxic action of heavy metals seemed to induce the expression of genes encoding γ -glutamylcysteine (γ-ECS) and glutathione synthase (GSHS) enzymes by enhancing GSH levels. Moreover, GSH can efficiently influence coordination of metals to the active sites of affected enzymes preserving their activity [16].

2.2 Glutathione reductase

Glutathione reductase (GR) is a flavo-protein oxidoreductase mediates the reduction of GSSG to GSH using NADPH as an electron donor. GR is predominantly located in chloroplasts but some isoforms were also detected in mitochondria and cytosol [17]. GR activity secure the redox potential of cells at highly reduced GSH/GSSG and AsA/DHA ratios under regular and oxidative stress conditions. Biotic and abiotic stress factors including toxic metals were found to influence GR activities in plants [17, 18]. Under Cd stress GR activities were increased in sugarcane callus cultures in time- and concentration-dependent manner [19]. Elevated GR activities were detected in response to CD-treatment in a variety of plant species such as *Solanum tuberosum*, *Raphanus sativus*, *Glycine max*, *Saccharum officinarum*, *Capsicum annuum, Arabidopsis thaliana, B. juncea, Brassica campestris, Vigna mung,* and *Pisum sativum* [12]. On the contrary significant reduction of GR activity was shown in *Ceratophylum demersum* treated with Cd [20]. A seedling age specific changes in GR activities were observed in *Oryza sativa* genotypes under Pb stress. After initial deacreases in GR activities in both roots and shoots up to 10 days a remarkable increase of enzyme activities was found after 15 days of treatment [21].

2.3 Glutathione peroxidases

Glutathione peroxidases (GPXs) are a large family of broad substrate spectrum multiple isozymes. Contrary to most of their counterparts in animal cells, plant GPXs contain cysteine instead of selenocysteine in their active site. Antioxidant GPX protecting cells from oxidative damage by reducing H_2O_2, organic and lipid hydroperoxides in association with the GSH pool [22]. Presence of GPXs were detected in cytosol, chloroplasts, mitochondria, peroxisome and apoplast.

Stress responses of GPXs are contradictory. Cd stress increased GPX activity in cultivars of *C. annuum* plants [23] but reduced activities were found in roots of Cd-exposed *P. sativum* plants [24] while no change was observed in GPX activities in maize roots exposed to Ni [25]. The activity of GPX activity was significantly enhanced in *Lolium perenne* shoots exposed to Se but chronic metal exposure decreased enzyme activity [26]. Externally supplied GSH to Cd treated barley genotypes was shown to counteract Cd-induced elevation of root GPX activity by reducing GPX activity to the control level [14].

2.4 Detoxification action of glutathione S-transferases

Glutathione S-transferases (glutathione sulfo-transferases, GSTs) are major phase II, GSH-dependent detoxication enzyme superfamily. GSTs catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds to form water soluble, non-toxic GSH conjugates [27, 28]. GSTs are divided into two distinct super-family members: the membrane-bound microsomal and cytosolic family members. Microsomal GSTs are structurally distinct from the cytosolic in that they homo- and heterotrimerize rather than dimerize to form a single active site [29]. In cytoplasm GSTs account for roughly 1% of soluble proteins in maize leaves [30]. Various plant GST izozymes were shown to possess with GSTs/GPX activity mediating lipid hydroperoxide metabolism to non-toxic alcohols [31].

Elevated GST activities were found in leaves and roots of Cd exposed pea plants [24] and in roots of *Phragmites australis* plants by Iannelli et al. [32]. Cadmium- and mercury-induced root growth inhibition is strongly correlated with increased GST and GPX activity in barley [33] while in maize seedlings, Cd treatment strongly induced (20-50-fold increase) GST Bronze2 and GST III [34]. A detailed summary of literature studies on GST induction by heavy metals such as Cd, Pb, Cu, As(III) and As(V) has been published [12].

3. Phytochelatins (PCs) as heavy metal chelators

One of the detoxication mechanisms in plants to overcome heavy metal stress is the production of thiol-containing oligomer peptides from a precursor glutathione (GSH) by phytochelatin synthase (PCS, γ-glutamylcysteine dipeptidyltranspeptidase) (**Figure 2**).

PC synthase-deficient mutants of *Arabidopsis and S. pombe* exhibited high sensitivity towards Cd and arsenite providing strong evidence for the role of PCs in heavy metal detoxification [35]. Phytochelatin level of plant can serve as biomarkers for the initial detection of heavy metal stress. Since the immobilized metals are less toxic than the free ions, PCs are considered as part of the detoxication mechanism of higher plants, [36, 37]. Phytochelatins, with $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ general formula (where n = 2-11), can act as chelators to bind heavy metals in the cytosol and the metal-phytochelatin complexes are compartmentalized to vacuoles. Varieties in structures of PCs include the replacement of glycine residue (Gly) with β-alanine (β-Ala), Ala, glutamine (Gln), serine (Ser) or glutamate (Glu) [38].

As a result of Cys residues PCs have high thiol (SH) contents and ability to strongly bind heavy metals exhibiting increased metal-binding capacity with increasing size [39]. PCs chain lengths varies with plant species and metal forms.

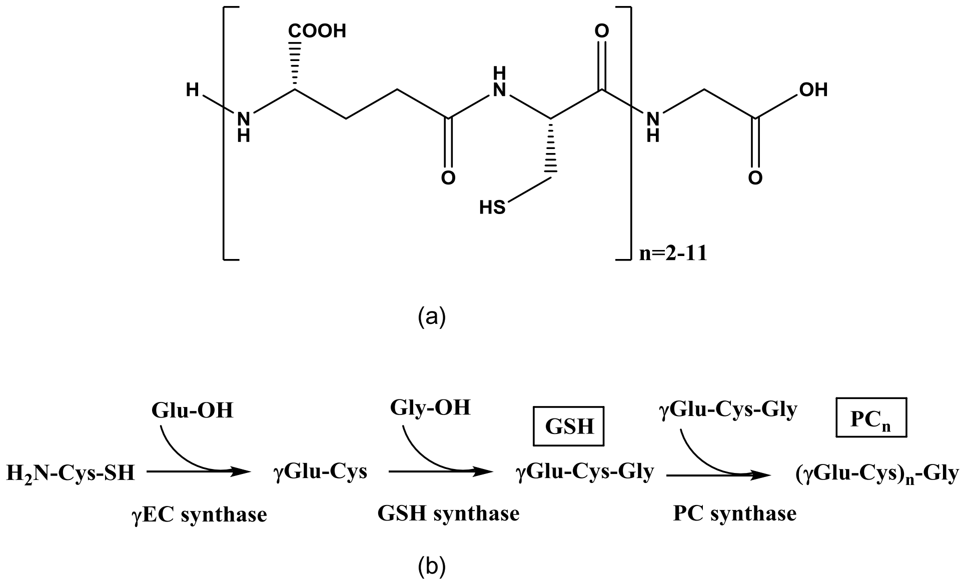


Figure 2.
General structure of (a) and biosynthesis (b) phytochelatins.

The relative affinity of metals such as Cd, Pb, Hg, Cu to GSH and PC_n oligomers increases with chain length (GSH < PC_2 < PC_3 < PC_4) [40]. After the formation, the high molecular weight metal-phytochelatin complexes are sequestered in the vacuoles by the involvement of ABC-type (ATP-binding casette) transporters [41]. PC synthase was shown to be activated by heavy metal ions such as Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}, Pb^{2+}, Ni^{2+}. Cd tolerance of *Arabidopsis* and tobacco was found to be mainly related to PC content. Contents of PCs and GSH in *Arabidopsis* were 3.5 and 3 times higher than in tobacco plants and the concentration of various PCs oligomers in the two species was different: PC_3 and PC_4 oligomers were prevalent in wild-type tobacco as compared to high concentration of PC_2 and PC_3 in *Arabidopsis* [42].

Cd^{2+} ions were found as the most effective stimulator of PCs biosynthesis and 4-6-fold higher induction of PCs were detected than with Cu^{2+} and Zn^{2+} compounds in cell cultures of Indian snakeroot (*Rauvolfia serpentina*) [43] and red spruce (*Picea rubens* Sarg) [44], respectively.

Biosynthesis of PCs takes place in the cytosol of root cells. PCs are produced from glutathione, homoglutathione, hydroxymethyl-gluthathione or glutamylcysteinyl-glutamate by a transpeptidase, the constitutive PC synthase enzyme [45]. In *Solanum nigrum* L. copper treatment enhanced the biosynthesis of PCs was shown followed by the immobilization of toxic Cu in the roots by inhibiting the translocation into the shoots [46]. Reduced transport of As from roots to shoots was found in rice cultivars subjected to the elevated levels of arsenic due to the stimulation of formation of phytochelatin-arsenic complexes [47]. Arsenite and arsenate anions are readily absorbed by plants and both anions were found to induce effectively the biosynthesis of PCs *in vivo* and *in vitro* [48]. A higher degree of production, accumulation, and transportation of PCs has a definite role in tolerance of plants to heavy metal stress. However, biosynthesis of PCs does not neccessarily take place in roots. In hyperaccumulator *Sedum alfredii* plants PC synthesis and Cd accumulation were most abundant in the leaves followed by the stems but hardly detected in the roots [49]. In a Cd hyperaccumulator wheat plant, Cd accumulation was not only the sequestration of Cd-phytochelatin complexes in the roots but their translocation to shoots also takes place. The translocation of Cd from root to shoot is through the xylem appears to be the main process for shoot accumulation. At a relatively high Cd treatment (20 μM) phytochelatin biosynthesis was enhanced more evidently in shoots [50]. The phytochelatin independent mechanism of tolerance of higher plants to Cd toxicity also exists and can be attributed to the highly developed apoplastic transport systems [51].

PCs enzyme activities were not only also induced Cd stress. The principal mechanism of intracellular metal detoxification by complexing and transporting metals into the vacuole was also established for stress by various heavy metals such as mercury, copper, arsenic, silver, nickel, gold, and zinc [52–54].

Stochiometry of complexes of Pb^{2+} formed with various PC_n (n = 2–4) were examined in details. Mass spectrometry analysis of Pb– PC_2 revealed four different complexes corresponding to $[Pb–PC_2]^+$, $[Pb_2–PC_2]^+$, $[Pb–(PC_2)_2]^+$, and $[Pb_2– (PC_2)_2]^+$. The coordination of Pb^{2+} with PC_2 was postulated via the thiol groups of cysteine residue of PC_2 and possibly by carboxylic groups. In case of PC_3 and PC_4, two complexes were detected for each metal such as $Pb–PC_3$, $Pb_2–PC_3$, $Pb–PC_4$, and $Pb_2–PC_4$ [55]. The metal-PC_n complexes formed with Cd^{2+} differed from those of Pb^{2+}. Higher metal/peptide molar ratios were estimated in Cd-PC_n complexes than in Pb-PC_n complexes (n = 3, 4), suggesting that phytochelatins of marine algae *Phaeodactylum tricornutum* are capable to sequester and detoxify more Cd^{2+} than Pb^{2+} ions forming complexes with a different structure and stoichiometry. As a confirmation 80% of Cd^{2+} was detected in a complex form, and only 40% of the absorbed Pb^{2+} was bound to phytochelatins in the cellular extract [56].

4. Metallothioneins (MTs), the metal-chelating proteins

Involvement of MTs has been reported in a number of physiological processes such as regulation of cell growth and proliferation, toxic metal protection and homeostasis, free radical scavenging or protection from oxidative stress, DNA damage repair [57]. For a long MTs was considered to be expressed only in mammals while in plants enzymatically formed PCs are the protective biomolecules against heavy metal toxicity. MTs is a cytosolic superfamily of cysteine-rich proteins capable to bind both physiological and xenobiotic heavy metals [58, 59]. While phytochelatins are formed enzymatically MTs are the products of mRNA translation [60, 61]. In the structure of MTs cysteine (C) residues representing about 30% of the constitutional amino acids. Primary structures of this low molecular weight protein family (M_w ranging from 5 to10 kDa) are characteristically rich in highly conserved CC, CXC (where X is a general amino acid) and CXXC motifs that render a unique ability to bind mono- or divalent metal ions, such as Cu^{2+}, $Zn^{2+}$and Cd^{2+} [62]. While in animal MTs no aromatic acids occur histidine (His) residues can be found in a number of plant MT sequences. The replacement a part of the Cys residues by His would be an increased selectivity for Zn^{2+} over Cd^{2+}, and thus the function of the respective MT is more selective in maintaining Zn^{2+} homeostasis than heavy metal detoxification [63]. In addition, the thiol(ate)s in MTs can act as powerful antioxidants, and therefore MTs have definite roles in protection against oxidative stress [64].

Based on the arrangement of Cys residues four types of plant MTs exist [59]. Type 1 MTs are mainly expressed in roots, while the expression of type 2 MTs mostly occurs in shoots, type 3 MTs are induced in leaves and during fruit ripening, and type 4 MTs are abundant in the developing seeds [65]. Regarding high level of sequence diversity of plant metallothioneins, type 1-4 MTs is further subdivided to various isoforms. All four types of plant MTs and their isoforms are able to chelate heavy metals. In general, the primary structures of plant MTs in type 1, 2 and 3 have a similar cysteine topology. The two Cys-rich domains (α and β) are attached by a 30-40 amino acid long cysteine-poor linker depending on plant species. Cysteine topology of type 4 MTs different from that observed in MTs of type 1-3. In angiosperm species three Cys-rich regions linked by two Cys-poor linkers containing 15 and 40 amino acids [58, 60]. Experiments with *Arabidopsis thaliana* MTs expressed in copper and zinc sensitive yeast mutants provided evidences that MT1a, 2a, 2b and MT3 function as copper binding MTs. The seed-specific type-4 MTs were more effective than other *Arabidopsis* MTs in providing protection against Zn toxicity and enhancing Zn accumulation [66].

Studies on copper tolerance and expression MT1 and MT2 genes in several *A. thaliana* species revealed that MT1 was uniformly expressed in all ecotypes and MT2 was copper inducible. In cross-induction experiments, Ag^{+}, Cd^{2+}, Zn^{2+}, Ni^{2+} significantly enhanced the levels of MT2 genes [67]. *A. thaliana* plants knocked down for MT1a and b isoform expression exhibited increased cadmium sensitivity. These lines accumulated less As, Cd and Zn in the leaves than wild-types indicating that MT1 have a definitive role in Cd tolerance and possibly involved in Zn homeostasis [68]. Lack of MTs increased Cd and Cu sensitivity in PC-deficient *Arabidopsis* plants suggesting that PCs and MTs contribute to Cu and Cd tolerance and may overlap in their functions [66]. Experiments with *A. thaliana* MTs expressed in copper and zinc sensitive yeast mutants provided evidences that MT1a, 2a, 2b and MT3 function as copper binding MTs. The seed-specific type 4 MTs were more effective than other *Arabidopsis* MTs in confering protection against Zn toxicity and enhancing Zn accumulation [66].

MTs typically bind metal ions in characteristic metal– thiolate clusters that provide high thermodynamic stability coupled with kinetic lability [69]. The large diversity in the metal binding regions of plant MTs confers the ability to bind a greater range of metals than in animals possessing a greater range of function [59]. A model of cadmium binding to mammalian MTs showed that all cysteine residues participate in the coordination of 7 mol of Cd per mol of MT. Two polynuclear metal clusters formed during binding with 3 and 4 metal atoms on β- and α-domain, respectively.

In one metal cluster requires 9 cysteine SH group to bind 3 Cd in a six-membered ring while the four-metal-cluster forms a bicyclo[3.1.3] structure with the participation of 11 Cys-SH groups (**Figure 3**) [70].

Experimentally and predicted stochiometries of metal-plant MTs are in agreement. Type 1 plant MTs with 12 Cys residues can bind 4-5 metal ions, type 2 with 14 Cys can coordinate 5 metal ions while type 3 MTs with only 10 Cys residues exhibit the lowest capacity for metal binding (4 metal ions) [58].

The key characteristics of metal-MT complexes are the high thermodynamic but low kinetic stability. Thermodynamically, MTs are the most stable zinc sites in eukaryotes but have appropriate kinetic lability for the protein to intermolecularly exchange zinc with proteins [72]. Metal binding affinities of MTs characterized by complex stability constants are hardly available in the literature. However, pH of half-displacement values (pH (1/2)) are available for several plant MTs indicating that the more stable protein-metal complex have lower pH(1/2) value [63]. pH(1/2) values for all MTs follow the order Cu(I) < Cd(II) < Zn(II). Accordingly, MTs will bind Cu(I) *in vitro* more strongly than Cd(II) or Zn(II), and Cd(II) will be bound more powerfully than Zn(II). Numerically The pH values at which MT1 of pea loses half of the initially bound metals are 5.6 for Zn(II), 4.0 for Cd(II), and 1.5 for Cu(I) [73].

Further studies on large and complex MT gene families in higher plants may exhibit beneficial metal binding and induction properties to enhance the phytoremediation capacity of plants used for heavy metal removal in soils. To understand the function and the mechanism of action of plant MTs requires further manipulations on the expression of this protein family.

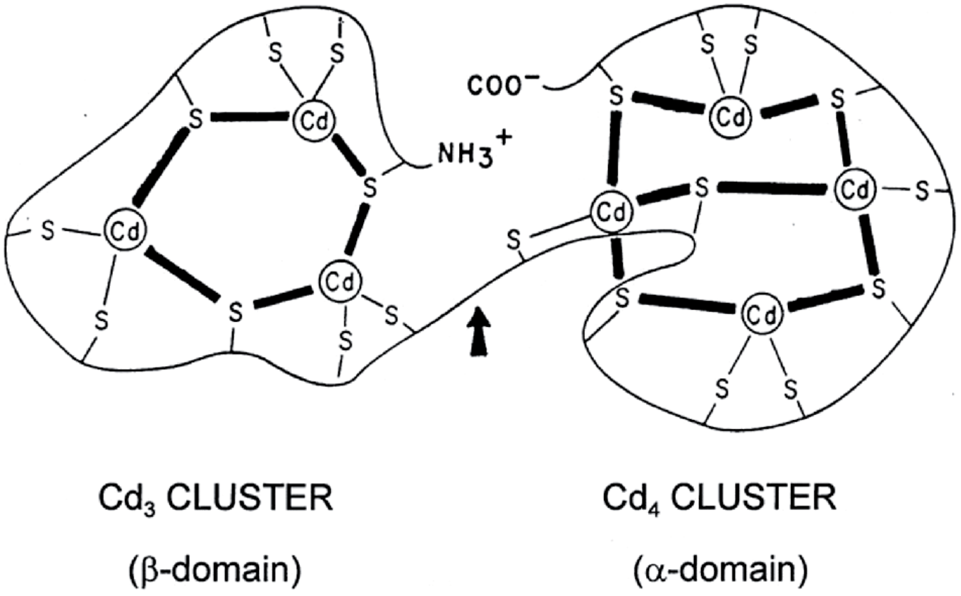


Figure 3.
Proposed structures of the four-metal and three-metal clusters of rat liver metallothioneins based on 113Cd NMR data [71]. Adapted from Klaassen et al. [70].

5. Protective action of proline (pro) against heavy metals toxicity

Proline (Pro) is an essential proteinogenic amino acid that fulfill several developmental functions in plants and has a fundamental role in responses to biotic and abiotic stress. In plants proline can protect cells from environmental stress factors by several protective mechanisms such as acting as osmoprotectant, functioning as chemical chaperone stabilizing proteins and antioxidant enzymes, chelating metals, scavenging reactive oxygen species (ROS), balancing the intracellular redox homeostasis ($NADP^+$/NADPH ratio, GSH pool) and participating in cellular metabolic signaling [74–76].

Protective mechanism of proline as ROS scavanger include direct reaction with ROS. Free and polypeptide-bound proline was demonstrated to react with hydrogen peroxide (H_2O_2) and hydroxy radicals (OH.) producing stable free radical adducts and hydroxyproline (**Figure 4**). However, the most important ROS-scavenging mechanism in the stress protection is the reaction with singlet oxygen ($^1O_2$). A direct reaction between H_2O_2 and proline plays a minor role in scavenging of cellular H_2O_2 and the formation of nitroxyl radical accumulate during is very sluggish as compared to that of proline and OH.. Nevertheless, proline effectively quenches $^1O_2$ via a charge transfer mechanism to form the ground triplet oxygen ($^3O_2$) as a ground state molecular oxygen. Due to its action as a $^1O_2$ quencher, proline may help stabilize proteins, DNA, and membranes [77].

Toxic action of lead, copper, and zinc on proline, malondialdehyde (MDA), and superoxide dismutase (SOD) has been studied in the cyanobacterium *Spirulina platensis*-S5. Parallel to growth reduction elevated MDA, SOD, and proline contents were found with increasing concentrations of metals. Elevated levels of MDA indicated the formation of free radicals in generated heavy metals stress while enhanced amounts of SOD and proline demonstrated the undergoing scavenging mechanism [78].

The molecular mechanism of proline protection of cells during stress may involve the effects on redox systems such as the glutathione (GSH) pool. Pro reduces heavy metal stress by detoxification of free radicals produced as a result of Cd poisoning. Pro may physically quench oxygen singlets or react directly with

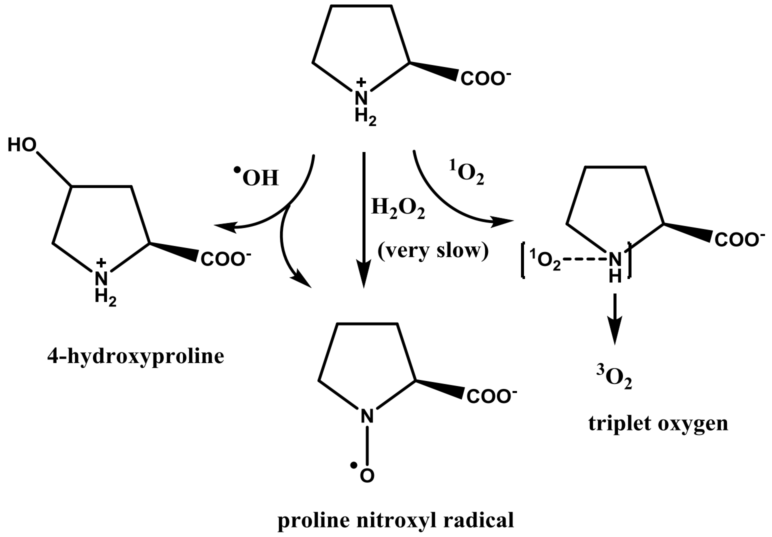
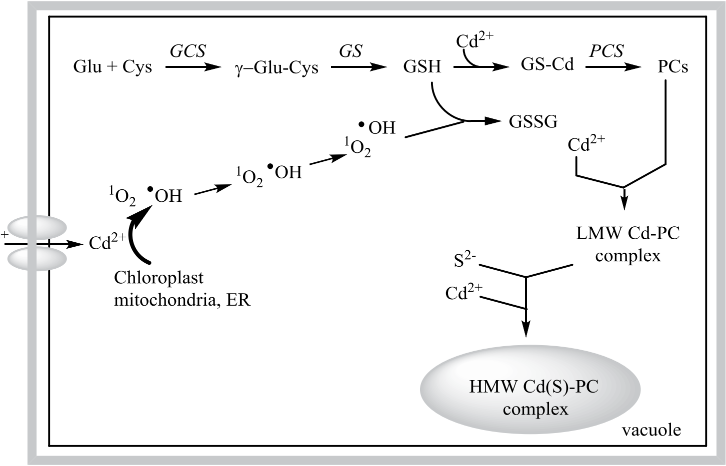


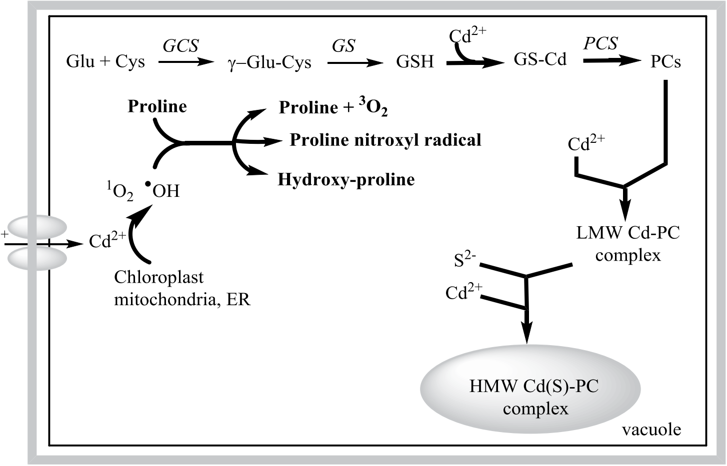
Figure 4.
Routes for scavenging reactive oxygen species (ROS) by proline. Adapted from Liang et al., 2013 [74].

hydroxyl radicals. These reactions result in reduced free radical damage (lower malondialdehyde levels) and a more reducing cellular environment (higher GSH levels). The high GSH levels in turn facilitate phytochelatin synthesis and sequestration of heavy metal phytochelatin conjugates in the vacuole. This enhanced sequestration of Cd-phytochelatin complexes in the vacuole accounts for the transiently increased Cd content of P5CS (Δ^1-pyrroline-5-carboxylate synthetase)-expressing cells in transgenic algae (**Figure 5**) [76].

The chelation of metals is also considered as possible mechanism responsible for the protective effect of proline in cells against stress. Reversal on Cd- and Zn-induced inhibition of glucose-6-phosphate dehydrogenase and nitrate reductase enzymes by proline supported the function of proline as a metal chelator by forming proline-metal complexes [79]. Nevertheless, the stability of metal-proline complexes was found to be relatively low [80]. to effectively influence the inhibitory



A) Wild type *Chlamydomonas reinhardtii*



B) P5CS transgenic *Chlamydomonas reinhardtii*

Figure 5.
*Participation of proline in reducing cadmium stress by detoxification of free radicals generated by Cd toxicity. In wild type algae (**Figure 5A**), cd^{2+} induces the production of reactive oxygen species that rapidly oxidize GSH to GSSG. In transgenic algae (**Figure 5B**) the resulting decreased GSH are reduced with free pro leading to increased GSH levels and therefore enhanced phytochelatin synthesis to coordinate and sequester Cd. Adapted from Siripornadulsil et al., 2002 [76].*

concentration of the metal ions in the assay mixtures. A copper–proline complex was found in the roots of copper-tolerant sea thrift (*Armeria maritima*) [75].

Accumulation of free proline plants in response to abiotic stresses has been demonstrated by Delauney and Verma [81]. In *Arabidopsis*, accumulation of Pro occurs after NaCl stress and can reach 20% of the total free amino acid pool [82]. The metal-tolerant plant species such as *Armeria maritima*, *Deschampsia cespitosa*, and *Silene vulgaris* were reported to contain substantially higher levels of constitutive proline than non-tolerant plants [46]. In hyperaccumulator artichoke (*Cynara scolymus* L.), Pro elevation in cells correlated with increasing lead concentration [83]. Enhancement of Pro concentration in root of rape seed (*Brassica napus* L.) was more significant than in shoot tissues as a result of increasing concentrations of Pb^{2+} [84]. Root specific accumulation of the proline was detected in Indian mustard (*Brassica juncea* L.) under lead and cadmium stress [85]. Similarly elevated prolin levels were detected in roots of a variety of plants such as black nightshade (*Solanum nigrum* L.) exposed to copper [4], in wheat stressed by cadmium [86], and mercury and cadmium stressed lemongrass (*Cymbopogon flexuosus* Stapf) [87]. In hybrid poplar (*Populus trichocarpa* × *deltoides*) Pro accumulation in roots was almost double than in leaf tissues due to highly toxic Cd^{2+} exposure [88]. In the roots of pepper plants (*Capsicum annuum L.)* elevated proline level was detected with increasing Cr concentration, while Pro decreased in leaves [89]. On the contrary, shoot specific accumulation of Pro was detected in sunflower (*Helianthus annuus* L.) seedlings as a result of copper stress [90]. In metal non-tolerant bladder campion (*S. vulgaris*) Cu was most effective inducer of the proline accumulation followed by Cd and Zn, respectively [46]. Treatment of cauliflower seedlings (*Brassica oleracea* var. botrytis) with Cd, Hg, and Zn resulted in the concentration dependent accumulation of Pro up to double. Among these heavy metals Hg was the most effective inducer of Pro biosynthesis [91]. Treatment of sal seedlings (*Shorea robusta*) with heavy metals increased the proline accumulation in plants in the order of $Cd^{2+} > Pb^{2+} > As^{3+}$ [92]. In wheat seedlings stimulation of Pro levels was more pronounced by copper than zinc [93]. Additionally, induction of Pro contents in shoots of black gram (*Vigna mungo* L.) seedlings treated with heavy metals changed in the order of highest to lowest as Hg > Pb > Co > Zn and followed the toxicities of heavy metals. The results suggest that heavy metal stress induced proline accumulation is strongly dependent on the concentration and type of heavy metal and plant species [94].

It seems that two mechanisms are responsible by which proline provides protection against heavy metal stressors. One of these, up-regulation of proline biosynthesis to accumulate proline to serve as an osmolyte, a chemical chaperone, and a direct scavenger of hydroxyl radical and singlet oxygen. Secondly, linkage of proline metabolic flux to metabolic pathways to maintain the intracellular redox homeostasis ($NADP^+$/NADPH ratio, GSH pool).

6. Oxylipin signaling under heavy metal stress

Oxidized fatty acids, oxylipins, are an important class of signaling molecule in plants in responses to abiotic stresses [95, 96].

Oxylipins regulate growth, development, and responses to environmental stimuli of organisms [97]. Lipoxygenases, allene oxide synthase and a series of cytochromes P450 related oxygenasesare involved in oxylipin biosynthesis. Enzymatically synthesized oxylipin, the jasmonic acid (JA) and a precursor molecule 12-oxo-phytodienoic acid (OPDA) were shown to accumulate in response to pathogen infection [98]. Jasmonate-dependent tolerance to heavy metals is mediated by defensins, small cysteine-rich proteins present in plant cells [99, 100]. Oxylipins are involved in stress signal transduction, regulate stress-induced gene expression, and interact with other signaling pathways in plant cells, including signaling pathways of the plant hormones auxin, gibberellin, ethylene, and abscisic acid (ABA) [101, 102]. However, the role of oxylipins in plant adaptation and defense mechanism to abiotic stress is less studied. Protective action of oxylipins at toxic levels of heavy metals is considered to activate detoxification processes. Non-enzymatic reaction of reactive oxygen species with lipidic substances results in hydroxy fatty acids which are biologically active oxylipins and play important role in protective action of heavy metal stress [103]. Spontaneously formed oxylipins are called phytoprostanes. Pretreatment of tobacco plants with phytoprostanes results in reduction of cell death in response to copper sulfate stress [104]. In roots of tobacco seedlings Al-induced accumulation of 2-alkenals formed from fatty acid hydroperoxides was detected [105]. ROS generated during aluminum stress formed toxic aldehydes in reaction with fatty acids and caused severe root growth inhibition. Removal of 2-alkenals from tissue through overexpression of 2-alkenal reductase reduced Al phytotoxicity. Stimulation the expression of a number of stress responsive by phytoprostanes [106] makes oxylipins promising tools for improving stress tolerance of plants to heavy metals.

7. Conclusion

Heavy metal stress on plants in contaminated soils due to increased anthropogenetic activities led to an intensive research on detoxification and sequestering mechanisms of plants to understand and manipulate defense mechanisms developed in plants to tolerate stress by metal ions. The accumulation of heavy metals in plant organs results in the damage of the structural components and disruption of cell metabolic processes leading to plant growth retardation. Increased biosynthesis of various plant biomolecules to confer tolerance during toxic action of heavy metals is an intrinsic ability of plants. Induced formation of low-molecular weight amino acids, peptides or proteines as chelators such as proline (Pro), glutathione (GSH), phytochelatins (PCs) or metallothioneins (MTs) under heavy metal stress enhances metal binding and detoxification capability of plants. In addition, proline and GSH related enzymes such as GSH reductase, GSH peroxidases and glutathione S-transferases are also key components of the antioxidant defense system in the cells to scavenge reactive oxygen species (ROS). Protective action of oxidized fatty acids oxylipins at toxic levels of heavy metals is considered to activate detoxification processes as signaling molecules.

Exploring and manipulating genes induced under heavy metal stress make possible to develop transgenic plants with enhanced detoxication properties for phytoremediation technologies in polluted soils. The use of hyperaccumulator plants [107] which can accumulate heavy metals in the leaves at 100-fold higher concentration than normal plant species can be the alternative solution to clean-up polluted soils.

Author details

Istvan Jablonkai
MTA TTK Lendulet Functional Organic Materials Research Group, Institute of Organic Chemistry, Research Centre for Natural Sciences, Budapest, Hungary

*Address all correspondence to: jablonkai.istvan@ttk.hu

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Chapter 6

Nutrients Deficit and Water Stress in Plants: New Concept Solutions Using Olive Solid Waste

Samir Medhioub, Slah Bouraoui, Ali Ellouze
and Hassen Sabeur

Abstract

Great efforts were deployed by researchers to mobilize water resources while is becoming rarer and to control with efficiency the water besides nutrient needs for the plant. Autonomous water and nutritional anti-stress device for plants (AWANASD) based on the recovery of rainwater patented by Medhioub et al. fits into this general framework. Scientific efforts were also dedicated to preserve the environment and minimize energy consumption through using agricultural waste materials in different fields. This chapter provides a new concept based on the use of the olive solid waste in AWANASD as water storage and nutrient elements for plants giving rise to the new system called AWANASD-OSW.

Keywords: stress, water, nutrient, olive solid waste, device

1. Introduction

Climate change and the excessive needs of human activities impact the water resource [1] and consequently its availability notably for the big water use of agriculture sector activity [2]. It accounts for 69% of global withdrawals [3], 2021. The irregularity of rainfall distribution and the faster and increasing water demand by 50% by 2030 [4] threat considerably some regions in the world. So, it is important to realize that in arid environments, challenges of preserving and saving water are crucial for achieving the Millennium Development Goals [5]. As the agriculture sector is the largest consumer of water, several researches and achievements aim to save water and ensure the minimum nutrient requirements for optimal growth of crops.

Reference to [3], **Table 1** resumes some irrigation systems based on saving water use. In fact, these irrigation methods have their advantages and disadvantages but all of them require water sources, storage tanks, installation, etc. and incorporating nutrients under different forms.

Agricultural sector activity is on the other side a source of renewable and valuable waste. Many research works were carried out to enhance this green waste in the different fields enjoying their specific performances mainly thermal, lightness, and its organic material characteristic. In France, regarding the fight against climate change and the strengthening of resilience in the face of these effects, deputies adopted an amendment (No. 7012) relating to the use of bio-based materials in construction. It indicates that from January 1, 2028, bio-based materials must be

System	Description	Reference(s)
Clay pot irrigation	A clay pot is buried and filled up with water to irrigate the plants placed around it. Water seeped through its wall, will be absorbed by the roots of the plants.	[6]
Drip irrigation	Water and nutrients are delivered to the field in pipes called "drip system lines" containing smaller units called "drip systems." Each drip system emits drops containing water and fertilizer, which allows a uniform application of water and nutrients directly to the root zone of each plant, over an entire field.	[7]
Continuous irrigation	It uses a porous tube qualified as a semipermeable membrane (SPM). It delivers slowly and continuously water directly into the plant root zone.	[8]
Hydrophilic polymers or hydrogels	Hydrophilic polymers or hydrogels were small granules that function like sponges: They retain water up to 500 times their weight with rain or watering then it will be released later slowly and in very small quantities when it is incorporated into the soil.	[9–15]

Table 1.
Overview of some irrigation methods.

used in at least 25% of renovations and constructions ordered by the public institute [16]. All green waste can be used as well in agriculture. It can be turned into humus and nutrients, which are essential for soil life and plant development.

