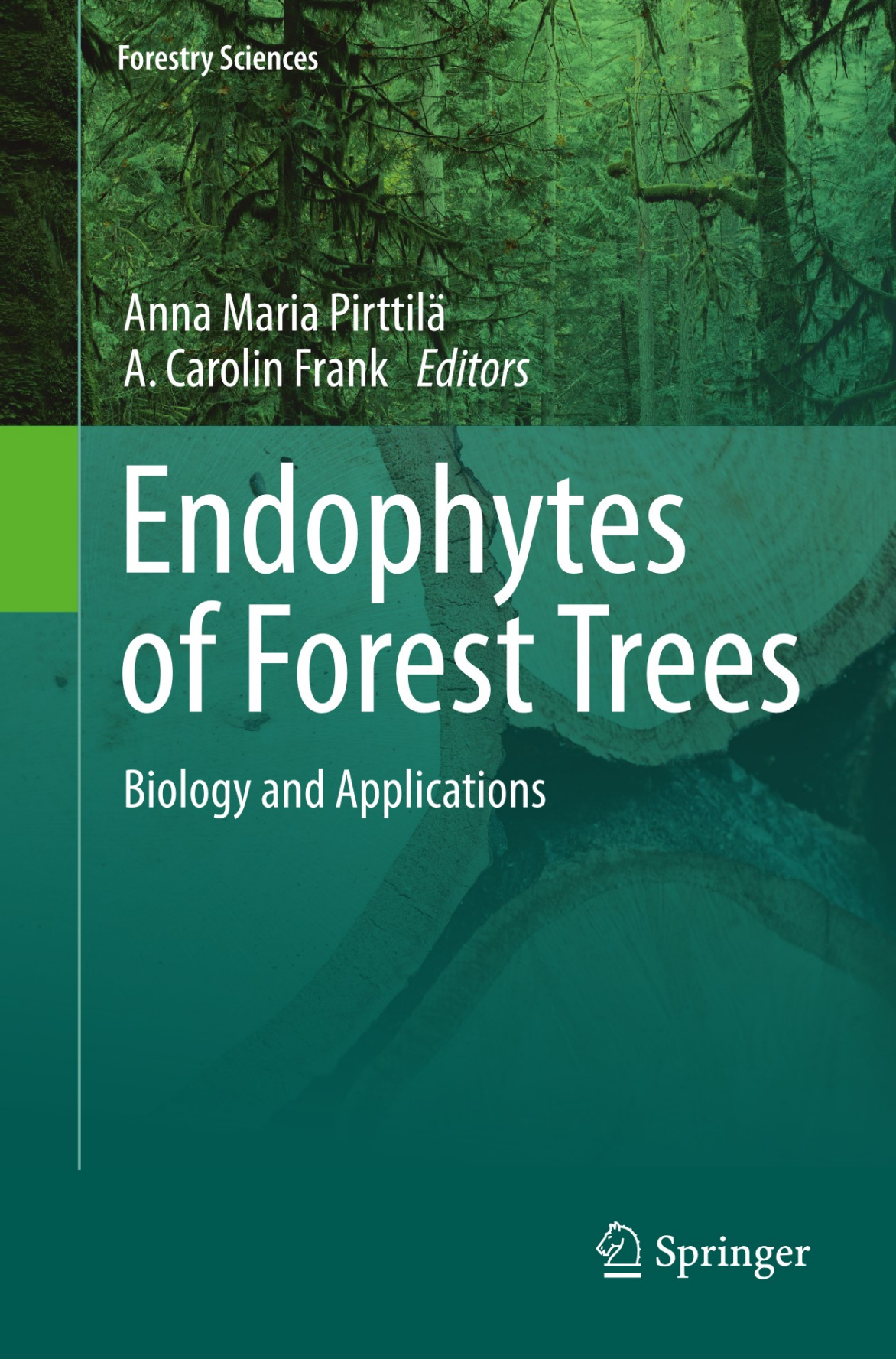
Forestry Sciences
Anna Maria Pirttilä
A. Carolin Frank Editors
Endophytes of Forest Trees
Biology and Applications
Springer

Endophytes of Forest Trees

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Editors

Endophytes of Forest Trees

Biology and Applications

Springer

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Preface

Although to the naked eye plants appear to be single organisms, it is now well established that they form associations with a consortium of incredibly numerous and diverse microbes that dwell on their surface, around their roots, and within their tissues. This book is the first full survey on what the forest trees hold within—endophytic fungi and bacteria. Interestingly, these endophytic microorganisms from two distinct domains of life have converged to perform some of the same functions in relation to their shared tree host, possibly allowing the long-lived trees to meet the challenge of variable or deleterious environmental conditions. Both bacterial and fungal endophytes can increase the growth of their host and provide increased tolerance towards biotic and abiotic stresses. Examples from this book include growth-promotion, protection against drought stress, resistance to insects and pathogenic fungi, and alleviation of metal toxicity.

Given the often profound beneficial effects that endophytes exert on their hosts, there are several fields where an improved understanding of forest tree endophytes is necessary. First, forest tree endophytes may be a more important part of host tree biology than previously recognized. Endophytes and other plant-associated microorganisms are often neglected when studying forest tree range shifts or the effects of stressors such as air pollution and disease on trees. However, endophytes have a pivotal role in, for example, the interaction between trees and insect pests, and can be susceptible to the same stressors as their tree host, with possible consequences for important mutualistic interactions. These examples illustrate the necessity of taking a holistic approach to the study of trees, including associated microbial assemblages.

Second, forest tree endophytes have great biotechnological potential. Whereas endophytic bioactivity is being harnessed for applications in agriculture worldwide, the progress in forestry is far behind. Advances are currently being made on biocontrol of spruce budworm by endophytic fungi, and the use of endophytic bacteria in phytoremediation, which may bring new biotech applications on the market within the near future. Endophyte-based applications could, and should, become much more extensive and important to forestry than they are today.

Third, endophytes of forest trees can be used for biotechnological applications on other arenas as well. The fact that they provide a widely unstudied and diverse source of completely new bioactive compounds has already raised the interest of pharmaceutical industry. The bioactive compounds produced by endophytes have unlimited prospects for development as leads for various drug compounds, cosmetics, food preservatives, components for wood and paper industry, drapery, and so on.

Essential for promotion of all three research fields is a better understanding of the evolution, diversity and patterns of host-endophyte interactions in the wild. Such knowledge is necessary for predicting endophyte behavior (mutualistic vs pathogenic) with respect to the host under specific conditions, or in new ecosystems. In general, the diversity and ecology is better understood for endophytes of grasses and agricultural crops than for endophytes of forest trees. Foliar endophyte diversity is reasonably well known for a few forest tree species, but many ecologically and economically important tree hosts have not yet been examined for endophytes, or lack a comprehensive understanding of endophyte diversity across tissues. Most of all, bacterial endophytes are practically unstudied in forest trees: only a handful of reports exist even though bacterial forest endophytes hold a great promise with respect to their biological activities.

Fortunately, new technologies and methods that are discussed throughout this book have the potential to close this knowledge gap and bring the 'endophytology' of forest trees to a new level. Thanks to the rapid progress in sequence technology, we are now poised to explore the uncultured portion of both fungal and bacterial endophytes across a large number of tree species, tissues, geographic locations and environmental conditions. Until we have methods for studying the role of each individual member in complex symbiotic microbial communities, we can use comparative analysis of large numbers of naturally occurring variant communities with extensive environmental metadata. This kind of holistic approach, usually applied in ecology, has significantly deepened our understanding of microbial communities in other ecosystems such as the phyllosphere and the human gut, and holds great promise for advancing the field of forest endophytes. Culture-independent approaches that allow characterization of a large number of samples at depth can partition the community in core versus transient endophyte species that may have different functional roles in regards to the host. Such information might prove useful when selecting isolates for e.g. in vitro assays or biocontrol studies.

Genome sequencing has the potential to validate and extend current models of endophyte entry, colonization, persistence and beneficial host-interaction. So far, only bacterial endophyte genomes have been sequenced, but the same is true for fungal endophytes. Finally, metagenomics, single-cell genomics and expression of pathways in heterologous hosts hold great promise to discover endophyte metabolites and yield insights into the ecological role of endophytes that elude current culturing attempts.

We can expect that the continuous development of these techniques for the study of endophytes will fully reveal their biology in the plant, as well as in the forest. As endophytes are proving to be the crucial, even essential components of plant life, their significance for the forest might equal, or even exceed that of other plant symbionts.

Oulu, Finland — Anna Maria Pirttilä
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Contents

Part I
Diversity of Forest Tree Endophytes

Structure of Diversity in Dark Septate Endophytes: From Species to Genes

Christoph R. Grünig, Valentin Queloz, and Thomas N. Sieber

Abstract Dark septate endophytes (DSE) are among the most abundant colonizers of plant roots. The form taxon DSE includes a broad range of fungal species that are only distantly related and the taxonomy of DSE has puzzled mycologists for years. In the following chapter we discuss the structure of diversity in dark septate endophytes. In the first part, we give an overview of the taxonomic placement of DSE and present a reference dataset of ITS sequences of well-characterized DSE taxa that can be used to classify DSE in future studies. The second part is dedicated to the diversity in the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex (PAC), which includes some of the most frequent and widespread DSE. Diversity of PAC is discussed on various levels ranging from single root segments to continents and from communities to genes.

Abbreviations

PAC	*Phialocephala fortinii* s.l. – *Acephala applanata* species complex
DSE	Dark septate endophytes
AMF	Arbuscular mycorrhizal fungi
BLAST	Basic local alignment search tool
MPV	*Mollisia, Phaeomollisia* and *Vibrissea* teleomorphs
ITS	Internal transcribed spacer
CSP	Cryptic species
MSR	Morphological species recognition
GCPSR	Genealogical concordance phylogenetic species recognition
RFLP	Restriction fragment length polymorphism
MLH	Multi-locus genotypes
AMOVA	Molecular variance analysis

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1 Introduction

Diversity of organisms is required to maintain the biogeochemical cycles of ecosystems in the long term, i.e. biodiversity is core to the survival of life. However, it is not known how many and which species are essential to minimize the risk of ecosystem "failure". To approach this important question, measurements of species diversity at constant time intervals and comparisons of these measurements with changes of abiotic environmental factors are needed. Whereas many studies have been dedicated to species diversity of plant and animal communities, diversity of microbial communities has received much less attention. This is especially true for fungal endophytes in apparently healthy roots (Sieber 2002). Endophytic fungi have repeatedly been isolated from mycorrhizal roots during studies on the diversity of ectomycorrhizal communities, but the systematics, diversity, and ecology of these non-classical mycorrhizal endophytes have only recently been more intensely addressed. Root endophytes can be found on all parts of the root system whereas mycorrhizal fungi are usually confined to primary, non-lignified roots. Thus, the biomass of root endophytes in the total root system is expected to be higher than that of mycorrhizal fungi. Consequently, the interactions of root endophytes with their host and with other microorganisms are assumed to be at least as significant as those of mycorrhizal fungi.

In the present chapter we give an overview of the diversity of dark septate endophytes (DSE), a group of ubiquitous and abundant root endophytes. Starting from the definition of the term DSE, we will give some insight in the taxonomy and biodiversity of DSE. Then, we will discuss the structure of biodiversity in DSE species belonging to the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex (PAC) over a multitude of spatial levels ranging from spatial units as small as single roots up to continents.

2 What Are DSE and What Is Their Distribution?

Read and Haselwandter (1981) introduced the name "DS hyphae" (DS = dark septate) for sterile, dark, septate hyphae and microsclerotia, which occurred in roots of various alpine plants. Stoyke and Currah (1991) implemented the form taxon "dark septate endophyte" (DSE) and used it for fungi which form partly or entirely melanized, septate thalli within healthy root tissues. The taxon DSE serves primarily to differentiate these fungi from endophytes with septate, hyaline hyphae and from fungi with sparsely septate, hyaline hyphae, which are characteristic for arbuscular mycorrhizal fungi (AMF). However, the distinction based on the degree of pigmentation is often arbitrary because pigment strength varies greatly in some species (Addy et al. 2005; Hambleton and Sigler 2005) especially that of mycelia in colonized roots (Yu et al. 2001; Barrow 2003). We propose to define DSE as those endophytic fungi, which form colonies that are at least partly dark brown, dark gray or black on 2% (w/v) malt extract agar when incubated at 20°C. DSE are an abundant group of symbionts distinct from mycorrhizal fungi. DSE are

widespread and an increasing number of reports show that DSE colonize roots from arctic to tropical habitats (Wilberforce et al. 2003; Fuchs and Haselwandter 2004; Kai and Zhao 2006; Weishampel and Bedford 2006; Liang et al. 2007; Chaudhry et al. 2009; Lehnert et al. 2009; Likar et al. 2009; Newsham et al. 2009; Zubek et al. 2009; Khidir et al. 2010; Wu et al. 2010; Yuan et al. 2010). However, the acronym DSE is a form taxon and includes a broad range of species that do not form a monophyletic group. The taxonomy of DSE has puzzled mycologists for years. Some DSE readily sporulate in culture, e.g. *Microdochium bolleyi* and several *Cadophora*/*Phialophora* species (Sieber and Grünig 2006), but complete absence of sporulation or sporulation only after prolonged incubation at low temperatures is a feature common to many DSE, hampering morphology-based identification. Colonial morphology of most species is indistinct and therefore does not allow reliable classification. Growth rates are sometimes helpful but do not suffice for a proper identification (Kowalski and Kehr 1995; Ahlich-Schlegel 1997; Grünig et al. 2008a). Thus, mycologists mainly rely on molecular methods for classification of DSE, especially on comparisons of DNA sequences of unknown DSE with those available from nucleotide databases. Accuracy of the information retrieved from these databases depends, however, on the quality of the data deposited. For example, public databases contain sequences from non-cultivated environmental samples that were tagged either with genus or species names retrieved by BLAST searches or with the substratum from which they were isolated/amplified. Although these sequences can give important information about the presence of fungi in substrates and ecosystems, or at geographical locations where they had never been detected before, they may lead to the propagation of erroneous names. The most reliable sequences are those originating from the type specimen or the type culture. Many reference sequences of DSE taxa represented by type strains are available from GenBank, but unfortunately some misnamed sequences have been deposited (Grünig et al. 2009). More rigorously curated databases such as aftol.org may serve as an alternative, but unfortunately they host only a limited number of DSE taxa. Another approach is to perform blast searches locally using the stand-alone software (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/LATEST/) against a custom database including only well-characterized strains of DSE species (e.g. type strains). We favour this approach and tried to gather data of the internal transcribed spacer (ITS) regions of properly characterized dark septate endophytes and close relatives deposited in GenBank and sequence data gained during our own studies (Table 1). A text file including the trimmed sequence data of GenBank Accessions included in Table 1 can be downloaded from the permanent link http://www.forestpathology.ethz.ch/research/pac-research/pac-systematics. However, examination of culture morphology and micromorphology of sporogenesis – if sporulation occurs – is always recommended to validate the results obtained by blast searches.

Many of the DSE taxa listed in Table 1 are placed in the Helotiales, one of the most diverse fungal orders in the ascomycetes that comprises more than 300 genera including over 2,000 described species (Kirk et al. 2001). Three major groups that include DSE can be found in the Helotiales. One group comprises species closely

Table 1 Collection of reference strains for the most abundant DSE and closely related taxa. A file containing the trimmed ITS sequences of these strains is available from http://www.forestpathology.ethz.ch/research/pac-research/pac-systematics

Strain	Collection number[a]	Classification[b]	Taxon	Geographic origin	Host/Substrate	Collector	Classification[c]	Reference[d]	GenBank accessions
K92_113	CBS_109321	DSE	*Acephala applanata*	Bödmeren, Switzerland	Root of *Picea abies*	K. Ahlich	type strain	Grünig and Sieber 2005	AY078145
K93_444	CBS_443.86	DSE	*Phialocephala fortinii* s.s.	Suonenjoki, Finland	Root of *Pinus sylvestris*	H.E. Wilcox	type strain	Grünig et al. 2008a	AY033087
UAMH_6816		DSE	*Acephala* sp. 1	Jasper National Park, Canada	Root of *Cassiope mertensiana*	G. Stoyke	mol	Grünig et al. 2009	EU434823
CBS_143.92		DSE	*Vibrissea truncorum*	Alpenpark Berchtesgaden, Germany	Decaying twigs of *Alnus viridis*	A. Bresinsky	fm/mol	Grünig et al. 2009	EU434855
CBS_258.91		DSE	*V. truncorum*	Algonquin Prov. Park, Canada	Submerged root of *Populus* sp.	R.G. Thorn	fm/mol	Grünig et al. 2009	EU434854
720-2		DSE	*Acephala* sp. 2	Kevo, Finland	Root of *Pinus sylvestris*	V. Queloz	mol	Grünig et al. 2009	EU434827
DSE10-8 s		DSE	*Acephala* sp. 2	Derborence, Switzerland	Root of *Sorbus aucuparia*	V. Queloz	mol	Grünig et al. 2009	EU434824
312-6v		DSE	*Acephala* sp. 3	Scatlé, Switzerland	Root of *Vaccinium myrtillus*	C.R. Grünig	mol	Grünig et al. 2009	EU434829
D_3-1		DSE	*Acephala* sp. 4	Redwater Natural Area, Canada	Root of *Pinus banksiana*	R.S. Currah	mol	Grünig et al. 2009	EU434830
D_3-6		DSE	*Acephala* sp. 4	Redwater Natural Area, Canada	Root of *Pinus banksiana*	R.S. Currah	mol	Grünig et al. 2009	EU434831
PFO-041		DSE	*Acephala* sp. 7	Czech Republic	Root of *Leucorchis albida*	M. Vohnik	mol	n.a.	HQ713749
agrKH079		DSE	*Acephala* sp. 7	Bavaria, Germany	Root of *Calluna vulgaris*	A. Pietrowski	mol	n.a.	FM172778

EW76	CBS_123555	DSE	*Acephala macrosclerotiorum*	Hubertusstock, Germany	Root of *Pinus sylvestris*	B. Münzenberger	type strain	Münzenberger et al. 2009	EU882732
AF207-5		DSE	*A. macrosclerotiorum*	Afforestation, Lithuania	Root of *Picea abies*	A. Menkis	mol	Menkis et al. 2004	EU434833
444-7v	UAMH_10852	DSE	*Phialocephala glacialis*	Creux du Van, Switzerland	Root of *Vaccinium myrtillus*	V. Queloz	type strain	Grünig et al. 2009	EU434843
CdV_5_3.5 s	UAMH_10853	DSE	*P. glacialis*	Creux du Van, Switzerland	Needle of *Picea abies*	C.R. Grünig	cm/mol	Grünig et al. 2009	EU434842
UAMH_10279		DSE	*Phialocephala sphaeroides*	Elk Island National Park, Canada	Root of *Aralia nudicaulis*	H. Addy	type strain	Wilson et al. 2004	AY524844
70-2		DSE	*P. sphaeroides*	Bödmeren, Switzerland	Roots of *Picea abies*	C.R. Grünig	cm/mol	Grünig et al. 2009	EU434852
UAMH_10206		DSE	*Phialocephala* sp. 8	Perryvale, Alberta, Canada	Roots of *Carex aquatilis*	M.N. Thormann	fm/mol	Grünig et al. 2009	EU434851
UAMH_10827		DSE	*Phialocephala urceolata*	Hazelwood, USA	Heparin solution	D. McGhee	type strain	Wang et al. 2009	EU155145
K93_453	CBS_300.62	DSE	*Phialocephala dimorphospora*	New Brunswick, Canada	Wood pulp	W.B. Kendrick	cm/fm/mol	Grünig et al. 2002a	AF486121
MYR5.2		DSE	*Phialocephala* sp. 9	Senge Khabab, Tibet	Root of *Myricaria prostrata*	K. Burri	fm/mol	Burri, unpublished	HQ713750
MYR3.4		DSE	*Phialocephala* sp. 9	Senge Khabab, Tibet	Root of *Myricaria prostrata*	K. Burri	fm/mol	Burri, unpublished	HQ713751
CBS_444.86		DSE	*Cadophora finlandica*	Suonenjoki, Finland	Root of *Pinus sylvestris*	C.J.K. Wang	type strain	Wang and Wilcox 1985	AF486119
aurim603		DSE	*Cadophora finlandica*	Varena, Lithuania	Root of *Picea abies*	A. Menkis	cm/mol	n.a.	AY606311

(continued)

Table 1 (continued)

Strain	Collection number[a]	Classification[b]	Taxon	Geographic origin	Host/Substrate	Collector	Classification[c]	Reference[d]	GenBank accessions
UAMH_8861	CBS_116122	DSE	*Meliniomyces variabilis*	Jasper National Park, Canada	Root of *Rhododendron albiflorum*	S. Hambleton	type strain	Hambleton and Sigler 2005	AY762619
UAMH_10381		DSE	*M. variabilis*	Vancouver Island, Canada	Root of *Tsuga heterophylla*	M.L. Berbee	cm/mol	Hambleton and Sigler 2005	AY394917
UAMH_10107	CBS_116122	DSE	*Meliniomyces bicolor*	North Yorkshire, Unitied Kingdom	Root of *Nothofagus procera*	A. Taylor	type strain	Hambleton and Sigler 2005	AJ430147
UAMH_10108	CBS_116123	DSE	*M. bicolor*	Kragero, Telemark, Norway	Root of *Quercus robur*	T. Vralstad	cm/mol	Hambleton and Sigler 2005	AJ292203
UAMH_10111	CBS_116126	RE	*Meliniomyces vraolstadiae*	Akershus, Eidsvoll, Norway	Root of *Betula pubescens*	T. Vralstad	type strain	Hambleton and Sigler 2005	AJ292199
UAMH_10112	CBS_116127	RE	*M. vraolstadiae*	Akershus, Eidsvoll, Norway	Root of *Betula pubescens*	T. Vralstad	cm/fm/mol	Hambleton and Sigler 2005	AJ292200
ARON3024.S		RE	*Meliniomyces* sp. 1	Hedmark, Norway	Root of *Betula pubescens*	T. Vralstad	mol	Hambleton and Sigler 2005	AJ430126
ARON3066.S		RE	*Meliniomyces* sp. 2	Hedmark, Norway	Root of *Pinus sylvestris*	T. Vralstad	mol	Hambleton and Sigler 2005	AJ430176
UBCtra264		RE	*Meliniomyces* sp. 3	n.a.	Root of *Gaultheria shallon*	M.L. Berbee	mol	Hambleton and Sigler 2005	AF149070