Our contribution in this chapter will be divided into three sections—the first one will review the new concept of irrigation method based on the recovery of rainwater given by [3], called autonomous water and nutritional anti-stress device (AWANASD) for plants; the second section is reserved to introduce the process of obtaining the olive solid waste (OSW), its fields of application and its physical and chemical characteristics. The last section gives a new vision to improve AWANASD by the use of OSW as the main component to respond at the same time to the minimum of water and nutrients required to the plant.

2. Autonomous water and nutritional anti-stress device (AWANASD)

The new concept of AWANASD, given by [3] (Video 1: https://vimeo.com/user163271525/review/665174900/0954b75438), is a genius new concept inspired by the ancient clay pot method of irrigation. The bottom line of AWANASD is collecting rainwater then storing them temporarily with soluble nutrients enrichment and delayed water transfer to the plant. It's a regular cyclical of water storage and transfer in order to overcoming the water stress of the plant in drought periods taking advantage of the rainy season (**Figure 1**).

AWANASD is made up of three compartments—the first one is a rainwater receiver exposed to open air and designed to filter and convey the collected water to the second compartment; the latter is buried in the soil near the maximum root density of the plant and in which the temporary storage water is enriched by nutrients. This water will forward to the last compartment which is the key piece of AWANASD. It has a defined permeability to ensure a deferred daily volume rainwater outflow and consequently, it will fill the lack of water needed to plant survival in the dry season. The calculated permeability is related to multiple parameters mainly the climate of the target crops region.

AWANASD will be able to spare the underground water tables from intensive exploitation and eventually from the poor-quality water [17]. It also reduces water consumption [18] and water loss by evaporation and deep percolation [19] and consequently improper management of water resources [20].

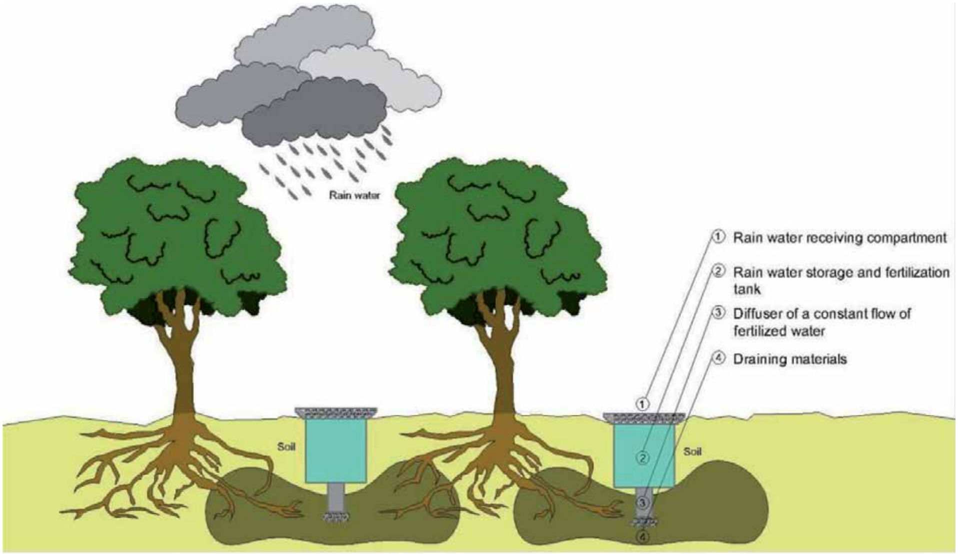

Figure 1.
Simplified AWANASD function.

The analytical model of AWANASD is based on the next water balance equation:

$$\textit{inflows volume} = \textit{change in storage volume} + \textit{outflows volume} \tag{1}$$

More numerical details were shown in Ref. [3].

3. Olive solid waste: origin, valorization, and characterization

3.1 Introduction

According to the International Olive Council, the olive sector takes great importance in the economics of a large number of countries (**Figure 2**) and had tripled its production in the last 30 years [22]. The annual production of table olive for the period 2018–2019 was closed at 3 million tons [22]. This would indicate that the sector is expanding. As a consequence of the activity of this sector, large volumes of waste and by-products are generated. Among these agriculture wastes, those resulting from classic pressure processes, batch processes (super press), and continuous processes (centrifugation).

3.2 Oil extraction process

3.2.1 Classic or traditional process

In classic (traditional) extraction units, the oil extraction process consists of the following different steps (**Figure 3**):

- Grinding: It is carried out by granite stone grindstones, which rotate in a tank whose floor is also made of stone. This grinding is carried out manually or through an animal. This step, therefore, makes it possible to obtain a paste that contains solid matter and fluids (oil and water from vegetation).

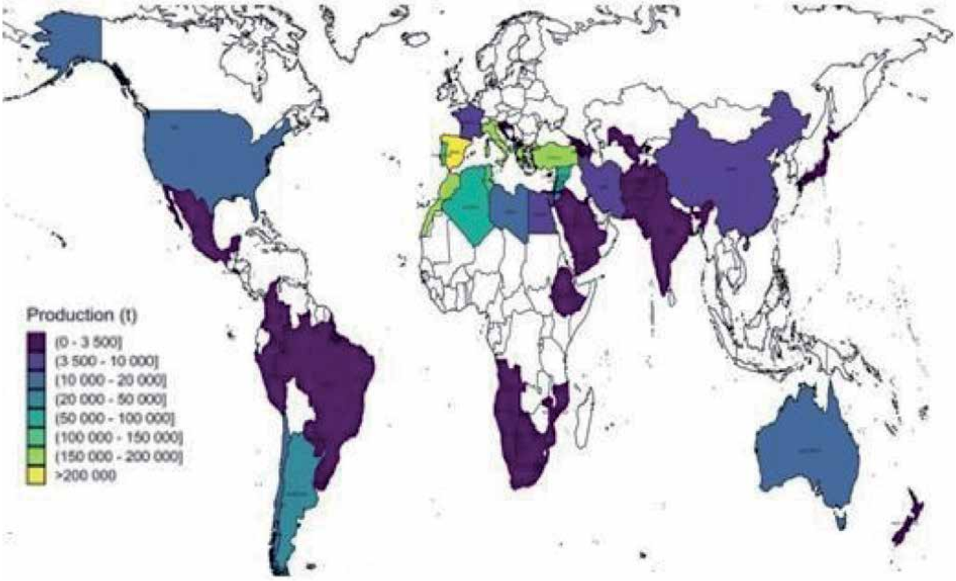

Figure 2.
World olive oil production, 2018/19 crop year [21].

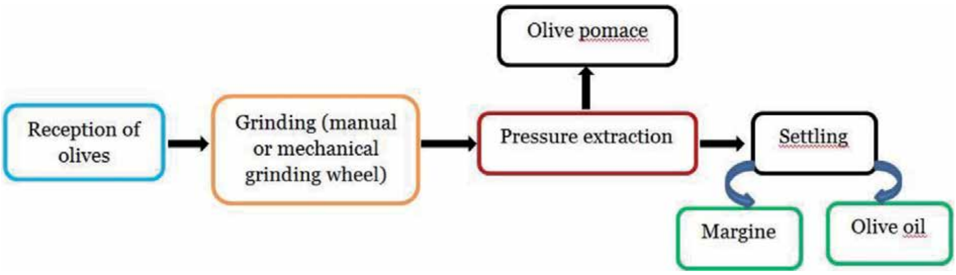

Figure 3.
Classic press and super press extraction systems.

- Phase separation: The pulp produced is placed on scourtins (fiber discs plants). Then, oil extraction is carried out by pressure. The pressing generates a solid by-product called olive pomace. These olives pomaces are the residues solids recovered following the first pressing or centrifugation. They are made up of residues of the skin, pulp, almond, and fragments of olive pits.

- A separation by settling of the liquid phases (oil and vegetation water) is performed. This separation takes place in the open air in cement, earthenware, or clay containers. A liquid by-product was generated at the end of this step, called vegetable waters. It is the brown aqueous liquid residue that separated from the oil by sedimentation after pressing or centrifugation. This liquid has a pleasant smell but a bitter taste. This effluent relatively rich in organic matter constitutes a pollution factor that creates a real problem for the olive industry.

3.2.2 Batch process or super press system

The olives received in the traditional oil mills go directly through the following steps:

- Grinding: It is carried out by grinding wheels. The grinding wheels used for grinding are slightly off-center with respect to the axis of rotation, which increases the possibility of crushing olives.

- Mixing: This step releases as much oil as possible. Raclettes bring back permanently the dough under the grindstones which then play the role of kneading machines. The dough is obtained after about half an hour.

- Phase separation: The dough is then placed in a layer approximately 2 cm thick on nylon fiber discs (the mats), themselves stacked on top of each other around a central pivot (called a needle) mounted on a small carriage. The set is placed on a hydraulic press piston which allows the dough to be subjected to a pressure of the order of 100 bars. The liquid phase flows into a tank. The pomace stays on the scourtins. This operation takes approximately 45 minutes. Then, each scourtin is cleared of its pomace by tapping it as when cleaning a carpet.

- Decantation: The oil, having a lower density than that of water, goes back to the area. This is the natural settling. However, this method is almost no longer used, due to its slowness and the difficulty in separating the oil from the water vicinity of the interface between the two fluids. These are vertical plate centrifuges that today make it possible to separate olive oil from vegetable waters (**Figure 3**).

3.2.3 Continuous process

There are two types of the continuous extraction process—three-phase centrifuge system and two-phase centrifuge system.

3.2.3.1 Three-phase centrifugal extraction system

The olives, once received, undergo preliminary treatments, such as stripping, stone removal, and washing to have good oil quality.

- Grinding: This is carried out by mechanical disc or hammer grinders. These grinders can work continuously; the dough is obtained almost instantly.

- Mixing: The dough is poured into a stainless-steel tank moderately thinned with water lukewarm, in which a spiral or worm turns, also in stainless steel.

- Phase separation: This consists of separating the solid part (pomace) from the fluid (vegetable waters). The kneaded paste is injected by a pump into a centrifuge whose axis is horizontal (horizontal settling tank).

- Decantation: Vertical centrifuges with plates are used which make it possible to separate olive oil from vegetable waters [23]. This extraction process is illustrated in **Figure 4**.

3.2.3.2 Two-phase centrifugal extraction system

The olives undergo the same stages of stripping, stone removal, washing and grinding, mixing, and settling as those of the previous three-phase system.

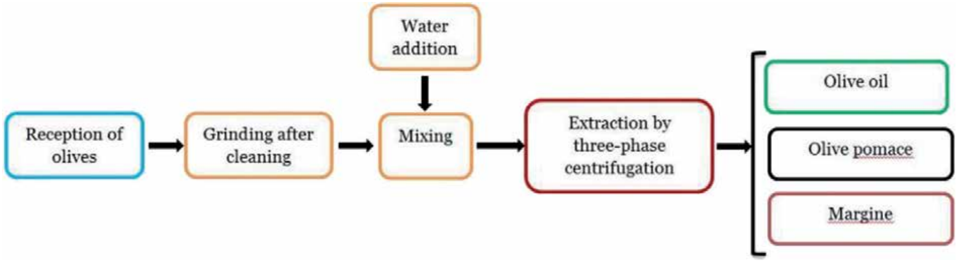

Figure 4.
Three-phase centrifugal extraction system.

However, this olive oil extraction process works with a new decanter with two-phase centrifugation (oil and moist olive pomace) which does not require the addition of water for the separation of oil and solid phases containing pomace and the vegetable waters. This two-phase decanter allows for slightly higher oil yields than those obtained by the conventional three-phase decanter and the press system. In addition, it does not increase the volume of vegetable waters.

Figure 5 shows the different stages of olive oil extraction by a two-phase centrifugal extraction system.

3.3 Valorization of olive pomace

As a renewable by-product source further its high added-value, the olive solid waste was valued in different areas. **Table 2** summarizes the most important uses. Each of these uses will be detailed succinctly later in the text.

3.3.1 Combustion of stone and whole stone

The olive stone is a biomass fuel that has low N and S percentages [24] with a minimum environmental impact. The important power heating combustion is converted to electrical sector and for heating buildings [25]. Rodrıguez et al. [34] and Arvanitoyannis et al. [46] detailed more in their study the thermal treated olive stone used.

3.3.2 Activated carbon from olive stone

Activated carbon was used in many fields (mining, pharmaceutical industries, food, etc.) [27, 28]. Activated carbon from olive stone is mainly used for the removal of contaminants, such as arsenic [47] or aluminum [48], odors, unwanted colors, and tastes [49].

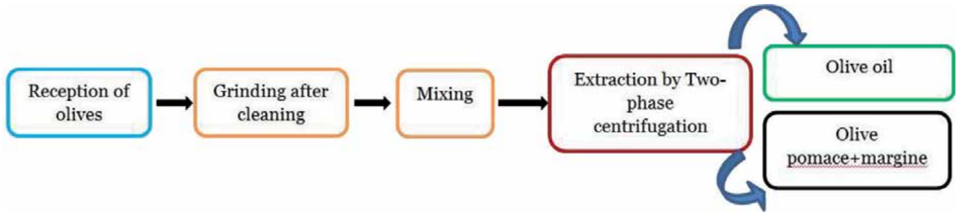

Figure 5.
Two-phase centrifugal extraction system.

Application	Raw material	Pretreatment	Application sector	References
Combustion	Stone and seed	Dried	All industries residential and commercial	[24–26]
Activated carbon	Stone and seed	Pyrolysis activation	Food, chemical, petroleum, nuclear, mining, pharmacological industry	[27–32]
Bio-oil	Stone and seed	Pyrolysis	Wide field of industries	[33]
Furfural	Stone and seed	Acid hydrolysis	Wide field of industries as solvent	[34]
Plastic filled	Stone	Grinding	Plastic and construction	[35–38]
Abrasive	Stone	Grinding	Cleaning	[39]
Cosmetic	Stone	Grinding	Cosmetic	[40–42]
Animal feed	Stone and seed	Grinding	Food	[43]
Resins	Stone and seed	Pyrolysis or liquefaction	Electrochemical	[44, 45]

Table 2.
Overview of some OSW uses.

3.3.3 Liquid and gas products from olive stone pyrolysis

Olive stone pyrolysis gives interesting bio-oil and gas products [33].

3.3.4 Furfural production

There are many processes to produce furfural such as acid hydrolysis of xylose and some of which present the olive stone. Several industrial uses of furfural are performed, such as solvent or as a base for synthesizing its derived solvent [34].

3.3.5 Olive stone as a plastic filled

The olive stone as a natural and biodegradable raw material [35, 36] was already studied to prepare a friendly environment product then a certain plastic structure by mixing it with a certain polypropylene to produce a new thermoplastic polymer [37].

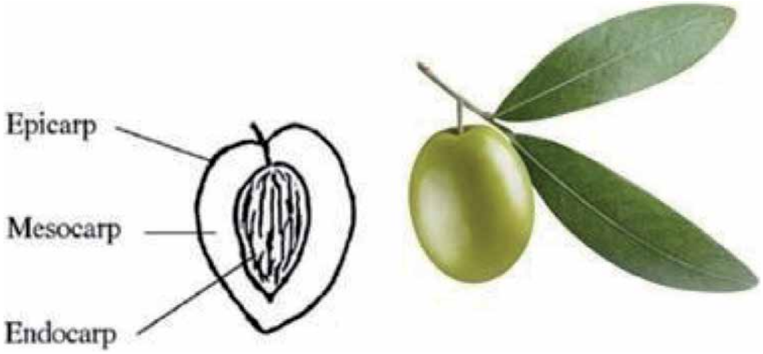

Figure 6.
Olive composition.

3.3.6 Olive stone as an abrasive

The interested proprieties of olive stone in terms of resistance to rupture and deformation confers an abrasive quality that let it wide use in the industrial sector [39].

3.3.7 Olive stone in cosmetic

Olive stone is incorporated as a component in many products to aid in skin exfoliation [40, 41].

3.4 Characterization

3.4.1 Composition of the olive

The olive composition depends on its variety (**Figure 6**), soil, and climate [23]. The contents olive is composed of epicarp (2–2.5% of weight) which is in fact the skin of the olive. It is covered with a waxy material, the cuticle, which is waterproof, then, the mesocarp (71.5–80.5% of weight) [50] which is the pulp of the fruit. It is made up of cells in which the drops of fat that will form olive oil will be stored, during the "lipogenesis" phase and finally, the endocarp or the stone (17.3–23% of weight).

3.4.2 Physical proprieties of olive solid waste

The olive solid waste (OSW) used in the tests reported in this chapter (**Figure 7(a)** and **Table 3**) was obtained from a three-phase centrifugal extraction process from "Botria oil" Tunisian company mills. After a centrifugal separation of the husk residue, the extracted olive solid waste (OSW) underwent a natural drying process in an open shelter.

The tested OSW showed 605 and 1490 kg/m^3, respectively to bulk and relative density and 24-hour water absorption capacity of 11.5%. **Figure 7(b)** shows its particle-size distribution.

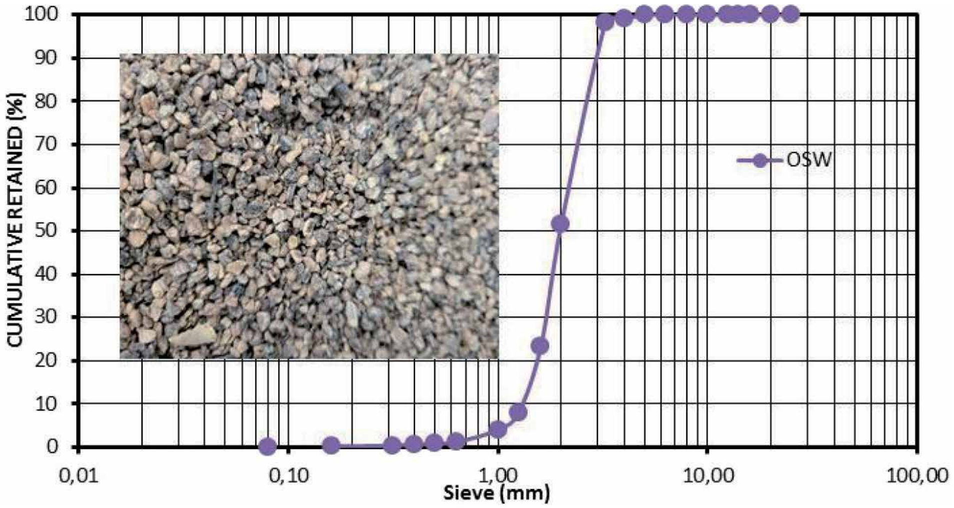

Figure 7.
Olive stone. (a) Sample used for testing. (b) Grain size distribution.

t (mn)	0	15	30	60	90	120	180	240	360	1440	2880
M_0 (g)	178	178	178	177	171	182	178	179	177	175	177
M_f (g)	178	257	272	293.1	295	318	319	324	325	330	336
V_0 (ml)	279	279	279	279	279	279	279	279	279	279	279
V_f (ml)	279	338	345	351	360	369	373.5	376	379	383	390
W (%)	0	44	53	66	73	75	79	81	84	89	90
f (%)	0	21	24	26	29	32	34	35	36	37	40

Table 3.
Water and profusion of OSW as a function of the immersion time.

3.4.3 Olive solid waste behavior in water

The organic nature and the porous structure of solid waste cores have been the subject of a specific study of their behavior in the presence of water and as a function of the immersion time. The following procedure has been adopted while not losing sight of RILEM recommendations [51]:

- Weigh 200 g of a raw OSW
- Dry the OSW sample in a 105°C oven and for 24 hours (until a constant mass of less than 0.1% is reached)
- Weigh the dried sample (M0)
- Place it in a graduated test tube and note the corresponding volume (V0)
- Fill the test tube with water and put the dried OSW sample in it until a given time t.
- Net volume (V_f) and weight (M_f) of wetted OSW corresponding to time t
- Repeat the above operations for each time t equals to 15, 30, 60, 90, 120, 180, 240, 360, 1440, and 2880 mn)

Table 3 shows all measured values.

The water content W is given by Eq. (2).

$$w = \frac{M_f - M_0}{M_0}(\%) \tag{2}$$

The volume occupied by a given weight of dry OSW material increases at the same time as its humidity. This phenomenon is, therefore, called profusion. This is characterized by expansion coefficient f (expressed in %; Eq. (3)) as the increase in the volume corresponding to a given humidity compared to the volume occupied by the same quantity of bio-sourced but in the dry state [52]:

$$f = \frac{V_f - V_0}{V_0}(\%) \tag{3}$$

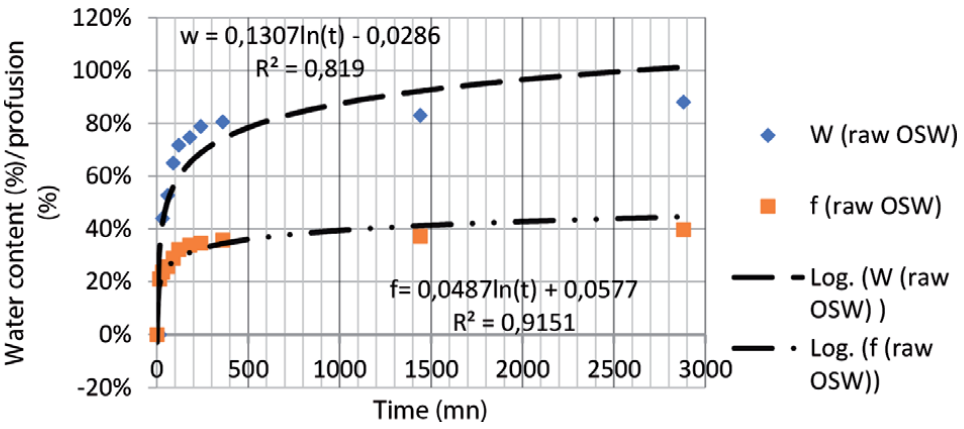

Figure 8.
Water content and profusion function of time of OSW.

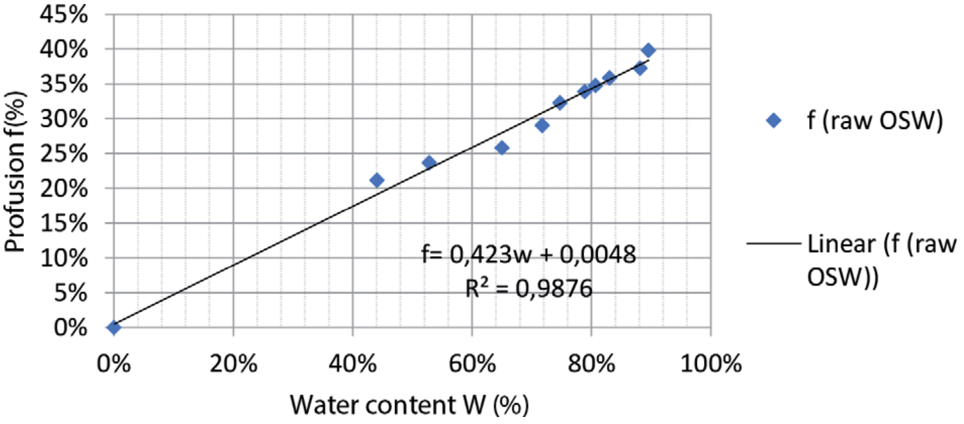

Figure 9.
Profusion function of the water content of OSW.

	Unit	Raw OSW	A	B	Mean	OSW after dissolving in water	A	B	Mean
N[*]	%		0.915	0.854	0.884		0.581	0.49	0.536
Mineral matter	%		4.72	5.2	4.96		1.06	1	1.03
Calcium[*]	mg/kg		0.276	0.277	0.276		0.159	0.132	0.146
Phosphor[*]			0.021	0.014	0.017		0.009	0.009	0.009
Potassium[*]			0.019	0.017	0.018		0.012	0.01	0.011
Sodium[**]			0.044	0.035	0.039		0.055	0.059	0.057
Copper[**]			7.488	7.186	7.337		4.893	4.695	4.794
Zinc[**]			7.289	4.284	5.786		5.06	6.792	5.926
Manganese[**]			10.28	9.482	9.883		3.395	3.496	3.446
Iron[**]			335.9	364.7	350.3		67.009	66.426	66.718

[*] *Macronutrients.*
[**] *Micronutrients.*

Table 4.
Elementary chemical elements in the raw and dissolving OSW.

Figure 8 shows by using regression equation the approximation curves and their equations of the water content and profusion function of time. We note that, according to correlations coefficients R^2, the two equations reflect well the tendency of W and f with time. We note as well that water saturation and the maximum profusion of OSW begin after 4 hours. In addition, we deduce the relation between w and f shown in **Figure 9**.

3.4.4 Chemical proprieties of olive solid waste

ICP technique, short for "Inductively Coupled Plasma" was used for measuring the content of an inorganic element in a sample. This technique is applicable to all types of elementary chemical elements.

The results of ICP sample analysis of OSW for two samples (A and B) were given in **Table 4**.

4. Olive solid waste used in AWANASD: a new concept for nutrients deficit and water stress

Adding to its organic material, the physical and chemical proprieties of OSW let confer it a potential and interesting material not only for its ability to stock water on it around the double of its weight but it is a useful nutrient element for plants even not with a big quantity but it can be required to thwart certain nutrition deficit. Medhioub et al. [3] gave a design of AWANASD for the governorate of Sfax (Tunisia). This design consists of filling the third compartments of AWANASD with grains sand of 3 mm in diameter to reach permeability equal to 10–7 m s^1 to give a water flow of 0.4 L day^1 at a depth of about 1 m. Nevertheless, the authors did not specify which and how the nutrient should be done. So, our proposed device concept named "AWANASD-OSW" is a new version of AWANASD which can be

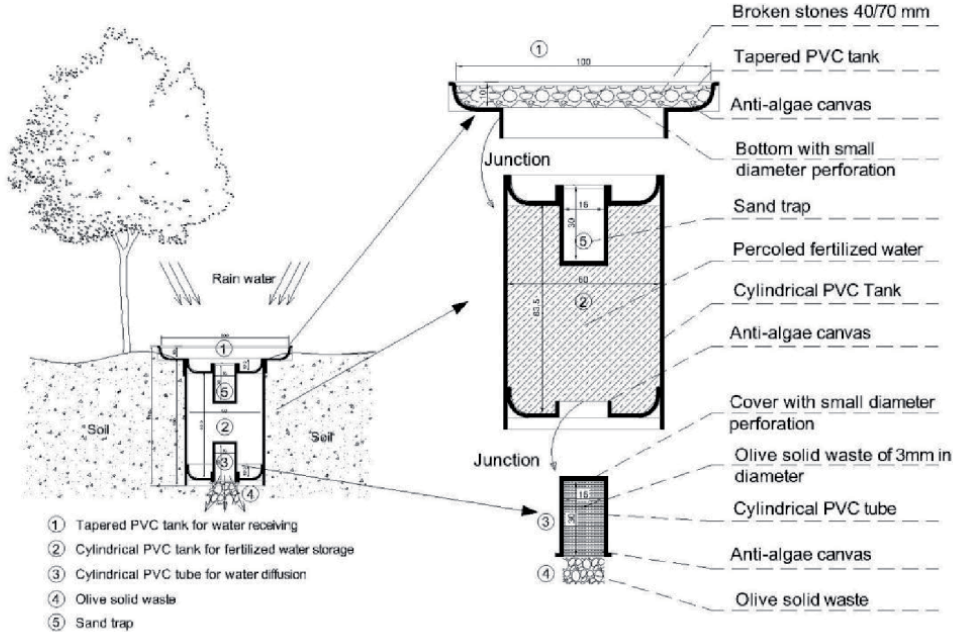

Figure 10.
AWANASD-OSW design.

applied to the same location. AWANASD-OSW includes the same number of compartments of AWANASD (**Figure 10**) and ensures the goal of delayed water transfer to the roots of plants. However, the third compartment which is a cylinder (32 cm height; 16 cm of diameter) will be filled by a specific volume of OSW (V_{OSW}) having a similar sand particular diameter. This is given by equation Eq. (4):

$$V_{OSW} = {}^{V_s}\!/_{f} * 10^{-3}\left(m^3\right) \tag{4}$$

where Vs is the sand volume equal to $6410^{-3}(m^3)$; f is the profusion of OSW taken for the maximum of water content (40%).

5. Conclusion

As it is a living organ, a plant's need is nutrition and a water supply. Different technical methods have been developed and applied to meet this need. The reliability of these methods varies in degree of performance. The recent one called AWANASD is given by Medhioub et al. [3], ensuring the minimum water flow and nutrition during drought months at the level of the maximum concentration of roots.

AWANASD applied for Sfax governate concluded the use of grain sand with a specific diameter to ensure the objective of delayed water transfer but it did not mention the nutrition issue. Our AWANASD-OSW new concept fully incorporates the said system but replaces the grain sand with olive solid waste with the same granulometry.

This renewable agriculture waste material has interesting physical and chemical properties besides its characteristic as a biodegradable organic material. It allows the release in the presence of water of nutrients for plants in addition to its role of water store.

A full-scale experimental device must be set up not only to ensure the expected theoretical performances but also to assess its longevity.

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Author details

Samir Medhioub[1]*, Slah Bouraoui[1], Ali Ellouze[2] and Hassen Sabeur[1]

1 LGC-ENIT, Tunis, Tunisia

2 ENIS, Sfax, Tunisia

*Address all correspondence to: samir.medhioub@gmail.com

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Chapter 7

Salt Stress and Plant Molecular Responses

Usman Khalid Chaudhry, Zahide Neslihan Öztürk Gökçe and Ali Fuat Gökçe

Abstract

Plants being sessile suffer from several abiotic stresses during growth. These include drought, salinity, heavy metal, temperature, and nutrient stress. Salt stress is one of the devastating abiotic stresses that plant suffers under natural growth conditions. It is more common in arid regions due to excessive evaporation, which causes the accumulation of inorganic salts disrupting the plant metabolism. It also triggers the influence of drought stress, as plants are unable to absorb water. Additionally, it also causes oxidative stress in plant tissues. Thereby, plant adaptation to salt stress, rely on signals and pathways that help plant in establishing cellular ionic and osmotic homeostasis. Stress-responsive transcription factors play crucial roles in the regulation of gene expression in responses to salt stress. Moreover, genome editing has gained much attention for the engineering of traits for the better adaptation of plants to salt stress. This chapter elucidated the plant's physio-biochemical responses and molecular mechanisms to salt stress.

Keywords: genome editing, oxidative stress, sensing, salt stress, transcription factors

1. Introduction

Abiotic stress is an external environmental factor that influences plant growth stages. It includes a fluctuation in temperature ranging from chilling to heat stress, deposition of heavy metals in soil due to anthropogenic activities also results in heavy metal stress/toxicity. Limited availability of water for a shorter or longer period causes drought stress [1]. Most importantly and thoroughly discussed is the salt stress that arises due to deposition of higher salt contents in the soil layers due to poor application of irrigation water, higher evaporation that also deposits salt in the upper surface layer of the soil [2]. Currently, climate change is also stimulating several abiotic stresses on a yearly basis and the salinity problem is one of them. Globally, the number of salt-affected soils is increasing and approximately 20% of the agricultural land has been affected [3].

Salt stress also causes secondary stresses such as osmotic, water, ionic, and oxidative stresses. Osmotic stress is formed as soil salinity decreases the soil and leaf water potential, that disrupts the water relation of plants and decreases the turgor pressure of plants [4]. Plant roots serve as a medium of nutrient uptake, but the excessive salt solution in the vicinity of the roots forces its uptake that causes ionic toxicity in plants. Additionally, plant roots are unable to extract/absorb water that also further aggravates plants growth by acclimatizing its water stress.

Plants start their journey from seed germination to seedling establishment, plant growth, and fruiting, however higher salt contents in the soil cause poor germination and weakened morphological growth leading to declined yield [5].

Salt stress also disrupts the physiological functioning of the plants, such as the closure of stomata, reduction in gaseous exchange, damage to photosynthetic machinery, decrease in chlorophyll contents, and distortion of PSII system [6, 7]. Salt stress also disrupts the biochemical functioning of the plants with the excessive production of reactive oxygen species (ROS) resulting in oxidative stress [8]. The ROS is favorable at a lower concentration that is crucial for cellular signaling processes for the induction of positive response in antioxidant defense likewise biological processes to help improve stress adaptation mechanisms [9]. However, higher ROS causes oxidative stress, and plants have the inherent ability to alleviate ROS species with the upregulation of genes that synthesize antioxidant enzymes [10]. Plants can tolerate salt stresses by maintaining antioxidant enzyme activity or increase their levels to cope with oxidative stress caused due to environmental adversities in various plant species [11]. Abiotic stress causes gene expression changes in plants and a comparison of these changes may provide information on adaptation and stress tolerance/sensitivity [12].

Transcription factors are involved in several biological processes in plants to regulate stress tolerance. Transgenic approaches have extensively used transcription factors that function in salt stress signaling pathways for the genetic engineering crops to confer stress tolerance. Most used transcription factors include ABA-binding factor (ABF), basic leucine zipper (bZIP), dehydration responsive element binding (DREB), myeloblastosis (MYB), NAC, and WRKY due to their significance in the signaling against salt stress [13]. Their specificity depends upon homodimerization, heterodimerization, and modifications at post transcription, post translation by small RNA (miRNA). Additionally, modification in histone modification and DNA methylation are involved to perform the main role for regulating gene expression in response to salt stress. The transcription factors bind to cis-acting elements in promoter regions to regulate gene expression. Therefore, they exhibited potential for controlling the expression of target genes, as some transcription factors are early stress-responsive genes [13, 14].

Currently with the introduction of novel genome editing techniques also assisted in developing stress-resilient genotypes. Generally, nowadays clustered regularly interspaced short palindromic repeats (CRISPR) is the most used genome editing technique due to simplicity and ease of availability. It is believed that unraveling of molecular mechanisms and genome editing approaches are the best options to pave the path to understand and develop stress tolerant genotypes to cope with future climate and ensure food security [15].

2. Salt stress and plant growth

Roots provide anchorage, access to soil water, and essential nutrients required throughout the growth stages. Its morphological characteristics play an important role in the growth and production of plants [16]. During stress, the plant roots are the first organ that senses adverse conditions such as excessive salt in the soil. The study of the root system is of great interest as the structure and distribution of roots assist in higher water uptake. It also maintains the productivity of plants under salt stress conditions [17]. Salt stress interrupts the normal growth of the plants as excessive salt accumulation in the vicinity of roots and its uptake cause a toxic environment for plants. The typical symptoms of morphological growth of plant include retardation of vegetative growth, increase in plant development, senescence sometimes resulting in death [8]. The inhibited growth is the first noticeable symptom of salt stress that might results by alteration of cell division due to salinity shock. Other obvious symptoms of sodium accumulation in plants are necrosis of older leaves, it starts from the tip leading towards the leaf base. Therefore, it also causes early senescence of leaves resulting in poor plant biomass and yield. We also observed that higher salt contents resulted in a decreased number of leaves, plant height, and reduction in bulb yield of onion [6, 18]. Plant growth reduction with the acclimatization to salt stress is due to disruption of normal physio-biochemical in plants with the development of secondary stresses that are discussed below.