ARON2962.S		RE	*Meliniomyces* sp. 3	Oslo, Norway	Root of *Vaccinium myrtillus*	T. Vralstad	mol	Hambleton and Sigler 2005	AJ430113
ARON3066.S		RE	*Meliniomyces* sp. 4	Hedmark, Norway	Root of *Pinus sylvestris*	T. Vralstad	mol	Hambleton and Sigler 2005	AJ430176
UAMH_6735	IMI182065	DSE	*Pezoloma ericae*	Bolsterstone, United Kingdom	Root of *Calluna vulgaris*	D. Read	type strain	Read 1974	AY762620
UAMH_8680		DSE	*P. ericae*	Athabasca, Canada	Root of *Ledum groenladicum*	S. Hambleton	cm/fm/mol	Hambleton et al. 1999	AY762622
ZT98022	UAMH_5422	DSE	*Leptodontidium orchidicola*	Devonian Botanical Garden, Canada	Root of *Platanthera hyperborea*	R.S. Currah	type strain	Currah et al. 1987	AF486133
UAMH_8152		DSE	*L. orchidicola*	Cardinal River Divide, Canada	Root of *Pedicularis bracteosa*	A.A. Fernando	cm/fm/mol	Currah et al. 1987	AF214576
TS_06_017		DSE	*Cadophora malorum*	Valbella, Switzerland	Root of *Picea abies*	T.N. Sieber	cm/fm/mol	n.a.	HQ713752
CBS_266.31	UAMH_11047	DSE	*C. malorum*	n.a.	n.a.	F.D. Heald	type strain	Harrington and Mcnew 2003	AY249057
02_09_2_3		DSE	*Cadophora* sp. 1	Etang de la Gruère, Switzerland	Root of *Picea abies*	V. Queloz	fm/mol	n.a.	HQ713753
K93_333		DSE	*Cadophora* sp. 2	Jura, Switzerland	Root of *Abies alba*	K. Ahlich	fm/mol	n.a.	AY664503
ZT_93_029		DSE	*Cadophora* sp. 2	British Columbia, Canada	Pith of *Alnus rubra*	C.E. Dorworth	fm/mol	n.a.	HQ713754

(continued)

Table 1 (continued)

Strain	Collection number[a]	Classification[b]	Taxon	Geographic origin	Host/Substrate	Collector	Classification[c]	Reference[d]	GenBank accessions
B24-2		DSE	*Cadophora* sp. 2	Stettbach, Switzerland	Root of *Pinus sylvestris*	S. Bachmann	cm/fm/mol	n.a.	HQ713755
CBS_307.49		n.a.	*Cadophora fastigiata*	Sweden	Blue stain from *Pinus* sp.	S.O. Pehrson	cm/fm/mol	Harrington and Mcnew 2003	AY249073
CBS_268.33	UAMH_11046	n.a.	*Cadophora melinii*	Sweden	n.a.	J.A. Nannfeldt	type strain	Harrington and Mcnew 2003	AY249072
CBS_141.41	UAMH_11053	n.a.	*Cadophora luteo-olivacea*	Munksund, Sweden	Waste water of Schleiferei Byske	Munksunds Sulfatfab-rik	type strain	Harrington and Mcnew 2003	AY249066
MYR1.1		DSE	*Cadophora* sp. 3	Senge Khabab, Tibet	Root of *Myricaria prostrata*	K. Burri	fm/mol	n.a.	HQ713756
UAMH_9445	CBS_120290	DSE	*Cryptosporiopsis ericae*	Priest Lake, Idaho, USA	Root or *Vaccinium membranaceum*	B. McCracken	type strain	Sigler et al. 2005	AY540126
01_08_2_5		DSE	*C. ericae*	Etang de la Gruère, Switzerland	Root of *Picea abies*	V. Queloz	cm/mol	Sigler et al. 2005	HQ713757
UAMH_10106	CBS_120291	DSE	*Cryptosporiopsis brunnea*	Vancouver Island, Canada	Root of *Gaultheria shallon*	T. Allen	type strain	Sigler et al. 2005	AF149074
CBS_898.97		DSE	*Cryptosporiopsis melanigena*	Patzmannsdorf, Austria	Root of *Quercus petraea*	E. Halm-schlager	type strain	Kowalski et al. 1998	AF141196

CBS_640.94	DSE	*Cryptosporiopsis radicicola*	Krakow, Poland	Root of *Quercus robur*	T. Kowalski	type strain	Kowalski and Bartnik 1995	AF141193
CBS_109839	DSE	*Cryptosporiopsis rhizophila*	Drenthe, Netherland	Root of *Erica tetralix*	J.D. Zijlstra	type strain	Verkley et al. 2003	AY176753
01_10_2_5	DSE	*Cryptosporiopsis* cf *melanigena*	Etang de la Gruère, Switzerland	Root of *Picea abies*	V. Queloz	cm/fm/mol	n.a.	HQ713758
01_13_2_8	DSE	*Cryptosporiopsis* cf *melanigena*	Etang de la Gruère, Switzerland	Root of *Picea abies*	V. Queloz	cm/fm/mol	n.a.	HQ713759
01_13_3_5	DSE	*Cryptosporiopsis* cf *melanigena*	Etang de la Gruère, Switzerland	Root of *Picea abies*	V. Queloz	cm/mol	n.a.	HQ713760
CBS_445.86	DSE	*Chloridium paucisporum*	Syracuse NY, USA	Root of *Pinus resinosa*	C.J.K. Wang	cm/fm/mol	Alberton et al. 2010	EU938675
CBS_491.70	DSE	*Cladophialophora chaetospira*	Denmark	Root of *Picea abies*	D.S. Malla	cm/fm/mol	Crous et al. 2007	EU035405
NZ_9_1	DSE	*Dermea* sp.	Arthur's Pass, New Zealand	Root	S. Ringger	mol	n.a.	HQ713761
03_08_2_3	DSE	*Didymella bryoniae*	Etang de la Gruère, Switzerland	From root of *Picea abies*	V. Queloz	cm/fm/mol	n.a.	HQ713762
3357	DSE	*D. bryoniae*	Spain	Leaf of *Holcus lanatus*	S. Sanchez Marquez	cm/fm/mol	Sánchez Márquez et al. 2010	FN394714
TS_04_050	DSE	*Didymosphaeria* sp.	Muri, Switzerland	Root of *Picea abies*	N. Brenn	cm/fm/mol	Brenn et al. 2008	HQ713763
Ca2_1.5	DSE	*Embellisia chlamydospora*	Senge Khabab, Tibet	Root of *Carex stenophylla*	K. Burri	cm/fm/mol	n.a.	HQ713765

(continued)

Table 1 (continued)

Strain	Collection number[a]	Classification[b]	Taxon	Geographic origin	Host/Substrate	Collector	Classification[c]	Reference[d]	GenBank accessions
Zeyer3		DSE	*E. chlamydospora*	Santa Rosa, Chile	Root of *Paja brava*	J. Zeyer	mol	n.a.	HQ713766
CBS_342.71		DSE	*E. chlamydospora*	Saskatchawan, Canada	Stem of *Linum* sp.	A.E. Barrett	cm/fm/mol	n.a.	AB120854
CBS_115143		DSE	*Exophiala* sp.	Australia	From botteled spring water	n.a.	cm/fm/mol	Crous et al. 2007	DQ008140
MYA-3385		DSE	*Gaeumannomyces cylindrosporus* (*Harpophora graminicola*)	Washington, USA	*Poa pratensis*	N. Tisserat	cm/fm/mol	Saleh and Leslie 2004	AY428772
MYA-3373		DSE	*Gaeumannomyces graminis* var. *graminis* (*Harpophora radicicola*)	Kansas, USA	*Oryza sativa*	J.F. Leslie	cm/fm/mol	Saleh and Leslie 2004	AY428779
WurzelC_13_4		DSE	*Herpotrichia* sp.	Zürichberg, Switzerland	Root of *Picea abies*	C.R. Grünig	cm/mol	n.a.	HQ713767
Island2_1		DSE	*Herpotrichia* sp.	Iceland	Root of *Vaccinium* sp.	B. Müller	cm/mol	n.a.	HQ713768
Island3_3		DSE	*Herpotrichia* sp.	Iceland	Root of *Vaccinium* sp.	B. Müller	cm/mol	n.a.	HQ713769
O25-1		DSE	*Leptosphaeria* sp. 1	Stettbach, Switzerland	Root of *Pinus sylvestris*	S. Bachmann	cm/fm/mol	n.a.	HQ713770

UAMH_10720	DSE	*Monodictys arctica*	Alexandra Fiord, Ellesmere Island, Canada	Root of *Salix oppositifolia*	M.J. Day	type strain	Day et al. 2006	EU686521
K94_019	DSE	*Monodictys arctica*	Antarctica	Lichen	C. Möller	mol	Möller and Dreyfuss 1996	EF485231
K._Tanaka_709	DSE	*Lophiostoma macrostomum*	River bank of Oowasawa river, Japan	From stem of unknown plant	K. Tanaka	cm/fm/mol	Tanaka and Hosoya 2008	AB433276
K27-1	DSE	*Macrophomina phaseolina*	Stettbach, Switzerland	Root of *Pinus sylvestris*	S. Bachmann	cm/fm/mol	n.a.	HQ713771
Mac_Pot2	DSE	*M. phaseolina*	Pianura Bolognese, Italy	Rhizosphere of *Solanum tuberosum*	L.M. Manici	cm/fm/mol	Manici and Caputo 2009	EF017208
4018	DSE	*Microdochium bolleyi*	La Coruna, Spain	Roots of *Elymus farctus*	S. Sanchez Marquez	cm/fm/mol	Sánchez Márquez et al. 2008	AM924150
Ca1.2	DSE	*Monodictys* sp. 1	Senge Khabab, Tibet	Root of *Carex stenophylla*	K. Burri	cm/fm/mol	n.a.	HQ713772
Ca5.2	DSE	*Monodictys* sp. 1	Senge Khabab, Tibet	Root of *Carex stenophylla*	K. Burri	cm/fm/mol	n.a.	HQ713773
CBS_605.92	RE	*Neonectria radicicola*	Hamburg, Germany	Root of *Tilia petiolaris*	R. Schröer	cm/fm/mol	Schroers et al. 2008	EF607078
B14-1	RE	*N. radicicola*	Stettbach, Switzerland	Root of *Pinus sylvestris*	S. Bachmann	cm/fm/mol	n.a.	HQ713774
CBS_183.36	RE	*Nectria tawa* (*Cylindrocarpon obtusisporum*)	Germany	From tuber of *Solanum tuberosum*	H.W. Wollen-weber	cm/fm/mol	Halleen et al. 2004	AY677292
01_13_03_10	RE	*N. tawa* (*C. obtusisporum*)	Etang de la Gruère, Switzerland	Root of *Picea abies*	V. Queloz	cm/fm/mol	n.a.	HQ713775

(continued)

Table 1 (continued)

Strain	Collection number[a]	Classification[b]	Taxon	Geographic origin	Host/Substrate	Collector	Classification[c]	Reference[d]	GenBank accessions
CBS_402.69		DSE	*Oidiodendron maius*	Ontario, Canada	From soil	G.L. Barron	cm/fm/mol	n.a.	AF307768
3465		DSE	*Periconia macrospinosa*	Spain	Root of *Holcus lanatus*	S. Sanchez Marquez	cm/fm/mol	Sánchez Márquez et al. 2010	FN393421
KS00100		DSE	*P. macrospinosa*		Root sample from tallgrass prairie	K. Mandyam	cm/fm/mol	Mandyam et al. 2010	FJ536207
CBS_166.42		DSE	*Phialophora cyclaminis*	Aalsmeer, Netherlands	*Cyclamen persicum*	J.W. Roodenburg	type strain	Schol-Schwarz 1970	HQ713776
CBS_328.74		DSE	*Phialophora* sp. 1	Lienden, Netherlands	Root of *Triticum aestivum*	C.L. de Graaff	mol	n.a.	HQ713777
O24-2		DSE	*Phoma chrysan-themicola*	Stettbach, Switzerland	Root of *Pinus sylvestris*	S. Bachmann	cm/fm/mol	n.a.	HQ713778
CBS_522.66		DSE	*P. chrysan-themicola*	Kent, United Kingdom	Root of *Chrysan-themum morifolium*	H.J. Wilcox	neotype	Aveskamp et al. 2009	FJ426985
03_08_1_1		DSE	*Phoma exigua*	Etang de la Gruère, Switzerland	Root of *Picea abies*	V. Queloz	cm/fm/mol	n.a.	HQ713781
CBS_431.74		DSE	*Phoma exigua* var. *exigua*	Netherlands	From tuber of *Solanum tuberosum*	N.A.K. Keuring	cm/fm/mol	Aveskamp et al. 2009	FJ427001
O16-4		DSE	*Phoma radicina*	Stettbach, Switzerland	Root of *Pinus sylvestris*	S. Bachmann	cm/fm/mol	n.a.	HQ713782

CBS_111.79	DSE	*P. radicina*	Wanssum, Netherlands	Twig of *Malus sylvestris*	G.H. Boerema	cm/fm/mol	Aveskamp et al. 2009	FJ427058
EF-37	DSE	*Pseudocercospora cantuariensis*	Tianshan Mountain, China	Root of *Saussurea involucrata*	L. Wu	cm/fm/mol	Wu et al. 2010	FJ843591
CBS_112.24	DSE	*P. cantuariensis*	n.a.	Leaf of *Humulus lupulus*	H. Wormald	cm/fm/mol	Radisek et al. 2009	EU346864
ATCC_42652	DSE	*Saccharicola bicolor*	Nairobi, Kenya	Leaf of *Saccharum officinarum*	W.J. Kaiser	type strain	Eriksson and Hawksworth 2003	U04203
Hp3.4	DSE	*Taeniolella* sp.	Senge Khabab, Tibet	Root of *Heteropappus semiprostratus*	K. Burri	cm/fm/mol	n.a.	HQ713779
TRN289	DSE	*Taeniolella stilbospora*	Atazar, Spain	Rock surface	C. Ruibal	cm/fm/mol	Ruibal et al. 2008	AY843127

[a] *ATCC* American type culture collection, Manassa, Virginia, USA, *CBS* Centralbureau voor Schimmelcultures, Utrecht, The Netherlands, *UAMH* University of Alberta, Microfungus Collection and Herbarium, Edmonton, Alberta, Canada, *MUCL* Mycothéque de l'Université Catholique de Louvain, Louvain, Belgium

[b] *DSE* dark septate endophyte, *RE* root endophyte with variable culture color

[c] Classification based on culture morphology (cm), morphology of fructifications (fm) and/or molecular markers (mol)

[d] *n.a.* not available

related to *Mollisia*, *Phaeomollisia* and *Vibrissea* teleomorphs (MPV) including *Phialocephala* and *Cystodendron* anamorphs (Wang et al. 2006; Grünig et al. 2009). Several non-sporulating DSE species were found in this group and accommodated in the newly erected taxon *Acephala* (Grünig and Sieber 2005; Münzenberger et al. 2009). Although several species were newly described in this group in the past years (Kowalski and Kehr 1995; Wilson et al. 2004; Grünig et al. 2009; Münzenberger et al. 2009; Wang et al. 2009), taxonomy is far from being settled and very likely additional species will be found in the future. In addition, several form taxa were introduced by Menkis et al. (2004) and Grünig et al. (2009) to designate not-yet described species. A second group of DSE that is closely related to the MPV group is formed by *Cadophora finlandia* (Wang and Wilcox 1985), *Pezoloma* (*Rhizoscyphus*) *ericae* (Anamorph: *Scytalidium* spp.) and *Meliniomyces* spp. (Hambleton and Sigler 2005). As for the MPV group, taxonomy is not yet settled. The third group clusters together with the plant pathogens *Rhynchosporium secalis*, *Oculimacula yallundae* and *Pyrenopeziza brassicae*. *Leptodontidium orchidicola* is probably the best-known DSE with affinities to these plant pathogens (Currah et al. 1987) but also several *Cadophora* species such as *Cadophora malorum* and *Cadophora fastigiata* / *Cadophora melinii* are placed in this group (Harrington and Mcnew 2003). Besides the Helotiales, the Pleosporales also include many DSE. Other widespread DSE occur in the Sordariales (*Trichocladium opacum*) or the Pezizales (*Wilcoxina* spp.).

3 Cryptic Speciation and Species Recognition in DSE

Another source of complexity in DSE taxonomy and research is introduced by recognizing that what was believed to be one morphospecies, or species defined based on ITS sequences, can in fact be an assemblage of reproductively isolated lineages i.e. cryptic species (CSP). The reason is that in mycology, species were defined based on morphology of asexual and sexual reproductive structures (morphological species recognition, MSR). Unfortunately, the number of morphological characters is often limited and the variability is pronounced (Brasier 1997; Petersen and Hughes 1999; Burnett 2003), and this is certainly the case with DSE. Moreover, induction of sporulation is cumbersome in some DSE and others, such as *A. applanata*, probably do not sporulate at all. These species are not accessible for the classical MSR concept. Not surprisingly, an increasing number of morphologically indistinguishable (cryptic) species were described using either the genealogical concordance of different sequence loci (genealogical concordance phylogenetic species recognition, GCPSR) (Koufopanou et al. 1997, 2001; Fisher et al. 2002; Dettman et al. 2003; O'Donnell et al. 2004), a population genetic analysis framework (Templeton 1981; Hartl and Clark 1989) or a combination of both. For example, GCPSR in conjunction with a population genetic approach improved resolution in PAC significantly, and allowed proper subdivision of *P. fortinii* in several CSP (Grünig et al. 2007).

4 Distribution, host specificity and dispersal in the *Phialocephala fortinii* s.l. – *Acephala applanata* Species Complex (PAC)

Members of the PAC dominate the endophytic mycobiota in roots of conifers and members of the Ericaceae in heathlands, forests and alpine ecosystems (Addy et al. 2000; Grünig et al. 2006) and can be found on all parts of the root system, e.g. from the mycorrhizal root tips to the root collar (Menkis 2005; Grünig et al. 2008b). Abundance of PAC species on several host species was extensively studied in Europe and North America (Grünig et al. 2008b), but there is only little information for other continents. PAC species were detected only a few times in the southern hemisphere. Although, the root endophytic communities of some ericaceous and orchid hosts in south-eastern Australia are well studied (Chambers et al. 2000; Midgley et al. 2002; Bougoure et al. 2005; Bougoure and Cairney 2005a,b), only one PAC strain could be isolated (Midgley et al. 2004). Similarly, PAC was rarely isolated from New Zealand forests (Ian Dickie, personal communication; C.R. Grünig, unpublished). Moreover, PAC were isolated from *Asterocedrus* sp. in undisturbed forests in Patagonia (South America) (C.R. Grünig unpublished). These findings indicate, that PAC are not restricted to the northern hemisphere but occur also in the southern hemisphere, yet, at lower frequencies.

Host specificity of PAC species is low or lacking because most species were isolated from a broad range of woody plant species. Only *A. applanata* shows a clear preference for hosts belonging to the Pinaceae in forest ecosystems with ericaceous ground vegetation (Grünig et al. 2006). Natural long-distance gene or genotype flow of PAC is assumed to be restricted (Grünig et al. 2008b) because (i) *A. applanata* was never observed to sporulate, other PAC species rarely sporulate, and the conidia do not germinate *in vitro*, (ii) PAC species have never been detected in arable soils (Ahlich-Schlegel 1997; Brenn et al. 2008) and (iii) PAC species were never detected in spore traps in contrast to other *Phialocephala* species (Kauserud et al. 2005). Trade with colonized nursery plants may serve as an alternative source for the dissemination of PAC (Brenn et al. 2008).

5 Structure of Species Diversity in PAC Communities: From Roots to Continents

5.1 Communities and Single Roots

Sympatry of PAC species in forest ecosystems seems to be a common pattern. The number and abundance of PAC species found in communities present within forest plots of approximately 200 m^2 varied greatly ranging from two to ten species (Grünig et al. 2004, 2006; Queloz 2008). Species abundance distributions within

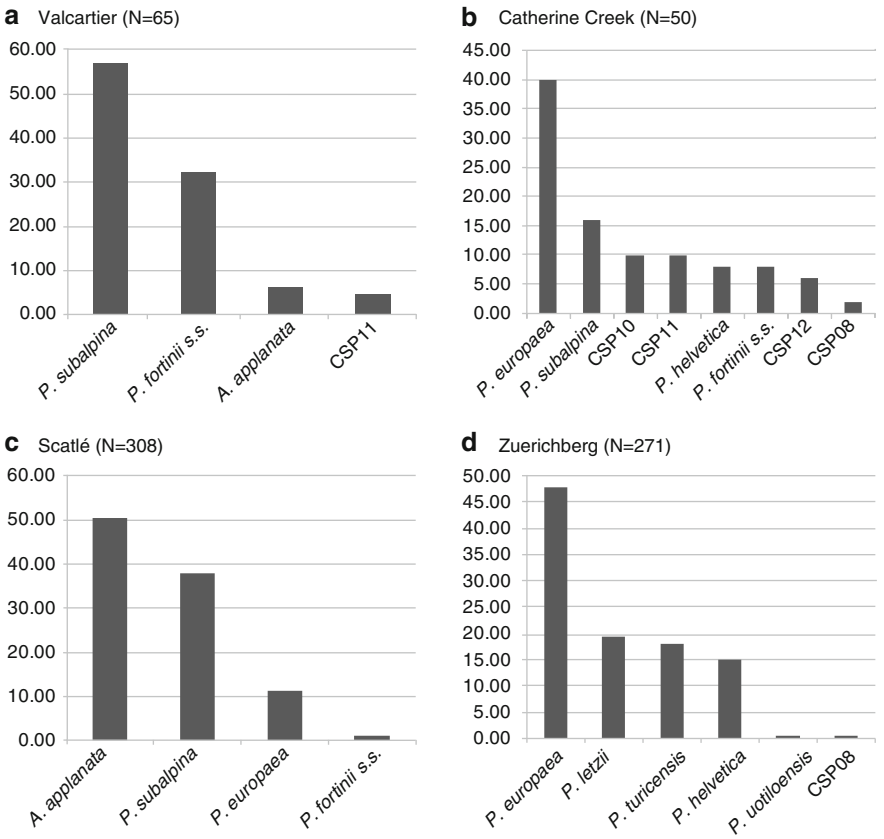

Fig. 1 Examples of species abundance distributions for PAC species. PAC communities follow a hyperbolic distribution with a few abundant species and many 'rare' species, consistent with the community structures of many other organismal groups

these communities normally follow a hyperbolic distribution with a few abundant species and many 'rare' species (Fig. 1), consistent with the community structures of many other organismal groups (McGill et al. 2007). Interestingly, community structures seem to remain stable for several years (Queloz et al. 2005).

On the plot level, PAC communities are spatially structured, but it is unclear whether the observed patterns are purely stochastic or the results of competition, dissemination patterns and habitat diversity and structure. Statistically, most PAC species seem to be "spatially exclusive" as indicated by significantly negative measures of association. However, this finding must be interpreted with caution: Pielou's measure of association Q (Pielou 1977) does not account properly for the spatial pattern of presence/absence of PAC species, and rarely isolated PAC species are possibly more abundant in reality but are masked by more frequent PAC species. In fact, in our studies, more than one cryptic species was present at one grid point and often two PAC species could be isolated from the same 5-mm-long root

segment (Piercey et al. 2004; Queloz et al. 2005; Grünig et al. 2006), supporting the observation of complex colonization patterns with up to five individuals belonging to three PAC species in single 8- to 10-cm-long roots (Grünig et al. 2008b).

5.2 Biogeographical Structure in PAC Species

The biogeography of microbial species is poorly understood and there is an open debate regarding if and how microbial biodiversity is structured (Green et al. 2004; Bell et al. 2005; Martiny et al. 2006; Yang et al. 2010). Key factors that shape biodiversity in microorganisms in space and time are the ability to disseminate, adaptive radiation, habitat differentiation and competition. Microorganisms including fungi have been regarded as cosmopolitan for many years (Fenchel and Finlay 2004), leading to the hypothesis of Baas Becking that "everything is everywhere, but the environment selects" (the EisE hypothesis) (Baas Becking 1934; de Wit and Bouvier 2006; O'Malley 2007). However, the EisE hypothesis was challenged following the advent of molecular genetic markers that illustrated that species of microorganisms defined by morphological characters and/or conserved molecular markers are often assemblages of cryptic species (Taylor et al. 2000) as in the case of PAC species. This observation led researchers to hypothesize that a hidden biogeography of microorganisms may exist, and the influence of species definition became a controversial issue in studying the biogeography of microbial species (Fenchel 2005; Taylor et al. 2006; Peay et al. 2008). Indeed, hidden biogeographical structures were reported for several fungal species when resolution of species assignments was high (James et al. 1999; Jacobson et al. 2006; Geml et al. 2008) and in only a few cases, no biogeographic structure has been found especially for free-living fungal microbes (Pringle et al. 2005).

Because of the limited ability of PAC species to disseminate and the high precision available to differentiate PAC species one would expect evidence for a strong biogeographical pattern. The biogeography of PAC was analyzed for 5,236 PAC isolates sampled from 44 study sites distributed across the northern hemisphere. Two well-known community ecological approaches were applied to analyze the biogeography of PAC species. First, the distance-decay relationship (Green and Bohannan 2007) was calculated for PAC communities. Second, resampling was performed to test the effects of sampling intensity (number of study sites) and distance separating study sites on the number of species detected. For truly cosmopolitan microorganisms, the similarity of communities should be independent of the geographical distance separating them, i.e. communities separated by a few hundred meters or by thousands of kilometres should have the same probability of being very similar (or very different). However, these analyses revealed no evidence for a cryptic biogeographic structure for PAC species (Queloz et al. 2011). Instead, the only predictor for species diversity was the sampling effort, determined by the number of exhaustively sampled study sites within a defined geographical area (Queloz et al. 2011). Given the fact that PAC species are symbionts of

non-motile hosts contradicts the idea that only free-living species are expected to be cosmopolitan (Taylor et al. 2006). Surprisingly, neither climate nor tree species of forest stands predicted the community structure of PAC species but PAC communities seem to be assembled randomly (Queloz et al. 2011).

5.3 Structure of Genetic Diversity in PAC Species

Many genetic analyses of fungal populations are based on the distribution of allele-frequencies at selectively neutral and unlinked loci of individuals (Milgroom 1996; Sunnucks 2000). Therefore, a minimum number of individuals (clones) per population are required to study the partitioning of genetic diversity over different spatial scales. However, the *in situ* delimitation of individuals of thalloid microorganisms is often impossible especially when the organisms either grow endophytically and/or occur below ground such as PAC (McDonald 1997). Hierarchical sampling designs and molecular methods must be applied to determine the size of single genotypes. Several highly polymorphic molecular markers such as microsatellites (Jarne and Lagoda 1996; Dutech et al. 2007), single-copy or multicopy RFLPs (Chen et al. 1994; Grünig et al. 2003) and/or PCR fingerprinting techniques (Weising et al. 1995) can be used to recognize individuals of endophytic fungi or below-ground organisms. For example, clones of PAC species were shown to colonize several square meters of forest soil using ISSR-PCR fingerprinting (Grünig et al. 2002b). Comparisons of different marker types can help to assess the marker's suitability to recognize individuals (Grünig et al. 2003; Queloz et al. 2010). Unfortunately, PAC species differ greatly in allelic diversity, which in turn influences detection of individuals by molecular markers. For example, only 26 and 20 multi-locus genotypes (MLH) could be detected among 166 and 155 strains of *A. applanata* collected from two 196m^2 study sites in Switzerland using single-copy-RFLP genotyping, but application of additional PCR fingerprinting helped to recognize 43 and 37 individuals (Grünig et al. 2006). Beside the recognition of individuals, population biology of species complexes such as PAC is challenging for several reasons: (i) the colonization frequency of roots by PAC varies between 6% and 95% among sites and (ii) the number of PAC species varies between 1 and 10 among sites. Unfortunately, the number and kind of species present at a site are *a priori* unknown, making an appropriate sampling strategy difficult. The only way to overcome these problems is to apply extensive samplings although the less abundant PAC species will often not yield enough individuals for population genetic analysis. Therefore, data about the structure of genetic diversity of PAC species is restricted to a few abundant species. Previous studies showed that *P. fortinii* s.s. and *P. europaea* are widely distributed and belong to the most abundant species in many PAC communities, making them suitable to study the partitioning of genetic diversity within species (Queloz et al. 2011). In addition, these two species show high allelic diversity by molecular markers, allowing proper characterization of individuals without the need to apply additional molecular markers (Queloz et al. 2010).

5.4 Genetic Diversity Within PAC Populations

Several fundamental measures have been developed to describe the diversity found in populations of species at the level of individuals and genes/alleles: The clonal fraction (Zhan et al. 2003), the genotypic diversity (Stoddart and Taylor 1988), Nei's gene diversity (Nei 1973), and the allelic richness (Hurlbert 1971; Kalinowski 2004). Whereas the clonal fraction and the genotypic diversity measure the diversity of individuals for a given number of strains, Nei's gene diversity and the allelic diversity estimate the number and frequency of alleles in a population. Sensitivity to differences in sample size is a drawback of these measures when applied to PAC collection, as the number of PAC individuals is often different between samples (Kalinowski 2004; Grünwald et al. 2003). Several software packages are freely distributed to calculated these measures (see also Excoffier and Heckel 2006) such as Arlequin (Excoffier et al. 2005), POPGENE32 (Yeh et al. 1999), ADZE (Szpiech et al. 2008) or GENEPOP (Rousset 2008), and some of them include rarefaction analyses, allowing to correct for uneven sampling sizes.

P. fortinii s.s. and *P. europaea* differ in the extent and distribution of diversity in their genomes as indicated by differences in the number of alleles and Nei's gene diversity at 12 microsatellite loci (Table 2). Whereas three highly polymorphic loci (mPF_138B, mPF_860B and mPF_142B) and no monomorphic loci were observed in *P. fortinii* s.s., *P. europaea* possessed only one highly polymorphic locus (mPF_644) and 11 low polymorphic loci. Interestingly, the highly polymorphic locus in *P. europaea* does not correspond to any of the three highly polymorphic loci in *P. fortinii* s.s. In summary, allelic richness was higher in *P. fortinii* s.s. populations than in *P. europaea* populations (Table 3).

The relative genotypic diversity and clonal fraction of both species are correlated with the number of strains collected: the more strains, the higher clonal fraction and the lower relative genotypic diversity, i.e. the probability of finding new individuals decreases with increasing number of strains. Besides dependence on the number of strains, probability of finding new individuals depends on the distance between sampling points: the closer the sampling points, the higher the probability of finding the same individual, indicating that local dispersal occurs by spreading mycelium. In fact, strains isolated from different roots collected at the same grid point more often belong to the same individual than those from roots collected at different grid points (Grünig et al. 2002b).

5.5 Genetic Diversity Among PAC Populations

Several analysis frameworks are available to study the structure of genetic diversity among populations, yielding insights into the evolutionary processes acting on species. First, hierarchical structuring of diversity can be analyzed using molecular variance analysis (AMOVA) (Excoffier et al. 1992). In AMOVA the portioning

Table 2 Number of alleles and gene diversity at 12 microsatellite loci for *Phialocephala europaea* and *P. fortinii* s.s

Species	Region	Country (State)	Study site	Number of isolates	Number of MLH	mPF_011	mPF_022	mPF_043	mPF_644	mPF_860A	mPF_138B	mPF_035A	mPF_672	mPF_068	mPF_860B	mPF_088	mPF_142B
P. europaea	Europe	Austria	Rothwald	86	38	1	2	2	12	1	3	1	3	1	1	1	3
		France	Alpes maritimes	73	34	1	2	2	11	3	3	1	3	2	2	1	4
		Switzerland	Boedmeren	28	17	1	2	2	9	1	2	1	3	2	1	1	3
		Switzerland	Creux du Van	121	43	1	2	2	18	1	4	1	3	1	2	1	3
		Switzerland	Derborence	184	48	2	1	2	17	2	4	2	2	1	1	1	4
		Switzerland	Runcaglia	97	41	2	2	2	10	3	3	1	2	2	1	1	4
		Switzerland	Scatlé	33	25	1	2	2	9	2	3	1	2	2	1	1	4
	North-America	USA (California)	Sierra Nevada	10	10	2	1	3	2	5	2	1	4	2	1	1	1
Total # Alleles						**3**	**2**	**3**	**26**	**7**	**6**	**2**	**5**	**3**	**3**	**1**	**5**
Gene diversity						**0.02**	**0.11**	**0.48**	**0.91**	**0.19**	**0.21**	**0.02**	**0.27**	**0.17**	**0.04**	**0**	**0.61**
P. fortinii s.s.	Europe	Finland	Kevo	108	63	3	3	1	4	2	20	3	3	2	9	4	5
		France	La Fage	102	38	4	3	1	4	1	16	2	3	1	7	4	6
		France	Le Pirou	13	11	2	3	1	3	1	8	2	2	1	2	3	3
		Poland	Bialowieza	92	51	4	3	1	5	1	17	3	3	1	10	4	11
		Scotland	Hill of Fare	23	13	1	2	1	3	1	11	1	2	1	4	2	3
		Switzerland	Etang de la Gruère	25	23	3	3	1	5	1	10	1	3	2	8	2	6
		Switzerland	La Chaux	202	139	4	3	1	5	1	34	2	5	2	22	3	18
		Ukraine	Tschornohora	78	43	2	3	2	4	1	15	2	4	1	5	4	9
	North-America	Canada (Québec)	Valcartier	21	15	3	2	1	5	3	9	1	2	1	5	1	9
		USA (Maine)	Marsh Island	17	10	3	2	1	3	1	8	1	3	2	3	1	7
Total # Alleles						**5**	**3**	**2**	**6**	**4**	**43**	**3**	**6**	**2**	**35**	**7**	**26**
Gene diversity						**0.56**	**0.62**	**0.00**	**0.71**	**0.02**	**0.96**	**0.26**	**0.60**	**0.04**	**0.74**	**0.61**	**0.74**

Table 3 Measures of gene diversity, genotypic diversity and allelic richness in populations of *Phialocephala europaea* and *P. fortinii* s.s

Species	Region	Country (state)	Site	Number of isolates	Number of MLH	Clonal fraction CF (%)	Relative genotypic diversity G/n (%)	Average gene diversity	Allelic richness
P. europaea	Europe	Austria	Rothwald	86	38	55.81	23.76	0.20	2.22
		France	Alpes maritimes	73	34	53.42	27.34	0.28	2.60
		Switzerland	Boedmeren	28	17	39.29	38.89	0.21	NA
		Switzerland	Creux du Van	121	43	64.46	18.64	0.23	2.63
		Switzerland	Derborence	184	48	73.91	11.54	0.21	2.56
		Switzerland	Runcaglia	97	41	57.73	21.60	0.23	2.34
		Switzerland	Scatlé	33	25	24.24	54.10	0.26	2.39
	North-America	USA (California)	Sierra Nevada	10	10	0.00	100.00	0.24	NA
Summary *P. europaea*			**Sum**	**632**	**256**	**46.11**	**36.98**	**0.25**	**2.46**
P. fortinii s.s.	Europe	Finland	Kevo	108	63	41.67	30.68	0.46	3.66
		France	La Fage	102	38	62.75	14.17	0.38	3.56
		France	Le Pirou	13	11	15.38	76.47	0.35	NA
		Poland	Bialowieza	92	51	44.57	27.71	0.41	3.75
		Scotland	Hill of Fare	23	13	43.48	35.38	0.28	NA
		Switzerland	Etang de la Gruère	25	23	8.00	86.21	0.42	NA
		Switzerland	La Chaux	202	139	31.19	53.16	0.47	4.32
		Ukraine	Tschornohora	78	43	44.87	38.61	0.42	3.63
	North-America	Canada (Québec)	Valcartier	21	15	28.57	51.22	0.41	4.32
		USA (Maine)	Marsh Island	17	10	41.18	37.78	0.38	
Summary *P. fortinii* s.s.			**Sum**	**681**	**406**	**36.16**	**45.14**	**0.49**	**3.88**

of the molecular diversity found within species across different spatial scales are computed. Second, the isolation-by-distance hypothesis can be tested for the species under study. The isolation-by-distance hypothesis predicts that populations that are separated by larger geographical distances are genetically more differentiated than closely located populations (Holsinger and Weir 2009). Third, effective population sizes and migration rates among populations can be estimated using coalescent-based approaches (Beerli 1998; Beerli and Felsenstein 2001).

AMOVA analyses for *P. europaea* and *P. fortinii* s.s. show that the two species deviate in the amount of diversity found at different spatial scales. While for *P. europaea* more than 96% of the genetic variation was found within populations and only 4% of the variation was found among populations, for *P. fortinii* s.s. 80% of the genetic variation is present within populations and more than 12% of the variation is found among populations within regions, and only a small fraction of variation is found among regions (∼5%) (V. Queloz, unpublished). Similar partitions of diversity were also reported for other ascomycetes including the plant pathogens *Phaeosphaeria nodorum* (Stukenbrock et al. 2006) and *Mycosphaerella graminicola* (Linde et al. 2002) and seem to be common even for humans (Holsinger and Weir 2009). Testing the isolation-by-distance hypothesis and analyzing the migration rates among populations of PAC species is still pending and should be one focus for future research.

6 Outlook and Concluding Remarks

In the present chapter we showed how biodiversity of dark septate root endophytes and in particular of the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex (PAC) is structured. We showed that PAC species do not show a biogeographical pattern in accordance with the long-standing idea of "everything is everywhere" in microbiology. However, these results suggest nearly unlimited dispersal of PAC species, fundamentally contradicting our current knowledge about the mechanisms of dispersal of PAC species, and further research to find explanations for this discrepancy is needed. In addition, it would be fascinating to know whether the observed structure of biodiversity in PAC represents a general pattern that can be observed also in other DSE species complexes. Finally, integrating the knowledge about the diversity of DSE species in general and PAC species in particular to understand the diversity in host-DSE interactions will be another fascinating topic that should be addressed.

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Diversity of Fungal Endophytes in Temperate Forest Trees

Martin Unterseher

Abstract Approximately 10% of around 1,000 temperate tree species have so far been investigated for the occurrence of fungal endophytes, world-wide. The observed diversity, mostly measured as species richness of cultivated strains, ranges from a few taxa to more than 100 species per tree species. Statistical species richness and molecular phylogeny analyses indicate however, that the observed species numbers are only a fraction of the effective endophyte richness of a host plant, and that the relevant unit of biological organisation may lie below the species level, at the level of populations and phylotypes. State-of-the-art high-throughput techniques such as dilution-to-extinction cultivation and massively parallel sequencing of target DNA loci could overcome the limits that have prevented exhaustive surveys so far. Further aspects of biodiversity, such as host preference and species turnover on different substrata, geographical regions, or collection times have rarely been addressed, but would reveal new patterns of biological and functional diversity of temperate forest endophytes. Furthermore, application of classical ecological theory to endophytology could lead to substantial progress in the field.

Abbreviation

NGS next generation sequencing

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1 Introduction

Many comprehensive reviews and articles about endophytic fungi in tropical and temperate forests have been published in recent years, providing heaps of data and information (e.g. Jumpponen 2001; Osono 2006; Arnold 2007; Sieber 2007; Saikkonen 2007; Slippers and Wingfield 2007; Albrectsen et al. 2010; Tejesvi et al. 2010). Whereas only a limited portion of the growing literature of forest endophytes is reviewed here, various theoretical and statistical papers about biodiversity and classical ecology are cited, because of their value to push also endophyte research forward (Prosser et al. 2007; Arnold et al. 2010).

The following review focuses mainly on fungal endophytes in the phyllosphere of temperate forest trees, whereas those groups that predominantly colonise the bark, wood, and the rhizosphere are addressed to a lesser degree. The reason for this is that non-foliar fungal endophytes generally receive less attention (e.g. Danti et al. 2002 for bark endophytes), commonly treated as non-endophytes, such as wood-inhabiting fungi (Boddy and Rayner 1983), and are reviewed separately within this volume (see chapter by Grünig and Sieber). The present diversity assessment of fungal endophytes in temperate forest trees addresses three central theses: (I) Knowledge of endophytic diversity is preliminary and largely biased towards cultivation-dependent approaches, (II) future studies of endophytic diversity would benefit from current and forthcoming high-throughput cultivation and next generation sequencing (NGS) technologies, and (III) from the incorporation of previously disregarded ecological theories.

2 Biological and Functional Diversity of Temperate Forest Endophytes

The term biological diversity, or biodiversity, is used in an inflationary way in both scientific and non-scientific literature and has gained entrance to all levels of societal life (Balmford et al. 2005). According to Bisby et al. (1995) biodiversity means "the variability among living organisms from all sources and the ecological systems of which they are a part. This includes diversity within species, between species and of ecosystems." Whittaker (1977) was one of the first to realise that biodiversity is scale-dependent, hierarchical in nature and that it incorporates different levels of diversity in a fixed order (Zak and Willig 2004; Jost 2007).

Translated to endophytology, point diversity, which is the first level on a considerably minute dimension, could mean the number of species (or any equivalent like phylotype or molecular taxonomic units) within one leaf of a particular tree species. Usually, α-diversity (species richness in the classical sense) would then be the number of foliar endophytes on that particular tree species. Another possible definition of α-diversity could be the number of foliar endophytes in leaves exposed to sunlight or shadow, or a season such as spring or autumn.

If then α-diversity of a particular tree species is compared with that of another tree species within the same forest, it is β-diversity or species turnover that is measured (Zak and Willig 2004). Gamma (γ)-diversity would be measured or estimated by comparing endophytic richness and composition between larger geographic areas.

2.1 Species Richness of Temperate Forest Endophytes

Around 10% of approximately 1,000 tree species in temperate forests (Latham and Ricklefs 1993) have been investigated for the occurrence of endophytes so far with obvious preferences for foliar endophytes in the European regions (e.g. Table 1 in Sieber 2007). Studies in temperate regions of Asia, Australia and New Zealand, South Africa and South America are clearly underrepresented (exceptions are e.g. Hata and Futai 1995; Hata et al. 2002; Li et al. 2007; Slippers and Wingfield 2007; Joshee et al. 2009).

Observed species richness of endophytes in temperate forest trees ranges from a few taxa in *Acer* spp. (Sieber and Dorworth 1994; Pehl and Butin 1994; Vujanovic and Brisson 2002; Unterseher et al. 2007), *Betula* (Barengo et al. 2000), *Quercus* spp. (Cohen 1999; Ragazzi et al. 2003; Gennaro et al. 2003), *Abies* spp. (Carroll and Carroll 1978), or *Pinus* spp. (Legault et al. 1989; Sieber et al. 1999) to 100 and more, in *Abies alba* (Sieber-Canavesi and Sieber 1993), *Picea abies* (Sieber 1988; Müller et al. 2001), or *Carpinus caroliniana* (Bills and Polishook 1991). However, the majority of these and other surveys did not apply statistics to evaluate sampling effort and to provide figures of projected total number of endophytes (Shipunov et al. 2008).

I therefore extracted data from five surveys of endophytes in temperate tree species (Sieber and Hugentobler 1987; Petrini and Fisher 1990; Collado et al. 1996; Müller et al. 2001; Unterseher et al. 2007) and analysed species accumulation curves and richness estimators. In all cases, species accumulation curves did not reach saturation, hence complete sampling or even oversampling of the endophytic assemblage was not achieved (Fig. 1). The three different estimators of species richness, Chao 2, Jackknife 2, and Bootstrap (e.g. Colwell and Coddington 1994), differed within the same data set and calculated survey completedness between 61% and 97% with a mean of 84% (Fig. 1, Table 1). As a consequence of this undersampling, the extrapolation of species richness beyond observed data becomes doubtful (O'Hara 2005; Chao et al. 2009). This exemplary analysis and recent studies (Hoffman and Arnold 2008; Joshee et al. 2009; Unterseher and Schnittler 2010) indicate that current knowledge of endophyte species richness is preliminary.