3. Salt stress and physiological processes

Salt stress disrupts the physiological functioning of the plant, as the first response of the plant is to limit gaseous exchange with the closure of stomata. It also helps in reducing the water loss of the plant. Higher salt accumulation along with stomatal closure limits CO_2 intake that disturbs the photosynthesis of the plants. Likewise, photosynthesis of the plant decreases that ultimately affects vegetative growth and harvest yield [19]. It also aggravates ionic toxicity and osmotic stress to the plants. It is reported that abscisic acid (ABA) results in stomatal closure. The ABA is the main chemical compound that is produced in the roots in response to stress. It sends signals for the closure of stomata. It is directly linked to the rate of photosynthesis, therefore, damage to photosynthetic machinery and pigments [20]. Additionally, the plant also accumulates sucrose that changes electron transport. Increased salt contents in photosynthetic tissues also cause shrinkage of the thylakoid membrane. There is another perspective that decreased stomatal conductance is beneficial for the plant as it helps in limiting the entry of toxic ions via roots. It was corroborated by a couple of studies that decreased stomatal conductance resulted in a reduction of the uptake of ions to the transpiration stream [21]. Plants uptake higher Na^+ contents from the soil under salt stress that decreases the uptake of other essential nutrients required by plants for their functioning. It causes an ionic imbalance in a plant that replaces K^+ loading by Na^+ to the chloroplast resulting in damaged photosystem II. It is the protein complex that functions in the catalyzation of light-induced water oxidation to the evolution of oxygen. Salt stress decreases the activity of PSII that decreases the quantum yield of PSII. It has been reported that NaCl inhibited the photochemical efficiency of PSII. It is sensitive to Na+ toxicity and directly reduces the photosynthesis process of the plant [7]. Osmotic stress is regarded as the deficiency of water in plant tissues due to excessive accumulation of salts. It is caused due to the inability of plant roots to absorb water. Higher Na^+ also decreases the turgor potential of the plant [22]. Resultantly the supply of water to the leaf decreases that causes lower leaf development. Decreased number of leaves or senescence directly reduces the photosynthetic rate and damage to chlorophyll contents. Salt-sensitive genotypes that are unable to cope with stress suffer from dehydration caused by a disturbance in turgor pressure resulting in the death of plant tissues [23].

4. Salt induced oxidative stress

Salt stress directly causes ionic imbalances and with the severity of the stress to ionic toxicity. Moreover, it also triggers oxidative stress with the higher production ROS. The toxic influence of higher ROS accumulation causes increased lipid peroxidation, membrane damage, in addition DNA and protein damage [24]. The ROS are mostly produced in chloroplast and mitochondria that include singlet oxygen ($^1O_2$), superoxide radical ($O^{2\bullet-}$), hydroxyl radical ($^\bullet OH$), and hydrogen peroxide (H_2O_2). It includes superoxide dismutase that works as a first in the antioxidant system by catalyzing the dismutation of superoxide radical ($O^{2\bullet-}$) to H_2O_2 and O_2 in the cytosol. Ascorbate peroxidase is the main player in detoxifying hydrogen peroxide in plant chloroplast, mitochondria, cytosol, and peroxisomes during stress conditions. It utilizes ascorbic acid as an electron donor for the reduction of H_2O_2 to H_2O. Catalase also alleviates oxidative damages by converting H_2O_2 to H_2O [25].

Secondary metabolites such as tocopherol play role in the stabilization of the membrane integrity. It also works as a signaling molecule in response to stress between cells [26]. Ascorbic acid is an important antioxidant that is found abundantly in the apoplast and cellular organelles. It scavenges $^1O_2$, $O^{2\bullet-}$, and $^\bullet OH$ for plant adaptation against salinity. Carotenoids are localized in the chloroplast that is reported to help plants in light reception to continue the process of photosynthesis, furthermore, they are also known as a protective compound for scavenging ROS species [27]. Putrescine supplementation is beneficial to restrain the production of ROS, as it speeds up the antioxygenic enzymes that ultimately help in protecting the chloroplast membrane [28]. In one of the study, putrescines are reported to enhance the level of carotenoids and glutathione in response to salt stress [29].

Several studies have reported the higher accumulation of ROS is a response to stress leading to cellular damages [7]. Additionally, it was also observed that higher production ROS due to salt stress caused decreased crop production. Therefore, it is essential to control the regulation of ROS to protect plant tissues from cellular damage caused by oxidative burst. Moreover, C4 plants have been reported to show higher resistance to salinity compared with C3 plants. As they have a better capability of preserving photosynthetic mechanisms against oxidative stress [30]. Salt stress influence on plant oxidative stress is shown in **Figure 1**.

Several genes have been identified that encode antioxidant enzymes SOD, CAT, APX, and glutathione reductases (GRs), that are involved to scavenge ROS in plants. These genes are described as T genes as they contribute to stress tolerance [9]. Contrarily S genes have been reported that result in excessive ROS production. They are responsible for higher oxidative stress, increased programmed cell death,

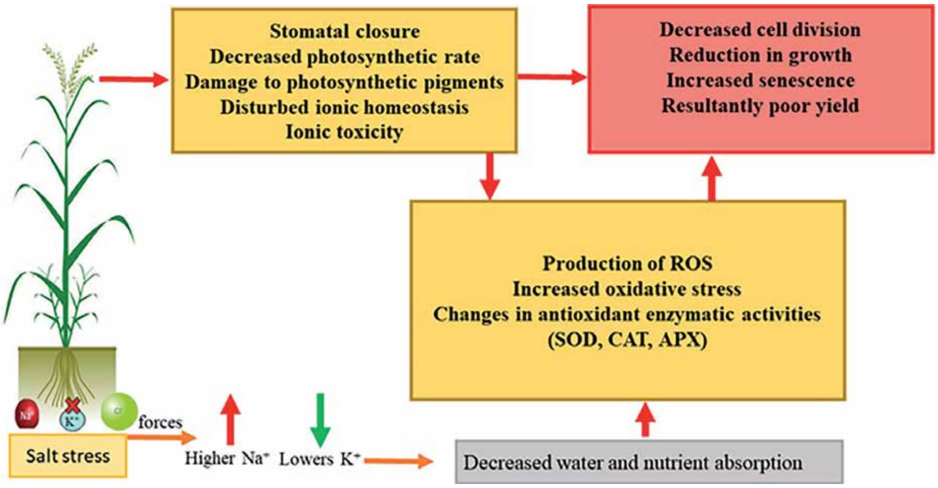

Figure 1.
Plant responses to salinity stress. Salinity favors the higher uptake of Na^+ and lower uptake of K^+ that causes ionic homeostasis leading oxidative burst and reduced growth and yield.

Crops	Gene	Function	Reference
Tobacco	CuZnSOD, APX	Reduced oxidative stress	[31]
Wheat	P5CS	Higher proline accumulation	[32]
Onion	Cat	Improved catalase activity	[7]
Rice	OsGR2	Alleviated oxidative stress	[33]
Tomato	SOD	Decreased ROS production	[34]
Maize	APX, SOD, CAT, POD	Higher antioxidant enzymatic activities to cope ROS	[35]

Table 1.
Genes responsible for the alleviation of oxidative stress in crops.

decreased antioxidant activity. Other gene group that causes disturbances in hormonal concentration, or ionic distribution have also been reported. Plant molecular breeders have utilized these identified T genes to incorporate into plants to confer salt stress tolerance. The list of some antioxidant genes is given in **Table 1**.

5. Salt stress inducible genes

Plant response to salinity stress is a complicated process that includes a various numbers of genes to finally activate physiological and biochemical mechanisms. The study of these genes provided a new horizon to identify their functions to cope with stress. These genes initiate a transcriptional response in plants with a set of several genes and their transcription also varies with the severity and duration of the stress. As they are categorized as early responsive genes that protect the plant by stimulating genes for free radical scavengers, detoxification enzymes, osmoprotectants, etc. The second ones are transcription factors, protein kinases, whereas the third group includes ion transporters and aquaporins [36].

6. Salt stress signaling

Plants being sessile must suffer from salt stress and suffer from several secondary stresses caused by Na^+. They undergo several morpho-physiological and biochemical changes to adapt/cope with stress that has been thoroughly studied. However, until now, the identification of Na+ sensors and perception by plants is still ambiguous. Under salt stress conditions, cellular organelles, and compartments such as the nucleus, chloroplast, peroxisome, plasma membrane, apoplast, and cell wall play crucial roles. Regardless of all the recent progress in unraveling complex stress mechanisms, there is still needed to find out how stress signals are generated, integrated, and coordinated in/from different organelles. Furthermore, scientists have identified specific genes, genomic regions, proteins, and metabolites against salt stress. The high-throughput transcriptomics and proteomics are potent functional genomic that can elucidate cellular networks for the perception of salt stress. Higher Na^+ causes a higher accumulation of ROS in plant tissues that disturbs the K^+ and Ca^{2+} homeostasis [37, 38]. These stress constraints theoretically may be sensed either by cytosolic or membrane-bound sensors that later translated into physio-biochemical and genetic modifications for the optimization of plant growth. There is also a high probability that a couple of sensory mechanisms might operate in the same cell, and at same time translating information related to the severity of stress. Therefore, it remained a great challenge to understand the mechanisms of signaling. Higher salt concentration disturbs the ionic homeostasis causing ionic stress. It is a general perspective that it triggers the elevated Ca^{2+} levels in the cytosol within seconds after exposure to salinity. It is further aided due to the activation of slow-activating vacuolar/two-pore channel 1 that results in the release of vacuolar Ca^{2+}. Therefore, the identification of proteins involved in the quick influx of Ca^{2+} is efficient for the discovery of salinity sensors. *Arabidopsis thaliana* a model plant has 20 different glutamate receptor-like proteins that are suggested for their function as cation channels [39].

Additionally, non-selective cation channels include a diverse group of ionic channels having the capability to discriminate several essential nutrient and toxic ions to plant under salinity conditions [40]. Increased cytosolic Ca^{2+} is largely accumulated in the cortex and endodermis of the root. Its higher influx also stimulates Ca^{2+}/calmodulin (CaM)-dependent kinases, that trigger plasma-membrane H^+ ATPase [41]. Higher ROS burst is also due to elevated levels of cytosolic Ca^{2+}. Therefore, both higher ROS and Ca^{2+} regulate the release of abscisic acid resulting in the initiation of transcriptional responses [42].

7. Salt stress and signaling pathways

Stress stimulus perception needs cell-surface harboring sensors or receptors to understand different stresses for the transduction of signal via signaling pathways. The main signaling pathways include receptor-like protein kinases (RLK), mitogen-activated protein kinase (MAPK) that are known as molecular switches for upstream signaling for the determination of the fate of plants in response to environmental stresses [43]. Thus, to understand how plants recognize high concentrations of salt and ultimately adapt themselves to salt stress is essential. The MAPK signaling cascade is the main pathway. It connects the external environment to the endogenic cellular activities of the plant. It is conserved in Arabidopsis which exhibited 20 MAPKs [44]. Their activation targets specific substrates i.e., transcription factors and other kinases. It confers salt stress tolerance by regulating salt-related genes for the homeostasis of oxidative burst and likewise osmotic stress [45]. With the exposure to salt stress, activated MAPK cascades initiated the varied transcriptional response of salt-related genes [46]. It suggested the link between salt sensors and target genes. Transcriptome analysis of Arabidopsis revealed that 152 genes changed their expression with the over-expression of MAPK. Modified genes can be clustered into different types. Some genes are regarded as stress marker genes, and others as ethylene, jasmonic acid, and auxin signaling [47, 48].

8. Salt stress and transcription factors

Transcription factor is a protein that attaches to the DNA-regulatory sequences usually found in the 5′-upstream region of target genes to initiate the rate of transcription of the gene. They help turn "on" or "off" the specific genes with the binding to the neighboring DNA. This results in increasing or decreasing the transcription of the gene, synthesis of protein, and ultimately modifies cellular functioning against salt stress. Additionally, they also perform a function to control cellular processes such as cell cycle mechanisms, intercellular regulation mechanisms, reproduction, growth [49]. Their most important salt stress responses make valuable for the characterization of their functions [50, 51]. Some transcription factors are highly conserved in plants that modify gene expression to confer stress tolerance against salt [52]. The main transcription factors that respond to abiotic stresses are bZIP, NAC, WRKY, AP2, MYB, DREB, and bHLH. They follow ABA-dependent and ABA-independent signal pathways. These pathways are highly involved in controlling gene expression [53]. They are essential for the association of signaling pathways for their involvement in gene expression, thereby it signifies the importance of these factors in the plant genome. As arabidopsis genome sequence revealed 1500 transcription factors [54]. The transcription factors play a crucial role in plant adaptation against different environments [55]. One transcription factor is responsible for controlling the expression of numerous genes. In this way, the engineering of these factors further highlighted their importance to upregulate or downregulate the gene expression for plant adaptation to salt stress. The union of these factors developed a transcription unit called regulon (a group of numerous genes that turns on/off as a unit). Very few regulons have been reported in plants in response to abiotic stresses. One of them is DREB regulon and its overexpression exhibited tolerance against major abiotic stresses such as cold, drought, and salt stress [56]. The wheat cultivars exposed to long-term salinity and bZIP gene expression was noticed significantly upregulated that conferred salinity tolerance in sensitive cultivars, contrarily its expression was lower in tolerant cultivars [57]. The other transcription factor NAC genes were overexpressed. Its higher expression alleviated the harmful effects and rendered salt stress tolerance [58]. Another study also reported that NAC5 gene showed upregulated gene expression in response to salt stress. Moreover, it might also regulate the production and aggregation of proline, sugar, LEA proteins to mitigate oxidative stress in plants [59]. Rice is a monocot model plant therefore it has been explored to unravel information about transcription factors. In rice SALT-RESPONSIVE ERF1 (SERF1), the gene showed root-specific stimulation after being acclimatized to salinity. The SERF1 gene is responsive against H_2O_2, which means that it has the potential to minimize ROS burden in plants [60].

9. Salt stress and ion transporters

Salt stress disrupts the ionic homeostasis in plant cells and causes ionic stress. The increased concentration of Na^+ and Cl^- ions disturbs the K^+/Na^+ ratio with the formation of a toxic environment for plant cells. It is mainly caused due to favoring higher uptake of Na^+ through the same pathway that uptakes K^+. It is also reported that it is difficult for transporter proteins. Another assumption is that it is hard for plant transporter protein to differentiate between Na^+ and K^+ due to similarity. Additionally, both ions are competitors of each other to occupy binding sites. Therefore, the plant uptakes higher Na^+ ions and results in Na^+ toxicity. The Na^+ influx in cytosol increases making plants difficult to thrive due to salt stress. Higher Na^+ efflux into the cytoplasm results in depolarization of the membrane potential, which also activates outward rectifier channels (K^+) i.e. guard cell outward rectifying K^+ channel (GORK) for the extrusion of K^+ [61].

Conversely, plants have adopted a mechanism to minimize the influx of Na^+ ions to avoid depolarization of membrane by retaining a negative potential to enhance retention of intracellular K^+ [62]. The antiporters Na^+/H+ helps in vacuolar Na^+ compartmentation and extrusion from the cell. It is considered as a vital process for the detoxification of Na^+ from the cytosol and helps in osmotic homeostasis to confer salt stress tolerance. In salt stress, several selective pumps activate to favor K^+ uptake instead of Na^+. They use high and low-affinity potassium transporters for the uptake of K^+. The transporters include Na^+/H^+ exchangers (NHX), salt overly sensitive 1 (SOS1) antiporter are crucial to decrease salt stress [62, 63].

High-affinity potassium transporter (HKT) protein was reported to be the first selective transporter that performs the selective role for K^+ uptake instead of Na^+ [64]. It plays an important role in the exclusion of Na^+ and maintains K^+ homeostasis. The transporter HKT2;1 has been reported for conferring tolerance against salt stress. The NHX that are localized in the tonoplast also performs a key role to restrict the entry of Na^+ by promoting the outer movement of Na^+ to the vacuole. The SOS1 functions as an exchanger in the plasma membrane. Its overexpression conferred salt tolerance [65].

10. Salt stress and epigenetics

In the field of functional genomics in plants epigenetics has gained much attention due to its potential for the adaptation of plants against stress. Salt stress significantly reduces the vegetative growth and yield of the crops. In past conventional breeding, approaches helped a lot for the selection and adaptation of plants but they are time-consuming [66]. Currently, new genetic approaches such as epigenetics that modifies plant genome without changing the sequence of DNA such as histone modification, methylation and demethylation of DNA, and gene imprinting are helping hand for the development of stress-resilient genotypes [67]. It also refers to the heritable variation in the regulation of genes leading to DNA modifications and protein. These modifications are reversible that can alter the phenotype of the plant. As methylation of cytosine is a conserved mechanism of epigenetics that is involved in several biological processes e.g., the proliferation of transposons, and regulation of gene expression in response to salt stress. Generally, it inactivates the genes whereas demethylation is responsible for the activation of the gene. It was further corroborated by genome-wide mapping that methylated genes were 8% that showed activation, whereas methylation of the transcribed region exhibited 31% expressed genes. Salt stress influence on cytosine methylation has been reported in many important crops. It is a conserved epigenetic mechanism that plays a crucial role in several biological processes in plants. It includes transposon proliferation, genomic imprinting, and gene expressional changes. Generally, it is related with the inactivation/suppression of genes expression, contrarily demethylation causes activation of genes [68, 69]. A study reported that salinity-induced methylation resulted in down-regulation of negative regulator, whereas it upregulated positive regulated [70]. The H3K9ac and H3K4me3 positively regulate gene expression and H3K9 methylation is reported as a repressive chromatin marker [71].

11. Salt stress and genome engineering

Change, any alteration, or target mutagenesis of the genome is known as genome editing. With the advent of this modern technology, it allows the introduction of specific changes with more precision at specific sites. It utilizes engineered nucleases that cleave the sequence of target DNA strand breaks, that are repaired by either homologous recombination or non-homologous end-joining. The customized modifications of DNA that include insertions, deletions, can be obtained via DNA repair mechanisms. Until now four different genome engineering techniques have been used in plants. These include meganucleases, zinc finger nucleases, transcription activator-like effector nucleases, and the most recently introduced is CRISPR/Cas RNA-guided nucleases [72]. CRISPR is a new versatile tool for genome editing derived from a bacterial system. This eminent technology showed promising results to edit genomes of different plants, resulting in improved plant biotechnology research. Currently is being employed for the development of new varieties with improved traits against salt stress shown in **Figure 2** [73].

This technology has the ability of multiplexing that can co-target multiple loci, therefore it has the great potential to knockout the complete cassette of non-desirable genes that halts plant growth in response to salt stress. It can be used to disrupt the functions of a negative regulators, such as RMC transcription factor negatively regulates in response to salt stress [74]. Several genes have not been explored that are responsible for the negative regulation of salt stress. The RING finger protein 1 is a ubiquitin ligase E3 that is a negative regulator that enhances the production of H_2O_2 and suppresses antioxidant enzyme activities. However, the knockdown of this negative regulator resulted in disruption of H_2O_2 synthesis and positively regulated antioxidant enzymatic activities [75]. Disruption of the functions of genes by CRISPR to confer salt stress tolerance is given in **Table 2**.

The CRISPR-based epigenome editing helps in protecting the plants from the harmful effects of salt stress [82]. The CRISPR/Cas9 protein has been used as a dCas9 is attached to the modifier of epigenetics for the alteration of gene expression [83].

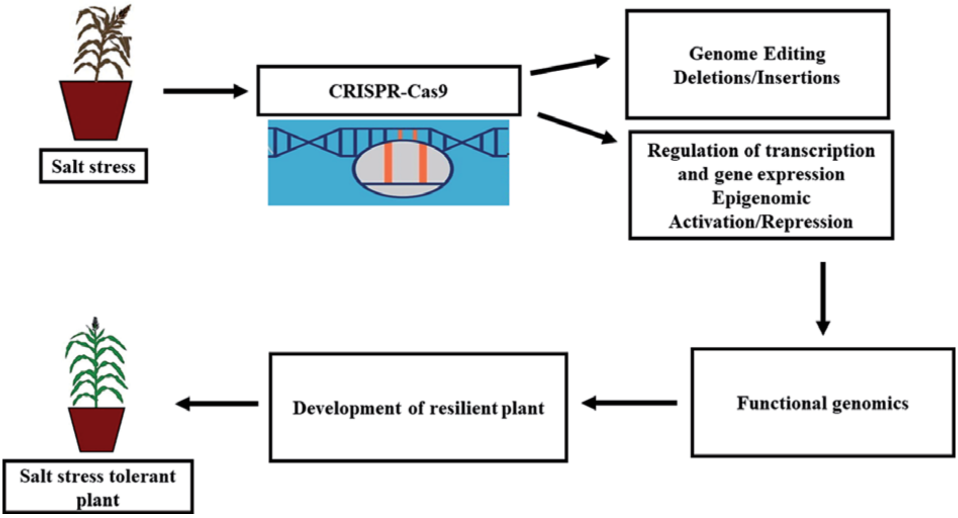

Figure 2.
Salt stress and role of genome editing for the development of salt tolerant plants.

Crops	Gene	Function	Reference
Rice	OsDST	Decreased stomatal density and tolerance to osmotic stress	[76]
Tomato	HyPRP1	Negative regulator of salt stress	[73]
Rice	OTS1	Regulator for salt stress response	[77]
Maize	HKT1	Potassium transporter	[78]
Tomato	CLV3	Regulates shoot development	[79]
Tomato	*SlARF4*	Tolerance to osmotic stress	[80]
Wheat	TaERF4	Transcription repressor of ABA	[81]

Table 2.
Genes knockdown by CRISPR/Cas for salinity tolerance.

12. Conclusion

Plant tolerance to salt stress involves several complex molecular mechanisms to respond for better morphological growth and maintain physiological functioning. Extensive research has been conducted to elucidate the physio-biochemical responses to control Na^+ uptake, its transport, and cellular homeostasis by minimizing oxidative stress. In this regard plant sensors and signaling played a crucial role in the adaptation of plants. Additionally, regulation of gene expression of the positive regulators also assists in salt stress tolerance. Conventional breeding techniques have been used for developing new varieties but that is time consuming however with the advent of genome editing as a tool for plant breeder, tolerant plants can be developed within a less period by disrupting the function of negative regulators or improving traits for salinity tolerance. Additionally, in the future to cope with the negative influence of harsh salt stress problems plant epigenetics can be explored for a better understanding of plant salt stress responses. It can also be potentially used for the regulation of gene expression that can confer salt stress tolerance.

Conflict of interest

The authors declare no conflict of interest.

Author details

Usman Khalid Chaudhry*, Zahide Neslihan Öztürk Gökçe and Ali Fuat Gökçe
Department of Agricultural Genetic Engineering, Ayhan Sahenk Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University, Niğde, Turkey

*Address all correspondence to: ukojla0455@gmail.com

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Chapter 8

Mitigation and Adaptation Strategies of Plants against Hailstorm under Changing Climate

Pralhad Jaybhaye

Abstract

The growth, development and yield of crops as well as animal and fishery are depending on the prevailing extreme weather events. Vulnerability of agriculture to climate change is becoming increasingly apparent in recent years. During 2014 and 2020, India experienced episodes of abnormally widespread and untimely hailstorm events. The conversion of number of thunderstorms into hail storm events in India shows increasing trend day by day. And the type and level of hailstorm stress must be properly quantified through proper scientific planning for present as well as future references for finding mitigation and adaptation solutions. Keeping above in view, this review paper has been prepared. This study includes aspects covering atmospheric stress (hailstorm), their challenges and coping strategies in various agricultural enterprises including crops and livestock. This review paper will ignite the minds of all stakeholders including students and researchers to explore more in finding proper adaptation and mitigation measures. This study will pave the way for developing food and livelihood systems that will have greater economic and environmental resilience to risk. Therefore, the available information has been reviewed on formation of hailstorms, its characteristics and damage potential, forecasting and control mechanisms, mitigation and adaptions through different innovations and interventions.

Keywords: climate change, hailstorm, Indian agricultural, impact, adaptation

1. Introduction

The agricultural commodities include field crops, horticultural crops, livestock, poultry and fisheries. The basic principle of agriculture lies with how crop/livestock interacts with atmosphere and soil/food as a growing medium. The system acts as pathway which regulates the intake of water/feed, nutrients and gas exchanges. Thus, any change in the quality and quantity of atmospheric variables will certainly affect the pathway through changes in atmospheric and edaphic/feed factors. Besides these, climate change is also adding salt to the wound by aggravating the extreme weather events [1].

Agricultural production in India is becoming increasingly vulnerable to climate variability and change characterised by altered frequency, timing and magnitude of precipitation and temperature. High rates of snow and glacial melting, frequent floods and droughts, heat waves, hailstorms, heavy frost and increased incidences of the pests and diseases cause widespread damage and losses to agricultural sector in India. With warmer climate, frequency and severity of extreme weather events would increase as indicated by incidences of heat waves, extreme rains, hailstorm, etc. during recent years. India's average annual economic loss due to natural disasters is estimated to be 10 billion dollars in which cyclone and flood accounts for 0.5 and 7.5%, respectively [2]. And other atmospheric stresses like hailstorm, heat wave, cold wave, frost, etc. cause huge losses to Indian agriculture. Since agriculture makes up roughly 14% of India's GDP, a 4.5–9% negative impact on production implies a cost of climate change to be roughly up to 1.5% of GDP per year [3]. And the impact may be of direct (mechanical) or indirect (physiological) depending on the type of stress, type of crop, stage of crop and mode of action of the stress on the commodity. However, there have been extreme weather events in the recent past including extended dry periods, floods, hailstorms, cyclonic rains and winds etc., which caused damage to standing crops and ultimately poor yield of crops. While, 11 states (viz., Karnataka, Chhattisgarh, Madhya Pradesh, Maharashtra, Odisha, Telangana, Uttar Pradesh, Andhra Pradesh, Jharkhand, Rajasthan and Uttarakhand) had submitted memoranda reporting crop loss due to natural calamities like drought, hailstorm, pest attack and cold wave/frost [4].

Hail is a solid, frozen form of precipitation that causes extensive damage to properties and growing crops. Hot, humid afternoon hours during the summer are the most congenial for development of hailstorms. These usually form over a relatively small area and pass over within a very short period, however, causes havoc even from a few minutes spell. Though hailstorm can occur in any part of the world, temperate zones are the most vulnerable. Among the countries, hail related losses are most prevalent in USA [5]. The damage with hails is determined by the size ranges and the number of hailstones that fall per unit area during a hail fall, wind force during the event and the property of the target. The extent of crop-hail damages also varies depending on the stage of occurrence of hail during the crop growing season. Even a short episode of hail can cause severe injury to crops, fruit trees, both downgrading the quality and causing subsequent losses due to diseases like blight, mould, canker and fruit rots. The hailstorms in a region often follow a definite pattern. However, the recent events in the country have surprised farmers and fruit growers when hail moved in from unexpected directions. Widespread unseasonal rainy spells accompanied by hail occurred in several states of India during February–March, 2014. It caused a large scale destruction of crops in Uttar Pradesh, Rajasthan, Madhya Pradesh, Maharashtra, Punjab, Gujarat, Uttarakhand, Haryana, Andhra Pradesh and Karnataka with central India (Maharashtra and Madhya Pradesh) being the worst hit. The country did not witness such a severe weather aberration since a long time [6].

However, hailstorm is a most divisive climatic hazard to Indian agriculture and it may be a product of changing climate. Hail being a very short term and localised phenomena, its prediction well in advance to inform all stakeholders for adequate preventive measures is a major challenge for even the most technologically advanced and hail affected countries like as USA. India, being situated in the tropical and subtropical region, the frequency of hail events is less than mid latitude and temperate countries. However, with climate change, the instances of severe weather aberrations are increasing the demand for strong institutional arrangements to combat such challenges. While occurrence, losses and post disaster management have been discussed extensively for the other climatic hazards such as excess rainfall, drought and flood, little attention has been given to hailstorm. The following sections elaborate on various aspects of hails for better hail preparedness and post hail management measures [7].

2. Hail storm, causes, forecasting and control mechanism

2.1 The science behind hailstorm and causes of recently observed hailstorms in India

2.1.1 Hail

Precipitation of small pieces of ice, with diameter ranging from 5 to 50 mm or something more, is known as hail, and hailstorm is the most dreaded and destructive form of precipitation produced during thunderstorms or from cumulonimbus clouds. These usually form over a relatively small area and pass over within a very short period, however, causes havoc even from a few minutes spell [8].

2.1.2 Science and causes of hailstorm

Hailstorms are the result of four atmospheric factors; strong convective instability creating strong updrafts, abundant moisture at low levels feeding into the updrafts, strong wind shear aloft, usually veering with height and enhancing updrafts. Some dynamical mechanisms that can assist the release of instability such as air flow over mountain ridges [8]. Hail is often associated with thunderstorm activity and changing weather fronts. Thunderstorm is a meso-scale system with space scale of few kilometres to a couple of 100 km and time scale of less than an hour to several hours. This is formed in huge cumulonimbus clouds, commonly known as thunderheads.

Hailstorms are the result of four atmospheric factors which are characterised as: (i) strong convective instability creating strong updrafts, (ii) abundant moisture at low levels feeding into the updrafts, (iii) strong wind shear aloft, usually veering with height, enhancing updrafts and (iv) some dynamical mechanisms that can assist the release of instability such as air flow over mountain ridges [8]. The worst hailstorms events have been reported in USA were mostly due to the squall lines and low pressure centres at the intersections of warm and cold fronts (41), cold fronts (21), warm fronts (14), stationary fronts (12) and unstable air mass (12) [9].

2.1.3 Causes of recently observed episode of hailstorm

In India unseasonal rain and widespread hailstorm during February–March, 2014 was prevailed due to the shifting of polar jet stream from its regular trajectory. It has been observed that due to the melting of Arctic ice, polar jet is shifting southwards, triggering extreme weather events all across the globe. It has pushed down the cold westerlies in India that usually flow over 30 latitude to 15° latitude, causing unprecedented hailstorms and rainfall [10]. The extended hailstorms in the non-conventional areas were resulted due to collision of winds from Bay of Bengal and Arabian Sea over Central India. Madhya Pradesh, Maharashtra (barring coastal districts) and also parts of interior peninsula such as north of Andhra Pradesh had witnessed such events during the aforesaid period. Clouds measuring up to 22 km in height and sub-freezing temperatures have been February–May 2014 and 2015 in northern, central and southern India was observed due to more convective activities following rise in temperature as the season progressed from winter to spring [11], and a agrometeorologist of the Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Parbhani (India) attributed the extreme events such as hailstorm instances experienced mainly due to conditional instability and such instabilities are commonly observed every year at isolated places in Maharashtra [12].

2.1.4 Climate change and hail events

The agricultural production, allied agribusiness and small scale agricultural business is sensitive to changing weather. Changing climate impacts on agriculture production is complex to understand. However, there is high level of agreement across studies that the impact in all probability is going to be negative for most crop categories. It is well established that there has been an increase in extreme weather events. Only 20% of these are due to natural causes; 80% are human-induced changes. India being mainly an agricultural country the economy and further its growth purely depends on the vagaries of the weather and in particular the extreme weather events [13]. Climate change implies higher frequency of extreme weather events such as hail to name a few—causing reduced crop yields and plant production and threatening the provision of other ecosystem services [14]. While, Intergovernmental Panel on Climate Change (IPCC) has predicted that in peninsular India, rainfall patterns will become more and more erratic, with a possible decrease in overall rainfall, but an increase in extreme weather events. What we are witnessing is certainly an extreme weather event [15]. While, at regional level, contradictory report was given by the Ministry of Earth Science, Government of India and in which clearly stated that there is no evidence of change in weather pattern over the country [4]. Extreme and unprecedented weather events have to be accepted as climate change phenomenon regardless of their frequency [16, 17]. However, such argument needs to be supported by more scientific evidences and a detailed analysis of past and recent events is the need of the hour.

2.1.4.1 Are the recent hail events a symptom of climate change?

Indian climatologist's majority opine that the unprecedented and untimely weather events were yet another sequence in the chain of freak weather events India has been witnessing in the last few decades. But many shy away from attributing it to climate change. As per Climate Change Centre, Indian Institute of Tropical Meteorology (IITM), Pune (India) it is premature to attribute it to climate change. More samples are required for the attribution. Temperatures are rising in the world everywhere and every year, so it is a symptom of climate change. But the current event of 2014 was an isolated one and has not been witnessed in the last 25–30 years. So, it is difficult to link it to climate change. It may be a part of natural variability of weather conditions.

While, hails or thunderstorms are extreme forms of weather events and deserve special attention in view of climate change. However, the homogeneity of existing reporting and data quality in many instances are questionable [18]. And due to this there is low confidence in observed trends so far in small-scale, however, few studies indicate that a strong positive relation exists between hailstorm activity and global warming. Fifth assessment report (AR5) of IPCC highlighted some studies with regard to trends of hailstorm occurrence over the years in different parts of the world [19]. A strong positive relation exists between hailstorm activity and subsequent hailstorm damage which is likely to be aggravated by global warming. By 2050, the estimated annual hailstorm damage to unprotected farming could increase from 25 to 50% [20].

2.1.5 Hail forecasting and control mechanisms

Radioactive Detection and Ranging (RADAR) technology in hail forecasting found most expedient and it is difficult to forecast all a hailstorm, since its occurrence is sporadic and confined to very limited areas in a thunderstorm.

While hail formation continues to elude scientists, sophisticated RADAR has been developed that can detect the presence of hail before it falls to the ground. Eventually, warnings may be issued as about 15 minutes before hail strikes, thus allowing pilots to avoid threatening air space, people to seek shelter, and property to be protected [9].

While, artificial hail control is an important measure in disaster prevention and mitigation. With the development of atmospheric science and related science and techniques, the ability of hail cloud identification and subsequent hail suppression technique has been improving continuously. There are two techniques available for suppression or control of hail, but mercy is that no one can able to prove it confidently on ground of validation still today.

i. Cloud seeding: This process of seeding of condensation nuclei into the system on or before the start of hail formation process can be done using either specialised aircrafts or anti-hail rockets.

ii. Creating shockwaves: The creation of shock waves can prevent the formation and growth of hail by melting altogether. Shockwaves are produced using hail guns/cannons.

3. Hail observations and monitoring

The data on temporal and spatial distribution of hailstorms as well as size of hailstones with the consequent damage have to be documented systematically which facilitates identification of vulnerable areas more scientifically. The size and density of hailstones that fall per unit area are being measured. The hailstone size and intensity are measured by using hail-pads and hailstone size measured by different ways; vernier scale, metric scale ruler, metric scale tape or by comparing with different shapes (e.g., grain, pea, mango, ball, coin, etc.) [9, 21–25].

3.1 Hail characteristics and damage potential

Usually, the extent of damage by hail is determined by the size and density of hailstones that fall per unit area and wind force during the hail fall event. There are three types of hail classified according to the occurrence and nature: (1) soft hail (less than 5 mm ice mixture when falling on the earth surface breaks into small pieces is called as soft hail), (2) small hail (small ice crystals falling on the earth along with rainfall but do not break down into small pieces is called as small hail) and (3) severe hail (hard hails having diameter of 5.5 μm commonly known as severe hail). Secondly, according to size, shape and nature, hailstones are classified into different classes with photographs and its damaging potential [9, 23–25]. However, in agriculture, hail is considered an extreme event of great destructive potential and a single hailstorm event in an orchard could result in the total loss of fruit production and would damage the trees [26]. The damage with hails is determined by the size ranges and the number of hailstones that fall per unit area during a hail fall, wind force during the event and the property of the target. The extent of crop-hail damages also varies depending on the stage of occurrence of hail during the crop growing season. Different crops are damaged in varying ways by hail. Tea, tobacco, broccoli, tomato, spinach and soybeans leaves are delicate and subject to serious damage even when 6.4 mm diameter hailstones fall. Fruit crops, such as apples and peaches, can be easily bruised by small- to moderate-sized hail and can lose great value because of reduced quality.