Species richness alone however does not allow conclusions about global species richness. Large part of the observed endophyte assemblages of temperate trees are composed of ubiquistic and opportunistic colonisers of the phyllosphere and of other habitats and substrata, such as *Alternaria*, *Asteromella*, *Aureobasidium*, *Cladosporium* (anamorphic *Davidiella*), *Geniculosporium* (anamorphic *Hypoxylon*), *Phoma*, *Phomopsis* (anamorphic *Diaporthe*), *Ramularia* (anamorphic *Mycosphaerella*),

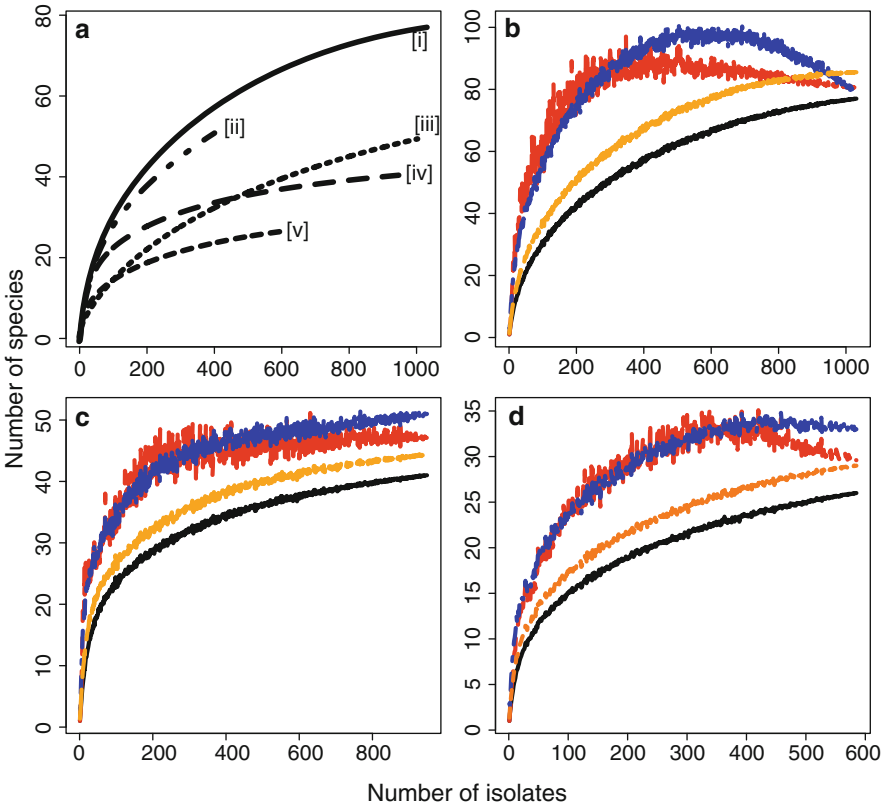

Fig. 1 Species richness analysis of fungal endophytes with species accumulation curves and species richness estimators. Species accumulation curves of five different surveys are re-scaled to the number of isolates for a better comparability in (**a**): [*i*] Collado et al. (1996); [*ii*] Unterseher et al. (2007); [*iii*] Sieber and Hugentobler (1987); [*iv*] Petrini and Fisher (1990); [*v*] Müller et al. (2001). Figures (**b**) (Collado et al. 1996), (**c**) (Petrini and Fisher 1990) and (**d**) (Müller et al. 2001) display accumulation curves (*black line*) and the three estimators Chao 2 (*red*), Jackknife 2 (*blue*) and Bootstrap (*orange*)

Sordaria or *Xylaria*. For some of these taxa "everything seems everywhere" (Whitfield 2005; O'Malley 2008) and at least at the species level, host preferences are rarely observed.

This overlap of endophytes, pathogens and saprobes in many species lists causes great uncertainties in the estimations of global fungal diversity based on fungi to plant ratios (Hawksworth 2001; Schmit and Mueller 2007). Carroll (1988) estimated the global species richness of fungal endophytes equal to mycorrhizal taxa. According to Dreyfuss and Chapela (1994) approximately 1.3 Mio endophytic fungi should inhabit our planet, whereas Thomas Sieber (2007) provided a lower-bound estimate of 500,000–600,000 endophytic fungal species. He extrapolated

Table 1 Observed and estimated species richness from five different surveys of foliar endophytes of temperate trees

				Species estimated			
	Host plant(s)	Species observed	Isolates	Chao 2	Jack-knife 2	Boot-strap	Completed-ness in%
Sieber and Hugentobler (1987)	*Fagus sylvatica*	64	2,337	70	78	71	82–91
Unterseher et al. (2007)	*Quercus robur* *Tilia cordata* *Acer pseudoplatanus* *Fraxinus excelsior*	49	384	75	80	57	61–86
Petrini and Fisher (1990)	*Quercus robur* *Salix fragilis*	41	949	47	51	44	80–93
Collado et al. (1996)	*Quercus ilex*	77	1,031	81	79	86	90–97
Müller et al. (2001)	*Picea abies*	26	585	30	33	29	79–90

from at least two host-specific endophytes per plant species on a global scale, however, this ratio is supposed to be much higher in temperate forests (Carroll 2006).

2.2 *Host and Substratum Preferences of Temperate Forest Endophytes*

Species turnover rates (β- and γ-diversity) determine the number of unique and shared species in different hosts, or at different scales and times. If the number of shared species is high, turnover rates are low. In such case, a focused investigation of few spatio-temporal niches would miss only few endophytes. If the number of shared endophytes is low, species turnover rates are high and the range of niche sampling largely determines the observed species richness and the perception of diversity.

Considering a single tree, endophytic fungi are found in all major tissues and organs. Arnold (2007) recently emphasised the distinctiveness of foliar endophytic fungi (e.g. Bayman 2006; Arnold and Lutzoni 2007) relative to other avirulent fungal plant associates, such as mycorrhizae and dark septate endophytes (e.g. Tedersoo et al. 2006; Tejesvi et al. 2010), wood and bark inhabiting fungi (Boddy and Rayner 1983; Sieber et al. 1995; Santamaría and Diez 2005; Simeto et al. 2005; Parfitt et al. 2010) and fungal epiphytes (Lindow and Brandl 2003; Santamaría and Bayman 2005; Weber and Anke 2006; Jumpponen and Jones 2009). This remarkable niche partitioning is repeated even on a smaller scale, though less

pronounced. Different species composition was observed within leaves when the tip or blade was compared with the base and petiole, respectively (Halmschlager et al. 1993; Lodge et al. 1996; Taylor et al. 1999).

Endophytic species richness and composition generally vary among tree individuals and are further influenced by the condition of the host plant (Sieber and Hugentobler 1987), by the time of sampling, and by the precise location of sampling units (Unterseher et al. 2007). Therefore, reliable figures of species richness and composition for a given host tree species requires repeated sampling of multiple individuals throughout an entire vegetation period (Bills and Polishook 1992; Hoffman and Arnold 2008). Own studies on *Fagus sylvatica* in north-eastern Germany and Switzerland (Unterseher and Schnittler 2009, 2010) and meta analysis of data from Switzerland (Sieber and Hugentobler 1987) manifested differences in endophytic diversity of the two regions. On the one hand *Rhodotorula* sp., *Isaria farinosa* (syn. *Paecilomyces farinosus*) or *Ramularia* sp. were abundant foliar endophytes in German beeches and absent in Switzerland, on the other hand, *Apiognomonia errabunda*, the foliar beech endophyte par excellence (Viret and Petrini 1994; Bahnweg et al. 2005), was the most abundant endophyte in Switzerland but rarely isolated from the northern beeches.

Unterseher et al. (2007) compared species richness and composition of foliar endophytes from four different temperate tree species, and identified several foliar endophytes with obvious preference for a single tree species, such as *Apiognomonia errabunda* in oak foliage (*Quercus robur*). The fact that the same species is also known as the dominant endophyte in leaves of *F. sylvatica* highlights the importance of the relevant level of biological organisation in endophyte diversity. It may lie below the species level–at the level of populations, genotypes (phylotypes) or ecotypes (Arnold 2007).

2.3 Functional Diversity of Temperate Forest Endophytes

Fungal endophytes can have profound effects on the host's biochemistry and physiology (Newsham et al. 1998), influence multitrophic networks e.g. as facultative insect pathogens (Vega et al. 2008), and entire ecosystems (Selosse et al. 2004; Rudgers and Clay 2007).

The functionality of an endophyte is often coupled with the location of its occurrence. Endophytic fungi in woody tissues often appear as primary wood decay fungi on dying or dead branches (Boddy and Rayner 1983; Boddy 1992; Parfitt et al. 2010), whereas dark septate endophytes of the rhizosphere (see chapter by Grünig and Sieber in this volume) functionally and ecologically overlap with soil fungi, saprotrophic rhizoplane-inhabiting fungi, obligate and facultative pathogenic fungi, and mycorrhizal fungi (Jumpponen and Trappe 1998). Fungi within living leaves of trees – the foliar endophytes – probably comprise the most heterogenous assemblage of mutualists, latent plant pathogens, facultative entomopathogens, parasites, saprobes and leaf-inhabitants with unknown function (Carroll 1995; Todd

1988; Petrini 1991; Butin 1992; Freeman and Rodriguez 1993; Wilson 1995a; Gange 1996; Bahnweg et al. 2005; Ganley and Newcombe 2006; Promputtha et al. 2010). They are often connected with fungi and bacteria on the leaf surface through multitrophic interactions (Santamaría and Bayman 2005; Bayman 2006; Weber and Anke 2006; Jumpponen and Jones 2009) and produce a wide array of secondary metabolites (Petrini et al. 1992; Strobel 2003; Zhang et al. 2006). It is probably the foliar endophytes in forest trees that most often are connected with the term "endophytic continuum" (Saikkonen et al. 1998; Schulz and Boyle 2005; Rodriquez et al. 2009).

Life strategies and the biochemical armoury of foliar endophytes may change as the plant tissue ages. Suddenly, and perhaps in response to external, abiotic stimuli (e.g. Bahnweg et al. 2005), they may become bio-necrotrophic or pathogenic, thus leading to a fungus-mediated aging or dying of leaf tissue (Bayman 2006). Subsequently, the fungus can switch to a saprobic lifestyle, enabling it to feed, grow and mate on the dying and dead tissue (Promputtha et al. 2007, 2010). It was recently demonstrated that primary wood decay fungi, which normally colonize and develop rapidly in branches and other woody tissues without contact to the forest floor (Unterseher et al. 2005; Unterseher and Tal 2006) may be latent endophytes (Parfitt et al. 2010). Up to now, the time and mode of entry of fungal propagules, their maintenance in sapwood, and the cues that trigger their development may only be speculated upon (Parfitt et al. 2010).

Horizontal gene flow from the endophyte to its host may have been a regular event during the evolution of the endophyte-host plant association (Strobel et al. 2004). This could be one explanation for the joint production of secondary metabolites, such as taxol from both *Taxus brevifolia* and its endophytic symbiont *Taxomyces andreanae* (Stierle et al. 1993). Of particular interest from a pharmaceutical point of view are endophytes that produce antimicrobial and antifungal compounds, which seem to have no negative effects to their eukaryotic hosts (Borges et al. 2009). Such metabolites are promising sources of new antibacterials and antifungals (Strobel et al. 2004).

3 The Interpretation of Endophyte Diversity is Method-Dependent

Species richness assessment is shifting from laborious morphological identification towards molecular identification and phylogeny. The internal transcribed spacer (ITS) region and the flanking 16 S and 28 S rDNA genes are mostly used as target loci nowadays because this multicopy rDNA region is well investigated in fungi (Hibbett et al. 1995; Nilsson et al. 2008; Nilsson et al. 2010), currently promoted as a fungal barcode (Begerow et al. 2010), and best represented in terms of absolute numbers in public nucleotide databases.

3.1 Cultivation of Fungal Endophytes

The method of choice to isolate fungal endophytes is to sterilise the surface of the plant tissue (leaves, needles: e.g. Schulz et al. 1993; Hahizume et al. 2008; twigs: e.g. Halmschlager et al. 1993; Fisher et al. 1986; Petrini and Fisher 1990; roots: e.g. Zhang and Dai 2009) and to plate differently-sized tissue fragments of it (Gamboa et al. 2002) onto one or several growing media (Bills and Polishook 1992). Complete media such as malt extract agar (MEA) with different antibiotics and selective growth inhibitors are often used for primary isolation (Sieber and Hugentobler 1987; Stone et al. 2004; Higgins et al. 2007; Unterseher and Schnittler 2009).

Surface sterilisation is done by treating the plant material with general disinfectants and strong oxidatives. Depending on the host plant species, the tissue type and the investigators experience, sterilisation procedures vary considerably (Schulz et al. 1993; Stone et al. 2004). After successful surface sterilisation, the size of the plated fragments determines the number of isolated endophytes. As a rule of thumb, the larger the substratum fragments, the lower is the number of outgrowing colonies (Carroll 1995; Gamboa et al. 2002).

A logical consequence of the correlation of tissue size with endophytic species richness would be to shred the tissue into very small particles. This has led to the most recent development of the dilution-to-extinction cultivation for fungal organisms. It was adapted from cultivation protocols for marine bacteria (Connon and Giovannoni 2002) first applied to leaf litter fungi by Collado et al. (2007), and modified for fungal endophytes by Unterseher and Schnittler (2009). Dilution-to-extinction culturing reduces interspecific competition and increases the possibility of detection of slow growing, weak competitors. At the same time, it still allows the isolation of ubiquitous and dominant taxa. Whereas the efficiency of cultivation-based studies of fungal endophytes was significantly increased, the observed species composition was different from that obtained with fragment plating (Unterseher and Schnittler 2009). This indicates that a combination of different cultivation protocols may be necessary for exhaustive isolation of the endophytic community (Unterseher and Schnittler 2010).

3.2 Cultivation-Independent Assessment of Endophyte Diversity

Although histological techniques have never fully been brought into play in studies of forest endophytes, a few cases of their application have provided important insights into distribution patterns of endophytes within host plant tissues. Early studies have demonstrated the highly localised intracellular growth of endophytes within single epidermal plant cells, such as *Rhabdocline parkeri* in Douglas-fir (*Pseudotsuga menziesii*) needles (Stone 1987a, b). These results help to understand the fragment size-dependent isolation frequency of foliar endophytes (Gamboa et al.

2002). More recently, Johnston et al. (2006) used a fluorescent immunolabelling method to detect endophytes in planta by staining the major fungal structural cell wall component (1,3)-β-D-glucan (Johnston et al. 2006).

PCR-based sequencing of environmental DNA and phylogenetic analysis are often the most appropriate ways to reveal unculturable taxa. In endophyte studies however, unanswered questions still prevail, such as the identification of unusual foliar endophytes like *Boletus* or *Thelophora* and the absence of abundantly cultivated taxa in sequence data (Arnold et al. 2007). According to my current knowledge, published protocols that create a substratum surface devoid of amplifiable DNA do not exist. Such DNA-free surfaces however would be required to assess diversity patterns of the "true" internal endophytes with direct amplification and sequencing of fungal DNA barcode regions, e.g. with massively parallel pyrosequencing (Hibbett et al. 2009).

4 Progress in Endophytology Requires a Broadened Methodic and Analytical Framework

Current endophytology is more and more integrated into various research fields, such as phyllosphere biology (Bailey et al. 2006) or general plant-fungus interactions (Peay et al. 2008; Comas et al. 2010). Besides the already mentioned "endophytic continuum", the integration is partly due to entirely molecular approaches with environmental PCR and sequencing clone libraries or metagenomic 454-sequencing, which currently make the separation of fungal endophytes and epiphytes impossible (Jumpponen and Jones 2010).

4.1 Integration of Ecological Theory and Statistics into Endophyte Diversity Studies

There is growing consensus among microbiologists, protistologists and mycologists, that current and future microbial ecology and biodiversity research urgently needs a theoretical framework in order to understand and predict the processes regulating the diversity, structure and evolution of microbial communities (Green and Bohannan 2006; Foissner 2008; Dumbrell et al. 2010; Arnold et al. 2010). However, prevailing ecological theories, which were mostly developed and tested on conspicuous macroorganisms are still rarely applied to microbial research (Forney et al. 2004; Prosser et al. 2007).

Species accumulation curves have been widely used in recent studies (Unterseher et al. 2007; Hoffman and Arnold 2008; Joshee et al. 2009) and provide an important tool to evaluate sampling effort and to compare the species richness of observed assemblages (Fig. 1a). However, a critical use of species richness

estimators (Unterseher et al. 2008) and a discussion of the rare species mystery (e.g. Novotny and Basset 2000; Ellison and Agrawal 2005) seem less common. Scarce data also exist for species abundance models and theories (Hanski 1982; Magurran and Henderson 2003; McGill et al. 2007) of microbial communities in general (Galand et al. 2009; Dumbrell et al. 2010; Unterseher et al. 2011). According to my knowledge, there is no published study on species abundance distribution models for endophytes.

4.2 Habitat-Specific Considerations for Temperate Forest Endophytes

From the perspective of fungi, forest ecosystems are highly complex and provide many different ecological niches (Lodge and Cantrell 1995; Stone et al. 1996; Unterseher and Tal 2006). These ecosystems affect the richness and species composition of wood decay fungi, which may have been wood-inhabiting endophytes, lichens, and thus lichen-inhabiting endophytic fungi (Li et al. 2007; Arnold et al. 2009), foliar endophytes, and further phyllosphere fungi, such as "sooty moulds" (Gilbert et al. 2007). Given that the species richness and composition of endophytes is largely dependent on the surrounding vegetation and the local climatic properties, sampling from the entire natural distribution range of a host plant species, especially from its climatic or geographic limits, will surely add new insights into the diversity of temperate forest endophytes.

5 Conclusions

Current knowledge of fungal endophytes makes common definitions of a fungal endophyte incomplete and out-dated, because they are largely influenced by human perception and methodic aspects (e.g. Carroll 1988: "... fungi forming *unapparent* infections within leaves and stems of *healthy* plants..."; Schulz and Boyle 2005: "... fungi that colonise a plant without causing *visible* disease symptoms at any specific moment..."; Cabral et al. 1993: ..." any fungi *isolated* from internal *symptomless* plant tissues..."). In order to benefit from the latest methodic and intellectual advancements, it may be time to refine some and abandon other still widely applied definitions of the term. With this respect, we should "not use too much of our time deciding whether an organism is an endophyte, a pathogen or saprophyte" and instead "get on with the biology" of these organisms (Wilson 1995b). This however would mean that we should also accept the ambiguity of those categories for many fungal groups.

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The Holomorph *Apiognomonia quercina/Discula quercina* as a Pathogen/Endophyte in Oak

Salvatore Moricca and Alessandro Ragazzi

Abstract This chapter summarises research carried out on the biology, ecology and the impact of the holomorph *Apiognomonia quercina/Discula quercina* in oak forests. The major life-history traits and aspects of the epidemiology (isolation, survival, reproduction, dispersal, host selectivity) and control of the oak anthracnose agent are elucidated. The role of weather patterns in disrupting the delicate interaction between the host tree and the microorganism is outlined. The evidence suggests that changes in the climate profoundly alter the plant-endophyte symbiosis, generating conflicts of interest between the partners in the interaction. When such competing interests arise, the survival and reproduction of one member of the interaction do not conform with that of the partner. The interaction becomes thus disadvantageous and harmful to one of the organisms and the symbiosis from mutualistic or neutral turns antagonistic. The fungal partner, which under normal conditions survives in quiescence, with a low biomass, resumes growth but now switches from a latent, asymptomatic occupier of inner oak tissues to an aggressive coloniser that sporulates profusely over the tree surface. The importance of investigating the functioning and the role of the plant-endophyte symbiosis in perennial host trees in natural forests is stressed.

1 Introduction

The circum-Mediterranean area is extraordinarily rich in plant species, and is regarded as a biodiversity hotspot (Cowling et al. 1996). Even though Mediterranean oak forests have been subjected to anthropic pressure and exploitation for millennia,

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they still represent an enormous reservoir of fungal diversity. The community of endophytic mycobiota inhabiting these forests remains largely unexplored.

The outstanding species richness of this area, which is now one of the most endangered in the world and is undergoing a severe loss of habitat, can be explained by the episodic glacial advances that occurred during the Quaternary period. In the last ice age, the southernmost regions of Europe served as a refuge for many organisms (Hewitt 2000). The Italian peninsula in particular, with its north-south mountain chain, the Apennines, represented a migrational route for a highly diverse forest flora. This is evident from the abundance of species and the high allelic richness of the plant populations located especially in the south of the country (Comps et al. 2001; Widmer and Lexer 2001). These plant species include several members of the *Fagaceae*. Microbes specifically colonising oaks, like the fungal endophytes harboured in the oak tissues, moved southwards with their hosts in a co-cladogenetic pattern, escaping the ebb and flow of the glaciers.

The ascomycete *Apiognomonia quercina* (Kleb.) Höhn., with its anamorphic state *Discula quercina* (Westend.) Arx, does not belong to the large group of still undiscovered Mediterranean fungal taxa. Like many other species of the *Gnomoniaceae* that cause anthracnose in a number of broadleaf hosts worldwide, the agent of oak anthracnose had such injurious effects that it could not go unnoticed for long.

The anamorph *D. quercina* was first reported by Saccardo at the end of the nineteenth century (Saccardo 1884). This fungus is now a prominent member of the endophytic fungal communities inhabiting Mediterranean oak forests. Its eye-catching symptomology and the severe damage it causes under certain circumstances render this microorganism very noticeable.

Oak anthracnose affects a number of hosts over extensive areas of the Northern Hemisphere. *A. quercina* has a large host range in the family of the *Fagaceae* and especially in the genus *Quercus* and is widespread in North America all the way from Canada to the Gulf of Mexico (Sinclair and Lyon 2005). In Europe it occurs in all countries bordering the Mediterranean and in many parts of north-eastern Europe (Morelet 1989; Halmschlager et al. 1993; Przybyl 1995; Ragazzi et al. 1999a).