3.2 Indian hailstorm scenario

Hailstorm hazard is not new to the Indians and it was recorded from the British emperor; one of the deadliest hailstorms of all time killed at least 230 people, and over 1600 sheep's and goats in Uttar Pradesh (India) on 30 April, 1888. The hailstones were reportedly as big as oranges, and in some places were as high as 600 gm. In 1898 Sir John Eliot first time in the Indian History complied hail storm record. Thereafter, Ramdas in 1938, hailstorm data of the period 1898–1935 analysed and published first time centimetres [9] and there after India Meteorological Department (IMD) as well as another all institutes and organisations were continued it through proper scientific planning with greater efforts till today. Regarding highest size of hail stones, the largest size hailstone occurred in association with a thunderstorm in April, 1888 at Moradabad a town near Delhi [13]. The hailstorm frequencies are highest in the Assam valley, followed by hills of Uttar Pradesh now known as Uttaranchal, South Bihar (now known as Jharkhand) and Vidarbha in the eastern parts of Maharashtra [27]. Whereas, the coconut size hailstones were recorded at Kondhwa in Pune, during 1908–1909. Further, during 1986, the hailstones of cricket ball size showered in Pune with observed mango size (750 gm) hails at Motala, Dist. Yevatmal (Maharashtra). The hailstone measured size of a pigeons egg in diameter (IMD 1888) [23, 28].

In some of the worst disasters associated with hailstorms, eight persons were killed in Kanpur (Uttar Pradesh) on 28 February 1992, and nine persons died near Nagpur (Maharashtra) in February 1979. Thunderstorm and squally winds killed 19 people in Patna on 29 May 1997, while in Kolkata nine people died on 26 March 1993 due to thunderstorm and lightning. Cataloguing of Disastrous Weather Events by the India Meteorological Department started in 1967. Prior to 1967, reports of deaths (loss of life) in association with thunderstorms are many [29].

The current history of hailstorm enlightens the devastative nature of hailstorm and which was clearly seen from last two decades; the affected area and losses of agriculture were documented by different workers and revealed that it was spreading on more no. of states increasing continuously in India from 1994–1995 to 1998–1999, 2014 and 2015 (0.46, 0.74, 1.2, 2.9, 1.8 and 6.3 m ha, respectively) [20, 22, 30].

3.3 Recent hailstorm episodes and damage

In India, an unprecedented 20 days was prevailed in different states, during February 24 to March, 2014, has left millions of farmers in a state of shock; surprisingly in 2014, almost 28 out of 35 districts in Maharashtra were severely affected by unseasonal rains and heavy hailstorm causing widespread damage to the agriculture (**Table 1**). The heavy damage to agriculture sector due to hailstorm in most of the affected villages of Maharashtra was experienced first time by elder and older peoples in their life during last 60 years [27]. Usually, hailstorm activity occurs during the months of April and May [30]. But this time it has occurred during February–March which is a severe weather aberration. Over 1.24 lac hectare (l ha) of farmland spread over 1086 villages in Maharashtra have been adversely affected in the on-going spell of unseasonal rain and hailstorm (February 10–13, 2018) which started on 10 February and at most affect 4–5% of the crop. Eleven districts mainly in Marathwada and Vidharba regions have reported hailstorm. The districts of Buldhana, Amavati, Akola and Washim in Vidharba have also got light showers. Two deaths were reported from Buldhana where farmers had taken shelter under trees to avoid the hailstones [31]. The comprehensively recent worst affected area by hailstorm events in India were reported by different organisations, media and personals are presented in **Table 1** [32–34].

Period of hailstorm aberration	Affected states of India	Damage to agricultural crops and losses
February 24–March 14	Punjab, Haryana, UP, Rajasthan, MP, Maharashtra, Karnataka and AP	Ready to harvest *rabi crops* such as wheat, pulses, potato, sugarcane, maize, groundnut and mustard and horticultural crops like grapes, papaya, mango, banana, onion and other vegetables damaged partially or completely. 4.65 million ha of standing crops were ravaged in the worst-hit Maharashtra and Madhya Pradesh alone.
February 11–13, 2014	Maharashtra	Over 3 lakh ha in Maharashtra were affected by thunderstorms and hailstorms. Overall, the crops of wheat, gram, sorghum, onion, grapes, oranges and cotton have been affected in 19 districts of the state. State Government, released nearly Rs. 313 crores from the state disaster response fund.
End of February 2014	Marathwada, Vidarbha, Northern Maharashtra and parts of Western Maharashtra	Absolutely destroying the farmer. Rabi crops like wheat, gram, cotton, sorghum, summer onion were lost, horticultural crops like papaya, sweet lime, grapes are battered and orchards which took years to grow are ridden to the ground. Turmeric was drying in the sun, grapes were waiting to be graded, and wheat was harvested and lying in the fields. A preliminary estimate and news reports, crops over 12 lakh ha thousands of livestock, animals and birds have succumbed to injuries and diseases, which threaten to spread. Around 21 people have lost their lives to the disaster.
March 14–16, 2016	Marathwada and Vidarbha region of Maharashtra State	Flattening rabi crops over 85,000 ha area.
February 11, 2018 at 09:15 am	Wakod Village, Resod, Washim District, Maharashtra	The mostly damaged of crops the area was horticultural and Rabi crops (viz., sorghum, cotton, pigeon pea, sunflower, etc.) and allied agribusiness was also partially damaged intensively.

Table 1.
Recent hailstorm episodes and damaged to agricultural sectors.

3.4 Hail storm frequency in India

Considering the resent hailstorm events, few of the scientists kept their views without data and they believe on some part of the Maharashtra may convert into hail prone area. Therefore, hail storm data are reviewed that the average number of days with hail is about 6–7 per year over Himachal Pradesh and its neighbourhood, but it decreases sharply to 1 in 2 per years over the adjoining plains. Over Bengal, Bihar, Uttar Pradesh and in central Indian states of Madhya Pradesh and Maharashtra hailstorm occurs on an average once a year. In the interior parts of southern peninsula, only one hail event may be expected once in 5 years. Hailstorms are comparatively rare over the coastal tracts of the Peninsula [9, 17]. However, similar observations were reported by another worker that the total number of thunderstorm and hail storm events in India during the period of 1967–2006 observed 1123 and 934, respectively with increasing trend of 1.90 and 1.00 per year, respectively [6]. While, hail is an extreme event out of the 10 selected key climate extreme events and recent past spatio-temporal pattern over India showed that highly significant increasing trend in the total number of events was observed [3]. While, hailstorm data for 38 years have been used for frequency analysis (1972–2011; excluding 1977 and 1984, for which data are not available). More than 61% of districts have experienced at least one hail event in this 38-year period. The highest frequency was noticed over districts in the northern parts of the Vidarbha region of Maharashtra adjoining the state of Madhya Pradesh. Vulnerability of agriculture to climate change is becoming increasingly apparent in recent years [29]. Regarding the frequency and trend of hailstorm in India, hailstorm data for 38 years have been used for frequency analysis (1972–2011; excluding 1977 and 1984, for which data are not available). More than 61% of districts have experienced at least one hail event in this 38-year period. The highest frequency was noticed over districts in the northern parts of the Vidarbha region of Maharashtra adjoining the state of Madhya Pradesh [22, 35].

4. Hail damage to agriculture

Optimal growth of the plant found under a certain quantity of each of the environmental factors, and any deviation from such optimal conditions adversely affects its productivity through plant growth and development. Effect of hailstorm on the plant depends on their intensity and duration of act. These stress factors hailstorm highly impacted on crop, thereafter on livestock and least on fisheries. Once hailstorm hazard prevailed, its impacts may be direct or indirect; due to direct impact causes mechanical damage or injuries, it may be total loss (e.g., death of human, animals and birds, lodging of plants, uprooted trees and crop, etc.) and partial loss (e.g., causality of human, animals and birds, heavy defoliation, shredding of leaf blades, breaking of branches and tender stems, peeling of bark, stem lesions, cracking of fruits, heavy flower and fruit drop in crops and plants, etc.). And due to indirect impact causes injuries or physiological disorder (depending on the type of stress, type of crop/animal/birds, stage of crop/animal/birds), it is based on stage of occurrence (damages in mature produce quickly become focal points for diseases like brown/grey rot smut); primary injuries (like dieback or wilting of damaged plant parts) and discoloration of damaged parts (like leaves and fruits affecting their quality and rotting of damaged fruits and/or tender stems and branches due to fungal and bacterial infections), etc.

Based on Post-Disaster Needs Assessment (PDNA) report of Food and Agriculture Origination (FAO), damage and loss in agriculture by agricultural sub-sector, percentage share of total (2006–2016) was highest 49% in crop and thereafter in decreasing order in livestock (36%), unspecified sectors (8%), forestry (4%) and fishery (3%) were observed. The type of the damages recorded during recently prevailed continues episodes of hailstorms (2013–2014–2017–2018) are given in **Table 1** [9, 36].

4.1 Hail damage to agricultural production and allied agribusiness

Hailstorm causes primary injuries due to direct impact of hails which causes heavy defoliation, shredding of leaf blades, breaking of branches and tender stems, lodging of plants, peeling of bark, stem lesions, cracking of fruits, heavy flower and fruit drop, etc. This is followed by secondary injuries which are nothing but the manifestations of primary injuries like dieback or wilting of damaged plant parts, loss of plant height, staining, bruises, discoloration of damaged parts like leaves and fruits affecting their quality and rotting of damaged fruits and/or tender stems and branches due to fungal and bacterial infections [9, 37]. While, heavy rain, hail and strong wind affect crop yield in different ways, especially depending on the development stage. Whereas roots may lose their contact and anchorage in soil when heavy either rain, hailstorm or in combination of both, decreases soil strength and increases the load which the plant must bear.

For rice, mainly reduced translocation of mineral nutrients and carbon for grain filling, increased respiration, reduced carbon assimilation within the canopy, rapid chlorosis and greater susceptibility to pests and diseases are reported as main mechanisms. Lodging reduces the yield of rice by self-shading and reducing canopy photosynthesis. Additionally, lodging of cereals provides a favourable environment for leaf diseases and causes harvesting losses. Lodging also adversely affects grain quality, for example lodging inhibits grain drying due to reduced air circulation and increased humidity. In addition to the type of crop, stage of growth, weather conditions and susceptibility to disease also determine the impact. Prolonged hot and wet conditions after the storm enhance losses by increase in incidences of diseases, particularly those caused by bacteria [20, 38–40].

An overview on yield reductions due to lodging are 31–80% in wheat, 4–65% in barley, 37–40% in oats, 5–20% in maize, and 5–84% in rice [41]. While, the huge loss of winter, rabi and annual as well as perennial crops was generated in 2014 due to fallen hailstorm during the month of February and March in India and crop wise affected area in 1000 ha of gram, wheat, sorghum, maize, summer rice, vegetables, banana and other crops are 149, 119, 20, 10, 9.0 0.6, 0.2 and 357, respectively in 2014 due to hailstorm in India were [27]. Similarly, recorded damaged area and entity due to hailstorm episode or hailstone events during the year 2014–2018 [9, 21, 30, 31, 33, 42, 43] and which is given in **Table 1**.

Though variation was observed in between official and the media reports of hailstorm damage in the February and March, 2014, a divisive nature of hail storm in one report uttered, nearly 20 L ha crops was under risk, death of poultry birds and animals observed around 39,000 and 4096, respectively. Whereas, 30,000 villages were affected from 28 districts of Maharashtra and economical loss was estimated 250 crores. While, due this episode of hailstorm, destroyed 1.45 L ha grape crop (a dollar crop) at maturity stage in Maharashtra state and district wise distribution was 0.65, 0.50, 0.20 and 0.10 L ha of Nasik, Sangli, Solapur and Pune, respectively [43]. Since, 2013–2014, hailstorms are striking the state every year during spring (February, March, April) when the rabi crops are getting ready for harvest. In March 2015, hailstorm damaged in general 7.5 L ha area and out of which 6.1 L ha comes under agriculture crops and 1.4 L ha under horticulture crops [32]. Whereas, the intensity and divisive nature of hailstorm uttered that the 4.55 l ha area of Marathwada region of Maharashtra state was affected by prevailed hailstorm during March–April, 2016, Which was perished partially or completely rabi crops and vegetables, and horticultural crops [36].

4.2 Damage to livestock and poultry

The basic principle of agriculture lies with how livestock interacts with atmosphere. Thus, any deviation of external optimal atmospheric conditions affects the pathway through changes in atmospheric and feed factors for animal growth, development and/or productivity. The quantum of impact on livestock and poultry mainly depends on the type of stress and animal, its age and mode of action of the stress [22]. Hailstorms can cause seriously injury to livestock, poultry. Livestock, mostly cattle and sheep, grazing in the open become the first victim of the hail. Poultry sector was another allied sector that was badly hit birds housed in temporary or semi-temporary sheds were the first casualty [34]. While, 1075 cattle have perished and 38,512 houses have been damaged due to a hailstone event [21]. In the year 2014–2017, according to a preliminary estimate and news reports, thousands of livestock, animals and birds have succumbed to injuries and diseases, which threaten to spread due to severe hailstorm [33].

4.3 Damage to biodiversity

Hailstorms causes severe damage or mortality of people and wildlife and it is not a new phenomenon, though the incidence may be growing in new areas. The deaths of 460 parrots due to hailstorm at Tumsar in Bhandara district of Maharashtra state, on 13 February 2018 [34]. The hailstorm events occurred from the end of February into May 2014 which were caused mortality of roosting birds such as rosy starling (*Pastor roseus*), house sparrow (*Passer domesticus*) and rose-ringed parakeet (*Psittacula krameri*). Mammals residing in open areas were also killed. During this period major events were occurred especially during 3–10 March, resulting in a huge loss to the wildlife and agriculture sector; Vidarbha and Marathwada regions of Maharashtra (2,00,000 km^2), owing to unusual hailstorms, 35 species of birds and nine species of mammals were found dead, totalling at least 62,000 birds and hundreds of mammals, in India, during 2014 and hail stones were up to 5 cm in diameter [44]. The unseasonal excessive rains along with hailstorms and lightning have also caused losses, 1075 of cattle have been perished [21].

Extreme weather events such as hailstorm in Cedar Rim in Fremont County, Wyoming, USA, was destroyed eggs and injure or kill juvenile and adult birds at the nest. Seventeen-percent of 128 nests failed due to the hailstorm; however, all failed nests were located where the hailstorm was most intense, and 45% of all nests on those plots failed due to hail. Mortality rates varied by species, nest architecture, and nest placement. Nests with more robust architecture and those more likely to survive the hailstorm, that natural history traits may modulate mortality risk due to hailstorms [45].

4.4 Damage to socioeconomics

Hailstorms can cause severe damage to farm structures apart from seriously injuring to humans and mortality of people due to intense hailstorms is not a new phenomenon, though the incidence may be growing in new areas [34]. And in the year 2014–2017, according to a preliminary estimate and news reports around 21 people have lost their lives to the disaster [33]. While, it was estimated that more than 20 lakh farmers of Madhya Pradesh are badly affected due to hailstorm calamity prevailed in 2014. Whereas, the survey reports show that there has been a loss of eight human lives and 38,512 houses have been damaged [21]. The number of farmers has committed suicide on account of failure/damage of agricultural crops, vegetables and fruits due to natural calamities including unseasonal rains and hailstorms during the year 2014–2015. And the amount of compensation/relief sought by the affected States and the amount of assistance provided by the Union Government during the said period; Uttar Pradesh, Rajasthan and Haryana demanding Rs. 744.48 crores, Rs. 8252 crores and Rs. 1135.91 crores from National Disaster Response Fund (NDRF), respectively. Assistance from NDRF for Natural Calamities (Drought) in the States from 2013 to 2014 to 2015–2016 was provided (Rs. in cores) and Assistance sought by the State Government are 39,180.41 and central assistance approved under NDRF are 7108.06. Whereas, the reports of State Government of Maharashtra confirm three cases of farmers suicide due to untimely rains and hailstorm during the period January–March, 2015 [46].

5. Hailstorm management/coping management

The substantial impact of hailstorm is mainly depending on the type of stress and crop/animal/fish, its stage/age and way of action of the stress. Management strategies for mitigation and adaptation of these stresses require both application of current interdisciplinary knowledge, development of a range of technological innovations and timely interventions. Considering its divisive nature this is a high time to update our knowledge regarding existing. However, the increased frequency of hailstorm events, especially in vulnerable ecosystem of Deccan Plateau region of India demanded appropriate measures to minimise adverse impact on agricultural crops. Hence, some of the pre hail, during hail and post hail possible management and adaptations revived.

Make use of location specific recommendations given in Agromet Advisory Bulletin (AAB) during pre/post hail disaster to maximise advantages and minimise losses in agricultural production. AAB is a most useful weather smart agriculture technological tool that can minimise climatic risks and build a resilient agricultural production [47] and the Maharashtra State Agricultural Department were aware and warned to the farmers through AAB in the year 2014–2015 about the prevailed hailstorms. But little that can be done to save the standing crops from hailstorms because of ignorance regarding alert message given in AAB [12], and the farmers, who followed the AAB are able to reduce the livestock rearing cost and increasing the net profit as compared to the non AAB farmers [47].

5.1 Pre hail management

It's high time to update our knowledge regarding existing technologies and side by side explores new avenues for managing atmospheric stresses in agriculture. As hail is the sudden event, and highly unpredictable, it is always better to take some precautions to minimise the hail damage.

5.1.1 Forewarning

Dissemination of forecast of thunderstorm/hailstorm through multimedia, special AAB/regular AAB are essential and it was found useful during the worst hailstorm of 2014–2015 [12, 47]. While, the state of affairs with regard to forecasting by RADAR technology, vulnerability zonation tools have been included [48].

5.1.2 Precautions

Prepare mind set of farmers for adaptation of hailstorm or fight against it and they need not be panic if the damage is less severe. Wait till the next sprout to decide whether totally abandon orchards or replacement of trees will suffer. While, plantations of tree shelterbelts can markedly reduce hail damage in their immediate vicinity since hails are usually associated with strong winds. Protective screens termed as anti-hail nets above the crop can be appropriately utilised especially for high value crops. These anti-hail nets are not effective against strong hail storms. While, shade nets can be a good option especially for high value crops in areas with higher probability of hailstorm occurrence. A range of anti-hail nets used to protect the apple orchards in Himachal Pradesh and similar suggestions are reported by [48], nylon nets intended for protection against birds actually gave good result towards protection against hail. Cropping pattern required to change of the hailstorm prone area and which are another way to abatement of hailstorm in regions with high frequency is to grow those crops that are less subject to hail damage. Some region-specific hailstorm resistance cropping pattern for Marathwada region are suggested [48]. Another approach to minimise the hail losses to the farmers is through insurance. Insurance is the most widely used adjustment tool on the economic front to crops and property damages due to hail and now it is supported by the government though it requires easier process to get relief [46].

Figure 1.
(a and b) Protecting grape fruits by silver coated hard board.

Adaptations of farmer's innovative interventions to fight against hailstorm in Maharashtra (cost effective technology to protect crop from hailstorm) viz., protecting young fruit seedlings and small horticultural tree by straw thatching/polythene tunnels, protecting grape fruits by silver coated hard board (**Figure 1a** and **b**), protecting banana fruits by using skirting's bag, which is manufactured from polythene (**Figure 2**) were found useful up to some extent. However, it requires validation for auditing exact protection efficiency and monitory benefit; through systematic documentation and conformation based on research in view of wide adaptability [36].

5.2 Precautions during hailstorm

5.2.1 Precautions for human beings

To avoid the casualty/damage/mortality, human being should be inside house or a covered shelter, avoid going outside unless absolutely necessary, listen to a local radio or television station or perceive authorised message from government institutes to see when it is safe to come out of cover, stay off of phones and other appliances during a hail storm, etc.

5.2.2 Precautions for crops

For the cash crop and horticultural farmer's mitigation of hailstorm through weather modification science viz., cloud seeding by using aircraft (**Figure 3**) and

Figure 2.
Protecting banana fruits Skirting's by polythene bags.

Figure 3.
Cloud seeding aircraft. Source: https://images.app.goo.gl/not4sup90BuQSoAP9.

Figure 4.
Anti-hail rocket. Source: https://images.app.goo.gl/TxEq1jzHaALvfg498 Ref. [9].

anti-hail rocket (**Figure 4**); creating shock waves through anti hail canon/hail gun (**Figure 5**) is possible up to some extent though it is very costly, but its reliability till today is not widely accepted. Therefore, it may be possible to commercial crop growers either on co-operative base or government provides this technology on subsidy/free of cost through relief fund provision.

5.2.3 Precautions for poultry and animals

5.2.3.1 Pre hail storm precautions

Hailstone creates abrupt high frequency sound; which have scaring birds and animals, such scaring condition are responsible to create depression of them. These depressions are caused damage to nervous system and finally its converted into death of such hazards birds and animals. To avoiding such disaster, if animal and poultry birds shed roof are of tin sheet, then which have covered by straw of sugar cane trash/rice/sorghum/Bajara/cotton/red gram/jute straw during the hailstone time. While, it was observed that the mortality and health problems of poultry birds and animals (especially kids of goat and sheep) reduced on large scale due adaptation of aforesaid technique at the time of hailstorm and post hailstorm disaster [36].

5.2.3.2 Post hail storm precautions

Take medical treatment/take care as per advice of veterinary doctors.

Figure 5.
Anti-hail cannon or hail gun installed in apple garden. Source: file:///C:/Users/user/Desktop/Hailstorm/Seminar_PRJ_2018_ROUGH/USED/improved%20hailgun/hagelkanon_en.pdf.

5.3 Post hail management

In fact, forewarning and preparedness for hailstorm is constrained mainly by the speed with which it occurs. Once damage occurs, specific management strategies are necessary in formulating the relief strategies for recovery, thus minimising the hailstorm impacts [49].

5.3.1 Coping strategies (in general)

Though, little information is available on measures for hastening recovery in hail damaged crops. These studies indicate the potential of technological interventions to cope with hailstorms. Therefore, some of the post-hail measures including nutritional supplement, plant bio-regulators and canopy management were revealed. Among these, pruning of the hardy and indeterminate eggplant crop induced effective branches, which produced more flowers and fruits. Nitrogen supplemented with urea drenching and stress alleviating effects of salicylic acid promoted recovery in maize while drenching with humic acid along with spraying of potassium nitrate improved productivity of onion. While, little information is available on measures for faster recovery in hail-damaged plants, application of additional nitrogen encourages new growth [50, 51].

To control of secondary damage due to insect pests and diseases, remove the fallen fruit to reduce the spread of disease and pests during their decay. Large wounds on trunks and branches should be covered with a water-based paint to avoid desiccation and disease infection. Fruit thinning by removal of hail-damaged fruits improves yield and quality of remaining fruit. Bud-breaking chemicals and growth/bio regulators may be applied to induce the vegetative growth in orchard crop along with fertilisers [9, 52]. Proper drainage facilities are to be provided to avoid waterlogging and to avoid secondary infection of diseases; near-maturity bulb crops like onion and garlic may be harvested to avoid rotting. In horticultural crops provide support to the bent/damaged tress with bamboo sticks and earthling up Provide light irrigation to soften the fields that are hardened/compacted due to hailstorm [36]. Select the proper contingent crops to take the advantage of soil moisture and to recover part of losses and pruning may be necessary to retain young trees and optimise new growth [9, 36, 48].

Hailstorm app to assess crop damage developed by Indian Space Research Organisation (ISRO) is one of another technique, it is not useful direct mitigation of hailstorm but it provides help to proper assessment through scientific way of hailstorm nature of damage and intensity, which provides base for agro technically advice to the farmers for adaptation and help in relief provision implementing by government [53].

5.3.2 Crop specific management

The certain crop specific management strategies that need to be followed for long term management of hailstorm damaged crops. In order to prevent further losses due to flower and fruit drop because of hailstorm, unseasonal rains and high winds, the mango orchards spraying with potassium nitrate (KNO_3) 1.0% was found beneficial. While, incidence of powdery mildew and insect pests like mango hoppers, thrips are very high due to hailstorm created favourable microclimatic condition (i.e., persistence of cloudy weather, increased humidity and wet conditions on foliage). Hence, affected orchards sprayed with fipronil (5% EC) @ 1.5 ml/L or spinosad (45% SC) @ 0.3 ml/L for aforesaid pest control and with hexaconazole @ 1.0 ml/L for powdery mildew disease control [9].

After hailstorm, (i) the chemicals used for foliar spray/drenching on onion crop at bulb initiation stage, improved the bulb yield of the onion when compared to water spray. The maximum benefit of about 40% higher yield was observed in case of drenching with humic acid (HA; 2 ml/L) and spray of KNO3 (1.5%) where the bulb yields were 23.4 mg/ha against 16.3 mg/ha when the crop was raised as such. (ii) Application of salicylic acid (SA), KNO_3 and urea drenching led to considerable improvement in grain weight of major cobs in maize especially when damage to cob was <20%. Recovery was not significant in case crop damage was more than 20%. Grain weight per cob was improved with application of plant bio-regulators (PBRs) compared to control plot in the range of 7–26.5% and 2.2–14.7% when damage of cob was <20% and > 20 (20–80)%, respectively, and among all treatments urea drenching and SA performed better. The yield improvement ranged between 14 and 26% with urea drenching being most effective. (iii) Pruning at 20 cm of two-month-old transplanted brinjal crop affected by hail had greater advantage than the same at 30 cm due to higher number of branches from basal nodes resulting in higher biomass/plant. It was noticed that deep pruning delayed flower bud formation only by 3–4 days in case of plants pruned at 20 cm height when compared to unpruned plants. It was also observed that timely removal of damaged fruits and dried twigs/branches from plants helped in faster recovery of the crop and also facilitated picking of fruits and other operations. Foliar spray of nitrogenous fertilisers such as urea (2%), KNO_3 (2%) and thiourea (10 μM) also resulted in improved plant growth and development that was monitored in terms of plant height, branch and chlorophyll index [20].

5.3.3 Provision of immediate rehabilitation and relief

After the hailstorm disaster, immediately needs rehabilitation and relief to the affected community. Therefore, at government level efforts has been made to post hailstorm disaster management and rehabilitation; and relief provided to the sufferers through NDRF and State Disaster Response Fund (SDRF) and by some non-government originations (NGO's) time to time. However, it was not sufficient and it is strongly supporting statements are available in public domain, which had discussed in both the both parliament of India [43]. In connection to this it is suggested that it is proposed to provide relief in the interest rate to be given on conversion of medium-term loans, exempted school and college fees to students from the affected families, another approach to minimise the hail losses to the farmers are through improvement in insurance rules, regulations and process. While, the households severely affected due to calamity should get the benefit of subsidised food grains, salt, sugar, gas and kerosene under Targeted Public Distribution System in the State under the National Food Security Act (NFSA)-2013 the Government of India, electricity bills for permanent agricultural pump consumers should be exempted, central government should make plain for start modified ‘Kanyadan Yojna’ to affected farmers and provide affordable amount of rupees for the marriage of the daughters of calamity affected farmers and conformity to this statement reports are mentioned in [46].

6. Conclusion

Climatic reason of hailstorm, its classification or characteristics and damage potential, control measures complemented with photographs are reviewed. It is concluded that the hailstorm events frequency increased in India and its cause is of climate change is not defined concretely. Causes of recent unseasonal hailstorm events have been discussed. The management techniques for recovery of different field and fruit crops have been reviewed. The crop specific management strategies discussed which should help in formulating the plans and strategies for minimising the impacts of such vagaries if these happen in future. Enlightened the need of research, relief and financial support to the hailstorm affected farming community.

7. Future scope

Thus, greater efforts are needed to improve the forecast skill and use these better forecasts in disaster management [13]. There is a need for formulation a pilot project involving IMD, SAUs, AICRPAM and progressive farmers to undertake studies on suppression of hailstorms and to explore economically viable protection strategies to minimise the losses from hailstorms; there is a need for adequate RADAR network in the country to forecast the occurrence of hailstorms over larger area [36, 48]. Other essentialities are the data on temporal and spatial distribution of hailstorms as well as size of hailstones with the consequent damage have to be documented systematically which facilitates identification of vulnerable areas more scientifically. As a way forward, suggesting to have very targeted and well-designed experiments on the regionally specific crop impacts of a hailstorm at the level of SAU’s and ICAER institutes and usable recommendations make it available to the farmers for cope up hailstorm through adoption of agro techniques and mechanisms.

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Author details

Pralhad Jaybhaye
Faculty of Agricultural Meteorology, Vasantrao Naik Marathwada Krishi
Vidyapeeth, Parbhani, India

*Address all correspondence to: agmetprj@gmail.com

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Chapter 9

Harnessing Beneficial Plant-Microbe Interactions for Enhanced Plant Adaptation to Abiotic Stresses

William Makaza and Casper Nyaradzai Kamutando

Abstract

Boosting crop production is a vital venture for enhancement of humanity. However, it remains a dream, especially in developing countries. To attain food security at household level, productivity is constrained by a several biotic and abiotic stresses. Yield losses are usually influenced by abiotic stresses, particularly drought and heat stress, and poor soil fertility. Optimal crop production under these stress factors requires substantial inputs, including irrigation and heavy fertilization, strategies which majority of farmers in poor countries lack capacity to exploit. Therefore, much more sustainable and accessible alternatives need to be developed in order to address the problem of food insecurity. Recently, research has proven that plant adaptation to abiotic stresses can be promoted by beneficial microbial species, especially those that reside in the rhizosphere. For instance, mycorrhizal fungi have been found to expand the root system of plants to access more water and nutrients. In-depth understanding of the mechanisms underlying beneficial plant-microbe interactions is key in development of holistic programs for boosting yields under abiotic stress conditions. This chapter seeks to unravel the mechanisms underlying beneficial plant-microbe interactions and the importance of these interactions in stress-adaptation.

Keywords: abiotic stress management, climate change impact, crop production and food-security, ecosystem, plant growth promoting microbes, role of plant-microbial interaction

1. Introduction

Food security is a basic human requirement that is being jeopardized by a rapidly booming demography, unsustainable agricultural practices, and a changing global environment. This puzzling divergence is drastically reducing agricultural productivity and is exacerbated by abiotic stresses, contributing to more than 70% global food loses [1, 2]. Nonetheless, the implications of the Green Revolution which intensified the use of high yielding varieties, synthetic pesticides, inorganic fertilizers and mechanization (eg., irrigation), significantly contributed to the reduction in microbial diversity within arable farmlands; and this phenomenon had a negative bearing on overall soil health [3, 4].

Abiotic stresses (including, high and low temperatures, salinity, flooding, drought, nutrient limitation, toxic metals and organic contaminants) contribute to unsustainable agriculture [5–7]. The potential costs of these abiotic stresses are significant, implying the need for sound, economical, and ecologically friendly measures to reduce their negative effects on plant growth and development [1].

Plants, unlike animals, cannot use avoidance and escape as stress-relieving tactics and as a survival strategy, their evolution has been defined by development of very advantageous relationships with their more mobile companions, microorganisms. Some of these relationships entail complex symbioses that provide stress tolerance, such as mycorrhizae and rhizobia, which aid in alleviation of nutritional and water deficiency [6, 8–10]. Beneficial microorganisms are increasingly being used in agriculture, with several research programs assessing microbial strains for their capacity to provide protection against a specific stress (eg., nitrogen and phosphorus deficiency) as well as cross-protection against numerous stresses [11–13]. Understanding the underlying physiological mechanisms by which the beneficial microbial taxa mediate stress tolerance is crucial to ensuring sustainable agricultural production under the current and predicted climatic conditions.

Plant growth and soil fertility are all aided by the beneficial microbes-interactions [14–16]. It has been now commonly recognized that certain unique and efficient microbial strains, known as plant growth promoting (PGP) microorganisms, improve plant growth, fitness, guard against pathogenic organisms, and aid to maintain soil health under diverse environmental conditions [4, 17, 18]. In addition, some microbial taxa are known to contribute to the biological processes of the soil formation [11, 19]. For instance, rhizosphere microorganisms contribute to the biogeochemical cycling of nitrogen (N), phosphorus (P), potassium (K) and sulfur (S) [10, 17, 20]. For the past 3 decades, knowledge on beneficial microorganism has dramatically increased and in some cases, PGP microbes were used as inoculants for enhancement of sustainable crop productivity [13, 21–23]. In this chapter, we provide a synthesis on the rhizospheric microbiome interactions; the influence of abiotic stresses on plant-microbe associations; as well as, describe roles performed by PGP bacteria in aiding plant adaptation to abiotic stresses.

2. Beneficial and harmful plant-microbe interactions

Plant-microbe interaction is a complex, dynamic, and ongoing process that dates back to Earth's first plant colonization. Plants and bacteria have been associated for millions of years, resulting in an assemblage of host and non-host species forming a holobiont [24–26]. A metagenomic study by Xu et al. [20] revealed over-representation of KEGG Orthology (KOs) involved in known plant–microbe and microbe–microbe interactions, such as bacterial secretion systems, flagella assembly, bacterial chemotaxis, bacterial toxins, bacterial motility, two-component system and biofilm formation. These KOs were responsible for transporting plant-derived nutrients such as; amino acids, peptides, urea, oligosaccharides and monosaccharides, into microbial cells [11, 13, 20].

Plants are regularly approached by both the beneficial and the harmful microorganisms (especially, fungi and bacteria) in both, the natural and cultivated lands. The phenomenon results in formation of beneficial partnerships between the plants and the microbes. These partnerships enhance both direct and indirect stimulation mechanisms. Indirect stimulation mechanisms such as those performed by mycorrhizal fungi and rhizobia, results in optimal availability of mineral nutrients and fixed nitrogen to plants [8, 27, 28]. The direct stimulation mechanims can be attained through phytohormones, antagonism towards pathogenic microorganisms and mitigation of stresses [4, 11, 29]. On contrary, the harmful interactions have detrimental effects on plant growth and development [12, 30]. Therefore, understanding the beneficial plant microbial interactions can recognize both positive and negative impacts of microbes on plants.

Beneficial microbial communities correlated with plant ecosystems can be classified as follows; phyllospheric, endophytic and rhizospheric [10, 31, 32]. Phyllospheric (above ground) microorganisms are known to significantly contribute to nitrogen fixation as well as phytoremediation [7, 24]. Most of the phyllospheric microbes have been encountered on plant surfaces in moss forests and were shown to tolerate abiotic stresses due to UV radiation and high temperature (35–50°C) [33]. Phyllospheric microbes belongs to different species of diverse genera including, *Achromobacter, Acinetobacter, Agrobacterium, Arthrobacter, Azotobacter, Bacillus, Delftia, Erwinia, Methylobacterium, Pantoea, Psudomonas, Pseudobacterium, Phytomonas, Sarcina* and *Xanthomona*s [1, 3, 24, 25]. Furthermore, in blue green algae (BGA), species like *Anabaena, Calothrix, Nostic, Scytonema* and *Tolypothrix* were observed very beneficial for plant adaptation under low N conditions [34].

Endophytic microbiomes characterize another useful plant growth microbes that infiltrate vertically or horizontally into the internal plant tissue (eg., root, stem, flower, fruits and seeds) [4, 25]. As for bacteria, they are from a diversity of taxa that include; *Azoarcus, Achromobacter, Burkholderia, Nocardioides, Herbaspirillum, Pantoea, Klebsiella, Gluconoacetobacter, and Enterobacter* [26, 35–37].

Above all, the most superior plant-microbe interaction is of soil microbes with the root ecosystems (i.e., rhizophere microbiome) (*see,* **Figure 1**). Historically, Morgan et al. [31] cited Hiltner (1904) as the first person to discover and introduce the term rhizosphere. This term was derived from '*rhiza*' and '*sphere*', the Greek names which means root and field of influence, respectively. The rhizosphere is 'home' to a wide range of microorganisms, many of which are unquestionably

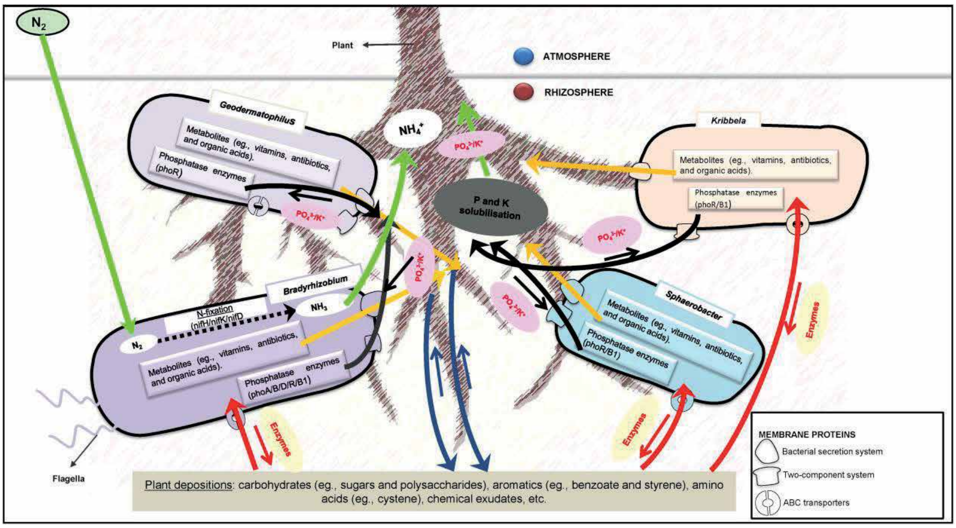

Figure 1.
Summary of putative PGP roles of the genomes reconstructed from A. dealbata rhizosphere metagenomes. Results are based on RAST and KAAS annotations of genome bins (≥ 70% completeness). Black arrows illustrate transference of phosphorus solubilizing enzymes from bacteria to the soil and the uptake of phosphate and potassium by the bacteria. Orange arrows denote conveyance of bacterial metabolites into the soil. Dark blue arrows depict plant depositions into the rhizosphere and re-uptake of organic nutrients by plant roots. Red arrows illustrate secretion of various enzymes involved in decomposition of plant deposits and uptake of organic nutrients by the bacteria. Light green arrows elucidate assimilation of nitrogen, phosphorus and potassium into the plant host (adapted from Kamutando et al. [38]).

beneficial to plants through preventing pathogenic infection and assisting in the acquisition of nutrients from the soil [31, 39, 40]. The taxonomic, genetic, and functional components of the rhizosphere microbiome must be understood in order to manage them for long-term crop productivity and agricultural sustainability under abiotic stress conditions [4, 6, 41]. Furthermore, the characterization of rhizosphere microbes as well as the determination of functional mechanisms mediating plant-microbe interactions has progressed in certain models, plant and non-plant species for sustainable agriculture [26, 29, 38, 42]. Considering the mobility and activity of the microbes in the rhizosphere, which is mediated by the rhizodeposits occurring around soil-root zone; *Methylobacterium, Pseudomonas, Serratia, Rhizobium, Paenibacillus, Erwinia, Enterobacter, Flavobacterium, Bacillus, Azospirillum, Burkholderia, Arthrobacter, Alcaligenes,* and *Acinetobacter*, have all been characterized and identified as rhizospheric microorganisms with plant growth promoting ability [6, 11, 29, 31]. These rhizodeposits include lost root cap, border cells, dead and lysed root cells, lost gases, passively and actively released solutes, gelatinous material from the mucigel; all stimulate microbial activity and enzyme production [9, 19, 43].

In establishment of the plant-microbe partnerships, both plants and microbe communicate through signals, such as volatile chemical chemicals, hormones and hormone mimics, as well as carbohydrate and protein-based signals [12, 40, 44]. For example, microbe and/or pathogen-associated molecular patterns (MAMPs or PAMPs) are carbohydrate-based and protein-based signals that are required for microbial survival [2, 30, 31].

3. Effect of abiotic stresses on plant-microbe interactions

Abiotic stress conditions are one of the most critical limiting factors for agricultural crops worldwide ([45], **Figure 2**). These harsh conditions include, low water availability due to salinity or drought, flooding, high or low temperatures, high or low soil pH, soil nutrient deficiency, and exposure to harmful chemicals including, toxic metals and organic pollutants [1, 4, 15, 26, 40, 47]. Impact of these stresses on food security is being studied more and more around the world and its effects were observed to be more on the disruption of metabolic homeostasis and pathways of the plant [5]. The habitats affected by these stresses may potentially generate a plethora of adapted PGP microbial strains that might be used as inoculants to alleviate the stress conditions [45].

For example, when exposed to abiotic stresses (e.g., heat stress), plant accumulate high levels of reactive oxygen species (ROS) in their tissues [9]. The ROS include hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{*-}), singlet oxygen ($^1O_2$) and superoxide radicles (O^{2-}), and these interact with the plant cell proteins, DNA as well as lipids, posing oxidative damage and cell malfunctioning [3, 48]. In addition, drought-induced moisture stress in the rhizosphere induces an increased plant respiration and carbon alterations assimilation [3, 47], which results in decreased ATP synthesis and an increase in ROS production; which have serious consequences on cellular metabolism [9, 12, 26, 49]. Under these circumstances, beneficial microorganisms can respond through synthesizing antioxidants and osmoprotectants [2, 19, 35].

Arid and semi-arid regions of the globe are more prone to salinity and drought stress conditions. Arid conditions are responsible for higher reductions of; crop productivity as well as arable land, especially in areas where sea levels are arising into agricultural lands [19, 29, 48, 50]. Plant growth is harmed by salinity in different ways [19]. From cereals to horticultural species, salinity can affect germination, plant vigor and crop productivity [21, 51, 52]. Nevertheless, it can lower nodulation,

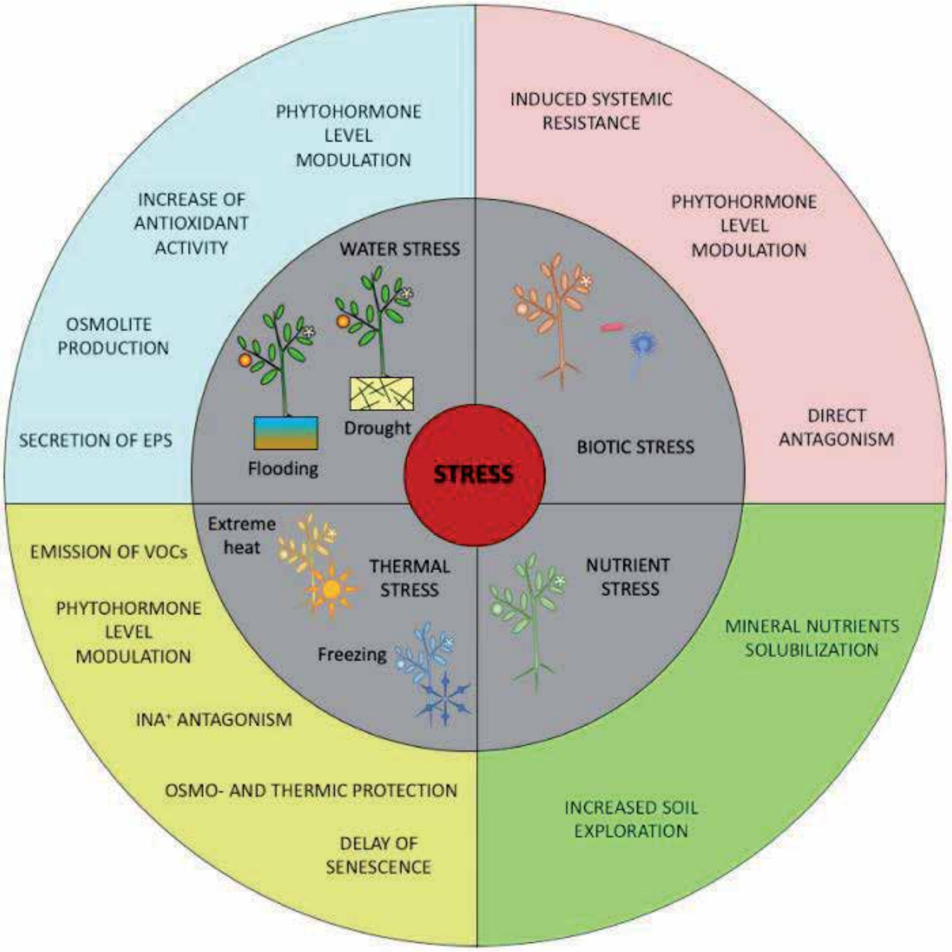

Figure 2.
Protective mechanism of plant microbial interactions under different levels of abiotic stress conditions. Exopolysaccharides (EPS), volatile organic compounds (VOCs), ice-nucleating activity (INA+). Sourced from Sangiorgio et al. [46].

nitrogen fixation and total nitrogen content in legumes, which has a negative impact on biological nitrogen fixation [1, 6, 9, 53]. Previous studies highlighted the sensitivity of symbiotic nitrogen fixation with global impact of salinity and drought stress, which occurs during both the development of symbiotic nodules and the succeeding period of nitrogen fixation and plant nitrogen intake [15, 29, 54].

In addition, presence of large levels of Na^+ and Cl^- on the roots modifies the functioning of uptake systems and changes the competitive interactions between ions for binding and transport into root cells thus limiting nutrient and water uptake [1, 45, 47]. In terms of nutrient uptake deficiency, this can be induced by saline conditions as Na and P uptake and accumulation is inhibited due to the formation of calcium phosphate precipitates which deter beneficial microbes away from its interaction with the plant roots [21, 51, 55]. Nonetheless, the performance of soil microbial enzymes such as nitrogenase, ureases or phosphatases can be inhibited, thus in turn, lowering biogeochemical cycling process [17, 53]. Also, non-calcareous soils of tropics were reported to harbor less beneficial bacterial taxa compared to neutral and calcareous soils of the deserts [45, 48]. A denaturing gradient gel electrophoresis profiling study of 16S rRNA by de Los Rios et al. [56] ascertained high microbial activity in desert conditions with high ability to survive hypersaline conditions. However, in general, abiotic stressors contribute to the visual appearance of sterile environment for the plant and microbial association [19, 40].

4. Beneficial plant-microbe interactions and sustainable agriculture

The principal factors that influence agricultural production by disrupting rhizosphere functioning are environmental pressures and their unpredictability [3, 20, 57]. A healthy plant rhizosphere not only aids in the provision of nutrients and water to plants, but it also gives long-term advantages to microbial diversity, which in turn aids plant health [22, 50]. The composition of root exudates, which account for microbial recruitment in the rhizosphere is mostly determined by plant genotypes [6, 29, 31]. These exudates, promote the multiplication of beneficial microbes of the plants at nanomolar concentration. Rhizobacteria, mycorrhizal fungi and other microbes are among the beneficial microbial diversity associated with the root zones that contribute to increased plant growth [4, 24, 48]. These beneficial microbes interact symbiotically or asymbiotically in promoting plant and soil health through a myriad of techniques including pathogen control, secondary metabolite synthesis and increased stress resistance [43, 45, 56, 58]. Also, many commercial products based on beneficial microorganisms or microbial consortiums, such as Subtilexfi (BeckerUnderWood, Inc., Ames, IA, USA), Kodiakfi (Gustafson, Inc., Plano, TX, USA), Biota Maxfi (CustomBio, Inc., Deerfield Beach, FL, USA), Trianum-Pfi (Koppert, Srl, Verona, Italy), express multiple functions and synergistic and additive effects on plant growth and development [46]. The various mechanisms in-with beneficial microorganism promote sustainable agriculture are detailed below.

5. Root-zone soil nutrient availability

Many of the nutrients required by plants are present in soil, but they are in insoluble precipitates or are bonded to inorganic and/or organic soil elements, making them unavailable to plants [7, 36]. Nutrient deficiency in plants is not only stressful, but can also increase the impact of other abiotic stresses. With the aid of plant-microbe associations, plant growth and development can be enhanced to sustain food production [6, 11]. Nutrient uptake activities facilitate this phenomenon [18, 59]. For instance, mycorrhizae interactions with plant roots increase the root surface area thus in turn improving efficient water and nutrient absorption form the bulk soil [9, 12, 57]. More than 80% of plant species form associations with glomeromycotan fungi which penetrate the root cortex and grow intercellularly before forming arbuscules [1, 45, 48].

Mycorrhizal fungi (AMF) are the main source for N, P and other mineral exchange (e.g., K, Mg, Cu, Zn and Fe) as initiated by the fungal hyphae in the soil [9, 15, 40, 60]. They enhance growth activities through symbiotic interaction with the host plants. Arbuscular mycorrhizae, ectomycorrhizae, ectendomycorrhizae, arbutoid mycorrhizae, ericoid mycorrhizae, monotropoid mycorrhizae, and orchid mycorrhizae are the main mycorrhizal classes studied which form symbiotic relationships with plants [6, 12, 33, 36, 43, 61, 62]. Their symbiotic relationship makes the host plants supply fixed C to AMF where in turn, greater nutrient uptake, drought and salinity tolerance, metal stress alleviation as well as resistance to pathogens and biotic stresses can be enhanced [45, 48, 57, 60, 61]. AMF such as *Glomus intraradices, Glomus mosseae,* and *Glomus caledonium* generally improve crop performance under drought conditions, alleviate salt and heavy metal-induced oxidative stress [45, 48, 59, 61].

Symbiotic microbes (e.g., rhizobia bacteria) migrate to the end of the absorbent hairs of the roots where they will attach, aggregate and respond to flavonoid signals excreted by the host root plant *via* the synthesis and excretion of lipochito-oligosaccharides (LCOs), called "Nod" factors [53, 61, 62]. Genetically, biological nitrogen fixation is controlled by three main genes; *nod, fix* and *nif* genes, and these are responsible for infection and nodulation as well as governing specificity for nodulation of specific species and specific genotypes, respectively [63–65]. *Fix* genes are specifically involved in the binding of N_2 and they include, *fixABCX, fixGHIS* and *fixKNR* [66]. Also, *nif* genes have three gene components (i.e., *nifH, nifD*, and *nifK*) code for polypeptide precursors of the two components of nitrogenase enzymes and facilitate N_2-binding process during BNF [53, 65].

Studies ascertained that about 60% copper (Cu), 25% nitrogen (N), 25% Zinc (Zn), and 10% potassium (K), were converted by microorganisms from their insoluble and/or organic forms into soluble and/or inorganic forms, available for plant uptake [1, 47]. The free-living nitrogen fixing PGP bacteria of the following genera; *Cyanobacteria, Azotobacter, Azospirillum* and *Beijerinckia* were reported to improve soluble sugars and electrolyte concentration in maize for its adaptation under drought stress [4, 45, 51, 57]. Considering N as the major nutrient required for plant growth and yield development, Morgan et al. [31] and Gupta and Sharma [57] revealed the implementation of 4R nutrient Stewardship for sustainable agriculture by exploiting biological nitrogen fixing microbes. Following the negative impacts of Green Revolution, anthropogenic N is drastically increasing and posing a cascade of large-scale problems on terrestrial and aquatic ecosystem [24, 33, 67, 68]. Biological nitrogen fixation (BNF) microbes were shown to be capable of contributing 180 x 10^6 t annually of in the world N, worthy approximately US$160-180 billion dollars [11, 15, 53, 61]. This finding justified the need of integrating BNF microbes for use as biofertilizers.

The intracellular PGP microbes (iPGP) form several nodule structures to improve N_2 fixation. For example, the endophytes like *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium* and *Rhizobium* of the family Rhizobiaceae, generally invade the root systems of crop plants to form nodules and all these contribute to N_2 fixation [13, 36, 37, 69]. In addition, extracellular PGP microbes (ePGP) including, *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia*, *Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas,* and *Serratia*, were also observed in close association with different plant species of leguminous species and a few cereals crop plants [4, 19, 45].

Globally, more than 90% of P in soil is unavailable for plant uptake [39, 70]. On contrary, P is being depleted in soils in spite of high chemical fertilizer uses [71]. The impact is brought by high weathering, prolonged warm and moist climatic conditions which deplete P sources, forming complexes with geochemical sinks like Calcium (Ca^{2+}), Aluminum (Al^{3+}) and iron (Fe^{2+}) ([60, 72, 73]. This immobilized P is metabolized by plants and microbial communities through the secretion of enzymes with high biological relevance, which are still almost exclusively recovered from cultured organisms [26, 56, 71]. More specifically, phosphate solubilizing microorganisms (PSMs) release phosphatase enzymes and organic acids and these activate innumerable biogeochemical processes which contribute to lowering of soil pH and in increasing chelation activities with additional P adsorption sites [74, 75]. Phosphatase enzymes are the central to the processing and stabilization of nutrients as well as P nutrient cycling in terrestrial ecosystems [57, 70]. They dephosphorylate phosphoester or phosphoanhydride bonds of both phosphoric acid (H_3PO_4) and organic matter [75, 76], thus producing soluble phosphate (mainly, PO_4^{3-}, HPO_4^{2-}, and $H_2PO_4^{-}$) which can be taken up by the plants and microbes. Increased activity of phosphatases occurs in response to P deficiency as part of P starvation responses

[39, 77, 78]. PSMs belong to various bacterial genera such as; *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* and these are highly associated with plants for P solubilization and mineralization [2, 19, 26, 61].

Apart from N and P, PGP microbes interact beneficially with plants in making K, Fe, Zn and sulfur (S) available for plant uptake. Studies revealed that more than 90% of K in the soil exists in insoluble forms (i.e., micas, illite, and orthoclases) [13, 79]. *Pseudomonas, Burkholderia, Acidithiobacillus, Bacillus* and *Paenibacillus* are known to use organic acids to solubilize K for plant uptake [13, 47, 50]. A strong correlation was observed between K solubilizing bacteria and eggplant grown under limited K soils [80]. Sattar et al. [22] and Verma et al. [69] postulated that K-solubilizing bacteria caused high productivity of food and/or feed crop production by enhancing nutrient uptake efficiency.

As for iron (Fe), soil pH changes towards alkaline conditions contributes to the conversion of Fe^{++} to Fe^{+++}, thus making it difficult for the plant uptake [11, 15, 18]. Plant-microbe associations that initiate efficient siderophore molecule synthesis for Fe chelation include *Pseudomonas, Burkholderia, Enterobacter* and *Grimontella* [45, 47, 80]. On the other hand, *Klebsiella*, *Stenotrophomonas, Rhizobium, Herbaspirillum* and *Citrobacter* are also known to generate siderophores in small quantities [6, 13]. A study by Wang et al. [81] and Fan et al. [37] ascertained that inoculating *Arabidopsis thaliana* plants with *Bacillus amyloliquefaciens* results in increased iron absorption.

In terms of Zn nutrient availability and its uptake, *Bacillus, Pseudomonas, Rhizobium, Burkholderia, Acinetobacter, Mycobacterium, Stenotrophomonas, Enterobacter* and *Xanthomonas* are among the bacteria identified as Zn solubilizers under Zn limited soils [82, 83]. In a wheat study by Shaikh and Saraf [84], inoculation of the crop with *Exiguobacterium aurantiacum* strain revealed 6-fold increase in Zn and Fe to 18.2 ppm and 24.67 ppm in grain, respectively which signifies its potential use as biofortification. For S, PGP oxidizing microbes convert unavailable S to available form for plant uptake through the synthesis of arylsulphatase enzymes [4, 15, 85].

6. Withstanding climate-induced abiotic stresses

Yadav [25] and Song et al. [68] revealed that extremophilic bacteria (e.g., psychrotrophiles and thermophiles) are linked to crop growth in harsh agro-ecosystems. For example, beneficial microbes that interacts with roots of calcareous and/or non-calcareous-growing plants were identified and these include; *Azotobacter*, *Flavobacterium, Bacillus*, *Bifidobacterium, Burkholderia, Methylobacterium, Pseudomonas, Proteobacteria* and *Serratia* [12, 26, 30]. In terms of soil salinity, adaptation of plants flourishing under hypersaline settings was shown to be aided by halophilic bacteria and haloarchaea [21, 51, 55]. These halophilic or halotolerant microbiomes aid PGP and adaptability to hypersalinity's abiotic stress [25, 40, 43, 48]. Additionally, *Paenibacillus yonginensis* DCY84^T strain was identified to efficiently protect ginseng plants of China under salinity stress conditions [86]. In the same study, high proline production was shown to be strongly associated with high transcription levels of *PgP5CS* gene that codes for salinity tolerance [87].

7. Phytohormone production as a stress-adaptation mechanism

Hormones like auxins, cytokinins (CK) and gibberellic acid (GA3) have a pivotal role in plant microbial signaling and plant growth [48, 68, 88]. These hormones contribute to BNF of rhizobia-plant interaction. For instance, cytokinins facilitate nodule organogenesis which has been exploited in diverse legume crops [14, 89]. In previous studies, exogenous cytokinin successfully stimulated amyloplast accumulation, cortical cell division and expression of early nodulation markers [4, 86].

Auxin producing PGP microbes like *Azospirillum, Acetobacter, Alcaligenes, Bradyrhizobium, Enterobacter, Pseudomonas, Rhizobium* and *Xanthomonas* synthesize indole-3-acetic acid (IAA) for sustained plant development [14, 32, 90]. In addition, *Pantoea sp., Marinobacterium sp., Acinetobacter sp.,* and *Sinorhizobium sp*., with potential to synthesize IAA, have been found to have a considerable impact on wheat germination and seedling growth in saline conditions [19, 21, 33, 87]. In salinity-stressed barley and oats, *Pseudomonas sp*. and *Acinetobacter sp.* have also been reported to boost IAA production [4, 6, 48, 86]. A strong association between IAA and GA3 producing microbes has been revealed, where both induce the proliferation of root architecture under abiotic stress conditions [45, 47, 69, 87, 88].

Nevertheless, increased quantities of endogenous GA3 in PGPR *Burkholderia cepacia* SE4-, *Promicromonospora spp.* SE188- and *Acinetobacter calcoaceticus* SE37-treated cucumber plants, increased plant growth under drought and salinity stress ([87, 88]. Also, drought tolerance in soybean plants was improved by *Pseudomonas putida* H-2-3 which secretes GA3 (Kang et al., 2014; Goswami and Deka, 2020). Transgenic canola plants were assessed for their response to submergence tolerance [91]. The findings showed that the plants expressed the *acdS* gene from *P. putida* UW4 under the control of the root-specific *rolD* promoter from *Agrobacterium rhizogenes*.

Cohen et al. [92] investigated the effects of *Azospirillum brasilense* Sp 245, an abscisic acid (ABA)-producing bacteria, on *A. thaliana* Col-0 and aba2-1 mutants. Results showed improved tolerance which was linked to increased IAA production in *Azospirillum* cells due to overexpression of the indole-3-pyruvate decarboxylase gene [14, 37, 93]. The study discovered that priming the two mutant plants with Sp 245 boosted the amounts of endogenous ABA under drought stress. Apart from increased chlorophyll content and water potential in drought-stricken plants, ABA was also involved in root growth, increased plant biomass and other required plant adaptations under drought stress conditions [94]. By inoculating soybean roots under drought stress circumstances in South Korea, Park et al. [95] investigated the efficacy of inoculating *Bacillus aryabhattai* SRB02 in producing gibberellin and other associated phytohormones associated with improved plant growth and development. Also, Ghosh et al. [96] ascertained that inoculating *A. thaliana* seedlings with GA3-auxin-CK-producing *Pseudomonas aeruginosa* PM389, *P. aeruginosa* ZNP1, *Bacillus endophyticus* J13 and *Bacillus tequilensis* J12 strains individually, resulted in a significant increase in plant performance under drought conditions.

Similar to IAA, bacterial 1-aminocycloprapane (ACC) deaminase aids in the relief of drought stress and the development of drought tolerance in plants [45]. The hormone lowers ethylene production levels which has negative effects on plant metabolic activities [88]. ACC deaminase enzymes cleave plant ethylene precursor thus promoting adaptability of plants to both biotic and abiotic environmental stresses [45, 47, 48]. Rice rhizobacteria which were found to harbor ACC deaminase were also found to be efficient in increasing salt tolerance and, as a result, promoting the growth and development of rice plants under salt stress [97]. ACC deaminase-producing rhizosphere-colonizing bacteria like *Achromobacter xylosoxidans* Cm4, *Pseudomonas oryzihabitans* Ep4 and *Variovorax paradoxus* C2, improve potato plant development and yield by reducing amino acid levels in the root zone [98].

Furthermore, using exopolysaccharides (EPS)-rich *Azospirillum* strain AbV5 in combination with polyhydroxybutyrate (PHB) increased maize output by 80% in nutrient stressed field trials, revealing a method for bio-inoculating non-legume crops with diazotrophic bacterial species [3]. The water holding capacity (WHC) of microbial EPS makes them even more important in water-stressed environments, where its presence can efficiently provide enough moisture supply in the rhizosphere [4, 9, 45]. With reference to Sandhya et al. [99], Sandhya and Ali [16] and Ghosh et al. [96], the *P. putida* strain GAP-P45, produced more EPS thereby indicating thermo-tolerance of the strain when exposed to abiotic stresses such as heat stress (≥50°C), drought stress (−0.73 MPa) and salt (1.4 M). The composition of polysaccharide chains varies by species; however, recurring monomers in polysaccharide chains include units of glucose, galactose, mannose, rhamnose, uronic acids (glucuronic and galacturonic acids), amino-sugars (N-acetylamino sugars), organic ester-linked substituents and pyruvate ketals [100–102]. EPS producing microbes are important because these strains can be used as biofertilizers in soil conditions that are under abiotic stress.

8. Functional roles of microbes in bio-control, phytoremediation soil renovations

The use of biocontrol agents in management of phytopathogens can contribute to an increased food yield and quality [44, 103]. These phytopathogens include nematodes, insects, bacteria, fungi, protozoa and viruses [41]. Some of the studied PGPR genera which act as biocontrol agents include; *Agrobacterium, Arthrobacter, Azoarcus, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Micrococcus, Rhizobium, Pantoea, Pseudomonas* and *Serratia. Pseudomonas* and *Bacillus* are highly preferred among them, because of their aggressiveness as invaders of the rhizosphere and have broad-spectrum antagonistic actions [41, 104]. *Bacillus sp.* had an antagonistic capacity against *Botrytis cinerea;* thus, minimizing nutrient competition [105]. The synthesis of salicylic acid, pyochelin and pyoverdine from siderophore-producing microbes suppresses illness by limiting pathogens' access to critical trace minerals in the rhizosphere [11]. *Bacillus subtilis* CAS15, has a biocontrol effect on *Fusarium wilt* of pepper and watermelon under controlled conditions, thus enhancing plant growth and development [11, 52, 106, 107].

Nevertheless, there are essential volatile and non-volatile organic compounds which have antagonistic effects against pests and pathogens [105, 107]. The volatile compounds include hydrogen cyanide, aldehydes, alcohols, ketones and sulfides whilst non-volatile ones have polyketides such as diacetylphloroglucinol (DAPG) and mupirocin [4, 8]. In addition, there are heterocyclic nitrogenous compounds such as phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN) and hydroxy phenazines; all these compounds contribute to sustainable biotic stress management [4]. As previously highlighted, the EPS-producing *Pseudomonas* strains were further reported as bio-control agents which increased sunflower crop productivity by defending it against the disease, *Macrophomina phaseolina* [108]. In the same study, the efficacy of salicylic acid (SA) which is produced by fluorescent pseudomonads in fungal phytopathogen biocontrol was also exhibited.

In other studies, potential of enzymes produced by PGP microbes with antagonistic mechanisms was assessed [24, 31]. Some studies showed that synthesis of enzymes guard against phytopathogens *via* induced lysis of their cell walls, which is accomplished by secreting hydrolytic enzymes like chitinases, glucanases, proteases, and lipases [4, 48].

Kamle et al. [109] reported the effect of induced systemic resistance (ISR) and systemic acquired resistance (SAR) on stress management by plants. These mechanisms can be initiated by microorganisms or chemical stimulants. For instance, lipopolysaccharides (LPS), siderophores, cyclic lipopeptides, DAPG, homoserine lactones and volatiles such as acetoin and 2, 3-butanediol, are some of the chemical elicitors produced by PGPR strains to trigger ISR and SAR [52, 107]. Bacterial species including; *Pseudomonas, Bacillus, Serratia* and *Azospirillum* have been reported as ISR inducers [45, 47, 48]. Conversely, SAR is triggered by the pathogenesis-related proteins like SA where it acts directly on invading cells to lyse them, reinforce cell wall borders to prevent infections, or cause localized cell death [4, 109].

Presently, more than 30% of arable land has been projected to be degraded by the year 2025 [49]. This is widely attributed to the negative effects of the Green Revolution [50]. The puzzling divergence of decreased fertile soil in relation to booming demography, pollution and perturbation of natural resources contribute to the drastic narrow range of soil microbial diversity and its activity [110]. EPS-producing microbes highlighted by Sandhya and Ali [16] and Ghosh et al. [96] were also reported to have contributed significantly to phytoremediation and/or land restoration. For example, these microbes react with cations like Na+, and due to the quorum sensing technique, rapid multiplication of EPS producing microbes occurs within the rhizosphere with the ability to decrease Na + availability [3]. This reaction was based on high sensitivity, tolerance, and the sequestration ability of the microbes against pollutants, as well as biotic and abiotic stress conditions [4]. With reference to Mishra and Arora [41], heavy metal pollutants in soils, with concentrations ranging from 1 to 100,000 mg/kg can be eliminated mostly *via* soil bacteria geoactive behavior [111]. Bacillus strain reported by Dary et al. [112] proved to have remediation power against copper (Cu), cadmium (Cd), and lead (Pb), and was also proven to contribute significantly to increased biomass of the *Lupinus* plant.

9. Climate change impacts on plant-microbe interactions

Climate challenges like increased CO_2 levels in the atmosphere, rising global temperatures and drought have had a global impact on plant and microbial ecology and physiology [90]. Since plants transfer some of the assimilated carbon to feed related microbial populations, a disruption in the C assimilation pathway as a result of climate change would have a significant impact on plant microbial interactions [45, 46]. Climate change has impacted on the interactions and dynamics of the plant-microbe responses, as well as, on the microbial communities associated with plants, thereby affecting their establishment and performance in regulating soil N and C dynamics [9, 29].

Under rising and/or elevated temperatures, species are migrating to higher elevations (altitudes) and latitudes [110]. As a result, early leafing and flowering time in the growing season have been discovered to modify the reproductive physiology of the host plant under the warming effect; thus altering plant phonological trait performances as well as multiple properties of the ecosystem [90, 110]. In terms of elevated CO_2 concentration, maximum biomass accumulated on both C_3 (45%) and C_4 (12%) plants has been observed as influenced by the changing climatic conditions, which initiate variations in C partitioning and distribution [29]. The differences in biomass accumulation levels reported between C_3 and C_4 plants could be attributed to the host's connection with beneficial microorganisms, especially arbuscular mycorrhiza (AM) fungi [94].

To profit from AMF, C_4 plants transfer more C to these fungi, and hence the selection force favors AM fungal growth over biomass accumulation by C_3 species in the case of C_4 plants [90, 113]. In another study, drought conditions impacted on plant growth and development such that photoassimilates partitioning were more biased towards rhizospheric microbes and AMF in the soil [110].

The relative abundances and diversity of microbial communities in soil can be used to infer the direct impact of climate change on microbial activities, response mechanisms and functional profiles [46, 110]. Furthermore, the disparity in behavior could be attributed by their differing growth rates and temperature sensitivity. As evidenced by microbial respiration, decomposition processes and C release from the soil, the influx of carbon to the soil has an impact on the activity and dynamics of microbial communities [90]. Explicitly, enhanced decomposition would produce a substantial amount of greenhouse gasses (GHGs), resulting in increased CO_2 efflux in the atmosphere and dissolved organic C export via the hydrologic leaching process as well as other physiological characteristics [114].

10. Future outlooks

Climate change impacts on plant-microbe, microbe-microbe interactions, as well as ecosystem functions are still poorly understood. One of the key limitations in this context is that the impact of global climate change has not been exploited further in plant-microbe interactions, yet this knowledge is key for harnessing beneficial plant-microbe interactions in promotion of crop productivity under climate change-induced abiotic stresses. For instance, understanding of cycles and doses of radiation exposure may impact the range of gene functional strategies viable in the soil. The functional potential of the electromagnetic radiation which could be strongly related to soil pH, total nitrogen, and organic matter can explain the effects of radiation on the phenological and genetic diversity of microbial populations as well as its interaction mechanisms (e.g., co-occurrence networks).

11. Conclusion

Climate change is expected to continue posing abiotic stresses globally, and if current trends continue, many parts of the planet will become hostile to agriculture. Investments in exploring and harnessing beneficial plant-microbial interactions for enhanced plant production under abiotic stresses as well as efficient agricultural production systems are required to ensure future food and nutrition security in the face of climate change. Stable and/or well adapting soil microbes and their mechanisms can thrive in challenging situations. This is associated to plant-microbial interactions ability which can contribute positively to root-zone soil nutrient availability as it can withstand climate-induced abiotic stresses. Also, plant-microbial interactions towards phyto-hormone production as a stress-adaptation mechanism as well as bio-control and phytoremediation soil renovations attributes can counterbalance any climate-related challenges. Therefore, plant-associated microorganisms have a principal important role in aiding productivity of plants under abiotically-constrained environments. For sustainable agriculture, developing microbial tools and technologies to exploit the beneficial plant-microbe-soil interactions is paramount.

Author details

William Makaza[1,2] and Casper Nyaradzai Kamutando[1]*

1 Department of Plant Production Sciences and Technologies, University of Zimbabwe, Harare, Zimbabwe

2 School of Agricultural, Fertilization and Environmental Science, Mohammed VI Polytechnic University, Benguerir, Morocco

*Address all correspondence to: kamutandocn@gmail.com

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Chapter 10

Responses of Spinach (*Spinacia oleracea* L.) to Acidic Saline Soils as Affected by Different Amendments

Sajal Roy and Nasrin Chowdhury

Abstract

Soil salinity impedes the normal growth of plants by a number of mechanisms, including osmotic stress and imbalance absorption of essential nutrients. The present study focused on holistic approaches to the production of spinach (*Spinacia oleracea* L.) in clay loam acidic saline soils. In connection with this, spinach was grown in soils with two salinity levels (hereinafter referred to as soil A: high salinity and soil B: extreme salinity) in the presence of vermicompost (VC), wood ash (WA), and zeolite (ZL) applied at the rates of 1% and 2% (w/w) both alone and in combination along with N-P-K fertilizer. Results indicated better growth as well as the uptake of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sodium (Na) over control with significant ($p < 0.01$) differences when VC and WA were applied in combination. The $C_{\text{shoot}}/C_{\text{root}}$ quotient of N, K, Ca, Mg, and Na was found greater than 1, whereas P was observed lower than 1. The Na: K, Na: Ca, and Na: Mg ratios were found to be highest in control that differed significantly ($p < 0.01$) from the rest of the amended soils. The present study suggests the combined application of VC and WA at the rate of 1% before cultivation to influence soil nutrient dynamics and plant growth in saline soils with acidic soil reactions.