The large range in which the pathogen thrives, comprising a variety of climates, suggests that it has different locally adapted populations with distinctive limits of tolerance to the various climates in which they occur. This is also suggested by its distribution in Italy, which ranges from semi-arid regions, such as Sicily (latitude 37–38° north, longitude 13–15° east) to areas with a temperate climate such as Tuscany (latitude 43–44° north, longitude 10–12° east) (Anselmi et al. 2002). Oak trees that are hosts of the fungus in this country are *Quercus cerris* L., *Q. frainetto* Ten., *Q. pubescens* Willd., *Q. robur* L. and *Q. suber* L. (Ragazzi et al. 1999a; Franceschini et al. 1993).

The present chapter deals with the ecology of the holomorph *A. quercina/D. quercina* in Mediterranean oak stands. The most important life-history traits of the fungus are described as they emerge from investigations carried out over the last 15 years. These investigations have clarified important aspects of the taxonomy, the infection cycle, variations in tissue colonisation over time and space, host and

host-organ preference, symptomology, disease epidemiology and control measures. These features, considered together and in the light of current changes in the climate, which are a major factor destabilising the already fragile Mediterranean forest ecosystem, provide a clearer understanding of the nature and the role of this endophyte in natural oak stands.

2 Taxonomy

Apiognomonia quercina (Kleb.) Höhn., (anamorph *D. quercina* (Westend.) Arx.) is one of a complex of species of *Apiognomonia errabunda* (Roberge ex Desm.) Höhn. This was initially classified as *Gnomonia errabunda* (Roberge ex Desm.) Auersw., but was transferred to the genus *Apiognomonia* by Barr (1978).

Apiognomonia errabunda sensu strictu, with its anamorph *D. umbrinella* (Berk. et Broome) M. Morelet, infects beech (*Fagus selvatica* L.), while the holomorph *A. quercina/D. quercina* infects various oak species. The *A. errabunda* complex also includes *Apiognomonia tiliae* (Rehm.) Höhn., with the anamorph *Gloeosporidium tiliae* (Oudem.) Petr., which infects *Tilia europea* L.; and *Apiognomonia veneta* (Sacc. et Speg.) Höhn., which, with the anamorph *Discula platani* (Peck) Sacc., attacks species of *Platanus*, especially *Platanus* x *acerifolia* (Aiton) Willd. However, Monod (1983) stated that *A. veneta* was distinct from A. *errabunda*. More recent investigations, based on morphological, ecological and sequence data from coding and non-coding DNA regions (rDNA ITS and actin, calmodulin, and the translation elongation factor 1-alpha [EF-1] genes), support the conclusion that *A. veneta*, the type species of *Apiognomonia*, is indeed distinct from *A. errabunda* (Sogonov et al. 2007).

The following epithets were provided for the holomorph *A. quercina/D. quercina* over time on oaks:

Gloeosporium quercinum Westend., 1854
Gloeosporium quercigenum Westend., 1854
Cytospora quercina (Westend.) Sacc., 1878
Fusicoccum quercinum (Westend.) Sacc., 1881
Discula quercina (Cooke) Sacc., 1884
Gloeosporium quercuum Harkn, 1884
Gloeosporium umbrinellum Berk. and Br., 1866
Gloeosporium canadense Ellis and Everh., 1889
Gloeosporium nervicola Massal., 1903
Gloeosporium intumescens Bub. and Kab., 1910
Gloeosporium divergens Peck, 1911
Gloeosporium cecidophilum Trotter, 1915
Gloeosporium marginans Bub. and SYd., 1915
Myxosporina quercina (Westend.) v. Höhn., 1921
Gloeosporium umbrinellum (Berk. and Br.) Petr., 1922
Discula westendorpii M. Morelet, 1968

Haemmerli et al. (1992) analysed by means of RAPD and RFLP the DNA of 30 isolates of *D. umbrinella* from larch, chestnut and oak. They found that isolates belonged to four groups each attacking a different host, confirming the high specialisation of members of the A. *errabunda* complex.

Apiognomonia quercina has as its basionym *Gnomonia quercina* Kleb., and its taxonomic position is as follows:

Gnomoniaceae
Diaporthales
Sordariomycetidae
Sordariomycetes
Ascomycota

3 Morphology

Apionomonia quercina produces minute perithecia, dark in colour tending to brown, on senescent or already fallen, usually over-wintered, leaves. The perithecia measure 330–450 × 580–850 μm. The asci are evanescent and measure 8–15 × 40–55 μm. The ascospores are two-celled, hyaline, tending to pale-yellow, and measure 3–7 × 13–22 μm. The acervula of *D. quercina* that develop on the shoots and twigs are round or elliptical, with a diameter often exceeding 250 μm. The conidia are one-celled, hyaline, ellipsoid, 3–6 × 8–15 μm. Colonies on PDA are very light-brown, with a sunken mycelium consisting of hyaline, septate hyphae.

4 Biology and Epidemiology

The distribution of the oak anthracnose agent does not seem to depend on the site or the nature of the soil, since it occurs on all types of sites and soils, irrespective of the stand altitude or its exposure to the sun.

The age of oaks that become infected ranges from 15 to 40 years, in coppices close to the time of harvest, and in ageing stands. Seedlings in the nursery also become infected, especially by *A. quercina*. In winter, the anamorph survives by differentiating its acervuli in cankers on the twigs and branches. When winters are mild the teleomorph can survive in the perithecia that form on the fallen leaves. Primary infection occurs after the winter season, when temperatures reach 18–20°C, which is high enough for the perithecia and the acervuli to mature. The ascospores and the conidia infect the leaves, buds, shoots and twigs. The teleomorph usually colonises the leaves, buds and shoots, while the anamorph colonises not only the leaves and buds but also the bark and xylem of the shoots (Ragazzi et al. 1999b). In the air the ascospores and conidia are dispersed by wind, rain and presumably insects, while in the litter the ascospores released by the perithecia are spread

by percolating water. The agent is also spread over long distances when already infected seedlings are shipped as material for planting.

In vitro, sporulation of the teleomorph peaks at 15,000 ascospores/ml after 14 days of incubation at 25°C, relative humidity (r.h.) 65%, under constant lighting at 15,000 lx. Sporulation of the anamorph under the same conditions peaks at 18,000 conidia/ml after 12 days.

When 2-year-old seedlings of *Quercus cerris*, *Q. pubescens* and *Q. robur* are infected with a 10^5/ml suspension of ascospores under controlled conditions, the incidence of infection is higher than when the seedlings are infected with conidia. *Q. cerris* is the most susceptible to anthracnose, followed by the other two species in that order. With both ascospores and conidia, the greatest incidence of infection is achieved with a 16-h day at 20,000/0 lx and a day/night temperature of 22°C/18°C, r.h. 40%/65%. Sinclair et al. (1987), examining *Quercus alba* L., *Quercus coccinea* Muench, *Q. rubra* L., and *Q. vetulina* Lam., found that *A. quercina* grew fastest at 16–20°C. As regards the effect of leaf age on anthracnose incidence, younger leaves of 18 days are more susceptible than 31-day-old leaves.

4.1 Symptoms

Oak anthracnose symptoms are typical and well characterised for each form. The teleomorph *A. quercina* essentially induces leaf spots, whereas the anamorph *D. quercina* causes shoot dieback. Symptoms vary depending on the climate, the oak species infected, the phenological phase of the tree at the time of infection, and also the tree organ infected.

In Mediterranean countries the first symptoms to appear are small brown spots on the leaves shortly after the annual resumption of growth. Spots gradually increase in size and become reddish-brown. These larger spots are usually irregular in outline, and bounded by the leaf veins. It is because of these symptoms that the diseases caused by this and other anthracnose agents are also called “angular leaf spot”. Those spots that are more nearly round are about 12–22 mm in diameter. As the disease progresses, spots may become confluent and in some instances they cover the entire leaf surface between the veins. Some part of the leaf blade however always remains green. When the spots cover about four-fifths of the leaf blade, the leaf withers, becomes papery, hazel-brown, and twisted. All the expanding leaves of the crown are often killed.

Older leaves are also affected, but their symptoms remain limited to brown spots. These spots likewise spread, but normally do not occupy almost the entire leaf area.

After colonising the leaves, the pathogen invades the petiole and through this reaches the shoot. Shoots can however also be directly infected by the anamorph. The shoot dies when it is completely girdled by the pathogen. The infection then advances to the twigs, which generally develop a 7–10-cm long, slightly depressed canker. This pattern of colonisation leads to the death of a number of twigs,

giving rise to the typical shoot dieback. The acorns are also often infected. As a consequence of infection, small black specks develop on the acorns.

When the fungus attacks repeatedly and with a rapid development of symptoms over the years, each time killing leaves, shoots and twigs, the final outcome is a general crown thinning, and eventually the death of the tree. Minute sexual fruiting bodies (perithecia) develop on the leaves, while the asexual state produces acervula on the twig cankers.

Disease incidence, severity and spread are closely linked to the weather. Rainy weather, especially in the spring and summer, strongly increases disease incidence in Great Britain (Grove 1937).

In North America the severity of the disease is also reported to vary with the host species. Parris and Byrd (1962) in Mississippi found that symptoms on *Quercus alba*, one of the most susceptible oak species, followed different patterns:

1. a very rapidly advancing spread of blight on leaves and shoots, with the young leaves becoming brown during the growth phase;
2. a dying-off of large and irregular portions the leaf blade; these portions tend to become twisted; although a part of the leaf often remains green;
3. only small necrotic spots form on adult leaves, which are less susceptible to the fungus than younger leaves.

These necrotic spots vary in their appearance depending on the tree species affected. In *Q. palustris* Muench. and *Q. shumardi* Buckl., spots are roundish and red-brown, with a yellow external halo on the upper leaf lamina, whereas on the lower leaf lamina they are slightly sunken. On *Quercus phellos* L. and *Q. laurifolia* Michx. spots are produced in great profusion, and are smaller, from brown to black in colour, with a green halo. On *Q. nigra* L. and *Q. falcata* Michx the spots are larger and have a yellow edge. When infection is severe the apical shoot dies and folds back on itself.

On *Q. virginiana* P. Mill. disease severity appears to be very low and the density of the leaf spots is normally also very low, only 1–2 per cm^2, compared with 15–20 spots per cm^2 on more sensitive species. *Q. durandii* Buckl. is much less susceptible than *Q. virginiana*, as is shown by the very low number of spots that appear on its leaves.

4.2 Production of Conidia

The rate of conidial production of the asexual state was examined by Ragazzi *et al.* (1999c) in an epidemiological report. Conidial production of *D. quercina* was determined on 2-year-old seedlings of *Q. cerris* in a controlled environment chamber with a 12 h day and day/night temperatures of 25°C/15°C; r.h. 50%/75%, and 25,000/0 lx. Seedlings were inoculated with a conidial suspension at a concentration of 10^5 conidia/ml. After the acervuli appeared, 34 days after inoculation, two spore traps (Rotorod Model 60A, Ted Brown Associates, Los Altos Hills, CA, USA) were

placed at a height of 1.80 m from the ground and 2.5 m apart. These samplers were operated from 6 a.m. to midnight with a pause of 25 s every 3 h, and from midnight to 6 a.m without interruption. Each spore trap had two silicone-coated rods (General Electric G-697) to trap the conidia. The number of conidia per m^3 was calculated according to the following formula:

$$\text{Number of conidia} = \text{conidia}/\,(\text{g}/\text{min}) \times (\text{K}) \times (\text{min})\,,$$

where g = no of revolutions/m^3 of air/min; K = coefficient for the type of rod used, equivalent to 0.050.

Conidial production increased steadily from dawn (06.00 h) to midnight (24.00 h) every day. The daily spore count was very low when the traps were initially turned on at 06.00 h. Spore counts gave a reliable indication of the number of conidia present in the air at any given moment. The spore count increased steadily over the various count days from the time of acervula appearance until day 14. On day 14, conidial production peaked, with lower values being recorded on subsequent days. This suggests that the greatest number of conidia were released by the anamorph in the first 2 weeks after the start of acervula formation.

The anamorph thus produces and disperses asexual spores constantly for at least 2 weeks. During this time the infection becomes greatly intensified. Disease severity is also reinforced by the release of the first ascospores, which differentiate on the leaves and constitute the primary infection.

The number of conidia released was the highest at midnight. The constant increase in the daily rate of conidial production, from dawn to midnight, observed under controlled conditions, doesn't exactly coincide with what happens in nature, at least when environmental temperature becomes too high. In the field it appears that the anamorphic state is inhibited by midday temperatures higher than 25°C and becomes more active during the milder temperatures and the higher humidity of the night and morning (Ragazzi et al. 1999c).

4.3 Isolation from Infected Tissue

To examine the endophyte population in a given ecosystem and make inferences about its role, it is essential to measure the size of the population accurately. But such measurements are difficult to make in forests, which are highly complex systems. The heterogeneity of the ecological context, the large surface of the trees, and the inconspicuousness of endophytic infections, often localised in particular niches, make the endophytes in forest trees difficult to detect, measure and explore in sufficient detail. For this reason, a sizable portion of the endophytic population may not be detected. The exclusion of this portion from the study may lead to an underestimation of the endophyte population size, and hence of its ecological impact (Moricca and Ragazzi 2008).

Depending on the weather, the oak anthracnose agent may be abundant or sporadic and erratic in oak stands. To make allowance for this variability, which can hide the true size of endophyte populations, sampling has to be as accurate as possible. Below are described some protocols developed at our laboratory to ensure an accurate census of the fungus in different oak organs and tissues.

4.3.1 From Twigs

Oak twigs (roughly 12 cm in length) were sampled from 1-, 2-, and 3- year-old oak branches. Samples were stored on ice and taken back to the laboratory within about 2 h. Here they were immediately surface-sterilised and processed. The two ends (about 1 cm) of each twig were cut off, and the remaining twig was cut into four equal parts of 2.5 cm each. All 1-year-old segments, plus 25% of the 2- and 3-yr-old twig segments, were placed on potato dextrose agar (PDA) and incubated for 3 weeks at room temperature. They were then inspected for the endophyte. The remaining 75% of the 2- and 3-year-old twig pieces were stripped off their bark with a sterile razor. The bark and the wood of these pieces were then placed separately on PDA, incubated as above, and scored for the endophyte.

4.3.2 From Buds

Two fragments of embryo tissue and two of bud scale were removed from each of two basal buds and two apical buds per twig.

4.3.3 From Shelled Acorns

Naturally abscised acorns were collected from mature oaks in October. Acorns were surface-cleaned, sterilised by immersion in 70% ethanol for 1 min, after which the shell was removed, leaving the two cotyledons attached to the embryo area. A single piece of each acorn (about a quarter of the total acorn) was surface-sterilised in 5% hydrogen peroxide for 5 min. Samples were placed in 22-mm-diameter sterile test tubes containing 2 ml sterile water, and incubated for 6 weeks at ambient temperature. The fungal colonies obtained were then scored for *D. quercina*.

4.3.4 From Increment Cores of the Tree Trunk

Core samples were taken from five trees (two cores per tree) by means of an increment borer at a height of 1.30 m from the ground. The increment borer was sterilised by flaming with ethanol just before each sampling. Since the hardness of oak wood often prevents borers from penetrating deeply into the wood, sampled cores were only about 10 cm-long. The bark was removed at the point where the

holes were bored, to prevent contamination of the cores by bark fungi. Cores were placed immediately on ice in sterile tubes and taken to the laboratory to be scored for *D. quercina*.

All tissue pieces were surface-sterilised in 3% sodium hypochlorite for 4 min, followed by immersion in 60% ethanol for 2 min. The fragments were placed in 9-cm-diameter Petri dishes containing 20 ml PDA supplemented with 30 ppm of streptomycin bactericide. The Petri dishes were then incubated in a controlled environment chamber at 22°C ± 2°C, 70% r. h., under constant lighting (15,000 lx).

Besides PDA, two other nutrient media were tested: oak leaf agar (OLA) (30 g oak leaves boiled in 350 ml water with 8 g agar [Difco] for 30 min, pH 6.6), and impoverished PDA (6 g/l potato dextrose broth [PDB] + 20 g/l agar), in order to define the colony phenotypes and to promote sporulation. Mycelium primordia were visible under the stereoscope after 2 days of incubation on all media, with distinct colonies that began to differentiate visibly after 4–5 days. Colonies were a very light brown, tending to yellow. The mycelium was submerged in the medium, with hyaline, septate, branched hyphae. Acervula that had a diameter of 150 μm were seen on impoverished PDA after about 5 days. They were epidermic, separate or confluent, light-brown, smooth-walled and with an irregular dehiscence. Conidiophores were 13.3–5 × 21 μm, hyaline, septate, single or branched (but only at the bottom), straight or slightly curved, elongated towards the tip. Conidia were 3.5–5 × 9–15 μm, hyaline, oblong, ellipsoid, not septate, with a smooth, thin wall, a blunt tip and a more or less truncated base.

A. quercina isolation on PDA was very successful. The best medium for colony growth was OLA. Optimal growing conditions in the controlled environment were: 30 W fluorescent lighting (15,000/0 lx), a 12-h day, with day/night temperatures 24°C/18°C; r.h. 50%/75%. Under such conditions colonies differentiated after only 3 days, and reached a diameter of 9 cm in 6 days (Ragazzi et al. 2002).

Similar investigations by other authors (Neely and Himelick 1967; Wilson and Carroll 1994) provided clear evidence of the importance of the nutrient medium in supporting fungal growth and sporulation.

Neely and Himelick (1967) tested various isolates of *Gnomonia quercina* Kleb. from white oak on 10 agar media (Difco) variously supplemented with bean pod, lima bean, corn meal, cabbage infusion, malt extract, potato dextrose, prune, and Sabourand-dextrose. Growth rates were determined at temperatures from 3°C to 30°C. Production of conidia was greatest on PDA with bean pod and malt extract, whereas none of the isolates differentiated many conidia on PDA with cabbage infusion, corn meal, or Sabourand-dextrose. All isolates produced a white to pale-grey aerial mycelium on all media, with colonies that formed grey to black zonation rings on many of the media. A dark-yellow pigment spread through the PDA medium after 4–6 weeks of incubation at 21°C. The greatest colony diameters (7–9 cm) were seen with corn meal, prune, Sabourand-dextrose, and PDA. The optimal growing temperature was 18°C.

Wilson and Carroll (1994) devised sterilisation protocols for the optimal isolation of *D. quercina* from various tissues of *Quercus garryana* Dougl (Table 1).

Table 1 Surface-sterilisation of oak tree tissues (from Wilson and Carroll 1994)

	Surface-sterilisation steps			
Plant tissue	95% Ethanol	70% Ethanol	Sodium hypochlorite[a]	Sterile distilled water
Acorn shells	60 s	60 s	5 min in 33% solution	Five washes
Naked seeds (shelled acorns)	Not used	30 s	10 min in 10% solution with 1% SDS[b]	Five washes
Twigs, bark, wood	60 s	2 min	8 min in 33% solution	Five washes
Leaves	50 s	60 s	5 min in 33% solution	Four washes
Prebud burst and young postbud burst leaves	25 s	30 s	2 min in 33% solution	Four washes

[a]Diluted (by volume) from a 5% sodium hypochlorite household bleach stock solution
[b]Sodium dodecyl sulphate (*SDS*) is a detergent used as a wetting agent

Table 2 *Spearman's coefficient of the isolation frequency of some endophytic fungi recovered from twigs of declining* Quercus cerris *and* Q. pubescens *trees*

	Year		
Taxon	2005	2006	
Apiognomonia quercina/Discula quercina	23.4	23.4	0.700***
Biscogniauxia mediterranea	14.0	15.6	0.425**
Colpoma quercinum	11.6	10.4	0.320*
Diplodia mutila	10.9	11.7	0.660***
Phomopsis quercina	11.1	11.7	0.400**

***Significant at 0.001; **Significant at 0.01; *Significant at 0.05

4.4 Incidence and Gradient of Anthracnose

4.4.1 Incidence

Disease incidence was calculated in a coppice of 18–20-year-old *Q. cerris* trees growing in east-central Tuscany by laying out four transects of 1,200 m^2 each. In each transect the infected trees were counted and the leaf area infected was calculated on 15 leaves from a sample of 10 infected trees. Anthracnose incidence varied from 40% to 55%. On *Q. pubescens* a similar investigation found that anthracnose incidence varied from 15% to 20%.

Table 2 shows the isolation frequency of the two anthracnose morphs in relation to some other endophytic fungi recovered from twigs of *Q. cerris* and *Q. pubescens* with signs of decline, expressed as Spearman's coefficient.

Since *A. quercina* is native to several regions of Europe, it is not included in the quarantine lists of the European and Mediterranean Plant Protection Organization (EPPO). However, if we assume that it is the geographic transposition to a new site the guiding principle for classifying an organism as exotic (Lockwood et al. 2007), then the holomorph *A. quercina/D. quercina* is to be considered non-indigenous in several European forest stands, where it is increasingly common in a number of previously uncontaminated areas, and where it often displays considerable

Fig. 1 *Chronology of reports of the holomorph* Apiognomonia quercina/Discula quercina *in Italy over the last two decades*

ecological fitness and invasiveness. Its high rate of spread and survival capability are due to a number of biological properties:

1. it generates a high biomass. Its asexual form produces 49,300 conidia per m^3 air at a distance of 10 m from the inoculum source after 10 days of sporulation. The inoculum load is still high (2,400 conidia/m^3 air) at 1,000 m from the source after 40 days of sporulation;
2. it is highly persistent because it is endowed with a triple survival capacity. The conidial form persistently differentiates on the twigs, leaves and buds of the tree; the sexual form remains vital by means of perithecia on fallen leaves; and in southern Europe on account of the mild winters the mycelium grows actively all the year round on oak leaves, buds and twigs;
3. it has considerable ecological plasticity, adapting to a wide variety of climates and environments. The fungus occurs in northern and eastern Europe, including parts of Russia, and southern Europe (the Mediterranean countries). It is found in North America from Canada down to the Gulf of Mexico on the Pacific coast;
4. it has an extensive host range.