Keywords: saline soils, spinach, vermicompost, wood ash, zeolite

1. Introduction

Saline soils contain substantial quantities of soluble salts giving electrical conductivity (EC) greater than 4 mS cm^{-1} at saturation extract [1, 2]. More than 1100 million hectares (mha) of lands worldwide are occupied by salt-affected soils, which are expected to rise due to several natural and anthropogenic factors [3–6]. In Bangladesh, saline soils are now estimated at 1.06 mha, which is about 26.7% higher compared to the past estimate of 0.83 mha in 1973 [7]. Soil salinity impedes the normal growth of plants by a number of mechanisms including osmotic stress, imbalance absorption of essential nutrients such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg), and toxicities of sodium (Na) and chlorine (Cl) [8, 9]. However, crop production in salt-affected areas is not impacted in the same manner as the degree and extent of salinity are minimum in monsoon during the months of July to August and reaches the maximum in dry periods during the months of March to April. The increase in the degree and extent of soil salinity in most of the periods of the year has raised the challenge of farmers on how to grow high-value and short-duration crops during cool and dry winter months of the year in the coastal regions of Bangladesh. Spinach (*Spinacia oleracea* L.) is a widely cultivated and consumed popular green leafy dietary vegetable which allows multiple short-duration production cycles (30–48 days) [10, 11]. Besides, spinach is a salt-tolerant and cool-season crop [12, 13] that grows best in slightly acidic to slightly alkaline (pH 6.0–7.5) soil reactions [14].

The application of different amendments has widely been practiced over the few years for the improvement of physico-chemical properties of soils toward sustainable production of crops [15–18]. Vermicompost (VC) is efficient, cost-effective, environment-friendly and sustainable organic fertilizer [19] that can be prepared by composting a variety of organic substances including animal dung, municipal sewage sludge, and domestic waste [20]. On the other hand, wood ash (WA) is the solid by-product of wood incineration and is utilized in soils as an acid-neutralizing material [21]. Besides, zeolite (ZL), an aluminosilicate, is characterized by large sorption and ion-exchange capacity because of its three-dimensional framework [22]. Although the effects of the aforementioned amendments have been studied separately on plant growth, their combined effects are rarely understood. Moreover, WA has not been studied yet as an acid-neutralizing alternative material to reclaim acidic saline soils. Therefore, the objectives of the present study were to study the effects of VC, WA, and ZL applications alone and in combinations on the performance of spinach in two different saline soils having an acidic reaction.

2. Material and methods

Spinach was grown under the influence of different amendments (e.g., VC, WA, and ZL) applied alone (i.e., VC, WA, and ZL) and in combinations (i.e., VC + WA, VC + ZL, and WA + ZL) at 1% (w/w) and 2% (w/w) rates in soils with high salinity (hereinafter mentioned as soil A) as well as extreme salinity (hereinafter mentioned as soil B) after being subjected to leaching with 2 pore volume (PV) of water.

2.1 Collection and processing of soils and amendments

Bulk soil samples from the top layer (0–15 cm) were collected from two different locations having two different levels of salinity (soil A: high salinity and soil B: extreme salinity). The physical and chemical parameters of soil A and soil B, which were analyzed before setting up for experiments are given in **Table 1**. After bringing to the laboratory, soil samples were dried at room temperature for few days, hard clods were broken with a wooden a hammer followed by sieving through 4 mm stainless steel mesh for the pot experiment. Representative subsamples were separated from the 4 mm sieved bulk soil samples and passed again through 2 mm stainless steel mesh for the initial analyses of the physical and chemical parameters.

Vermicompost of cow manure (hereinafter referred to VC) was collected from a VC producing farm and calcium-type ZL ($CaAl_2Si_4O_{12}·nH_2O$) was collected from a market distributed by National Agricare, while WA was prepared by burning of woods in mud stoves. All the amendments were passed through a 2 mm stainless sieve. The properties of amendments are given in **Table 2**.

Parameters	Soil A	Soil B
pH	5.01	5.22
ECe (mS cm^{-1})	9.25	37.64
Organic carbon (%)	1.20	1.11
Organic matter (%)	2.07	1.91
Sand (%)	34.0	31.0
Silt (%)	39.0	42.0
Clay (%)	27.0	27.0
Textural class	Clay loam	Clay loam
Available N (mg kg^{-1})	63.54	57.86
Available P (mg kg^{-1})	2.56	5.99
Available K (mg kg^{-1})	145.35	433.64
Available Ca (mg kg^{-1})	583.33	850.00
Available Mg (mg kg^{-1})	590.00	1610.00
Available Na (mg kg^{-1})	782.24	1797.08

Table 1.
Physical and chemical characteristics of soil A and soil B.

Parameters	VC	WA	ZL
pH	7.89	11.77	7.47
EC1:10 (mS cm^{-1})	1.92	11.27	3.09
Organic carbon (%)†	18.06	0.17	0.17
Total N (%)†	2.01	0.1	0.1
Total P (%)†	0.49	1.16	0.12
Total K (%)†	2.34	4.13	1.81
Total Ca (%)†	2.56	10.4	3.04
Total Mg (%)†	0.42	1.28	0.88
Total Na (%)†	—	0.11	0.47

†*On dry weight basis.*

Table 2.
Characteristics of amendments used in the present study [23].

2.2 Set-up of the pot experiment

In our previous pot experiment without incorporation of any amendment indicated strong inhabitation of germination of spinach seeds by extreme soil salinity (soil B), while leaching with 2 PV of non-saline water resulted in germination and growth of spinach [23]. From the knowledge of previous pot experiments, extreme saline soils (soil B) amended with VA, WA, and ZL as single and in combination (i.e., VC + WA, VC + ZL, and WA + ZL), both at 1% (w/w) and 2% (w/w) rates were subjected to leaching with 2 PV of water using the Eq. (1) [24]. In this regard, the PV was adjusted to 925 cm^3 for all the pots. The characteristics of the water used for the leaching of soils are given in **Table 3**.

$$PV = Vs \times \Phi s \qquad (1)$$

where,

Vs is the volume of the soil in the column and

Φs is the porosity

For the cultivation of spinach, plastic pots with 17 cm height and 13 cm of average diameter were taken. An amount of 2 kg soil (dry weight basis) was placed in each plastic pot having holes in the bottom. The required amount of amendments was weighed in a balance. The amendments were mixed homogeneously with previously weighted soil samples. There was a set of control where neither N-P-K fertilizer nor any amendment was applied. The application of different amendments yielded a total number of 14 treatments (**Table 4**). All the treatments were taken in triplicates. The pots were arranged in a completely randomized way. The recommended dose of N-P-K (60–18-0 kg ha^{-1}) fertilizer was applied in both soil A and B as basal doses [25].

Parameters	Properties
pH	6.70
EC (mS cm^{-1})	0.12
Total K (mg L^{-1})	2.85
Total Ca (mg L^{-1})	4.10
Total Mg (mg L^{-1})	3.18
Total Na (mg L^{-1})	3.37

Table 3.
Characteristics of water used for leaching of soil B and irrigation in pot experiment.

Treatment legend	Treatment description
T1	Non-amended soil (control)
T2	Soil + N-P-K
T3	Soil + N-P-K + VC (1%)
T4	Soil + N-P-K + WA (1%)
T5	Soil + N-P-K + ZL (1%)
T6	Soil + N-P-K + VC (1%) + WA (1%)
T7	Soil + N-P-K + VC (1%) + ZL (1%)
T8	Soil + N-P-K + WA (1%) + ZL (1%)
T9	Soil + N-P-K + VC (2%)
T10	Soil+ N-P-K + WA (2%)
T11	Soil + N-P-K + ZL (2%)
T12	Soil+ N-P-K + VC (2%) + WA (2%)
T13	Soil+ N-P-K + VC (2%) + ZL (2%)
T14	Soil + N-P-K + WA (2%) + ZL (2%)

Table 4.
Treatment legends and their description for the pot experiment.

2.3 Sowing and harvesting of plants

Spinach seeds were soaked in water for 12 hours before sowing in the soil. Twelve seeds were sown in each pot. The seedlings were later thinned and five vigorous plants were maintained in each pot until harvesting. The soil in each pot was watered with water to maintain soil moisture roughly constant at 60 ± 5% of WHC. It is worth mentioning that the quality of irrigation water is important as irrigating with saline water can worsen the condition of soil salinity [26]. The water used for irrigating plants is classified as non-saline (**Table 3**) on the basis of EC value [27].

Plants were harvested manually at 35 days of growth after outpouring the soils on the floor so that root damage can be minimized during the uprooting of plants. The plants were cut at the root-shoot junction to separate the plants into two portions. At harvest, some measurements such as fresh weight of shoot and root were taken.

2.4 Processing of harvested plants

The shoot and root were washed first with tap water several times to remove adhering soil particles and then with distilled water. The shoot and root were lapped between tissue papers to absorb excess water adhering to plant parts. After a few days of air drying, the plant parts were separately put in envelopes and allowed to dry in an oven at 65 ± 5°C for 72 hours. After drying to constant weight, plant parts were weighted to determine the moisture content. Oven-dried root parts were ground manually, while shoot parts were ground to a fine powder with an electrical grinder and sieved through a 0.5 mm stainless sieve for chemical analyses [28].

2.5 Methods of analysis

The pH and EC of all the soil samples were measured by pH meter (Seven CompactTM pH/Ion S220) and EC meter (Adwa AD 330) meter after preparing the suspension at 1:5 soil to water ratio (w/v). The pH and EC of VC and WA were measured at a 1:10 ratio (w/v) [29]. The $EC_{1:5}$ of soil samples was converted to ECe by multiplying a conversion factor as mentioned in Hazelton and Murphy [30]. The particle size of soil was analyzed by the hydrometer method as described in Huq and Alam [28]. The contents of organic carbon were determined by Walkley and Black wet oxidation method and converted to organic matter by multiplying the organic carbon content by the *Van Bemmelon factor* of 1.724 [31]. Available N (NH_4^+-N) and available P in soils were extracted by standard methods as given in Keeney and Nelson [32] and Gupta [33], respectively. The concentrations of exchangeable Na, K, Ca and Mg were extracted with 1 N NH_4OAc (pH 7.0) at 1:5 ratio (w/v) [34]. For the analysis of total concentrations of elements in plants and amendments, a suitable amount of the samples was first digested by a digestion mixture solution as mentioned in Parkinson and Allen [35].

The available and total N concentrations were determined by collecting NH_3 which was liberated by distillation of the extract and/or digest with strong alkali (usually 40% NaOH) containing boric acid- mixed indicator in an Erlenmeyer flask and the titration of the distillate with $p < 0.01$ N H_2SO_4 [36]. The available P in the extract and total P in the digest was measured by the ascorbic acid blue color method and vanadomolybdo yellow color method using UV–visible spectrophotometer at a wavelength of 880 nm and 490 nm, respectively [28]. The contents of Na and K in extract and digest were measured by atomic absorption spectrometer (Agilent Technologies 200 Series AA), whereas Ca and Mg concentrations were determined by the ethylene di-amine tetra acetic acid (EDTA) method as described in Huq and Alam [28].

2.6 Analysis of data

The uptake of elements in spinach was determined by multiplying the concentration of that element with the dry mass of plant parts [37].

$$Uptake = Concentration\ in\ shoot\ or\ root \times dry\ weight$$

The shoot-root quotient (C_{shoot}/C_{root} quotient) was calculated to estimate the transfer of the element from the underground part to the aerial part by using the following formula [38].

$$Cshoot\ /\ Croot\ quotient = \frac{Cshoot}{Croot}$$

Where,

C_{shoot} = Concentration of the element in shoot.

C_{root} = Concentration of that element in root

Pearson's correlations and Duncan's Multiple Range Test (DMRT) were performed by using statistical packages for social sciences (SPSS) to measure significant differences between pairs of means of the obtained results. Standard deviations were determined using Microsoft Excel 2016.

3. Results and discussion

The performance of spinach grown in soil A and soil B in the presence of different organic and inorganic amendments applied alone and in combination at two different rates (i.e., 1% and 2% w/w) was evaluated in response to yield,

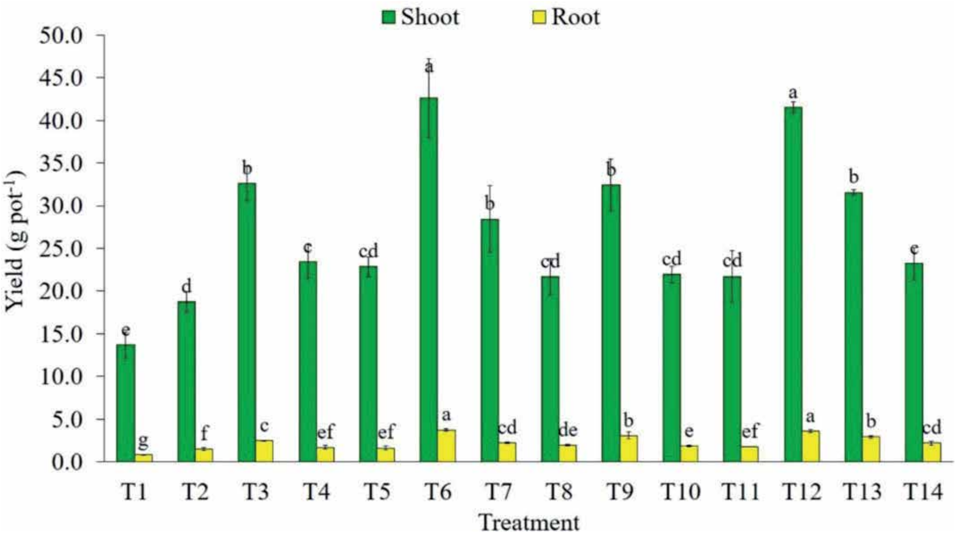

Figure 1.
Fresh weight (g pot^{-1}) of different parts of spinach grown in soil A. Mean (s) followed by the same letter within the bar do not differ at 5% level of significance. Treatment legend description is given in ***Table 4****.*

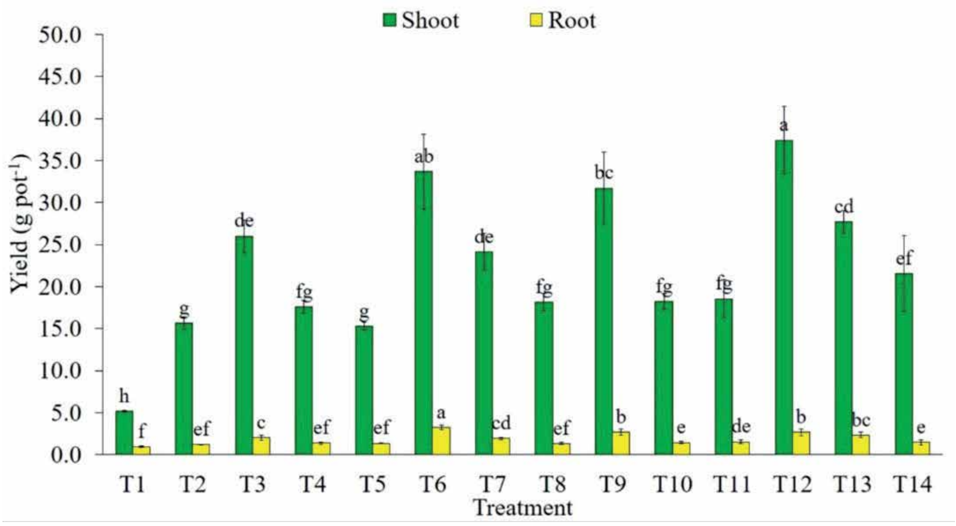

Figure 2.
Fresh weight (g pot^{-1}) of different parts of spinach grown in soil B. Mean (s) followed by the same letter within the bar do not differ at 5% level of significance. Treatment legend description is given in ***Table 4***.

concentration, and uptake of several elements. In addition, the $C_{\text{shoot}}/C_{\text{root}}$ quotient and different ratios among Na, K, Ca, and Mg are presented sequentially.

3.1 Yield of spinach

The fresh weight of shoot and root of spinach grown in soil A and soil B is shown in **Figures 1** and **2**, respectively. The application of amendments had a significant effect ($p < 0.01$) over control on the growth and yield of spinach. The fresh weight of shoot and root of spinach grown in both soil A and soil B increased significantly ($p < 0.01$) when VC and WA were applied in combination either at a 1% or 2% rate. The fresh weight of shoot and root of spinach grown in soil A was found 212.14% and 350.81% higher in T6 in comparison to T1. In soil B, the fresh weight of shoot in T12 increased by 622.67%, whereas of root in T6 increased by 235.17% compared to T1. When the biomass of spinach growing in soil A and soil B was compared (**Figures 1** and **2**), the adverse effect of salinity was found to be highest in control and lowest in amended soils especially when VC plus WA were applied in combination. The fresh weight of the shoot decreased by 2.30–62.08%, while of root decreased by 12.02–33.74% when grown in soil B compared to soil A.

3.2 Concentration, uptake and $C_{\text{shoot}}/C_{\text{root}}$ quotient

The concentrations of N, P, K, Ca, Mg and Na in spinach grown in soil A and soil B are shown in **Tables 5** and **6**, respectively. There was a significant variation ($p < 0.01$) among the treatments in the concentration of N and K in shoot and root for both soil A and soil B. The application of amendments also increased the concentration of P, Ca, and Mg with significant differences both for the shoot ($p < 0.01$) and root ($p < 0.01$) of spinach grown in both soil A (**Table 5**) and soil B (**Table 6**). The maximum concentrations of P, Ca and Mg in both shoot and root were found when VC along with WA was incorporated in soils. The application of amendments resulted in significant variation in the concentration of Na in the shoot ($p < 0.05$) and root ($p < 0.01$) of spinach grown in soil A (**Table 5**). In soil A, the concentration of Na in the shoot decreased by 24.61% in T3 relative to T1, whereas

Treatment	N		P		K		Ca		Mg		Na	
	Shoot	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**
T1	2.02^{d}	1.11^{h}	0.35^{e}	0.85i	1.89^{g}	1.11^{h}	0.45^{g}	0.27^{g}	0.87^{h}	0.35^{g}	1.39^{ab}	1.09^{a}
T2	3.01^{abc}	2.19^{cd}	0.57^{cd}	0.94^{ghi}	2.74^{f}	1.41^{g}	0.51^{fg}	0.30^{fg}	1.05^{fgh}	0.40^{fg}	1.35^{abc}	0.81^{de}
T3	3.34^{a}	2.14^{d}	0.72^{bc}	0.92^{hi}	3.41^{abcde}	1.86^{f}	0.54^{f}	0.38^{d}	1.21^{cdef}	0.50^{e}	1.05^{d}	0.71^{fg}
T4	3.38^{a}	2.63^{a}	0.60^{cd}	1.02^{fgh}	3.40^{abcde}	2.49^{ab}	0.55^{ef}	0.42^{c}	1.15^{defg}	0.65^{ab}	1.25^{abcd}	0.97^{bc}
T5	3.44^{a}	2.21^{cd}	0.79^{ab}	1.19^{cde}	3.06^{def}	2.09^{de}	0.56^{def}	0.34^{ef}	1.12^{defg}	0.56^{cde}	1.24^{abcd}	0.88^{d}
T6	2.53^{c}	1.56^{g}	0.92^{a}	1.19^{cd}	3.80^{a}	2.13^{de}	0.76^{ab}	0.56^{a}	1.28^{bcde}	0.70^{a}	1.14^{cd}	0.64^{g}
T7	3.15^{ab}	2.23^{c}	0.72^{bc}	1.06^{efg}	3.70^{abc}	1.98^{ef}	0.53^{f}	0.33^{ef}	1.37^{abc}	0.48^{ef}	1.12^{cd}	0.81^{de}
T8	3.22^{a}	1.97^{e}	0.67^{bcd}	1.14^{def}	3.23^{bcde}	2.11^{de}	0.63^{cde}	0.37^{de}	1.18^{cdef}	0.50^{e}	1.28^{abcd}	0.84^{de}
T9	3.16^{ab}	2.36^{b}	0.87^{a}	1.38^{ab}	3.44^{abcde}	2.16^{de}	0.53^{f}	0.33^{ef}	1.50^{a}	0.51^{e}	1.16^{bcd}	0.70^{fg}
T10	2.95^{abc}	2.30^{b}	0.57^{cd}	1.28^{bc}	3.21^{cde}	2.53^{ab}	0.58^{def}	0.44^{c}	1.07^{efg}	0.56^{cde}	1.26^{abcd}	1.04^{ab}
T11	3.38^{a}	2.17^{cd}	0.60^{cd}	1.08^{def}	2.98^{ef}	2.20^{cd}	0.63^{cd}	0.32^{f}	1.15^{defg}	0.53^{de}	1.08^{d}	0.90^{cd}
T12	2.62^{bc}	1.85^{f}	0.92^{a}	1.42^{a}	3.73^{ab}	2.37^{bc}	0.72^{ab}	0.49^{b}	1.28^{bcd}	0.63^{abc}	1.14^{cd}	0.75^{ef}
T13	2.93^{abc}	2.15^{d}	0.68^{bc}	1.04^{fg}	3.85^{a}	2.09^{de}	0.78^{a}	0.53^{a}	1.44^{ab}	0.52^{de}	1.19^{bcd}	0.86^{d}
T14	2.94^{abc}	1.85^{f}	0.53^{d}	1.10^{def}	3.52^{abcd}	2.58^{a}	0.69^{bc}	0.41^{cd}	0.97^{gh}	0.61^{bcd}	1.43^{a}	1.07^{a}
p value	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.05	0.01

Mean (s) followed by the same letter within the column do not differ at 5% level of significance. Treatment legend description is given in ***Table 4***.

Table 5.
Concentration (%) of different elements in shoot and root of spinach grown in soil A.

Treatment	N		P		K		Ca		Mg		Na	
	Shoot	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**
T1	1.92^{c}	1.01^{g}	0.18^{g}	0.45^{c}	2.61^{h}	1.91^{g}	0.36^{c}	0.22^{h}	0.72^{f}	0.29^{e}	4.32^{a}	3.87^{a}
T2	2.71^{ab}	1.97^{ab}	0.32^{bcde}	0.52^{c}	3.38^{defg}	2.34^{de}	0.38^{c}	0.26^{gh}	0.83^{ef}	0.35^{de}	2.97^{b}	2.77^{b}
T3	2.59^{ab}	2.07^{a}	0.34^{bcde}	0.47^{c}	4.04^{a}	2.65^{abc}	0.48^{ab}	0.28^{fg}	1.16^{abc}	0.42^{bcd}	2.32^{de}	1.70^{fg}
T4	2.55^{ab}	1.79^{d}	0.34^{bcde}	0.49^{c}	3.27^{efg}	2.06^{fg}	0.42^{bc}	0.31^{def}	1.04^{bcde}	0.61^{a}	2.65^{bcd}	1.99^{d}
T5	2.93^{a}	2.03^{ab}	0.24^{fg}	0.47^{c}	3.72^{cde}	2.38^{cde}	0.47^{ab}	0.32^{cde}	1.04^{bcde}	0.42^{bcd}	2.44^{cde}	1.72^{fg}
T6	2.40^{b}	1.51^{f}	0.44^{a}	0.88^{a}	3.66^{cde}	2.24^{def}	0.48^{ab}	0.37^{b}	1.20^{abc}	0.40^{bcd}	2.18^{e}	1.34^{h}
T7	2.62^{ab}	1.74^{de}	0.39^{abc}	0.75^{b}	3.78^{abcd}	2.19^{ef}	0.53^{a}	0.32^{cde}	1.34^{a}	0.40^{bcd}	2.51^{cde}	1.61^{g}
T8	2.58^{ab}	1.91^{bc}	0.42^{ab}	0.89^{a}	3.28^{efg}	2.47^{bcde}	0.46^{ab}	0.34^{bcd}	1.10^{bcd}	0.44^{bc}	2.81^{bc}	2.19^{c}
T9	2.47^{b}	1.82^{cd}	0.37^{abcd}	0.92^{a}	3.92^{abc}	2.52^{bcd}	0.43^{bc}	0.31^{cdef}	1.36^{a}	0.48^{b}	2.29^{de}	1.80^{ef}
T10	2.49^{b}	1.68^{e}	0.28^{def}	0.97^{a}	3.17^{g}	2.35^{de}	0.46^{ab}	0.34^{bcd}	1.00^{cde}	0.43^{bc}	2.54^{bcde}	1.94^{de}
T11	2.79^{ab}	1.80^{d}	0.26^{ef}	0.68^{b}	3.53^{cdef}	2.69^{ab}	0.42^{bc}	0.30^{ef}	1.08^{bcd}	0.41^{bcd}	2.35^{de}	1.89^{de}
T12	2.47^{b}	1.72^{de}	0.33^{bcdef}	0.89^{a}	3.49^{cdef}	2.65^{abc}	0.47^{ab}	0.42^{a}	1.16^{abc}	0.38^{cd}	2.15^{e}	1.55^{g}
T13	2.70^{ab}	1.98^{ab}	0.30^{cdef}	0.91^{a}	3.97^{ab}	2.82^{a}	0.52^{a}	0.33^{cde}	1.24^{ab}	0.45^{bc}	2.46^{cde}	1.98^{d}
T14	2.78^{ab}	1.82^{cd}	0.25^{fg}	0.94^{a}	3.22^{fg}	2.41^{bcde}	0.48^{ab}	0.35^{bc}	0.87^{def}	0.46^{b}	2.58^{bcde}	2.01^{d}
p value	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Mean (s) followed by the same letter within the column do not differ at 5% level of significance. Treatment legend description is given in ***Table 4****.*

Table 6.
Concentration (%) of different elements in shoot and root of spinach grown in soil B.

root concentration decreased by 41.25% in T6 compared to T1. The maximum concentration of Na in both parts of spinach grown in soil B was also observed in control which was significantly higher ($p < 0.01$) from the rest of the treatments (**Table 6**). The concentration of Na in T12 and T6 decreased by respectively 50.31% and 65.46% compared to T1 for shoot and root.

When the concentration of N of spinach grown in soil A (**Table 5**) was compared with that of spinach grown in soil B (**Table 6**), it was found that the concentration of N increased by as highest as 41.33% in shoot and 46.67% in the root of spinach grown in the soil A. The concentration of P, Ca, and Mg in both shoot and root decreased in response to increased soil salinity for all the respective treatments (comparison between **Tables 5** and **6**). The concentration of P increased by as highest as 227.80% in shoot and 151.57% in the root of spinach when grown in soil A compared to soil B. Similarly, the concentration of Ca and Mg respectively decreased by as highest as 37.19% and 26.77% in shoot and 38.19% and 73.91% in the root of spinach grown in soil B compared to soil A. However, the concentration of K in response to salinity showed a variable trend depending on the types of the treatments. The K concentration in the shoot of spinach grown in soil B was found higher than soil A for all treatments excluding T4, T6, T10, T12, and T14. The concentration of K in root was also found higher in soil B in comparison to soil A for all treatments except T4, T10, and T14. On the other hand, it was evident that the concentration of Na decreased in all the respective treatments in soil A compared to soil B. The Na concentration decreased by as maximum as 67.80% in shoot and 71.86% in root when spinach was grown in soil A relative to soil B (comparison between **Tables 5** and **6**).

The uptake of N, P, K, Ca, and Mg in both aerial and underground parts of spinach grown in both soil A (**Table 7**) and soil B (**Table 8**) was found to be lowest in control and highest when VC and WA were incorporated in combination, with significant differences among the treatments both for the shoot ($p < 0.01$) and root ($p < 0.01$). The uptake of Na in both shoots was also found lower in control and higher in soils amended with VC along with WA. The uptake of Na in T1 decreased by 66.10% and 59.12% respectively in shoot and root for soil A (**Table 7**) and 77.64% and 33.88% for soil B relative to T12 (**Table 8**).

The uptake in both parts of spinach decreased in all the respective treatments in soil B compared to soil A (comparison between **Tables 7** and **8**). The uptake of N, P, Ca, and Mg decreased by as highest as 63.36%, 80.51%, 68.71%, and 67.50% respectively in shoot and 57.11%, 71.75%, 65.20%, and 60.59% respectively in root when spinach was grown in soil B. However, the uptake of K in both shoot and root with respect to salinity did not found in any definite trend. The shoot K uptake decreased in all treatments except for T2, T5, T7, and T9 whereas root uptake decreased in all treatments except for T1, T7, and T11 when spinach was grown in soil B. By contrast, the uptake of Na in both shoot and root of spinach was observed higher in soil B for all the respective treatments. The uptake of Na decreased by as highest as 54.42% and 61.38% respectively in shoot and root when grown in soil A.

The C_{shoot}/C_{root} quotient of N, K, Na, Ca, and Mg was found greater than 1, whereas P was observed lower than 1 both in soil A (**Table 9**) and soil B (**Table 10**). The C_{shoot}/C_{root} quotient of N, K, Na, Ca, and Mg were found in the range of 1.28–1.89, 1.27–1.95, 1.20–1.79, 1.29–1.97, and 1.61–2.96 for soil A, whereas 1.26–1.90, 1.32–1.73, 1.08–1.64, 1.11–1.74, 1.70–3.03 for soil B. On the contrary, the C_{shoot}/C_{root} quotient of P ranged from 0.41 to 0.78 and 0.27 to 0.72 for soil A and soil B, respectively.

3.3 Na: K, Na: Ca and Na: Mg ratios

The Na: K, Na: Ca, and Na: Mg ratios of shoot and root were found to be highest in control that significantly differed ($p < 0.01$) from the rest of the treatments for

Treatment	N		P		K		Ca		Mg		Na	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
T1	3.79^{f}	0.29^{d}	0.65^{f}	0.22^{f}	3.53^{i}	0.29^{h}	0.83^{h}	0.07^{g}	1.61^{f}	0.09^{f}	2.59^{h}	0.28^{f}
T2	7.26^{e}	0.99^{c}	1.38^{ef}	0.43^{def}	6.56^{hi}	0.64^{g}	1.23^{gh}	0.14^{efg}	2.51^{ef}	0.18^{e}	3.21^{gh}	0.37^{def}
T3	15.80^{ab}	1.43^{b}	3.36^{c}	0.61^{cde}	16.08^{cd}	1.24^{cde}	2.56^{cd}	0.26^{c}	5.67^{c}	0.33^{cd}	4.95^{cdef}	0.47^{cd}
T4	9.43^{de}	1.08^{c}	1.68^{e}	0.42^{def}	9.51^{fgh}	1.03^{ef}	1.52^{efg}	0.18^{def}	3.22^{de}	0.27^{de}	3.48^{fgh}	0.40^{def}
T5	9.58^{de}	0.88^{c}	2.20^{de}	0.47^{de}	8.41^{gh}	0.84^{fg}	1.54^{efg}	0.14^{efg}	3.12^{de}	0.22^{e}	3.42^{fgh}	0.35^{def}
T6	14.72^{ab}	1.59^{ab}	5.34^{ab}	1.21^{ab}	22.00^{b}	2.16^{a}	4.42^{ab}	0.57^{a}	7.41^{b}	0.71^{a}	6.62^{ab}	0.65^{ab}
T7	13.06^{bc}	1.11^{c}	2.93^{cd}	0.53^{de}	15.30^{de}	0.98^{efg}	2.21^{cde}	0.17^{def}	5.73^{c}	0.24^{e}	4.67^{cdefg}	0.40^{def}
T8	10.78^{cd}	1.06^{c}	2.27^{de}	0.62^{cd}	10.97^{fg}	1.14^{def}	2.14^{cde}	0.20^{cde}	4.00^{d}	0.27^{de}	4.33^{defg}	0.46^{cde}
T9	16.11^{ab}	1.85^{a}	4.49^{b}	1.09^{b}	17.64^{cd}	1.70^{b}	2.74^{e}	0.26^{c}	7.58^{ab}	0.40^{c}	5.97^{bc}	0.55^{bc}
T10	9.55^{de}	0.94^{c}	1.85^{e}	0.52^{de}	10.42^{fg}	1.03^{ef}	1.87^{def}	0.18^{cdef}	3.46^{de}	0.23^{e}	4.09^{efgh}	0.42^{cde}
T11	10.13^{cde}	0.80^{c}	1.80^{e}	0.39^{ef}	8.97^{gh}	0.81^{fg}	1.91^{def}	0.12^{fg}	3.42^{de}	0.20^{e}	3.21^{gh}	0.33^{ef}
T12	17.59^{a}	1.74^{ab}	6.22^{a}	1.34^{a}	25.15^{a}	2.23^{a}	4.87^{a}	0.46^{b}	8.64^{a}	0.59^{b}	7.65^{a}	0.70^{a}
T13	14.21^{b}	1.57^{ab}	3.33^{c}	0.76^{c}	18.68^{c}	1.52bc	3.81^{b}	0.39^{b}	7.00^{b}	0.38^{c}	5.86^{bcd}	0.62^{ab}
T14	10.63^{cd}	1.05^{c}	1.91^{e}	0.62^{cd}	12.75^{ef}	1.46bcd	2.48^{cd}	0.23^{cd}	3.51^{de}	0.35^{cd}	5.18^{bcde}	0.61^{ab}
p value	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Mean (s) followed by the same letter within the column do not differ at a 5% level of significance. Treatment legend description is given in ***Table 4****.*

Table 7.
Uptake (mg plant^{-1}) of different elements in shoot and root of spinach grown in soil A.