The invasiveness of the agent clearly appears from the various locations where it has been reported to occur in Italy over the last two decades (Fig. 1). After being first reported in Tuscany (central Italy) in 1990, this tendentially

thermophilic microorganism was mainly reported from southern and central Italy, but in the last decade it has also been reported from locations in the north, which were formerly pathogen-free. The fungus is thus spreading northwards.

4.4.2 Anthracnose Gradient

The anthracnose gradient was determined in a mixed stand of *Q. cerris* and *Q. pubescens* located at Ulignano, Province of Pisa, 400 m. a.s.l. (UTM co-ordinates x, 1655324,73; y, 4813251,52). A transect containing 68 *Q. cerris* trees and 14 *Q. pubescens* trees was laid out in the stand in a north-southerly direction, the same as the prevailing winds. Of these trees, 44 *Q. cerris* trees and 8 *Q. pubescens* trees were declining, and *A. quercina* was isolated from 32 of the declining *Q. cerris* trees and five of the declining *Q. pubescens* trees. Declining trees were taken to be emission sources of ascospores and conidia, since endophytes normally begin sporulating in declining trees, i.e. trees that have come under stress or that are senescent (Wilson and Carroll 1994; Faeth and Hammon 1997).

To measure the inoculum concentration at various distances from the transect, or source of inoculum, virtual lines were traced running 10, 100, 500, and 1,000 m from the southern end of the transect, and parallel to it (and perpendicular to the prevailing direction of the wind). Each recording line passed through a number of trees and spore traps (Rotorod Model 60A, Ted Brown Associates, Los Altos Hills, CA, USA). Each spore trap had a cylinder rotating at 2,400 revolutions and was operated from 12 noon until 2 p.m. once every 10 days (from day 10 to day 40). Each recording line determined the inoculum concentration at that distance from the transect. To measure the number of cankers, all cankers longer than 2 cm were counted on eight twigs from each tree (two twigs from each compass point) from the trees at each recording line. To measure the inoculum concentration (number of ascospores/conidia) at each distance from the transect the following formula was used:

$$\text{Number of propagules} = \text{ascospores/conidia/ (g/min)} \times (\text{K}) \times (\text{min}),$$

where g = number of revolutions/m^3 of air/min; K = coefficient for the type of rod used in the trap, equivalent to 0.050.

The disease gradient was calculated based on the number of sporulating cankers. All cankers on the twigs were taken to be sporulating cankers on the basis of the exponential model of Campbell and Madden (1990). The recording distance of 10 m from the end of the transect consistently yielded the greatest number of propagules trapped, compared with the other distances, and the number of propagules at 10 m was greatest after 10 days, when 49,300 propagules/m^3 of air were trapped. The disease gradient and the corresponding linear regression are shown in Fig. 2.

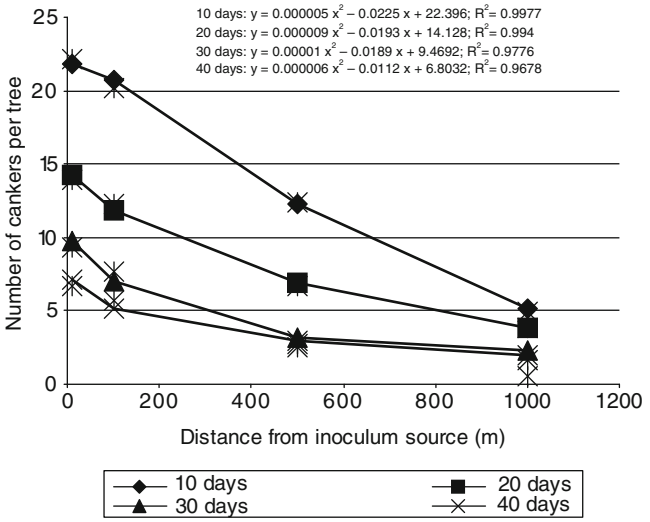

Fig. 2 Disease gradient of *Apiognomonia quercina* on *Q. cerris* and *Q. pubescens.* Average number of cankers per tree, as determined on the twigs of 15 trees (2 twigs per compass point = 8 twigs per tree) at each of four virtual recording lines (at 10, 100, 500 and 1,000 m from the inoculum source) (from Ragazzi et al. 2007)

The number of cankers per tree diminished with increasing distance from the source of inoculum. The linear regression was as follows: time, $y = 23.109 - 0.92x + 0.01x^2$ $R^2 = 0.4655$; distance, $y = 0.018x$ $R^2 = 0.40966$. Increasing the distance from the inoculum source from 10 to 1,000 m decreased the number of cankers by 71.2%.

The number of propagules (ascospores + conidia) captured decreased steadily with increasing distance from the recording lines (10, 100, 500 and 1,000 m from the transect) and this regular decrease was found at all recording dates (10, 20, 30 and 40 days). The greatest decrease occurred between 100 and 500 m.

In a disease control perspective all potential inoculum sources (i.e. declining trees) at all distances from a given population should be inspected, and any declining trees within these distances should be felled to eliminate the mass of inoculum.

5 Specificity of the Anthracnose Agent to Hosts and Host Organs

The oak anthracnose agent occurs on several Mediterranean oak species. *D. quercina* has been isolated from *Q. cerris* L., *Q. frainetto* Ten., *Q. ilex* L., *Q. pubescens* Willd., *Q. robur* L., *Q. suber* L. and from other members of the *Fagaceae*: *Castanea sativa* Mill. and *Fagus sylvatica* L. Other Mediterranean oak species, such as

Q. coccifera L., *Q. congesta* Presl., *Q. dalechampii* Ten., *Q. macrolepis* Kotschy, *Q. pyrenaica* Willd., *Q. troiana* Webb., *Q. virgiliana* Ten., whose fungal endophytic assemblage has not been investigated sufficiently or at all, may also be hosts. The fungus was isolated from beech and chestnut only when these trees grew in stands mixed with oak. It is conceivable that tree species other than oak are non-preferential hosts for this microorganism, which infects them only in particular circumstances, such as when the air inoculum reaches particularly high levels due to favourable environmental conditions.

A number of forest tree species have been investigated for their proneness to infection by fungal endophytes (Chapela 1989; Sieber et al. 1991; Petrini 1996). Some of these studies explored the specificity of fungal endophytes to oak hosts (Petrini and Fisher 1990; Kowalski and Kehr 1992; Collado et al. 2000). A wide range of relations, ranging from a loose host-generalism to a tight host specificity, has been found. Terms like host-exclusivity or host-recurrence were also used to describe extreme or intermediate points not properly fitting within those two categories (Zhou and Hyde 2001). Clearly it is impossible to limit all host-fungus interactions to a rigid binary scheme of specific or not specific, since they are dynamic and vary over time. The holomorph *A. quercina/D. quercina* presents such a difficulty since the teleomorph and the anamorph differ in their host preference. The teleomorph colonises a wide range of oak species, whereas the hosts of the anamorph are limited almost entirely to *Q. cerris, Q. pubescens* and *Q. suber*; its occurrence on other hosts is rare. More study is needed to elucidate the factors governing this difference in host colonization.

The teleomorphic and anamorphic states not only differ in their host preference, but also in the host organ they prefer, and this also needs to be elucidated. Host organ preference (a sort of niche restriction) occurs in both the sexual and the asexual forms of *A. quercina/D. quercina*, on *Q. cerris* and on *Q. pubescens*. The teleomorph *A. quercina* occurs only on the leaves, on which it over-winters, differentiating its perithecia early in the spring. The anamorph *D. quercina* survives from year to year on cankered twigs and branches, and in some stands, where conditions are favourable, on the buds (Ragazzi et al. 2003; Anselmi et al. 2004).

This divergence between host preference and organ preference supports the hypothesis that the competitive interaction between the two forms, exploiting almost the same resource, has in time led them to diversify their niches, albeit with frequent overlaps. In this connection, then, the two metagenetically related morphs act as distinct, competing entities.

Differences in host and organ preference are classical examples of resource polymorphism. The phenomenon is found in several holomorphs in the kingdom Fungi and it is also common among morphs of taxonomic groups in other kingdoms (Smith and Skulason 1996). On the other hand, specialisation is a basic strategy of organisms to ensure their survival. Species develop distinct evolutionary pathways in order to survive in different geographic areas, climates, seasons, habitats and niches that offer different food resources to them. Consequently, it is difficult to find any two species in nature whose niches overlap completely (i.e., all species tend to have restricted niches). Research across diverse taxa suggest that

resource polymorphism is a diversifying force that may play an important role in population divergence and speciation, yet little effort has gone towards examining the mechanisms and conditions that produce and maintain this phenomenon in fungi.

Host specificity and organ specificity in any case evolve along a coordinated series of steps that include reciprocal recognition between the host and the fungus, followed by penetration of the host epidermis by the fungus, and colonisation of the host tissue. For these steps to occur, the two interacting partners must develop matching traits. Matching traits can only arise through a close adaptation between the plant and its microbial partner, and this can be reached only by a longstanding cohabitation and coevolution (Moricca and Ragazzi 2008).

6 Control

The control of oak anthracnose represents a difficult task, with effective measures that are scarce or nonexistent. In any case, this objective is pursued through different strategies that must take into account, first of all, the context in which we are called to operate.

In arboretums and in urban and peri-urban forests, the problem of control is exacerbated by the fact that other microorganisms are also often involved, especially on adult trees.

The problem is even more intractable in natural habitats, especially large forested areas, where the only control measures are prophylactic, aimed at preventing or slowing down the disease. Such prophylactic measures include using healthy plant material in new plantations, and planting healthy seeds or at any rate seeds from healthy trees. Such measures limit the risk of future disease outbreaks. Another good practice is to eliminate any oak and beech trees that are heavily infected. All these measures are based on evidence that destroying the pathogen biomass limits disease incidence. It is also strongly recommended to maintain adequate spacing between trees. In stands where tree density is not too high, and where the relative humidity is generally below 40–50%, production of *D. quercina* conidia is much reduced (Ragazzi et al. 1999c).

Gathering up and burning infected plant residues such as fallen leaves, twigs, and branches significantly reduces pathogen inoculum in the nursery, in ornamental stands (urban parks) and in arboretums. In these places, the rate of infection is also substantially decreased by keeping the trees in good condition with proper watering and fertilising, with good ventilation and, where possible, by always ploughing parasite propagules into the soil.

Pesticides should be sprayed only in particular circumstances on seedlings and young trees in the nurseries, or on isolated oak individuals in parks and villas. Pesticides are also justified in exceptional cases on young trees in the field, when they grow in small and well circumscribed areas.

Application of 1% bordeaux mixture, followed by three applications of a Thiram-based fungicide at 10-day intervals, effectively contained oak anthracnose in the nursery (Morelet 1989). In the rare cases of intense attacks, when the disease causes heavy defoliation for a number of years in succession, two applications of Thiophanate-methyl, spaced 7–10 days apart, can be given to start off vegetative growth. Additional spraying is required if the growing season is cool and humid. Treatments with benomyl, topsin and zineb at 15–20 day intervals starting from late May reduced *Gloeosporium quercinum* in Azerbaijan, and complete control was achieved with 0.4% polycarbacin (Guseinov 1975).

All the above are good practices in artificial systems, where the equilibrium between plant and pathogen populations can be precisely manipulated, varying the inputs to control the disease, but they cannot control oak anthracnose in the forest. In oak stands ecological concerns and health hazards make chemical control prohibitively expensive and not warranted environmentally. Non-chemical control methods, on the other hand, would be totally ineffective, since they are too labour-intensive, complicated by operational problems, uneconomical and unfeasible over large areas.

A new and promising means of control is by exploiting natural antagonists to oak anthracnose. Many fungi, such as *Epicoccum nigrum* and various species of *Trichoderma*, occur syntopically on the same oak or oak organs as the anthracnose agent, and are currently being tested for their capacity to inhibit or suppress *A. quercina/D. quercina* growth and reproduction. Another benefit of antagonistic microbes is that they improve host fitness and performance (Harman 2000; Kiss 2001). Plants treated with antagonists generally have a greater photosynthetic efficiency, better growth, and produce a yield of better quality. Not only are trees more vigorous, but they are also more resistant to parasites.

7 Conclusions

The scientific community has long debated whether fungal endophytes of woody tree species are defensive plant mutualists or latent parasites. The question has given rise to much discussion, speculation, and conjecture. Experimental evidence in support of either view has been adduced (Arnold et al. 2003; Slippers and Wingfield 2007).

The uncertainty was essentially caused by the innate human tendency of cataloguing all elements of nature and categorising the living world in a variety of contexts. But giving a fixed definition for the complex role of endophytes appears to be a mere artifice. The plant-endophyte symbiosis is the result of an intimate and continuous interaction between a long-lived, phototrophic organism and a short-lived, heterotrophic microbe. By its very nature, symbiosis is a dynamic process that changes over time (Saffo 2002). In even the most stable and reciprocally beneficial symbioses, the interest of one member may not always continue to agree perfectly with the interest of the other. The misalignment of interests thus caused generates

a conflict between the members of the interaction, with one organism beginning to be detrimental to the other. To cope in advance with these potential conflicts, a plant fungal endosymbiont can select different lifestyles, ranging from mutualism to parasitism, depending on the circumstances it finds itself in.

Apiognomonia quercina, is a prominent member of the *Gnomoniaceae*. Like other members of the genus *Apiognomonia,* and like other endophytic species in the genera *Botryosphaeria*, *Ophiovalsa*, *Pezicula*, *Phomopsis* and *Sphaeropsis*, it has long been recognised as a tree pathogen (Smith et al. 1996; Sieber 2007).

The pathogenic nature of the oak anthracnose agent was confirmed by our studies, in which numerous lines of evidence supported the belief that this agent is uncooperative to tree defence. That this endophyte also had a parasitic lifestyle that it could fall back on at need, was demonstrated by the high anthracnose incidence and severity that it caused in oak under certain circumstances, when it also released a large amount of air-borne inoculum. Oak tissue colonization was always increased and more widespread when the trees became more strongly impaired by water stress. Oak anthracnose outbreaks, though caused by the endophyte, were thus strictly triggered by another factor, which was extraneous to the oak-endophyte symbiosis, and this was a changed climate.

These findings received confirmation when healthy oak stands not suffering from water stress were examined in epidemiological investigations and field surveys. With these unstressed trees the effect of the microorganism was quite different from what it was with the water-stressed trees, being almost negligible. In healthy and unstressed oak woods the occurrence of the fungus was sporadic and its virulence was not high. It tended to infect only the vegetative organs of the hosts, and in many oaks it remained metabolically inactive and absolutely non-pathogenic. It survived indefinitely as a harmless coloniser of leaves, sprouts and twigs, and formed only invisible infections.

It is therefore concluded that *A. quercina* encompasses a number of lifestyles, and that the selection of a lifestyle essentially depends on the health of the tree, and on climate patterns that affect the symbiotum as a whole. The host-fungus relationship thus changes in response to seasonal variations in the weather and in climate. Adverse changes in the weather such as prolonged droughts affect the physiology, phenology and biochemistry of oak trees (Hughes 2000). During a drought the water content and nutrient availability within the apoplast may fall dramatically. This generates a conflict of interest between the partners, with the microbial symbiont no longer finding suitable conditions for life within the senescing leaves and decaying branches of the stressed and declining trees. When this happens, the microbe is forced to modify its lifestyle to ensure its own survival. As a consequence it moves to the tree surface, where it begins to sporulate profusely to escape from the dying branches and inner tissues of the declining oaks where it had found a haven before.

The life-history strategy adopted by the microbial partner is a clear example of the instability of a symbiosis whenever one of the members, in this case the tree, is impaired by physiological stress and no longer able to ensure the continued, symptomless survival of the other. The microorganism then reacts by modulating

its own virulence, switching from a latent, asymptomatic life-style within the host to an aggressive exploitation and saprobic colonisation of the tree (Moricca and Ragazzi 2008).

Abiotic stresses of plants and trees are becoming ever more common in the circum-Mediterranean area as a consequence of climate change. The main effects of global warming in this area are an increase in the annual mean temperature, a greater rainfall in winter, prolonged drought in spring and summer, and a greater frequency of extreme weather events throughout the year (Moricca et al. 2009). The mild winters of recent decades no longer kill enough *A. quercina* propagules to reduce its biomass. The early start of vegetation makes trees more prone to late spring frost damage. Exceptional windstorms with hail cause branch and twig ruptures, producing a multitude of bark and leaf lesions that become entry points for the microorganism. To these physical injuries must be added the physiological injuries oaks suffer because they are exposed to multiple adverse environmental factors (hot winds, heat stress, scorch injury). To repair its damaged structures and functions, the tree has to utilise its reserve compounds. When the demands of energy become excessive, as in the case of prolonged drought for many years, the tree eventually exhausts its resources and collapses. Climate change thus limits tree vigor and predisposes oaks to anthracnose infection.

In this perspective, the holomorph *A. quercina/D. quercina* acts like an ecological player. It naturally prunes the senescent branches and leaves of declining oaks. It flexibly tailors its own degree of pathogenicity to the environmental conditions and to the physiology of the host. Clearly, it is not so much the agent itself, as the health of the oaks, the conditions of the site, and the weather patterns that dictate both the prevalence and the magnitude of the microorganism's pathogenic action.

Lastly, the importance of carrying out this type of studies with perennial trees growing in their natural habitat should be stressed. The ecological integrity of many forest biocenoses can shed light on the dynamics regulating the subtle plant/endophyte interaction. As has been shown, a better understanding of these complex ecosystems can elucidate the causes and processes that upset a tree-endophyte relationship and cause a symbiosis to evolve from a neutral or at least a harmonious relation to an antagonistic one.

These investigations also provide some useful clues to help explain why some fungal agents, which in an undisturbed ecosystem usually live without causing any visible damage, start spreading epidemically when they migrate to agricultural crops, which grow in high densities and are characterised by genetic uniformity and scarcity or lack of genetic resistance (Cohen 2004).

Furthermore, since the endophytic mycobiota living in unmanipulated forests have not been subjected to artificial selection pressures, they constitute an ideal material for testing hypotheses on fungal population biology, speciation and evolution (Stone and Petrini 1997).

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Diversity of Fungal Endophytes in Tropical Trees

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Abstract Our knowledge of the taxonomic diversity of fungi is not commensurate with their importance in ecosystem functions and biotechnology. While fungi may not be as appealing as some of the charismatic species, it is imperative that we get at least a near-real estimate of global fungal species diversity as early as possible to facilitate conservation and take full advantage of their technological potential. As indicator and surrogate species are used for estimating the diversity of species-rich groups such as fungi, their fidelity in reporting the diversity has to be confirmed before relying on them. In this context, tropical endophytes have been used as a reporter group to estimate global fungal diversity. I discuss here the diversity and host specificity of tropical foliar endophytes and the suitability of this ecological group of fungi as indicators of global fungal biodiversity.

Abbreviations

(DT)	dry thorn forest
(EG)	montane evergreen forest
(DD)	dry deciduous forest
(MD)	moist deciduous forest
CE-SSCP	(capillary electrophoresis single–stranded conformation polymorphism)
CE-FLA	(capillary electrophoresis fragment length polymorphism)
(ITS rDNA)	internal transcribed spacer region
(DGGE)	denaturing gradient gel electrophoresis

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1 Introduction

Global estimates of the number of fungi range from a very conservative 712,000 species (Schmit and Mueller 2007) to a staggering figure of 9.9 million species (Cannon 1997) with the 1.5 million estimate of Hawksworth (1991) being accepted by many mycologists. Currently, about 93% of the estimated 1.5 million species remain undescribed (Hawksworth 2004). While cogitating on where the undescribed fungi are found, Hawksworth (2004) identifies endophytes of tropical trees as one of the categories to be explored. The use of endophytes as a benchmark for biodiversity estimation is appealing, as the sampling procedures are standardized and easily quantified (Sieber 2007). Even though foliar endophytes are ubiquitous in tropical trees, several questions regarding the nature of their association with hosts, host specificity, geographic distribution, and contribution to global fungal diversity remain unanswered or, at best, partially answered. However, the information that has accumulated so far on tropical tree endophytes, when viewed *in toto*, provides some clues regarding their diversity and distribution and reveals aspects that need consideration.

This paper addresses issues such as host specificity, tissue preference, and the concepts of morphospecies and molecular species of tropical foliar endophytes, which are central to the understanding of the biology of endophytes and their robustness in serving as a reference group for fungal diversity estimates.

2 Host Specificity and Diversity

According to Dreyfuss and Chapela (1994), Fröhlich and Hyde (1999), Arnold et al. (2001), Arnold and Lutzoni (2007) and Shipunov et al. (2008), the hyper-diversity of fungal endophytes of tropical plant hosts suggests that the generally accepted number of 1.5 million species greatly underestimates fungal diversity. Host specificity concomitant with the high plant host diversity found in the tropics will result in a prolific diversity of plant-associated organisms. However, extrapolation from measured endophyte diversity on a few host species to global tropical fungal diversity depends strongly on assumptions of the degree of host specificity. Although extrapolations of host specificity data have been used to estimate biodiversity of plant-associated fungi and insects (Erwin 1982; Arnold et al. 2001; Novotny et al. 2006), extrapolation from regional endophyte surveys to a pantropical scale may not give the true picture without correction factors for endophyte taxa with loose host affiliations. It is now established that multi-host endophytes (generalists) such as *Colletotrichum*, *Pestalotiopsis*, *Phomopsis*, *Phyllosticta* and *Xylaria* occur routinely in taxonomically unrelated tropical tree species growing in unconnected geographical locations (Bayman et al. 1998; Okane et al. 2001; Baayen et al. 2002; Davis et al. 2003; Pandey et al. 2003; Lu et al. 2004; Jeewon et al. 2004; Murali et al. 2006; Wei et al. 2007; Tejesvi et al. 2009).

Table 1 Dominant endophyte taxa in representative tropical trees from different parts of the world

Host	Dominant sp.	Place	Reference
Hevea brasiliensis	*Pestalotiopsis* sp.	Peru	Gazis and Chaverri 2010
Tairira guianensis	*Phomopsis* sp.	Brazil	de Abreu et al. 2010
Tectona grandis	*Phomopsis* sp.	India, Thailand	Murali et al. 2006 Chareprasert et al. 2006
Guarea guidonia	*Phomopsis* sp. *Phyllosticta* sp.	Puerto Rico	Gamboa-Gaitán et al. 2005
Garcinia mangostana, G. parviflora	*Glomeralla* sp. (*Colletotrichum*) Guignardia sp. (*Phyllosticta*) *Phomopsis* sp.	Malaysia	Sim et al. 2010
Kandelia candel	*Guignardia* sp. (*Phyllosticta*)	Hong Kong	Pang et al. 2008
Euterpe Oleraceae	*Xylaria cubensis*	Brazilian Amazon	Rodrigues 1994
Manilkara bidentata	*Xylaria* spp.	Puerto Rico	Lodge et al. 1996
Spondias mombin	*Phyllosticta* sp. *Phomopsis* sp.	Brazil	Rodrigues and Samuels 1999
Trachycarpus fortunei	*Phomopsis* spp. *Glomerella cingulata* (*C. gloeosporioides*)	China, Australia, Switzerland	Taylor et al. 1999
Citrus lemon	*C. gloeosporioides*	Argentina	Durán et al. 2005
Malus domestica	*Colletotrichum* spp.	Brazil	Camatti-Sartori et al. 2005
Plumeria rubra	*Colletotrichum* sp. *Phyllosticta* sp.	India	Suryanarayanan and Thennarasan 2004

Invariably, these multi-host endophytes also dominate the endophyte assemblage of their tree hosts (Table 1). Since endophyte association with plants has existed for millions of years (Sieber 2007), it is highly probable that host range expansion has occurred frequently in the multi-host endophytes resulting in loss of host specificity. For instance, Mohali et al. (2005) studied populations of *Lasiodiplodia theobromae,* a cosmopolitan endophyte and pathogen isolated from various tree species in Venezuela, South Africa and Mexico and found no evidence for host specificity; there was very high gene flow between populations from different hosts within a region, though geographical isolation was indicated by unique alleles. As an extreme case of host acquisition, closely related *Xylaria* endophytes infect hosts belonging to three divisions (including liverworts and higher plants) and occurring on two continents (Davis et al. 2003).

One of the generalizations regarding fungus-plant interaction is that closely related plant hosts harbour the same fungal species, such that host sharing among fungi declines as a function of phylogenetic distance between the plant hosts (Webb

et al. 2008). This is true for the needle endophytes of conifers (Sieber 2007) and tropical foliar pathogens (Gilbert and Webb 2007), but not for epifoliar fungi in a tropical forest (Gilbert et al. 2007). A similar situation is observed for foliar endophytes of tropical forests, as many are multi-host forms and colonize trees of different lineages (e.g. Pandey et al. 2003). Although fungal pathogens generally have the phylogenetic pattern of closely related hosts sharing the pathogens (Parker and Gilbert 2004), certain ecological factors such as rainfall pattern and physical proximity of hosts, can broaden their host range such that pathogens can occur in phylogenetically distant but co-occurring hosts (Roy 2001; Farr et al. 2004). This was also evident in our long-term study on foliar endophytes of Western Ghats where we observed a pattern regarding the distribution of dominant multi-host endophytes in different forest types. The four forest types studied occur along a rainfall gradient with the dry thorn forest (DT) receiving the lowest annual rainfall and the montane evergreen forest (EG) experiencing the maximum annual rainfall with the dry deciduous (DD) and moist deciduous (MD) forests lying between these two extremes. We screened 25 dicotyledonous tree species from each forest type for their foliar endophytes. The EG forest shared one tree species with the MD forest, the DD and MD forests had nine common tree species, the DD and DT had three common tree species and DT and MD had three common tree species. *Phomopsis* spp. *Phyllosticta* spp. and *Colletotrichum* spp. were present in different tree hosts of all forest types. *Phomopsis* spp. were present in all the forest types but its dominance of the endophyte assemblage gradually decreased while moving from the dry to the relatively wet forests; the infection frequency of *Colletotrichum* spp. increased with increasing annual rainfall and reached its maximum in EG which receives the maximum rainfall (Fig. 1). This pattern of domination of endophyte assemblage in different forests could also be due to host preference as the tree species sampled varied with the forest type. However, an ordination analysis of the endophyte assemblage of five conspecific trees growing in DD and MD forests showed little host specificity among the endophytes. The clustering was based on the forest type indicating that the environment, rather than the tree host species, determines the endophyte distribution in these forests (Fig. 2). Hoffman and Arnold (2008) also observed a similar trend with endophytes of Cupressaceae. Host acquisition leading to host range expansion is not infrequent among endophytes. Indeed, overlapping ecological niches of taxonomically unrelated hosts can facilitate even cross kingdom host acquisition by endophytes (Nikoh and Fukatsu 2000; Shipunov et al. 2008). Therefore, perhaps due to a high degree of host acquisition by some multi-host endophytes, taxonomically disparate tree hosts harbor endophyte assemblages overlapping in species composition such that the high tree species diversity in tropics may not mirror endophyte diversity (Cannon and Simmons 2002; Suryanarayanan et al. 2004; Rojas et al. 2010).

According to May (1988, 1991), host specificity among plant-dependent organisms (such as insects and fungi) is likely to be less common in tropical forests where plant (host) diversity is high and, consequently, the density is low. Where the plant diversity is high, the opportunity for a plant-associated organism to become specialized on a host is limited as the probability of finding the host decreases

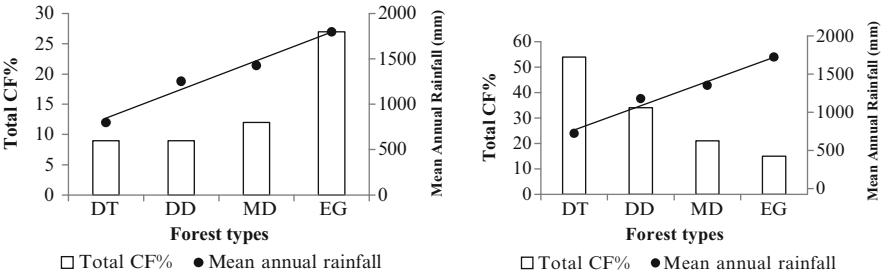

Fig. 1 Colonization Frequency (CF%) of foliar endophytic *Colletotrichum* spp. and *Phomopsis* spp. in different forest types of the Western Ghats (25 tree species were sampled for each forest type). *DT* dry thorn forest, *DD* dry deciduous forest, *MD* moist deciduous forest, *EG* montane evergreen forest

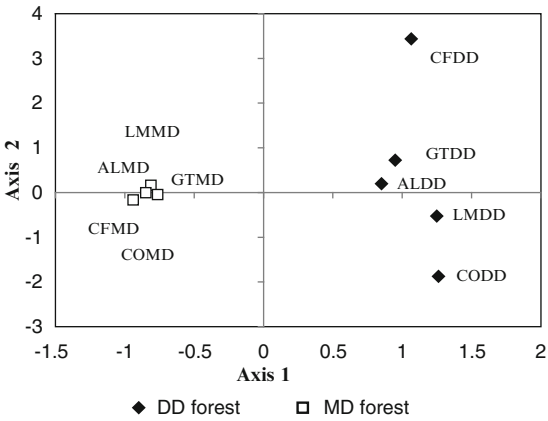

Fig. 2 Correspondence analysis of foliar endophyte assemblages of five conspecific tree hosts growing in *DD* and *MD* forests. *LM Lagerstroemia microcarpa, GT Grewia tiliaefolia, AL Anogeissus latifolia, CF Cassia fistula, CO Cordia obliqua*

due to non-contiguous distribution of the host. Empirical support for this comes from studies by Basset et al. (1996) and Novotny et al. (2006) on plant-associated insects of the tropics. Lack of host specificity is also observed in several plant-associated groups of tropical fungi including wood-rotting fungi (Lindblad 2000; Gilbert et al. 2002; Parfitt et al. 2010), ectomycorrhizal fungi (Diédhiou et al. 2010; Tedersoo et al. 2010) and arbuscular mycorrhizal fungi (Zhao et al. 2003). Thus, it appears that low host specificity is a rule among many guilds of tropical fungi including epifoliar fungi, wood rot fungi, mycorrhizae and leaf endophytes (Gilbert et al. 2007). Low host specificity reduces the plant-fungus ratio, which would in turn reduce estimates of fungal diversity that are based on plant diversity (Ferrer and Gilbert 2003). Extending this argument, unless the host specificity patterns of endophytes of different tropical forests are known, generalization for the entire tropics based on the apparent diversity of endophytes gleaned from a few tropical tree species is bound to give exaggerated results, as has been demonstrated for herbivorous insect species (Basset et al. 1996).

3 Tissue Preference

An interesting feature of tropical endophytes is that they show a higher degree of tissue preference than host preference. Different tissues of trees rather than the same tissue from different tree species have higher β diversity of endophytes. For instance, the endophyte assemblages of aerial root and leaf of *Ficus benghalensis* (Suryanarayanan and Vijaykrishna 2001), propagule and petiole of *Rhizophora apiculata* (Kumaresan et al. 2002) and leaf and bark of *Kandelia candel* (Pang et al. 2008) do not significantly overlap. Huang et al. (2008) also report tissue specificity among endophytes in their study of 29 Chinese medicinal plants. There is little overlap between foliar endophytes, bark fungi, and the non-symbiotic microfungi residing inside the lichen thalli growing on the bark of a tree species, indicating tissue or organ preference among endophytes (Suryanarayanan et al. 2005). Hence, endophytes of different tissues of tropical tree species have to be screened to get more complete information of their diversity and distribution. Suryanarayanan et al. (2009) observed tissue or substrate preference among different ecological groups of fungi such as foliar endophytes, bark and leaf litter fungi of trees in tropical forests of southern India and concluded that stratifying samples across different substrates provides a much better estimate of overall diversity than when foliar endophytes alone are used to report their diversity. Generally, although tissue preference is not absolute among endophytes, they may be adapted to the conditions present in the same tissue of different plants, resulting in the selection of a suite of fungi for each tissue. For example, foliar endophytes of mangrove trees tolerate high concentrations of salt or tannin in the growth medium, indicating their adaptation to survive in tannin and salt rich mangrove leaf tissues (Kumaresan et al. 2002). Similarly, the endophytes from tannin-rich mango leaves tolerate high levels of tannin in the growth medium, whereas conspecific endophyte taxa isolated from leaves of tree species poor in tannin content are unable to grow on such medium (Mohan Doss and Suryanarayanan, unpublished). Interestingly, Suryanarayanan and Venkatachalam (unpublished) observed a within-leaf stratification of endophyte occurrence in the leaves of *Pedilanthus* sp. where most of the endophytes were concentrated in the upper and lower epidermal peelings while very few species were present in the deeper mesophyll tissue. Precise methods, such as fluorescent tags, will confirm existence of microhabitats within plant organs being colonized by different endophytes.

4 Morphospecies and Molecular Species

There are two distinct paths of molecular approaches to study endophyte diversity. One complements the morphological method of study and the other involves direct sequencing independent of fungal isolation to account for non-culturable and slow-growing endophytes. The first approach is very useful in characterizing cultured

endophytes that are unidentifiable (sterile isolates or cryptic isolates with missing key taxonomic characteristics). The presence of non-sporulating forms makes species enumeration an incomplete exercise in endophyte studies. The common approach to account for such forms is to group them into morphospecies based on cultural characteristics (e.g. Dobranic et al. 1995). This method however, may not reflect the true diversity of endophytes of a given tree species; when and if morphologically dissimilar sterile isolates sporulate, they may be identified as members of a single taxon. We induced sporulation in a few sterile isolates by culturing them on a disk of cellophane overlying agar medium with glycine as the nitrogen source (Suryanarayanan and Swamy 1980) and found that what appeared to be distinct morphospecies had to be grouped with one or two commonly occurring species (Suryanarayanan, unpublished). Alternatively, sterile isolates that are similar and grouped under one morphospecies may actually belong to different taxa (Guo et al. 2003). Thus, when many isolates fail to sporulate, the species diversity of endophytes supported by a given tree host can be higher or lower depending on whether these forms are different from the sporulating and identifiable forms or not. Estimation of diversity merely based on morphology by categorizing morphospecies is fraught with other difficulties. Certain physiological factors alter the colony morphology and patterning of fungal growth that are used for characterizing morphospecies. Growth pattern in a single species of anamorph can change due to changes in nutrition diffusion and local hyphal populations (Matsuura 2002) or due to heterokaryon breakdown (Deacon 2006). Such phenomena are rarely considered when characterizing the morphospecies and consequently can lead to incorrect diversity values. Hence, diversity determination using morphospecies as functional taxonomic units, though rapid (Unterseher and Schnittler 2010), can lead to overestimation of diversity. In this context, the nuclear ribosomal internal transcribed spacer region (ITS rDNA) has been used to estimate endophyte diversity (Arnold and Lutzoni 2007; Unterseher and Schnittler 2010), understand phylogenetic relations among endophytes (Pandey et al. 2003; Promputtha et al. 2007) and even to discern sub-species level lineages among them (Arnold 2007). Molecular methods being better suited than morphological approaches for diversity estimation is evidenced by the fact that taxon accumulation curves for molecular species of loblolly pine foliage endophytes were steeper than those plotted for morphotaxons (Arnold et al. 2007). These observations notwithstanding, the molecular methods that are currently in vogue cannot entirely replace identification of endophytes based on morphology. Arnold et al. (2007) state that the direct PCR method they used for studying endophytes of loblolly pine revealed the presence of Basidiomycetes but failed to report the presence of Sordariomycetes that appear frequently by culturing.

Extraction of fungal DNA directly from plant tissues and identification of the fungi by comparing the amplified DNA with sequences available in the databases is another facet of molecular method to gain a holistic insight into endophyte diversity (Arnold et al. 2007). This method is very useful in documenting unculturable forms and confirms that reliance on culture studies alone can under-represent total diversity (Arnold et al. 2007). In addition, molecular investigations can elucidate the evolutionary history of endophyte groups and the nature of their relationship

with plant hosts. However, certain inherent problems such as contamination by epiphytic fungal DNA while sampling plant tissues (although this problem exists in culture studies also, testing the efficacy of surface sterilization method (Schulz et al. 1998) ensures to a great extent that endophytes alone are isolated), databases that are incomplete or having erroneous sequences and sequences representing misidentified fungi (Sieber 2007; Crouch et al. 2009) have to be considered when attempting molecular species identification. PCR followed by DGGE (Denaturing Gradient Gel Electrophoresis) is a useful tool, but careful choice of primers is essential to avoid overrepresentation of ascomycetes and amplification of a section with insufficient resolving power (Duong et al. 2006). This approach is also very useful for evaluating mixed environmental DNA samples, but limited by the problem of co-migration of sequences (Guerin et al. 2009). A more recent molecular tool that is rapid and efficient for endophyte species diagnosis includes the use of 5'-*tef1* intron sequences (Samuels et al. 2006; Rojas et al. 2010). The CE-SSCP (capillary electrophoresis single–stranded conformation polymorphism) and CE-FLA (capillary electrophoresis fragment length polymorphism), which were found very reliable in the study on soil fungal communities (Zinger et al. 2008) may also be used for environmental sampling of endophytes. Recent methodologies such as pyrosequencing approaches hold much promise for describing fungal communities including endophytes (Jumpponen and Jones 2009). Pyrosequencing techniques are very efficient in identifying many hitherto unreported fungal species and hence are superior to the conventional culture methods for discovery of new taxa (Hibbett et al. 2009). In this context, Hibbett et al. (2009) advocate a major shift in fungal taxonomy by developing a sequence-based species description so that information obtained from molecular investigations can effectively be used for estimating diversity.

5 Endophyte Diversity in the Dry Tropical Forests

Foliar endophyte communities of certain neotropical forests are exceedingly diverse (e.g. Arnold and Lutzoni 2007). The endophyte diversity in different types of tropical forests of southern India is considerably lower when compared to that of the neotropics, perhaps owing to low floristic diversity, presence of relatively open canopies, highly variable annual rainfall and dry-season ground fires (Murali et al. 2007). Species accumulation curves of foliar endophytes for these forest communities show that, while individual tree species have rich endophyte diversity, similar endophyte species are shared by different tree species (Suryanarayanan et al. 2002, 2003, 2011). In these forests, even when conditions favoring endophyte colonization such as precipitation prevails, only the infection frequency and not the diversity of endophytes increases as the trees recruit more endophytes from the same taxa (Murali et al. 2007). The higher isolation frequency of endophytes in the wet season can also be due to the enhanced growth of the fungi within the host tissues. Assuming that the usual practice of sampling portions of broad leaves might

not account for the actual endophyte diversity due to their possible non-uniform distribution in the leaf lamina, Suryanarayanan et al. (2002) sampled whole leaves of tree species such as *Bauhinia racemosa*, *Ixora nigricans*, *Elaeodendron glaucum*, *Anogeissus latifolia*, *Cassia fistula*, *Terminalia tomentosa*, *Grewia tiliaefolia* and *Erythoxylon monogynum* growing in the dry tropical forests of southern India and confirmed that only a few endophyte species colonize the leaves (low diversity) but densely (high colonization frequency). As the presence of endophytes can induce the resistance of plant host against pathogens (Arnold et al. 2003; Ganley et al. 2008; Saravesi et al. 2008), it would be worthwhile to investigate if a suite of polyphagous endophytes in the leaves render them resistant to infection by other endophyte taxa, leading to reduced endophyte species diversity in the leaves. This argument is conceivable as endophyte infection is known to alter the gene expression of plant hosts, also related to enhancing their resistance (Bailey et al. 2006). When colonization of resident endophytes is reduced by systemic fungicide treatment in leaves of *Mangifera indica*, new taxa are recruited as endophytes due to competitive release resulting from the elimination of competitors (Mohandoss and Suryanarayanan 2009). This result suggests that the native endophytes in a leaf can determine the endophyte diversity that the leaf supports. Further studies are essential to know if the multi-host endophytes ward off other potential endophyte species thus maintaining a near-uniform species assemblage in different tree hosts.

5.1 Multi-Host Endophyte Taxa

The genera of multi-host endophytes (e.g. *Colletotrichum*, *Pestalotiopsis*, *Phomopsis*, *Phyllosticta* and *Xylaria*) also include common pathogens or facultative parasites (Photita et al. 2004; Murali et al. 2006) which may be latent until a change in the host or environment status triggers the growth and disease induction (Rogers 2000; Hendry et al. 2002). They may also become active after leaf fall growing as pioneer litter-degrading saprobes (Kumaresan and Suryanarayanan 2002; Osono and Hirose 2009; Promputtha et al. 2010). In our study on microfungi of forests of the Western Ghats, we observed that the dominant and multi-host foliar endophytes belonging to *Colletotrichum* spp. and *Pestalotiopsis* spp. persisted as pioneer leaf litter degraders (Prakash and Suryanarayanan, unpublished). As the leaf litter can function as endophyte inoculum reservoir (Kumaresan and Suryanarayanan 2002; Osono 2002; Promputtha et al. 2010), the presence of a few dominant foliar endophyte genera with an extended host range in a forest community results in a high local inoculum density of these fungi thus reinforcing infection by these taxa and ensuring their persistence as foliar endophytes in the forest. According to the threshold model of Sieber (2007), senescence of conifer needles begins after reaching a threshold in endophyte infection density. In this context, although the multi-host endophytes can produce phytohormones *in vitro* (Suryanarayanan, unpublished), their influence on angiosperm leaf senescence and abscission are not known. Since the multi-host endophytes are common pioneer litter fungi, I propose

that these fungi have life cycles that consist of (i) a latent endophyte stage which orchestrates leaf senescence and abscission followed by (ii) an active saprobic stage as pioneer degraders of the litter, leading to sporulation and inoculum build-up for subsequent infection of the host, to initiate the endophyte stage again. Exception to this general scheme occurs when the benign endophyte turns pathogenic, perhaps due to alterations in the environment or host status.

In conclusion, as some tropical endophytes have loose host taxonomic affiliation and wide geographic distribution, the ratio of generalists and specialists among endophyte genera in different types of tropical forests has to be evaluated before using endophytes as an indicator group of global fungal diversity. These cosmopolitan taxa should be analyzed from phylogenetic angles to understand their evolution and spread. The higher diversity of some herbivorous insects in the tropics compared to temperate regions is explained by increased ecological specialization. In tropical forests, the secondary metabolite composition of trees differs more widely among species than in temperate forests resulting in many restricted niches that can be exploited by insects; consequently the diversity of plant-associated insects such as Lepidoptera is greater in the tropics (Dyer et al. 2007). Taking a cue from this, it would be worthwhile to determine how multi-host endophytes have evolved to occupy the myriad narrow niches offered by leaves of various tree species that differ in their defense metabolite profiles. The question of whether endophytes are attenuated pathogens or evolving pathogens is extremely relevant to such studies (Rojas et al. 2010). More focused studies including improved culture conditions and molecular methods for identification are essential to understand how corrections can be made to use endophytes of tropical trees as a benchmark for biodiversity estimation and whether ‘endophytism’ is a mode-of-life strategy among certain litter-degrading fungi.

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Tree Endophytes and Wood Biodegradation

Jaime Rodríguez, Juan Pedro Elissetche, and Sofía Valenzuela

Abstract In nature, wood-decaying fungi play an important role in global carbon and nitrogen cycling by promoting the bioconversion of organic matter. Wood-decaying fungi colonize and degrade wood by attacking cell components with enzymatic and non-enzymatic systems. Some fungal endophytes can become activate and express the wood-decay machinery under suitable conditions. It has been proposed that endophytic fungi are involved in triggering the development of early stages of wood decay. The understanding of fungal endophytes in wood biodegradation, including invasiveness, colonization strategies and degrading mechanisms, could help to understand forest carbon cycling. The potential uses of fungal endophytes could involve biopulping and wood pretreatment for bioethanol production, among others.