Treatment	N		P		K		Ca		Mg		Na	
	Shoot	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**
T1	1.39^{h}	0.19^{g}	0.13^{f}	0.09^{f}	1.90^{f}	0.36^{g}	0.26^{i}	0.04^{i}	0.52^{g}	0.05^{g}	3.12^{h}	0.74^{cd}
T2	5.89^{g}	0.46^{f}	0.70^{e}	0.12^{ef}	7.33^{e}	0.55^{fg}	0.83^{h}	0.06^{hi}	1.79^{f}	0.08^{fg}	6.47^{fg}	0.64^{cde}
T3	9.38^{cd}	0.93^{bc}	1.22^{c}	0.21^{e}	14.63^{d}	1.20^{c}	1.74^{de}	0.13^{def}	4.16^{cd}	0.19^{cd}	8.33^{de}	0.77^{cd}
T4	5.89^{g}	0.54^{ef}	0.78^{e}	0.15^{ef}	7.62^{e}	0.62^{fg}	0.98^{h}	0.10^{fgh}	2.44^{f}	0.19^{cd}	6.12^{g}	0.60^{de}
T5	7.08^{efg}	0.64^{def}	0.58^{e}	0.15^{ef}	9.17^{e}	0.78^{ef}	1.16^{fgh}	0.10^{efg}	2.60^{f}	0.14^{de}	6.00^{g}	0.55^{e}
T6	12.64^{b}	1.44^{a}	2.35^{a}	0.84^{a}	19.32^{b}	2.14^{a}	2.51^{b}	0.35^{a}	6.35^{ab}	0.39^{a}	11.47^{b}	1.28^{a}
T7	10.69^{bc}	0.88^{c}	1.58^{b}	0.38^{d}	15.43^{cd}	1.11^{cd}	2.16^{bc}	0.16^{cd}	5.46^{bc}	0.20^{c}	10.25^{bc}	0.81^{c}
T8	6.06^{fg}	0.46^{f}	0.96^{cde}	0.21^{e}	7.62^{e}	0.59^{fg}	1.08^{gh}	0.08^{ghi}	2.57^{f}	0.11^{ef}	6.54^{fg}	0.52^{e}
T9	11.72^{b}	1.10^{b}	1.73^{b}	0.55^{c}	18.64^{bc}	1.52^{b}	2.03^{cd}	0.18^{c}	6.45^{ab}	0.29^{b}	10.82^{bc}	1.08^{b}
T10	7.48^{defg}	0.69^{de}	0.83^{de}	0.39^{d}	9.51^{e}	0.96^{cde}	1.39^{efg}	0.14^{de}	3.00^{ef}	0.18^{cd}	$7.65e^{fg}$	0.79^{c}
T11	8.07^{def}	0.62^{ef}	0.76^{e}	0.23^{e}	10.22^{e}	0.93^{cde}	1.22^{fgh}	0.10^{efg}	3.11^{ef}	0.14^{de}	$6.78e^{fg}$	0.65^{cde}
T12	16.01^{a}	1.27^{a}	2.12^{a}	0.66^{b}	22.84^{a}	1.95^{a}	3.04^{a}	0.31^{b}	7.53^{a}	0.28^{b}	13.97^{a}	1.13^{ab}
T13	10.61^{bc}	0.82^{cd}	1.18^{cd}	0.38^{d}	15.61^{cd}	1.17^{cd}	2.04^{cd}	0.14^{def}	4.84^{cd}	0.19^{cd}	9.64^{cd}	0.83^{c}
T14	8.76^{cde}	0.70^{de}	0.77^{e}	0.36^{d}	10.11^{e}	0.92^{de}	1.51^{ef}	0.14^{def}	2.74^{f}	0.18^{cd}	8.10^{de}	0.77^{cd}
p value	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Mean (s) followed by the same letter within the column do not differ at a 5% level of significance. Treatment legend description is given in ***Table 4***.

Table 8.
Uptake (mg plant^{-1}) of different elements in shoot and root of spinach grown in soil B.

Treatment	N	P	K	Ca	Mg	Na
T1	1.83^{a}	0.41^{f}	1.70^{abcd}	1.66^{abc}	2.47^{abc}	1.28^{cd}
T2	1.38^{bcdf}	0.61^{cd}	1.95^{a}	1.71^{ab}	2.74^{ab}	1.66^{ab}
T3	1.57^{bcd}	0.78^{a}	1.84^{ab}	1.42^{bcd}	2.44^{abcd}	1.48^{abcd}
T4	1.29^{ef}	0.60^{cd}	1.37^{ef}	1.29^{d}	1.78^{ef}	1.29^{cd}
T5	1.55^{bcde}	0.67^{abc}	1.47^{def}	1.65^{abc}	2.02^{cdef}	1.41^{bcd}
T6	1.62^{abc}	0.77^{ab}	1.78^{abc}	1.35^{cd}	1.82^{def}	1.79^{a}
T7	1.41^{bcdef}	0.68^{abc}	1.87^{a}	1.59^{bcd}	2.85^{a}	1.39^{bcd}
T8	1.64^{ab}	0.59^{cd}	1.54^{cdef}	1.70^{ab}	2.38^{abcde}	1.52^{abcd}
T9	1.34^{def}	0.63^{bc}	1.59^{bcde}	1.63^{bc}	2.96^{a}	1.67^{ab}
T10	1.28^{f}	0.45^{ef}	1.27^{f}	1.33^{cd}	1.92^{cdef}	1.22^{cd}
T11	1.56^{bcde}	0.56^{cde}	1.36^{ef}	1.97^{a}	2.17^{bcdef}	1.20^{d}
T12	1.42^{bcdef}	0.65^{abc}	1.58^{bcde}	1.48^{bcd}	2.04^{cdef}	1.53^{abc}
T13	1.36^{cdef}	0.66^{abc}	1.85^{ab}	1.48^{bcd}	2.84^{a}	1.40^{bcd}
T14	1.59^{abcd}	0.48^{def}	1.37^{ef}	1.69^{ab}	1.61^{f}	1.34^{bcd}
p value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Mean (s) followed by the same letter within the column do not differ at a 5% level of significance. Treatment legend description is given in ***Table 4****.*

Table 9.
C_{shoot}/C_{root} quotient of different elements for spinach grown in soil A.

Treatment	N	P	K	Ca	Mg	Na
T1	1.90^{a}	0.39^{cde}	1.37^{cde}	1.65^{ab}	2.51^{bcd}	1.12^{de}
T2	1.38^{bc}	0.62^{ab}	1.45^{bcde}	1.51^{abc}	2.34^{bcde}	1.08^{e}
T3	1.26^{c}	0.72^{a}	1.52^{abcde}	1.74^{a}	2.77^{abc}	1.36^{bc}
T4	1.42^{bc}	0.72^{a}	1.59^{abc}	1.35^{bcd}	1.70^{e}	1.33^{cd}
T5	1.46^{bc}	0.51^{bc}	1.57^{abcd}	1.46^{abc}	2.51^{bcd}	1.42^{bc}
T6	1.59^{b}	0.51^{bc}	1.64^{ab}	1.29^{cd}	2.96^{abc}	1.64^{a}
T7	1.50^{b}	0.52^{bc}	1.73^{a}	1.64^{ab}	3.34^{a}	1.56^{ab}
T8	1.35^{bc}	0.47^{bcd}	1.33^{de}	1.34^{bcd}	2.50^{bcd}	1.29^{cde}
T9	1.36^{bc}	0.40^{cde}	1.56^{abcde}	1.40^{bcd}	2.88^{abc}	1.27^{cde}
T10	1.48^{bc}	0.29^{e}	1.35^{cde}	1.34^{bcd}	2.30^{cde}	1.31^{cd}
T11	1.55^{b}	0.39^{cde}	1.32^{e}	1.41^{bcd}	2.65^{bc}	1.24^{cde}
T12	1.44^{bc}	0.37^{cde}	1.33^{de}	1.11^{d}	3.03^{ab}	1.40^{bc}
T13	1.37^{bc}	0.33^{de}	1.41^{bcde}	1.59^{abc}	2.78^{abc}	1.24^{cde}
T14	1.53^{b}	0.27^{e}	1.34^{cde}	1.36^{bcd}	1.89^{de}	1.28^{cde}
p value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Mean (s) followed by the same letter within the column do not differ at 5% level of significance. Treatment legend description is given in ***Table 4****.*

Table 10.
C_{shoot}/C_{root} quotient of different elements for spinach grown in soil B.

both soil A (**Table 11**) and soil B (**Table 12**). The Na: K, Na: Ca, and Na: Mg ratios of shoot increased by 146.02%, 107.91%, and 105.87%, whereas of root increased by 226.29%, 256.53%, and 244.05% in T1 compared to the lowest values of the respective parts of spinach grown in soil A. On the other hand, in the case of soil B,

Treatment	Na: K		Na: Ca		Na: Mg	
	Shoot	Root	Shoot	Root	Shoot	Root
T1	0.74^{a}	0.98^{a}	3.12^{a}	4.05^{a}	1.61^{a}	3.13^{a}
T2	0.49^{b}	0.57^{b}	2.61^{b}	2.69^{bc}	1.30^{bc}	2.08^{b}
T3	0.31^{c}	0.38^{c}	1.94^{cde}	1.86^{fg}	0.88^{fg}	1.43^{def}
T4	0.37^{bc}	0.39^{c}	2.29^{bc}	2.29^{de}	1.09^{def}	1.49^{def}
T5	0.41^{bc}	0.42^{c}	2.23^{c}	2.60^{bcd}	1.10^{cde}	1.58^{cde}
T6	0.30^{c}	0.30^{d}	1.50^{f}	1.14^{i}	0.89^{efg}	0.91^{g}
T7	0.30^{c}	0.41^{c}	2.11^{cd}	2.42^{cde}	0.82^{g}	1.67^{cde}
T8	0.39^{bc}	0.40^{c}	2.04^{cd}	2.27^{e}	1.09^{def}	1.68^{cde}
T9	0.34^{c}	0.32^{d}	2.18^{cd}	2.13^{ef}	0.78^{g}	1.39^{ef}
T10	0.39^{bc}	0.41^{c}	2.19^{cd}	2.36^{cde}	1.18^{cd}	1.85^{bc}
T11	0.37^{bc}	0.41^{c}	1.73^{def}	2.81^{b}	0.95^{efg}	1.71^{cde}
T12	0.31^{c}	0.32^{d}	1.59^{ef}	1.53^{h}	0.89^{efg}	1.19^{fg}
T13	0.31^{c}	0.41^{c}	1.51^{ef}	1.62^{gh}	0.82^{g}	1.71^{cde}
T14	0.41^{bc}	0.42^{c}	2.10^{cd}	2.63^{bcd}	1.48^{ab}	1.78^{bcd}
p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Mean (s) followed by the same letter within the column do not differ at a 5% level of significance. Treatment legend description is given in ***Table 4****.*

Table 11.
Na: K, Na: Ca and Na: Mg ratios in shoot and root of spinach grown in soil A.

Treatment	Na:K		Na:Ca		Na:Mg	
	Shoot	Root	Shoot	Root	Shoot	Root
T1	1.68^{a}	2.02^{a}	12.35^{a}	17.65^{a}	6.08^{a}	13.61^{a}
T2	0.90^{b}	1.18^{b}	7.78^{b}	10.91^{b}	3.68^{b}	7.93^{b}
T3	0.58^{d}	0.64^{fg}	4.82^{c}	6.15^{c}	2.00^{d}	4.08^{cd}
T4	0.81^{bcd}	0.96^{c}	6.27^{bc}	6.36^{c}	2.55^{cd}	3.24^{d}
T5	0.66^{bcd}	0.73^{ef}	5.21^{c}	5.33^{c}	2.42^{cd}	4.15^{cd}
T6	0.60^{d}	0.60^{g}	4.58^{c}	3.61^{d}	1.86^{d}	3.31^{d}
T7	0.67^{bcd}	0.74^{f}	4.83^{c}	4.99^{cd}	1.90^{d}	4.01^{cd}
T8	0.86^{bc}	0.89^{cd}	6.13^{bc}	6.37^{c}	2.58^{cd}	5.07^{c}
T9	0.58^{d}	0.71^{f}	5.32^{c}	5.87^{c}	1.68^{d}	3.80^{cd}
T10	0.80^{bcd}	0.83^{de}	5.50^{c}	5.66^{c}	2.54^{cd}	4.49^{cd}
T11	0.66^{bcd}	0.70^{f}	5.54^{c}	6.33^{c}	2.18^{cd}	4.64^{cd}
T12	0.62^{cd}	0.58^{g}	4.61^{c}	3.68^{d}	1.87^{d}	4.05^{cd}
T13	0.62^{cd}	0.70^{f}	4.73^{c}	6.07^{c}	1.99^{d}	4.45^{cd}
T14	0.80^{bcd}	0.84^{d}	5.41^{c}	5.66^{c}	2.98^{bc}	4.38^{cd}
p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Mean (s) followed by the same letter within the column do not differ at a 5% level of significance. Treatment legend description is given in ***Table 4****.*

Table 12.
Na: K, Na: Ca, and Na: Mg ratios in shoot and root of spinach grown in soil B.

the Na: K, Na: Ca, and Na: Mg ratios increased by 191.75%, 169.46%, and 261.22% in T1 compared to the lowest values for the shoot, while the Na: K, Na: Ca and Na: Mg ratios increased by 247.88%, 389.11% and 319.93% in T1 compared to the lowest values for root. The Na: K, Na: Ca and Na: Mg ratios of both shoot and root of spinach grown in soil B was observed higher in comparison to corresponding ratios of the concerned part of plants when grown in soil A (comparison between **Tables 11** and **12**). The Na: K, Na: Ca, and Na: Mg ratios increased by as highest as 126.82%, 295.95%, and 227.32% for the shoot, while 146.49%, 335.27%, and 334.74% for root when grown in soil B relative to soil A.

3.4 Correlation co-efficient (r) of plant nutrients

Tables 13 and **14** show the correlation matrix among different parameters in shoot and root of spinach grown in soil A and soil B, respectively. In the shoot of spinach grown in soil A, the relationship of Na with all other elements was negative. Similarly, the relation of Na with all other elements except K was also found negative in the root. In both shoot and root of spinach grown in both soil A and soil B, the relations among K, Ca, and Mg were found positive. In both shoot and root of spinach grown in soil B, the concentration of Na was negatively correlated with all other elements. Similar to spinach grown in soil A, the relationships between K-Ca, K-Mg, and Ca-Mg were found positive for both shoot and root of spinach grown in soil B.

3.5 Discussion

The soil samples collected for the present experiment were saline in nature and acidic in reaction. Soils having EC greater than 4 mS cm^{-1} at saturation extract are regarded as saline soils [1, 39]. Saline soils with acidic character are rare in nature which could be ascribed to the dominance of S [40]. The acidic character of the soils collected from the area was also reported by LRUG [41].

The growth of spinach in regards to the fresh weight was adversely inhibited by extreme soil salinity which is in agreement with Panuccio et al. [42]. Furthermore, the fresh weight for all the respective treatments was found lower at relatively higher levels (soil B) of soil salinity in the present study. The reduction in the growth of plants in saline soils might be due to the result of the deficiencies of essential nutrients as well as toxicities of Na and Cl [43, 44]. However, in both levels of soil salinity (soil A and soil B), the application of amendments especially VC alone and VC in combination with WA had been found to increase the growth

	N	P	Na	K	Ca	Mg
N	1	0.149	−0.216	0.241	−0.103	0.262
P	0.203	1	−0.428**	0.619**	0.424**	0.616**
Na	−0.119	−0.311*	1	−0.324*	−0.116	−0.387*
K	0.490**	0.562**	0.035	1	0.614**	0.617**
Ca	−0.012	0.324*	−0.236	0.524**	1	0.302
Mg	0.185	0.459**	−0.159	0.726**	0.636**	1

[*] Correlation is significant at the 0.05 level (2-tailed).
[**] Correlation is significant at the 0.01 level (2-tailed).
Values above 1 indicate for shoot.
Values below 1 indicate for root.

Table 13.
Correlation coefficient (r) values for shoot and root of spinach grown in soil A.

	N	P	Na	K	Ca	Mg
N	1	0.042	−0.420**	0.211	0.365*	0.052
P	0.061	1	−0.426**	0.375*	0.294	0.527**
Na	−0.542**	−0.414**	1	−0.674**	−0.535**	−0.584**
K	0.560**	0.347*	−0.407**	1	0.387*	0.720**
Ca	0.165	0.639**	−0.668**	0.287	1	0.392*
Mg	0.399**	0.142	−0.411**	0.006	0.209	1

**Correlation is significant at the 0.05 level (2-tailed).*
***Correlation is significant at the 0.01 level (2-tailed).*
Values above 1 indicate for shoot.
Values below 1 indicate for root.

Table 14.
Correlation coefficient (r) values for shoot and root of spinach grown in soil B.

and uptake of nutrients in spinach. Several authors observed maximum growth and yield of plants when VC was applied in soils [45, 46]. The positive response of plants to salt stress had also been observed as a result of the application of amendments [21, 47]. However, instead of a high concentration of K, Ca, and Mg in WA, spinach growing in WA treated soils did not show considerable growth in the present study which may be due to a lack of N in the ash. In an experiment, Augusto et al. [48] stated that the absence of N in the WA did not increase plant growth growing on mineral soils of Nordic countries.

As a consequence of increased soil salinity, the uptake of N was inhibited which is in support of the findings of other authors [49, 50], and might be due to the antagonistic relation of Na with NH_4^+ [51]. The high concentration of N in different parts of spinach amended with VC either alone or in association with other amendments might be due to the high inherent N concentration and its release through decomposition of VC. A similar result was found by Erenoglu et al. [52], where the concentration of N in the shoot of wheat increased as the N content in soil was enhanced.

The concentration and uptake of P also decreased with increasing soil salinity. The reduced uptake of P in spinach with increasing salinity of soils might be due to high Cl^- concentration which is a common problem in saline soils. Several authors reported that high soil salinity is attributed to the reduction in P uptake by plants due to the antagonistic relationship between $H_2PO_4^-$ and Cl^- [9, 51, 53, 54].

A high concentration of total K in spinach for most of the treatments with increasing soil salinity might be due to high K content in the growth medium. The result was consistent with the findings of other authors [55, 56], where the concentration of K in plants increased with the increase of salinity. Al-Karaki [57] also reported that the concentration of K in the growth medium determines the net uptake of K and observed higher translocation of K from root to shoot in plants to a greater extent in the saline environment in comparison to non-saline conditions with the increase of K concentration in the medium. However, low uptake of total K by spinach for most of the respective treatments at relatively higher salt stress could be ascribed to a lower amount of total dry matter.

Both the concentration and uptake of both Ca and Mg in different parts of spinach decreased with the increase of soil salinity. A similar trend was found for saltmarsh grass, where the concentration of Ca and Mg decreased significantly with increasing salinity [53]. Sahin et al. [9] also observed significant negative correlations of Na with that of the concentrations of Ca and Mg. Consistent with the present findings, similar results were reported by other authors where the salt stress resulted in an increased accumulation of Na and decreased amounts of Ca and Mg in plants [51, 58]. The increase in the concentrations of Ca and Mg in treated soils compared to control might be due to the greater affinity for these cations relative to Na under different amendments.

The concentration of Na in shoot and root increased as the salinity of soil increased which is in agreement with other findings [9, 53, 59]. However, the decline in Na concentration in both shoot and root of spinach growing in soils especially amended with VC and VC plus WA can be explained by a dilution effect, that is, an increase in dry matter accumulation. A similar finding was reported by Kaya et al. [60]. Moreover, the significant increase in the uptake of Na in VC plus WA amended soils over control could be due to higher biomass content under amended conditions. In an experiment, Ferreira et al. [61] also found that the accumulation of Na in spinach increased by 1.3–3.0 times and increased tissue Na was neither a hindrance nor a benefit for the growth of spinach.

The C_{shoot}/C_{root} quotient of N, K, Ca, and Mg greater than 1 indicated their mobility from root to shoot, while P less than 1 indicated its immobility. In the present study, the ratios of Na: K, Na: Ca, and Na: Mg were found higher in the control. Hadi et al. [62] also stated that the uptake and transport of K, Ca, and Mg can be adversely affected by the high concentration of Na in saline soils, resulting in higher Na: K, Na: Ca, and Na: Mg ratios in plants. Consistent with the present findings, in another experiment, the ratios of Ca: Na and Mg: Na in cabbage were found lower with the increase of salinity levels [9]. Furthermore, the addition of amendments decreased Na: K, Na: Ca, and Na: Mg ratios which could be the consequence of better uptake of K, Ca, and Mg under different amendments.

4. Conclusion

The production of the crop is restricted due to soil salinity throughout the world possessing one of the greatest threats to food security. Soil salinity is also one of the most severe environmental constraints limiting the production of crops in the coastal areas of Bangladesh. Soil salinity coupled with acidity is a major challenge in coastal areas all over the world. The present experiment provided approaches toward sustainable use of saline soils with acidic reaction through the integrative approaches of leaching and application of different amendments. The growth and uptake of nutrients by spinach decreased with increasing the levels of soil salinity irrespective of the treatments. The response of spinach varied depending on several factors such as salt content in the soils, types, and rates of amendments. It can be summarized from the present experiment that the application of VC alone or in association with WA or ZL can be well suited for better performance of spinach, whereas the application of WA and ZL either alone or in combination (i.e. WA plus ZL) had the least effects in enhancing the yield of spinach in a saline environment. The application of VC and WA in combination resulted in maximum yield and uptake of nutrients at different levels of soil salinity while the ratios of Na: K, Na: Ca, and Na: Mg of shoot and root were found to be highest in un-amended soils. Moreover, the application of VC in association with WA with acid-neutralizing capacity presents an interesting source to enhance the performance of spinach by improving the physico-chemical properties of acidic saline soils. It can be suggested from the present work that the application of VC plus WA at 1% of each can be practiced before cultivation to influence soil nutrient dynamics, thereby augmenting growth and uptake of nutrients by plants in acidic saline soils.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Sajal Roy* and Nasrin Chowdhury
Faculty of Biological Sciences, Department of Soil Science, University of Chittagong, Chattogram, Bangladesh

*Address all correspondence to: sajal.roy@cu.ac.bd

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Chapter 11

Morpho-Anatomical Adaptation against Salinity

Smita Srivastava

Smita Srivastava

Abstract

Plants tolerant of NaCl, implement several adjustments to acclimate to salt stress, such as biochemical, physiological, and morphological modifications. Besides, plants also adjust to saline circumstances by altering their anatomical structure of roots, leaves, and morphological modifications. The leaf and roots are among the essential plant organs and are involved in the transport of water and minerals used for photosynthesis. From a plant physiology perspective, water use efficiency in the quantity of CO_2 fixed in photosynthesis compared to the leaf anatomy. In this review, we provide a comparative account of the morphology of the leaf and root under normal and salt stress circumstances. There is little information on the ultrastructure changes elicited in response to salt stress. The analysis expands our knowledge of how salt may impact the leaves and root anatomy.

Keywords: adaptive mechanisms, arbuscular mycorrhizal fungi, trichomes, stomata, mesophyll

1. Introduction

Salt stress is among the leading abiotic causes of modifications in many physiological, anatomical, and biochemical processes [1]. Anatomical and morphological changes in leaves under restricted moisture availability play a significant role in salt stress, and they are an indication of the level of tolerance. The epidermis is the external tissue of every plant organ and serves as the initial point of contact with its environment. It is essential to preserve physiologically appropriate circumstances in all plant and environment interactions for normal metabolism [2].

In salt-stressed plants, stem vascular cell thickness was much larger than control treatment; the salinity effect was concentration-dependent. Generally, plants grown in saline solution showed higher thickness in the cuticle, vascular tissues, and vessel than unstressed plants (**Figure 1B–C**). Furthermore, we observed that, in salt-stressed plants, the number of trichomes was increased from epidermal stem cells. In other words, an increase in salinity level led to more trichomes on the epidermal layer compare with control plants [3]. There are several reports on increased trichomes density under environmental stresses such as drought and salinity [4, 5]. An increase in trichome density may be a mechanism to increase tolerance to salt stress.

The importance of the cuticular layer in regulating a plant's water status and providing protection from environmental challenges has been recognized for a long time. The cuticular layer in plants restricts non-stomatal water loss and protects plants against damage from biotic and abiotic stress [6]. Due to their role

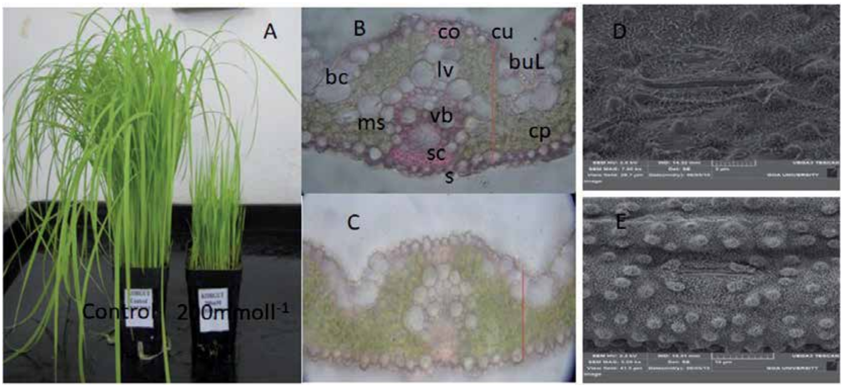

Figure 1.
Salt stress effects on rice plant growth under control and at 200 mM NaCl treatment(A): rice leaf anatomy, Oriza sativa; (B–C): light microscopy (cross-section) in control; (C): light microscopy (cross-section) in 200 mM NaCl; cu = cuticle, ms: mesophyll cell, Bul: bulliform cells, cp: parenchyma cells, m: mesophyll, s: stomata, vb: vascular bundles, s: stomata stomata, lv: largr vascular bundle and red line indicates the thickness of parenchyma; and scale bar = 100 μm; (D): scanning electron microscopy of the adaxial surface in control rice leaves; and (E): under stress condition (200 mmol/l), showing epicuticular wax deposition.

in controlling water loss, specialized epidermis structures have the potential to enhance the drought tolerance and WUE ("water use efficiency") of critical crops [7–10]. The cuticular wax on the leaves of the control (non-treated rice leaves) showed less than compared with the treated rice leaves (200 mmol/l NaCl), indicating adaptation against salinity to maintain photosynthesis to control water loss (**Figure 1D–E**).

Trichomes study has conventionally centered on specialized metabolic processes understanding in glandular trichomes [11–13]. Our data showed that in salinity tolerance, rice varieties have developed dense trichomes and increased in size with the help of a layer of air trapped in trichomes to reduce the rate of water transpiration as compared to susceptible varieties under differing concentrations of salt (40–160 mmol/l) [3].

The epidermis also includes stomata, which constitute epidermal pores that directly control the exchange of gases and also water status management. They may be found on a single surface (hypostomatic) and both leaf surfaces (amphistomatic) [14]. Stomata regulate water absorption through modifications in the stomatal opening, conductance, along with density. Shortly, plants alter their stomata closure to minimize water loss and a moderate absorption of CO_2 for changing circumstances [15]. Our data indicated that tolerant cultivars of rice closed stomata at the highest concentration (160 mmol/l NaCl) of salt [3]. Plant leaves can rise in stomatal density and a decreased saline area indicating a change to saline stress [16, 17]. Thus, the equilibrium between the density of stomatal and site could lead to stomatal conductance control and evaporation water loss [18, 19], which establishes equilibrium in photosynthesis [20]. Similar behavior was found for other species like "*Leptochloafusca*" [21] *Imperata cylindrical* [22], and *Triticum aestivum* [23].

Salt tolerant plants exhibit leaves thickening [23–25] which may contribute to the maintenance of turgor and content of leaf water. Wankhade et al. [26] and Hameed et al. [17] identified an optimistic association between salt stress tolerance and epidermal cells' thickening. The epidermal thickness increases the water effectiveness of plants and offers more area in which NaCl is effective for the epidermis of the leaf [27]. It can also be important to increase the region of sclerenchyma with rising salinity since it provides organ rigidity and this may be an essential characteristic for salt resistance [16]. The area of the photosynthetic leaf, parenchyma tissues exhibited a progressive reduction in salinity which is likely to influence CO_2 diffusion [18].

The most challenging issues to assess by traditional microscopy methods are rice mesophyll tissues as their cells are lesser and have a greater density of chloroplast [28, 29] comparison to other plants. Thus, precise mesophyll morphology evaluation is essential for assessing photosynthetic capability [30] and maintains the leaf structure against salinity via following ways;

1. *Salt exclusion*: It inhibits salts from entering the vascular scheme.

2. *Salt elimination*: Salts glands and hair actively remove salts and therefore maintain the concentration of salt below a specific threshold in the blades.

3. *Salt succulence*: If the amount of cells storage gradually rises with salt ingestion (since the cells raise water constantly), the salt level may be maintained for long durations reasonably consistent.

4. *Salt redistribution*: Na^+& Cl^- may easily be transported to phloem to allow redistribution across the plant of higher concentrations in actively transpiring leaves.

Significant modifications were detected in salt stress chloroplast in rice leaf contrast to tolerance variety. These include; (1) Modifications in the chloroplasts number & size, and starch level. (2) Disordered membranes of the chloroplast. (3) Variations in plastoglobuli numbers and sizes. (4) Loss of the disorganization and envelope of thylakoids and grana which directly affect the chlorophyll fluorescence and photosynthetic rate, and reduced the productivity of rice (12). Chloroplast is recognized as an organelle susceptible to environmental stress [31], and its pockets [32] are known to be present in salt. Even though earlier TEM investigations have shown alterations in the chloroplast ultrastructure to salt that influence photosynthetic [32], these structural variations are specifically noticeable in the thylakoids [28] that swell under salt stress [3–32]. Therefore, mesophyll conductance decrease was correlated with the olive leaf mesophyll thickening [33]. A substantial reduction in mesophyll cell size was also a notable leaf anatomical characteristic found in *A. gomboformis*. Comparatively, small cells were found to better withstand turgor pressure than massive ones and more efficiently help to maintain turgor [34]. Thus, the decline in cell size was relatively considered to sustain tissue turgescence in *A. gomboformis* as a leaf tolerance mechanism.

On the other side, improved porosity by raising the parenchyma intercellular space did not promote the propagation conductivity of stressed plants [35]. The existence of highly vacuolated epidermal cells with poor metabolic seemed to function as a dumping mechanism in preventing mesophyll cells from stress [36]. However, decreasing the diameter of the xylem tube contributed in decreased hydraulic and ionic conductance [37] and therefore reduced photosynthesis and plant development.

The influence of salinity on leaf ultrastructure changed with the plant tolerance to NaCl, as reported in two rice species, (*Oryza sativa* L.). The sensitive rice species ('Jaya') indicated variations in the chloroplast integrity compared to the tolerant species ('Kogut'). Specifically, the majority of chloroplasts of 'Jaya' exhibited indications of damage in reaction to elevated NaCl. In comparison, 'Korgut' tolerance chloroplasts variety did not exhibit any salinity impact. This response was associated with a 53% decrease in PN in Jaya, while no substantial variation in PN was found in 'Korgut' [3] (**Figure 2**). The impact of NaCl on the ultrastructure

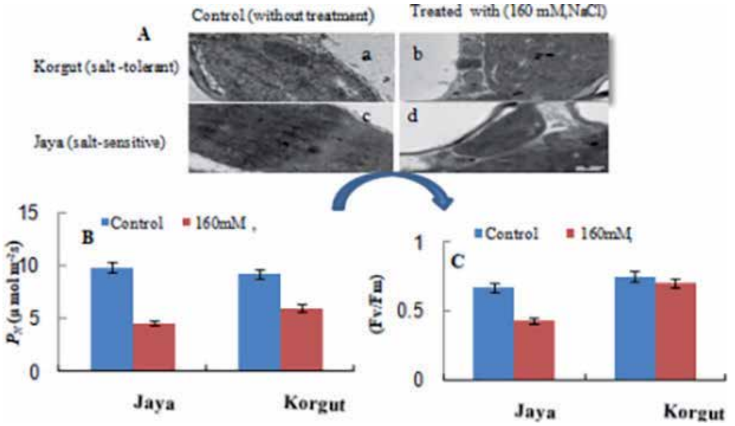

Figure 2.
(A) Transmission electron microscope images of salt-tolerant variety, (a) under control (without treatment) and (b) treated with 160mM Nacl, showing thylakoid system of chloroplasts remained unaffected in control as well as under treatment. However, salt-sensitive variety, transmission electron microscope images of leaf, (c) under control, Chloroplasts had a well-developed system of thylakoids, (d) Thylakoid were damaged with loss of grana stacking under treatment; (B) Gas Exchange measurements; (C) Light reaction of photosynthesis [3].

of leaf chloroplast was investigated in two distinct pea varieties with numerous sensitivity concentrations to NaCl, one sensitive to salt ("cv. Challis") and another relatively [38].

Aranda-Romero et al. [39] investigated anatomical disturbances generated with chloride salts (NaCl, $CaCl_2$, KCl) in both tolerant (*Cleopatra mandarin*) and sensitive (*Carrizo citrange*) citrus cultivars. Salts in P_N were associated with alterations in leaf anatomy, like the decrease in the thickness of the leaf and decreased mesophyll cell area (**Figure 1B–C**), enhanced leaf succulence, and decreased intercellular air space, surface, and density of the tissue. The anatomical alterations related to *Arbutus unedo* leaves in semi-thin sections were found by Navarro et al. [40]. A relationship between control plants and saline plants revealed that the cell size was not significantly changed in the 1st layer of palisade cells. Hernández et al. [38] observed in the same research that the NaCl concentration of salt and salt-tolerant pea plants had a distinct impact. Moreover, *Argyranthemum coronopifolium*, as demonstrated by Morales et al. [41], is similarly susceptible to high levels of salt. These researchers discovered that the salt in these plants does not cause modifications in the chloroplast's number, while in palisade & mesophyll cells, chloroplasts have risen significantly owing to a rise in the quantity of starch in palisade parenchyma compared to mesophyll tissues. A smaller formation of sucrose and hexoses and high activity of phosphate – sucrose synthase may explain the development of starch in leaf-chloroplasts in saline condition, which may lead to the triose-phosphate path towards the synthesis of starch or/and enzymes damage included in starch deterioration through modifications in the ionic chloroplast's composition [41, 42]. The variations in the sizes of a chloroplast, as well as starch accumulation among palisade and mesophyll, parenchyma was explained by various photosynthetic leaf rate values observed by Morales et al. [41].

2. Anatomy of roots

The roots under salinity stress are essential to stress management studies since the root surfaces are initially exposed to environmental stress [43]. The root system

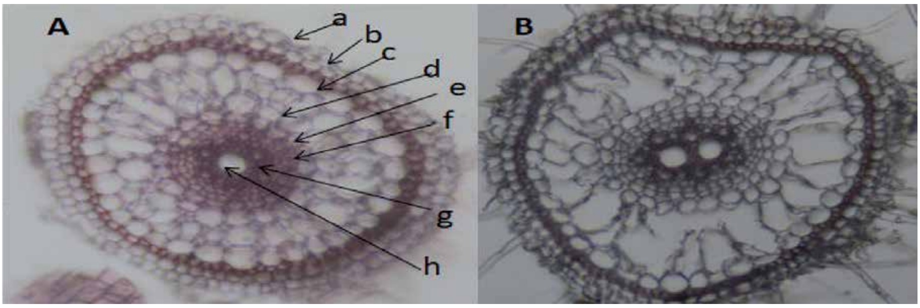

Figure 3.
Effect of salinity on root anatomy (10x, magnification) of oryza sativa L.(A): anatomical structure of rice roots under control (without treatment); and (B):under salinity stress (200 mM NaCl). The different letter in the figures represents; a; epidermis, b; exodermis, c; sclerenchyma layers, d; mesodermis, e; endodermis, f; pericycle, g; pholem, h; metaxylem.

anatomy correlates to root efficiency and permits plants to get nutrients and water, thus increasing the degree of replacement for lost plant water [44]. The anatomy system also prevents the salt build-up from roots, so that water from salty soils may continue [45]. Salt stress mainly controls root hair production and growth [46]. Root epidermal development demonstrates flexibility because external stimuli affect epidermal cells and root hair commencement [47]. The roots cross-section of rice species seedlings studied, in absence of stress, a greater roots thickness and well-organized tissue were noted in rice but root thickness decreased in salt condition (**Figure 3**). Growth of the plasticity root epidermis suggests a role of root hairs in detecting environmental signals that plants adapt to stressful circumstances as a reaction to different environmental conditions [48]. Optimal root systems promote plant development and increase plant output, as roots interface plants with the earth [47]. A plant root system that is increasing thus seems better since it enables it to reach deeper soil layers and get water and nutrients [49]. Moreover, soil environmental variables (temperature fluctuations, salinity, mechanical impedance, lack of O_2) may also have significant effects on the root morphology. Salt stress at a lower concentration induced plentiful root hairs, but progressively less root hair counts were calculated at increased salt levels [48]. Under the stress of salt, the root hair length and the root hair density were below 25 & 40% in comparison with untreated hydroponically cultivated wheat genotypes [36]. Two determinants of the total root surface area are total root duration and branching density – improved during moderate drought stress by comparison with "*Silene vulgaris* seedlings" [43]. Moreover, the morphology of certain plants indicates their salt sensitivity. Salinity inhibits the development of plants via osmotic & toxic effects and high sodium absorption levels because sodium raises soil tolerance, decreases root growth & root water flow by decreasing hydraulic conductivity [50]. The same behavior was also found in "*Euonymus japonica*" plants treated with a solution of NaCl and retrieved water comprising various levels of salt, which reduced the entire plant's root length, most particularly in thin ("$Ø \leq 0.5$ mm") as well as average thickness ("$0.5 < Ø \leq 2.0$ mm") roots [51]. In other varieties of the plant (*Portulaca oleracea* &*Pinus banksiana*), Croser et al. [52] & Franco et al. [46] have reported a rise in root diameter as a result of salinity. The higher root density found in plants indicates better tolerance and perhaps greater reserved accumulation [45, 46] that might enhance plant resistance and accelerate the plant's establishment for farming and landscape objectives, particularly ornamental plants [53]. Root hydraulic conductivity can vary according to the salt concentration of irrigated water used in cultivated fields [54]. The root hydraulic behavior usually expresses the entire root dry weight, regardless of the root architectural function in water absorption capacity. However, the number of fine roots that define the root and the area could vary significantly in any given relatively dry weight value, therefore influencing the degree of water absorption [55]. The detrimental impacts of abiotic stress may be reduced more by salt-tolerant rootstocks than by rootstocks sensitive to salt. Navarro et al. [54] reported lower fruit production and attribute in Carrizo grafting of the *Clemenules mandarins* ("salt-sensitive rootstock") in comparison with *Cleopatra* ("salt-tolerant rootstock"), both exposed to a solution of NaCl (30 mM). According to Penella et al. [56], the output of commercial pepper cultivars during salt irrigation was shown to be raised in salt-tolerant rootstocks. Nassar et al. [57] noted that the widths of vascular bundles and rice stems diameters reduced in NaCl. NaCl treatment in mung bean seedlings was noted by Khan et al. [58] to suppress the development of the vascular system. Gal et al. [59] found that shoot/root ratio was an essential indicator in predicting water consumption, water loss, and hydraulic conductivity in the C3 plants. The roots permeability to water is also measured by other root features, like the number of root hairs, root cortex width, xylem vessels number & diameter, and the suberin deposition in root endodermis and exodermis. This xylem structural modification may influence the capacity of water movement [60]. The xylem's capability to resist negativity depends on the specific environmental limitations [60]. Many studies show that every plant uses the same basic effectors and regulatory systems for salt tolerance and that the variation in glycophytic & halophytic species is quantitative rather than qualitative [61].

3. AMF ("arbuscular mycorrhizal fungi")

The inoculation of roots AMF (that is considered an essential bio-ameliorators) for salt soils and facilitating host plants' strong growth under stressful circumstances through various complicated events of communication between the plants as well as the fungus, leading to enhancing photosynthetic activity and other features linked to gas exchanges [62] and enhanced absorption of water. Several investigations have shown that AMF's effectiveness imparts development and increase in salinity stress plants [63, 64]. AMF enhances plant nutrition by improving the availability and transport of different nutrients [65]; and also enhances soil quality by affecting its texture and structure, and therefore plant health [66, 67] and reduces Na and Cl uptake, resulting in a growth boost [68]. Good interactions between AMF-soil plants may allow reusing of recovered water, especially when roots develop in saline soil [69]. Mycorrhizal inoculation prominently increased photosynthetic rate with other gas interchange characteristics, the content of chlorophyll, and water usage effectiveness in "*Ocimumbasilicum* L." in saline circumstances [70]. AMF-inoculated plants with "*allium sativum*" exhibited increased development rates, such as the leaf area index, dry & fresh biomass in saline circumstances [71] Wang et al. [72] observed a substantial improvement in fresh & dry weights and N levels of Root & Shoot owing to inoculation with mycorrhizal in mild saline conditions.

4. Conclusion

With a burgeoning population estimated to reach around 1.43 billion by 2030, India requires approximately 311 million tons of cereals and pulses to achieve food security. To meet the future food security target, it is expected to increase food grain production by 2 million tons per annum. To increase food grain production, there is a dire need to expand agricultural land and increase crop productivity. One of the possible solutions to address this problem is the genetic improvement of rice varieties in order to enhance their tolerance to salinity. In this review we have discussed morphology and anatomy review that indicates, high salinity is characterized by an increase in the leaf size, trichome and stomata size, and number, thickening of epidermis, area of vascular bundles, maintained thylakoid structure as adaptive characters for salinity stress; these characters are used as an indicator of the salinity of the soil. In addition, AMF is considered essential bio-ameliorators, which can enhance soil quality and maintain better productivity under salinity. Therefore, understanding different mechanisms enable crops to be sustained in hypersaline conditions; this may eventually contribute in improving rice yield on saline lands.

Author details

Smita Srivastava
Department of Botany, Goa University, Taleigao, Goa, India

*Address all correspondence to: srivastavasmita13@gmail.com

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Chapter 12

Intra-Annual Variation in Leaf Anatomical Traits of an Overwintering Shrub of High Elevations of Himalaya

Nikita Rathore, Dinesh Thakur, Nang Elennie Hopak and Amit Chawla

Abstract

Trait variability in response to seasonal variations can be hypothesised as an advantageous strategy for overwintering shrubs. This hypothesis was tested by elucidating patterns of trait variation in an evergreen alpine shrub, *Rhododendron anthopogon* D. Don. The study site was established at Rohtang (3990 m a.s.l.) in western Himalaya. Its leaves were sampled at 10 time points spanning a period of 1 year (beginning from 22-August-2017 to 14-August-2018) for estimating anatomical traits using light and scanning electron microscopy. The data were analysed using one-way analysis of variance, and the trait-temperature relationships were analysed using linear regression. The results indicated a lower variability in the anatomical traits. A few traits (e.g. cuticle thickness and epidermal scales) were found to be significantly correlated with temperature ($p < 0.05$). Our analysis revealed increase in cuticle thickness and a decrease in epidermal scales (size) during low-temperature conditions. The lesser variability found in anatomical traits of overwintering shrub could be explained as 'evolutionary gained adaptive traits'.

Keywords: acclimatory responses, broadleaved evergreen shrub, *in situ* analysis, glandular scales, seasonal variations, temperature regime

1. Introduction

Temperature, as one of the major abiotic factors in high altitudes limiting plant growth and distribution, varies on seasonal basis to a greater extent [1]. Therefore, the plants growing in such environments must be able to respond to these changes by actively acclimating their biology [2, 3]. Most exposed in this regard are the overwintering woody perennials, which get subjected to substantial variations in temperature in the course of a year, from temperatures reaching as high as up to 30°C the during summers to severe freezing conditions (dropping below −30°C) in the winters [4]. Because the overwintering evergreen woody perennials are exposed to seasonal shifts in temperature (favourable to harsh), they are expected to have evolved better to exhibit transitory acclimatory responses [5, 6]. Therefore, these represent an excellent system to study plant persistence strategies in harsh environments.

At high altitudes, plants must develop their 'defences' (during harsh conditions) necessary to survive in a highly variable environment [7]. It has been proposed that persistence in such environments is likely to be facilitated by plasticity in 'anatomical traits' of plants [3, 8]. For instance, the changes in leaf anatomical traits (e.g. increased leaf thickness along altitudinal gradient) have been reported to be linked to the plant's adaptation to changes in environmental conditions (e.g. decrease in temperature, etc.) [9, 10]. However, so far little is known about differential anatomical responses of overwintering evergreen shrubs to seasonal variability. An understanding of the patterns of variation in these traits, while they experience seasonal shifts, will provide insights regarding adaptive strategies in varying environmental conditions (from favourable to harsh).

Rhododendron anthopogon is a dominant evergreen woody shrub occurring in the alpine regions in Himalaya (**Figure 1**) [11]. It is also among those shrub species which are reported to occur at the highest elevations in Himalaya. The species has a broad niche width [12]. In its natural environment, this shrub often gets subjected to variations in environmental conditions along spatio-temporal gradients. All these characteristics indicate well adaptability of this species to grow in the high-altitude environment.

Studying adaptive responses of species growing in their natural habitat could provide invaluable information about how plants prepare themselves to persist under changing environmental conditions. The common approach to investigate plant responses to environmental variability has been to resample the same plant population(s) and directly make comparison between them [13]. *R. anthopogon* with overwintering foliage is appropriate for studying intra-annual adaptive responses, as its leaves have a life span of more than 1 year, thus enabling repeated measurements on a given leaf type to be conducted across multiple time points. This evergreen species provides an opportunity to study the cold acclimation physiology in overwintering foliage without any interference of any endo-dormancy transitions that are reported to occur in other perennials [14]. So, this alpine dwarf shrub could be utilised to understand plant adaptation strategies in harsh environmental conditions of high elevations.

In the present study, the aim was to understand the extent of variations in leaf anatomical traits that enable plant survival in the harsh climate of high elevations. This study will shed light on strategies of plants to persist in high-elevation natural ecosystems. Specifically, it was hypothesised that in response to seasonal variability of high-elevation environments, (i) the evergreen species will show conspicuous changes in anatomical traits (towards optimal values for a given temperature) if their foliage has to last throughout the year including the overwintering phase, (ii) changes

Figure 1.
R. anthopogon *in flowering stage.*

in trait values will show reverse trends once the conditions shifts towards growth optima. Specifically, the objectives of the study were: (i) to elucidate the patterns of variability in leaf anatomical traits along the seasonal gradient and (ii) to test for a relationship between traits and temperature.

2. Plant sampling

The study was conducted at a site near Rohtang Pass which lies in the east of Pir Panjal Range of western Himalaya, India. This place is characterised by severe cold and long winters with plenty of snowfall. The study site was established at an elevation of 3990 m a.s.l. (32°37′41″ N latitude and 77°25′65″ E longitude). At the study site, the vegetation remains under snow cover from mid-November to the end of May, and thus, has a short growing season.

To have accurate estimation of trait variability, the study necessitated repetitive trait measurements to be conducted on leaves developed in the same year. As observed in the field, the newly developed leaves of *R. anthopogon* become fully expanded during mid-August. At this stage, the abaxial surface of these leaves is yellowish-green in colour. Thereafter, a transition to characteristic brownish colour of abaxial surface could be observed towards the onset of winters. Afterwards, the brownish colour remains as such, as the plants progress through overwintering phase till senescence in the following year. Likewise, the leaves developed in the same year could be identified by looking at the position and colour pattern on abaxial surface of leaves. If the leaves developed in two consecutive years are compared during August, leaves developed in first year will occupy the lower position and will have brownish colour on the abaxial surface, whereas leaves developed in the following year will occupy the uppermost whorl position and will be completely yellowish-green (**Figure 2**).

Considering all these facts, the sampling was started in the third week of August, 2017, and continued till November in the same year. Further measurements were not possible as the plants got covered under a thick layer of snow for approximately 240 days. Sampling was again resumed after the snow-melt in mid-June which was continued till second week of August, 2018, to complete a full annual cycle. Sampling was done multiple times in a successive manner with an interval of 2–3 weeks, depending on availability of clear sunny days. Thus, the

Figure 2.
Leaves of R. anthopogon *in the month of August [leaves developed in previous year occupy the lower position (brownish colour of the abaxial surface; blue arrow), whereas leaves developed in the present year occupy the uppermost whorl position and are yellowish-green (red arrow)].*

sampling was accomplished for a total of 10 different time points: 22-August-2017, 12-September-2017, 29-September-2017, 11-October-2017, 23-October-2017, 4-November-2017, 15-June-2018, 28-June-2018, 14-July-2018 and 14-August-2018. At every sampling time point, leaves were cut under deionised water and fixed immediately in FAA [comprising Formaldehyde: Glacial acetic acid: Absolute ethanol in 1:1:18 ratio [15]] to measure the anatomical traits, such as total leaf thickness and thickness of cuticle, epidermis (both adaxial and abaxial), palisade and spongy parenchyma and total mesophyll. Also, sun-exposed, healthy, fully expanded leaves of *R. anthopogon* were collected to perform scanning electron microscopy (SEM) to observe the adaxial and abaxial surfaces of a leaf.

Temperatures (for both air and soil) were recorded at the study site during the entire study period, i.e. from August-2017 to August-2018 using temperature data loggers (M-Log5W, GEO Precision, Germany) [16]. Further, to extract the temperature values for each sampling time point, data values of 3 days (sampling day and the 2 days preceding this day) were used as suggested by Lee et al. [17]. The extracted temperature values were used to calculate the mean, minimum and maximum for a particular 'sampling time-point' for use in regression analysis.

3. Leaf anatomical measurements

Leaf sample preparations for light microscopy and SEM analysis were performed following the method of Tripp and Fatimah [15]. For light microscopy, rectangular pieces of leaves fixed in FAA were cut transversely avoiding the midrib. The samples (transverse sections) were passed through tertiary butyl alcohol (TBA) series (50–100%) and infiltrated with paraffin wax (58–60°C). The sections were embedded in small blocks of paraffin wax, and leaf samples of 12-μm size were obtained with a rotary ultra-microtome (Shandon™ Finesse™ 325, Thermo Scientific). The samples were progressively dehydrated in an ethanol series (30–100%), followed by double staining with 1% aqueous safranin and 0.5% fast green. The sections were permanently mounted on to slides with di-butyl phthalate polystyrene xylene (DPX). Micrographs were taken with camera (Nikon Digital Camera, D5300, Nikon Inc., Japan) mounted on light microscope (Nikon Eclipse E200, Nikon Inc., Tokyo, Japan), focussed at 40×. Total leaf thickness along with thickness of cuticle, epidermis (both adaxial and abaxial surface), palisade and spongy parenchyma and mesophyll tissue were measured (in μm) in randomly selected microscopic fields using ImageJ software. Maximum of three values were taken from three microscopic fields, respectively. These were later averaged for a given trait making one replicate. A total of five such biological replicates were estimated. ImageJ was calibrated with an image of ocular micrometre scale (taken at 40×).

For SEM, five leaves from samples collected at every sampling time point were used to determine the size of epidermal scales. Single leaf tissue was cut into two small rectangular pieces (about 4 × 4 mm) from either side of midrib in order to have representations of both the surfaces (adaxial as well as abaxial) of leaf, making it a replica. Both the surfaces of a leaf were mounted immediately on a single aluminium stub and then coated with a thin film (~30 nm) of gold-palladium for 3 minutes (15 kV, 20 mA) in a sputter coater (Hitachi coating unit E1010). The images were taken using a scanning electron microscope (Hitachi S3400N) at scales of 400 and 200 μm. SEM micrographs taken at 200 μm were used to determine the size of abaxial epidermal scales using image J software. Ten scales per micrograph were selected to calculate the diameter (twice), followed by their area estimation (assuming scale to be circular).

4. Statistical analysis

The mean ± standard deviation was calculated from five independent replicates for all the variables considered for the samples collected at each sampling time point. Linear model assumptions of normality and homoscedasticity were tested using Shapiro-Wilk test and modified Levene's test, respectively. After the data met basic requirements of analysis of variance, one-way ANOVA was performed, and the means were compared to understand the variations in anatomical leaf traits across the 10 time points. This was followed by Tukey's *post hoc* test for identifying the significant pairwise differences ($p < 0.05$). All the analyses were performed with R 3.6.1 statistical software [18]. Linear regression was performed to test the dependence of all the traits on temperature using 'ggscatter' in 'ggpubr' package [19] of R.

5. Anatomical trait variability and temperature relationships

The overall range of leaf anatomical traits of *R. anthopogon* was estimated across the different time points of the growing season (**Table 1**). The total leaf thickness was estimated to be between 24.45 and 30.36 μm during the whole study period. A thick layer of cuticle (0.69–0.84 μm) was present above the adaxial epidermis. Thickness of adaxial and abaxial epidermis varied from 0.93 to 1.16 μm and from 1.12 to 1.26 μm, respectively. The mesophyll tissue (21.14–27.45 μm) comprised of elongated palisade (12.74–16.17 μm) and isodiametric spongy parenchyma cells (8.56–11.03 μm). The transverse section (T.S) of a leaf of *R. anthopogon* is shown in **Figure 3**.

Further, the change in mean values of some of the leaf anatomical traits of *R. anthopogon* across different sampling time points was found to be statistically significant ($p \leq 0.05$), as revealed through one-way ANOVA (**Table 2**). In particular, there were significant differences in total leaf thickness as well as thickness of mesophyll and palisade parenchyma [However, no specific patterns were observed in these anatomical traits along the seasonal gradient (**Figure 4**)]. The cuticle thickness was found to be the highest during onset of winter season (4-November-2017) and the lowest in August (22-August-2017 and 14-August-2018) (**Figure 4**), which is the peak growing season. No significant differences in adaxial and abaxial epidermal thickness were observed for leaves sampled during different time points (**Figure 4**).

The adaxial and abaxial surfaces of leaf showed the presence of glandular scales (**Figures 3** and **5**). These were typically distributed throughout the abaxial surface of leaves during whole of the study period. However, these were mainly observed on the adaxial surface during August (22-August-2017 and 14-August-2018). The size of these glandular scales was estimated to be in the range from 0.015 to 0.025 mm^2. Variations in the size of abaxial epidermal scales due to changes in temperature regime were significantly more pronounced in comparison to other studied anatomical traits. Their size decreased during the early winter time points (i.e. 23-October-2017 and 4-November-2017) (**Figure 6**).

It was found that the air and soil temperatures were found to be positively correlated with each other. Moreover, similar correlation was observed between majority of the studied traits and temperature (both air and soil) ($p < 0.05$); therefore, the results for air temperature only are presented here (**Figure 7**). A positive correlation with temperature was observed for total thickness, mesophyll thickness and spongy parenchyma thickness. However, the thickness of cuticle increased with decreasing temperature (**Figure 7**). Thickness of epidermis (adaxial and abaxial), palisade

Time-point	Total thickness	Cuticle thickness	Adaxial Ep. thickness	Abaxial Ep. thickness	Mesophyll thickness	Palisade thickness	Spongy thickness	Scales size (mm^2)
22-August-2017	26.44 ± 1.058	0.712 ± 0.051	0.927 ± 0.043	1.215 ± 0.107	23.50 ± 1.430	13.12 ± 1.308	9.989 ± 1.198	0.025 ± 0.001
12-September-2017	25.53 ± 2.216	0.750 ± 0.016	1.028 ± 0.150	1.172 ± 0.089	22.19 ± 2.256	13.08 ± 1.272	8.560 ± 1.121	0.023 ± 0.007
29-September-2017	24.45 ± 1.686	0.743 ± 0.059	0.927 ± 0.136	1.146 ± 0.208	21.14 ± 2.215	13.29 ± 1.525	8.727 ± 1.118	0.023 ± 0.003
11-October-2017	25.39 ± 0.728	0.765 ± 0.042	1.075 ± 0.130	1.161 ± 0.070	21.22 ± 1.430	12.74 ± 1.379	9.066 ± 0.731	0.022 ± 0.003
23-October-2017	26.32 ± 2.139	0.774 ± 0.057	0.941 ± 0.047	1.233 ± 0.076	23.44 ± 2.543	13.92 ± 2.906	8.966 ± 1.540	0.018 ± 0.005
04-November-2017	26.98 ± 3.391	0.838 ± 0.075	0.977 ± 0.174	1.234 ± 0.117	23.63 ± 3.775	14.21 ± 2.468	8.891 ± 1.693	0.017 ± 0.002
15-June-2018	29.99 ± 5.396	0.841 ± 0.041	1.045 ± 0.098	1.244 ± 0.168	27.08 ± 6.036	14.82 ± 1.514	10.18 ± 2.268	0.015 ± 0.003
28-June-2018	28.87 ± 1.689	0.789 ± 0.069	1.043 ± 0.154	1.263 ± 0.246	25.47 ± 1.484	14.77 ± 0.426	10.16 ± 1.611	0.015 ± 0.002
14-July-2018	30.21 ± 4.268	0.778 ± 0.090	1.000 ± 0.088	1.146 ± 0.072	27.45 ± 5.181	15.90 ± 2.215	11.03 ± 2.527	0.017 ± 0.003
14-August-2018	30.36 ± 3.295	0.695 ± 0.050	1.159 ± 0.105	1.122 ± 0.099	27.20 ± 3.583	16.17 ± 1.828	10.69 ± 1.827	0.021 ± 0.004

Values are given as mean ± standard deviation. Ep., epidermis.

Table 1.
Variability in anatomical traits [thickness in μm (n = 5)] of R. anthopogon during the study period.

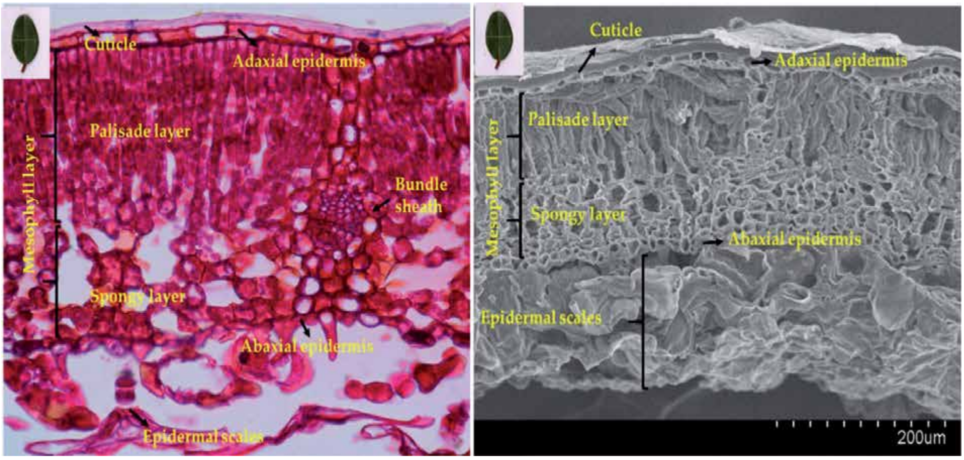

Figure 3.
View of transverse section of leaves of R. anthopogon *using light microscopy (left) and scanning electron microscopy (right).*

Parameter	Df	TSSq	MSSq	*F*-value	*P* value
Total thickness (μm)	9	220.0	24.45	2.825	0.011
Cuticle thickness (μm)	9	0.101	0.011	3.332	<0.001
Adaxial epidermis thickness (μm)	9	0.243	0.027	1.869	0.085
Abaxial epidermis thickness (μm)	9	0.111	0.012	0.645	0.752
Mesophyll thickness (μm)	9	265.7	29.52	2.610	0.018
Palisade parenchyma thickness (μm)	9	64.72	7.190	2.190	0.040
Spongy parenchyma thickness (μm)	9	35.26	3.917	1.442	0.204
Abaxial Epidermal scales (mm^2)	9	0.006	0.0007	27.18	<0.001

Df, degree of freedom; TSSq, total sum of squares; MSSq, mean sum of squares.

Table 2.
Summary of one-way ANOVA results showing the effect of 'seasonal gradient' factor on anatomical traits of R. anthopogon*.*

parenchyma and size of abaxial epidermal scales did not show any significant correlation with variation in temperature across the study period.

6. Anatomical traits can be explained more as 'evolutionary gained adaptive traits'

Plants often exhibit considerable variations in their anatomical traits enabling them to adapt to changing environments [20]. Therefore, the analysis of anatomical traits is crucial for understanding of plant functioning and survival at high elevations. It has been suggested that leaf anatomical structures are associated with physiological functionality in *Rhododendron* spp. and provide more competitiveness in variable environments [21]. In this present study, anatomical traits were not found to be with much variability. Some of the anatomical traits (e.g. thickness of adaxial and abaxial epidermis and spongy parenchyma) did not show any significant variations across different sampling time points during the study period. However, statistically significant differences were found for other traits such as total leaf

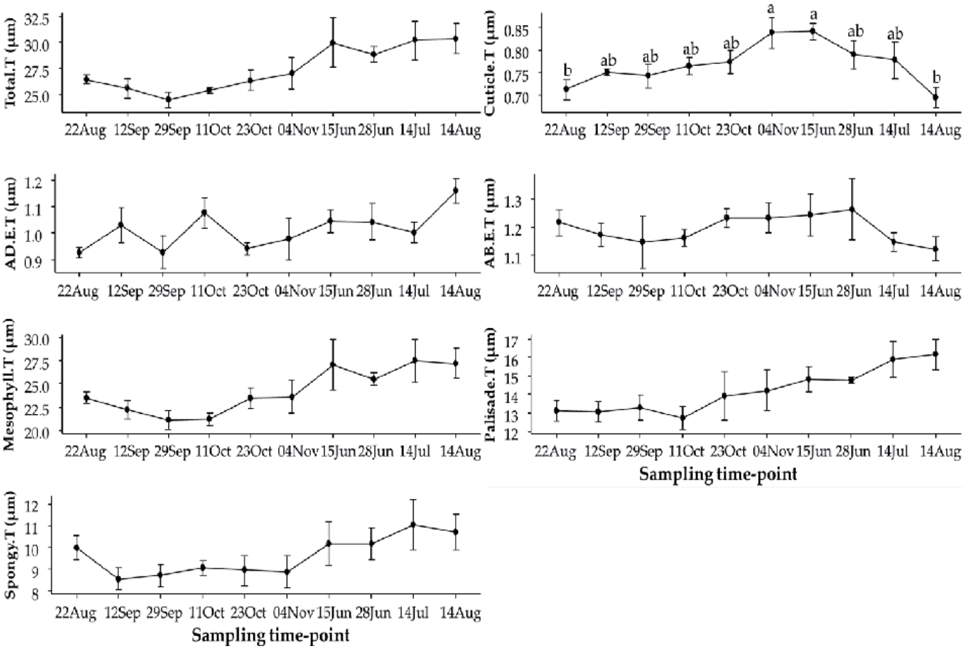

Figure 4.
Variability in leaf anatomical traits of R. anthopogon *during the study period. Vertical bars indicate standard error around the mean. Different alphabets (a, b) represent statistically significant values (p ≤ 0.05) as determined by Tukey's* post-hoc *test. Legend on x-axis, i.e. from 22-August to 04-November are the sampling time points for the year 2017, whereas from 15-June to 14-August represent the sampling time points of year 2018. Total.T, total thickness; Cuticle.T, cuticle thickness; AD.E.T, adaxial epidermal thickness; AB.E.T, abaxial epidermal thickness; Mesophyll.T, mesophyll thickness; Palisade.T, palisade parenchyma thickness; Spongy.T, spongy parenchyma thickness.*

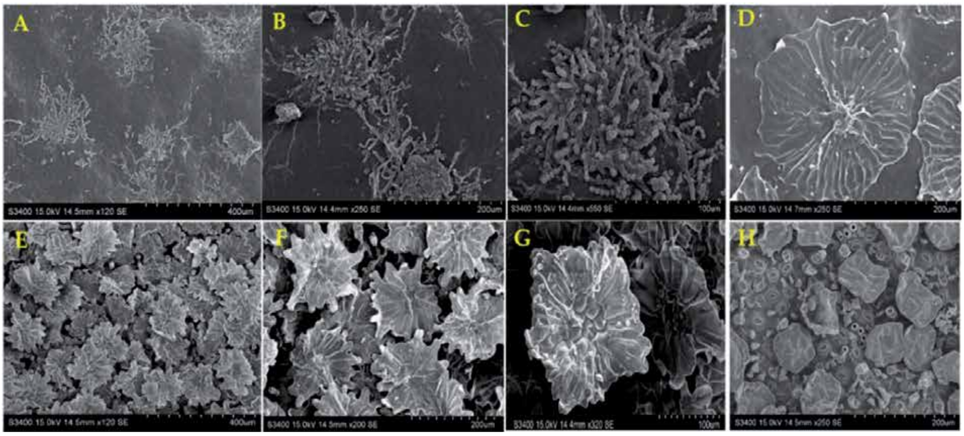

Figure 5.
SEM images showing epidermal scales on the adaxial (A–D; images take at a resolution of 400 µm, 200 µm and 100 µm, respectively) and abaxial surface (E–G; images take at a resolution of 400 µm, 200 µm and 100 µm, respectively) of leaves of Rhododendron anthopogon. *The stomata on the abaxial surface of a leaf (taken at a resoultion of 200 µm) can also be observed (H).*

thickness, cuticle thickness and thickness of mesophyll and palisade parenchyma. An increasing trend for cuticle thickness of leaves was observed with decreasing temperature. The cuticle thickness was found to be maximum during the onset of winter, which thereafter remained constant till snowmelt in the following year (i.e. 15-June-2018). Higher cuticle thickness in leaves helps reduce the transpiration

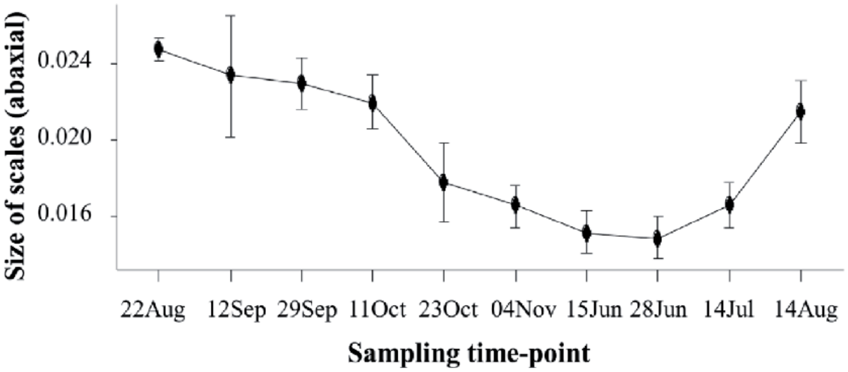

Figure 6.
Variability in the size of abaxial scales (mm^2) of R. anthopogon *during the study period. Vertical bars indicate standard error around the mean. Legends on x-axis, i.e. from 22-August to 04-November, are the sampling time points of year 2017, whereas from 15-June to 14-August represent the sampling time points of year 2018.*

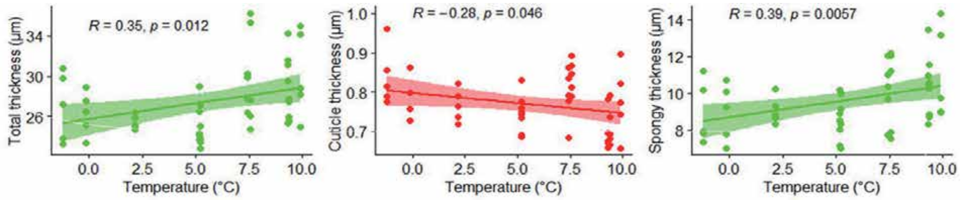

Figure 7.
Regression plots for leaf traits of R. anthopogon *and air temperature. The x-axis corresponds to temperature, whereas y-axis represents the values of anatomical traits estimated at different sampling time points. The red and green coloured regression lines in the graph represent negative and positive correlations between the two variables, respectively. Spongy, spongy parenchyma.*

losses from leaf to atmosphere under low-temperature conditions, when solar radiations are also high [21, 22]. A significant positive correlation of total leaf thickness, mesophyll thickness and thickness of spongy parenchyma tissues with air temperature was also found. Also, these anatomical traits displayed a positive correlation with each other. So, a decrease in total leaf thickness with decreasing temperature could be attributed to simultaneous reduction in thickness of both mesophyll and spongy parenchyma tissues. The reduced thickness of different tissues might be an adjustment for protection from 'dehydration conditions induced due to low temperatures' (by decreasing the surface area of leaf tissues on exposure to low temperatures) [23, 24]. The spongy parenchyma have large inter-cellular spaces which have a role in mesophyll conductance (gas transportation and exchange) [25]. Due to slower metabolism in plants during low-temperature conditions, there is lesser requirement for mesophyll conductance, which probably explains the cause of reduction in the thickness of spongy parenchyma in this species during early winter conditions.

The peculiar anatomical structures such as epidermal appendages, act as evolutionary adaptive traits and help plants in protection against harsh environmental conditions [26]. In the present study, the presence of unique overlapping glandular scales on abaxial side of leaves (covering the entire surface) was observed (**Figures 3**–**5**). Scales of similar shape but larger in size could also be recognised on the adaxial surface of leaves which, however, were evident only during August. On this side of leaf, these scales were unnoticeable during rest of the time period, which could be due to deposition of epi-cuticular wax on the adaxial surface of leaves. The presence of such leaf scales has also been reported in other *Rhododendron* species and have a specified function related to water relations, energy balance and gas exchange [27, 28]. The leaf surface topography, primarily represented by leaf hair/scales and the cuticular wax layer, is reported to shield it from the effects of low temperature and high solar radiations [29]. Similar physiological function of epidermal scales could be assigned for the studied species (i.e. protection from low temperature and high solar radiations). Further, the size of scales was observed to be lower during the onset of winter, which remained constant thereafter. This has not been reported in previous studies, which further evokes interest to investigate their functional role and dynamics. However, it can be proposed that the reduction in size could be due to increase in compactness, which may be the result of slight leaf curling as reported in other *Rhododendron* species [1, 6].

Overall, our results suggested that the leaf anatomy is relatively less sensitive to seasonal variations, as depicted by low variability observed in majority of the anatomical traits. The less variability could be attributed to the fact that the plant may not invest considerably in structural adjustments to counter seasonal variations. This can be explained by the fact that this evergreen shrub, occupying the highest elevations in the Himalaya, survives the harsh environmental conditions throughout the year, which is likely to be achieved via consistency in leaf anatomical traits. Thus, the results reinforce the idea that structural traits 'in general' are less variable and are 'evolutionary gained adaptive traits' [30, 31]. The low investment in structural adjustments may be due to their higher construction cost leading to diminishing returns [32, 33].

7. Conclusions

The findings presented here contribute to the understanding of intra-annual plant trait variability (the type of response and its magnitude) in an overwintering evergreen shrub. The evidences outlined above indicate that the leaf anatomy is less sensitive to seasonal variations. Low variability in leaf anatomical traits of *R. anthopogon* supports the concept of low-cost mechanism for attaining tolerance to harsh conditions and conservative use of resources. The results also indicated that certain evolutionary acquired adaptive traits such as epidermal glandular scales are supportive to successfully persist in harsh climates. Overall, the findings suggest that the plants in varying environments, such as high altitudes, reconfigure their anatomy to a little extent to sustain climatic variabilities.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author's contributions

N.R. carried out the plant sampling, anatomical trait measurements, SEM analysis, statistical analysis and drafting of the manuscript. D.T. performed the plant sampling, SEM analysis and statistical analysis. N.E.H. performed the plant sampling and anatomical trait measurements. A.C. conceived the study, designed the experiment, provided statistical guidance, edited and finalised the manuscript.

Author details

Nikita Rathore[1,2], Dinesh Thakur[1,2], Nang Elennie Hopak[1,2] and Amit Chawla[1,2]*

1 Environmental Technology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

2 Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India

*Address all correspondence to: amitchawla21@gmail.com

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Recent human migrations, technological advances, agricultural activities, and climate change-induced phenomenon have forced plants to increasingly adapt to new environments. This book highlights current morphological, anatomical, physiological, molecular, and genomic advances in plant defense mechanisms. These advances, including epigenetic mechanisms, have been linked to observed phenotypic plant plasticity. Researchers have found intriguing plant interactions and novel mechanisms, which have increased our understanding of how sessile plants adapt to and thrive in challenging environments. The studies in this book consider the resilience and sustainability of plant genomes and epigenomes and the role they will play in the next generation of food systems.

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