Abbreviations

BRF	Brown-rot fungi
DHBs	Dihydroxybenzenes
LP	Lignin peroxidase
MnP	Manganese peroxidase
PCR	Polymerase chain reaction
PO	Phenoloxidase
SRF	Soft-rot fungi

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SEM Scanning electron microscopy
TEM Transmission electron microscopy
WRF White-rot fungi

1 Wood Morphology and Chemical Composition

In higher plants, one of the most important morphological structures is the vascular system, composed of xylem and phloem. The vascular system is commonly known as wood (i.e. secondary xylem), which is a complex biological material composed of various cell type that, unlike animal cells, are surrounded by a strong and rigid cell wall. Depending on the point of view, trees are classified in two main groups: botanically in gymnosperms and angiosperms, or industrially in softwood and hardwood. Most of the gymnosperms are tall evergreen trees that retain their foliage for most of the year (i.e. conifers such as pine, fir, spruce and hemlock). Angiosperm trees (i.e. deciduous or broad-leaf trees such as birch, aspen, eucalypts, acacia and oak) of the temperate regions are mainly deciduous, but some of these species, such as some of the southern beeches (*Nothofagus* sp.) and the majority of tropical trees retain their green foliage for most of the year and are regarded as evergreen (Fengel and Wegener 1989; Butterfield 2007; Karlsson 2006).

The cell structures of softwood and hardwood differ in their anatomy and morphology. Softwood is comprised of a limited number of uniform cell types, in contrast to hardwood, which is typically comprised of a greater variability of cell types with different and heterogeneous cell morphologies. However all are derived from two meristematic cell types: vascular cambium and phellogen.

Wood is produced by a thin zone of dividing cells near the outside of the trunk or branch, just beneath the bark, known as vascular cambium. The vascular cambium is a layer of thin-walled cells that undergo constant division (meristematic tissue) and are essential for the continued growth of the tree, especially in diameter. As the crown of the tree gets larger with more leaves and branches, the stem increases in diameter to support this extra load. More wood is added by the cambium to the trunk and the stem thickens. In temperate climates, the cyclic production of new cells each spring and summer and the subsequent cessation of cambial divisions during autumn and winter leave the familiar pattern in the wood known as annual growth rings. Trees growing in milder climates with warm winters do not produce growth rings that are as sharp as and distinct as trees growing in climates where the winters are more severe and extreme.

Gymnosperms (softwood) are composed by up to 90% of long, tapering cells called tracheids, which are responsible for structural support and water transport. The hole in the middle of the tracheid is called lumen. The tracheids are connected to each other through pores. There are other cell types present, for example resin channels that hold resin, and ray cells. These cells are principally binded by lignin, forming a stable connection. Ray cells are oriented in a radial direction from the outside of the tree trunk towards the center. They transport waste material (extractives)

toward the heartwood and may be used for storage of various nutritional compounds. The ray cells accomplish horizontal transport of liquids across the annual rings. Tracheids of softwood are long and strong. The strongest papers are manufactured from chemical pulp made of softwood. In the mature wood, tracheids range between 2 and 5 mm in length but longer tracheids can be found in some tree species. With diameters of about 15–60 μm, tracheids are about 100 times longer than their width. Because they originate from the dividing cambial cells, they tend to remain in radial files in the wood. As a result of their tangential dimensions, they remain fairly uniform. The radial width of the tracheids is larger in earlywood than in latewood, where the cells appear radially flattened. The tracheids overlap one another along their thinner, wedge-shaped ends, which appear sharply tipped in tangential view but are more rounded in outline radial view. This cellular arrangement, which is a direct result of the pattern of the fusiform cambial cells in coniferous species, provides softwoods with a high 'along the grain' strength as well as with the maximum sidewall cell contact for the movement of water up the stem or the branch. Softwood of some species has axially-elongated cells termed axial parenchyma cells that sometimes are referred to as longitudinal or wood parenchyma. These cells differ from tracheids by having thinner walls and a protoplast that may live for several years (Walker 2006; Fengel and Wegener 1989).

Hardwood of angiosperm species has a more complex structure than softwood with different cells for water transport and support. Elongated libriform fibers have supportive function and are thick-walled in proportion to the diameter. These cells differ from softwood tracheids, as they are comparatively short (0.25–1.5 mm long, generally less than 1.0 mm), more rounded in transverse outline and play virtually no role in the ascent of sap. Shorter and wider cells called vessels are responsible for water transportation. Hardwood also contains a vertical parenchyma system and a horizontal ray parenchyma system. Hardwood fibers are shorter and thinner, producing paper with a smooth printing surface and higher opacity than softwood fibers. Because there is less lignin in hardwood than in softwood, it is also easier to bleach the pulp to high brightness. These qualities make the hardwood fibers appropriate for use as the raw material for printing paper. The density of hardwood is largely determined by the proportion of fibers compared to other cell types present in the wood. In low-density wood, the vessels occupy the major proportion of the wood volume, whereas denser woods have a larger proportion of thick-walled fibers (Walker 2006). Generally, hardwood contains more cellulose and hemicellulose and less lignin than softwood, whereas the proportion of extractives is higher. Fibers are usually classified into fiber tracheids, libriform fibers, and septate fibers. Libriform fibers are longer than fibers tracheids and have moderate to very thick walls and simple pits, having a supportive role. The shorter fiber tracheids have moderately thick walls and bordered pits. They function in both conduction and support although their occurrence in vesseled wood suggests that their function is primarily in support. It is likely that they represent an intermediate evolutionary form between the softwood tracheid and the true libriform fiber. The fibers in some woods have their fiber lumens divided into chambers by septa. Such fibers are known as septate fibers. The septa only cross the fiber lumen and do not connect to the

primary wall. They are produced by a late sequence of division in the fiber prior to death of the cytoplasm. Septate fibers resemble axial parenchyma in the wood of some species and are most abundant in such wood tissues where the latter is poorly represented. This has led to the general belief that septate fibers have evolved as an alternative site for the storage of starches, oils and resins (Walker 2006). Vasicentric tracheids are found close to the vessels in some hardwood species, particularly in the earlywood of ring porous species. They are short tracheid-like cells with profuse sidewall pitting. They are often longitudinally bent and flattened transversely on account of the lateral expansion of the adjacent vessels. Axial parenchyma cells (also called longitudinal parenchyma) are generally very abundant in hardwood. Similar to vessel elements and fibers, axial parenchyma cells derive from the axially elongated fusiform initials of the vascular cambium with the exception that vessel elements and fibers (except septate fibers) remain unsegmented. Axial parenchyma cells have relatively thin walls interrupted by small circular simple pits that are arranged irregularly. Helical thickening has been recorded in wood tissues of a few species. The rays in hardwood are generally larger and more variable than those in softwood. Rays are classified as uniseriate or multi-seriate, depending on whether they are one or several cells wide. Whereas the rays in the softwood are predominantly uniseriate, partially biseriate and rarely fully biseriate, the hardwood commonly has broad multi-seriate rays (Walker 2006).

The modular structure and wood properties enable a tree to grow from a structurally simple seedling to a complex, large, heavy structure with a single stem. Cell walls are a complex and dynamic mixture of components that form a nanomaterial performing many functions. While the phloem mainly allocates photoassimilates, the role of the xylem is the transport of water and inorganic nutrients from roots to all the aerial parts of the plant (Kehr et al. 2005). Wood cells of xylem are composed of a number of cell wall layers that form the primary (one layer) and secondary cell walls (two to three layers) (Daniel 2009). Wood cell walls are comprised of three major chemical components, namely cellulose, lignin and hemicelluloses. Cellulose forms a skeletal matrix surrounded and encrusted by the hemicelluloses and lignin. The polysaccharides, cellulose and hemicelluloses together with the aromatic polymer lignin and the extractives form the cell wall at different proportions and structures.

Cellulose is the main constituent of wood. Approximately 40–45% of the dry substance in most wood species is cellulose, located predominantly in the secondary cell wall. It is a linear homopolysaccharide of up to 15,000 D-glucose units linked by β-(1 → 4) glycosidic bonds and has a strong tendency to form intra and intermolecular hydrogen bonds. Bundles of cellulose molecules are thus aggregated together in the form of microfibrils in which highly ordered (crystalline) regions alternate with less ordered (amorphous) regions (Sjöström 1993).

Hemicelluloses are heteropolysaccharides, most of them have a degree of polymerization of only 200. Similar to cellulose, these compounds function as supporting material in the cell walls. Hemicelluloses are relatively easily hydrolyzed by acid to their monomeric components D-glucose, D-mannose, D-xylose, L-arabinose, and small amounts of L-rhamnose, D-glucoronic acid, 4-O-methyl-D-glucoronic acid,

and D-galacturonic acid. The content of hemicelluloses in wood is usually between 20% and 30% (dry weight based). Hemicelluloses of softwood are different than those of hardwood, as they contain mainly galactoglucomannans and hardwood hemicelluloses have glucuronoxylan as the main constituent (Sjöström 1993).

Lignin is a hydrophobic polymer that fills up the space between the cellulose microfibrils and hemicelluloses, fixating them towards each other and thus giving the cell wall its "woody" properties. The lignin content in softwood is 15–35% and around 20% in hardwood. Lignin is a complex tridimensional aromatic heteropolymer derived mainly from three hydroxycinnamyl alcohol monomers, called monolignols that differ in their degree of methoxylation, p-coumaryl, coniferyl and sinapyl alcohols.

Wood extractives are non-structural compounds of low molecular mass that are extractable from wood using solvents. Their composition varies considerably between tree families and genera. Some extractives play a role in the metabolism of the living cells (the parenchyma cells) in the tree. Other extractives are produced to protect the tree against fungi and insects. The total amount of extractives is normally only a few percent of the wood, but it can be considerably higher in tissues such as bark and branches, and is normally also higher in wounded wood (Jansson and Nilvebrant 2009).

2 Wood Biodegradation

In nature, wood-decaying fungi play an important role in global carbon and nitrogen cycling by promoting the bioconversion of organic matter. Wood-decaying fungi are the most important microorganisms that can colonize and degrade wood by attacking cell components with enzymatic and non-enzymatic systems.

The breakdown of wood polysaccharides requires a combination of enzymes, which break glycosidic linkages between β-D-xylopyranosyl and glucopyranosyl residues. A complete cellulase system consists of three classes of enzymes; 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91), which cleave cellobiosyl units from the ends of cellulose chains; endo-1,4-β-D-glucanases (EC 3.2.1.4), which cleave internal glycosidic bonds; 1,4-β-D-glucosidase (EC 3.2.1.21), which releases glucose units from cellooligosaccharides. The hemicellulolytic system is more complex because hemicellulose varies from plant to plant, being composed of various sugar units and having a substituted backbone. The enzymatic degradation system involves among others endo-1,4-β-D-xylanase (EC 3.2.1.8), which cleaves internal bonds in the xylan chain; β-xylosidases (EC 3.2.1.37), which cleave xylooligosaccharides to produce xylose; endo-1,4-β-D-mannanase (EC 3.2.1.78), which cleaves internal bonds and β-mannosidases (EC 3.2.1.25), which cleave mannooligosaccharides to mannose (Heidorne et al. 2006).

Lignin is degraded by phenol-oxidase enzymes, which include peroxidases, laccases and tyrosinases, but being the latter only produced intracellularly, it does not have a role in the polymer degradation.

Laccases (EC 1.11.3.2) are copper-containing oxidases that have a low redox potential that allows direct oxidation of only phenolic lignin units, which often comprise less than 10% of the total polymer. These enzymes have the ability to oxidize substrates with high redox potential in the presence of synthetic mediators, which allows the degradation of non-phenolic lignin. Natural mediators involved in lignin biodegradation remain to be identified, although some lignin-derived phenols could act as efficient laccase mediators.

Lignin peroxidase (EC 1.11.1.71, LP), and manganese peroxidase (EC 1.11.1.13, MnP), were discovered in the mid-1980s in *Phanerochaete chrysosporium* and described as true ligninases because of their high redox potential. LP is a hemoprotein that catalyzes the oxidative cleavage of C-C bonds in lignin model compounds. It is involved in the oxidative breakdown of lignin in white-rot basidiomycetes. LP catalyzes the oxidation of non-phenolic aromatic rings into aryl cation radicals by H_2O_2. MnP generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units via lipid peroxidation reactions. More recently, versatile peroxidase (VP) has been described in *Pleurotus* and other fungi as a third type of ligninolytic peroxidase that combines the catalytic properties of LP, MnP, and plant/microbial peroxidases that oxidize phenolic compounds (Martínez et al. 2005).

Based on their decay patterns, it is possible to establish three main types of wood-decaying fungi: white-rot fungi (WRF), soft-rot fungi (SRF) and brown-rot fungi (BRF).

White-rot fungi can be divided into two additional sub-types. For one subtype the decay of all wood components simultaneously is typical. In this case, the wood decay occurs by formation of erosion troughs and by progressive thinning of wood cell walls. This type of decay is consistent with a model in which several polymer-degrading enzymes act on the exposed surfaces of the wood cell walls and produce a progressive erosion of the wood components from the lumen to the middle lamella. The second sub-type of white-rot account for a relatively small number of fungi, which are selective for lignin degradation. In this case, removal of lignin and polyoses from the wood cell walls occurs without progressive thinning of the wall. For this type of white-rot, it has been demonstrated that even after long biodegradation periods when significant amounts of lignin and polyoses have been removed, the wood cell walls remain inaccessible to enzymes of molecular masses around 40 kDa. This indicates that the selective white-rot processes do not result from the direct action of the enzymes on wood cell walls (Blanchette et al. 1997).

Although only white-rot and brown-rot basidiomycetes can degrade wood extensively, some ascomycetes and their asexual states, the so-called deuteromycetes, can colonize wood in contact with soil. This results in a decrease in the mechanical properties of wood, giving rise to the so-called soft-rot, which involves a slow rate of polysaccharide degradation and a limited degradation of lignin. SRF can degrade wood under extreme environmental conditions (high or low water potential) that inhibit the activity of other fungi. Moreover, some basidiomycetes also cause a soft-rot-type decay pattern (Martínez et al. 2005).

In BRF the polysaccharides are primarily removed and lignin is degraded (transformed) only to a limited extent. To initiate the wood degradation process, BRF have developed a mechanism that is based on the reduction of Fe (III) to Fe (II) and production of hydrogen peroxide which are the Fenton reaction reagents. Fungal low-molecular mass iron reducing compounds are able to diffuse through the cell walls and initiate the degradation process by promoting a Fenton reaction. In the Fenton reaction, •OH radical is produced by the reduction of H_2O_2 and oxidation of Fe(II) in an acidic medium. BRF have developed two mechanisms to promote the Fenton reaction; the first and the most studied one is based on the involvement of dihydroxybenzenes (DHBs), and the second one is based on glycopeptides (Goodell 2003; Rodriguez et al. 2003). Besides these mechanisms, the participation of cellobiose dehydrogenase, which also reduces Fe(III) to Fe(II), has been proposed for *Coniophera puteana* but, as this is not a typical brown rot fungus, it can be considered a specific mechanism (Baldrian and Valaskova 2008). BRF produce endoglucanases but not exoglucanases, and they do not produce lignin-degrading enzymes.

Some ascomycetous fungi, called stain fungi, can colonize wood through parenchymatic rays and resin channels, which causes discoloration of softwood tissues but a very limited degradation. This type of degradation mainly affects extractives and water-soluble materials and does not affect the wood polymeric matrix structure.

Even though wood is designed by nature to resist biotic and abiotic stresses and presents many characteristics to avoid fungal attack, wood decay in industry results in huge financial losses.

3 Endophytes and Wood Biodegradation

Contrary to previous assumptions on the aseptic conditions of the plant interior, it is now widely accepted that plant tissues commonly contain cryptic infections without apparent impairment of the plant functions. These microbes can produce effects ranging from beneficial to pathogenic (Strobel and Long 1998; Neubert et al. 2006). These infections can be detected in diverse plant tissues including leaves, petioles, fruits, spines, seeds, bark and wood (Petrini 1991). Endophytic fungi have been found in almost all vascular plant species examined to date (Arnold et al. 2000) and they are considered important components of fungal biodiversity that until now has been underestimated (Hawksworth and Rossman 1997; Hawksworth 2000; Arnold et al. 2001; Mueller and Schmit 2007).

The niches occupied by fungal endophytes in plant tissues such as sapwood, deserve more attention (Hutchinson 1999; Hoff et al. 2004). Despite the efforts, the full range of ecological functions of endophytic fungi of woody plants is poorly understood, but it likely correlates with their species diversity (Purvis and Hector 2000; Hendry et al. 2002). A hypothesis on the role of endophytic *Xylaria*, which are among the most studied endophytic fungi, proposes that these fungi are simply

waiting for their host to senesce, to begin the decomposition of the host cell wall material (Petrini and Petrini 1985; Whalley 1996). Endophytic fungi employing this strategy would have an advantage over competing saprophytes, having "claimed" the place before decomposition begins (Carroll 1995; Davis et al. 2003). The overall explanation for the mechanisms by which decay fungi invade and degrade wood in standing and dead trees is still incomplete (Eaton 2000). It has been proposed that endophytic fungi could be involved in triggering the development of early stages of wood decay (Schwarze et al. 2000; Baum et al. 2003). Therefore, there is a need to further explore the role of wood-inhabiting endophytes especially in natural process such as wood biodegradation (Hoff et al. 2004).

The Compartmentalization of Decay in Trees (CODIT) theory suggests creation of barriers that resist invasion by microorganisms (Shigo and Marx 1977; Shigo 1984). However, these physical and morphological barriers do not make the tree immune to microbial colonization. As long as a wound remains open, microorganisms can invade through its surface. Compartments can fail when microorganisms become established in the wounded tissue, resulting in generation of new barriers at greater depths and distances from the wound (Shigo and Marx 1977; Shigo 1984). The anatomical structure of wood together with physical phenomena occurring at the wood surface (capillary action, changes in temperature and air pressure, rain splash) may be responsible for differences in the infection and colonization processes of wood of different tree species (Hintikka 1987). A single fungal infection could spread through the tree via xylem sap where cell vessels could be used as "highways" by endophytic fungi for long-distance dispersal within the xylem (Boddy 1994).

It has been demonstrated that the incubation of freshly cut branches under various drying regimes allows the growth of active mycelia. Emergence of active mycelia is assumed to originate from latent infections, which were naturally present in healthy tissues in the field (Chapela and Boddy 1988a,b). The water loss and the increasing oxygen concentration are proposed to trigger the switch from the latent to active phase of the mycelia (Rayner 1986). The presence of inactive endophytic propagules such as dormant chlamydospores inside of living standing trees has been associated to wood decay (Schwarze et al. 2000). Baum and collaborators (2003) isolated fungi from xylem of European beech (*Fagus sylvatica*) immediately after cutting, as well as after incubation of the wood tissues for different periods of time. Only a few isolates were obtained from freshly cut wood, but after 8 weeks of incubation under sterile conditions, a large number of isolates were recovered. Basidiomycetes required a longer incubation period than Ascomycetes for isolation from the tissue samples (Oses et al. 2008).

Fungal endophytes isolated from the Chilean native trees *Prumnopitys andina* and *Drimys winteri* had lignocellulolytic activity and also promoted wood biodegradation. Endophytic fungi were isolated from core samples obtained using an increment borer, from *D. winteri* a Basidiomycete identified as *Bjerkandera sp*., and a Deuteromycete corresponding to *Mycelia sterilia*, and from *P. andina* an unidentified Basidiomycete and *M. sterilia.* On agar solid media, the Basidiomycetes displayed positive reaction to phenoloxidase (PO) and cellulase tests and showed

no iron-reducing activity (BRF characteristics). A weak reaction to the cellulase and iron-reducing activities but none for PO activity was detected for both *M. sterilia* isolates. PO activity was detected for liquid cultures of both basidiomycetes. As an example it can be mentioned that after treating *D. winteri* wood chips with *Bjerkandera sp*. for 45 days, it presented the following weight and component losses: weight of 13.3% +/− 1.5%, total lignin of 13.2% +/− 1.2%, glucan 16.9% +/− 4.4%, polyoses 22.6% +/−3.8% and extractives 16.0% +/− 1.7%. These results indicate that the isolated fungal basidiomyceteous endophytes are able to develop a non-selective white-rot wood decay pattern (Oses et al. 2006).

Xylem colonization by fungal endophytes was observed to occur as clamydospores in the lumen of all four tree species studied *P. andina*, *Podocarpus saligna*, *D. winteri* and *Nothofagus obliqua* (Oses et al. 2008). The passive entry and distribution of fungal spores into the wood may be determined by analysis of anatomical structure of the host and the interactions with physical phenomena such as changes in temperature and air pressure, capillary action and rain splash (Hintikka 1987; Baum et al. 2003). On the basis of such analyses, it is possible to predict that when a freshly cut surface is exposed to spores or other types of fungal propagules, the infection processes vary in different tree species. In the case of gymnosperm wood, the spores remain at the outer surface, but in angiosperm the wood may become infected throughout its length at several centimeters of depth (Hintikka 1987).

Fungal adaptations such as a gelatinous layer on spores or propagules may be advantageous for penetrating the wood under suitable temperature and air pressure conditions. It would be interesting to investigate whether the spores and propagules of certain wood-decomposing species are adapted to invasion through specific anatomical features, such as perforation plates in different trees, and if such adaptation is related with the extensivity and selectivity of the wood decay patterns performed by white rot and brown rot fungi (Hintikka 1987; Baum et al. 2003).

Baum et al. (2003) have pointed out that xylem infections could occur through the thin periderm, lenticels, leaf scars, or scars of bud scales, followed by a subsequent dormant phase. Early reports have shown that once inside the xylem, the mycelium either infects single cells or establishes small propagules in a similar way to those formed by *Rhabdocline parkeri* Shrew in Douglas fir needles (Stone 1987) or *Ophiostoma ulmi* (Buisman) Nannf (Oulette et al. 1995).

Endophytic fungi might be involved in triggering of the development of early stages of wood decay (Schwarze et al. 2000; Baum et al. 2003). Limited oxygen ratios and/or nutrient availability are suspected to control pathogenic behavior of xylem endophytes such as *Fomes fomentarius* and *Nectria coccinea,* both considered forest pathogens (Sieber 2007).

A survey of fungal endophytes associated with xylem of presumably healthy trees was conducted. Wood-inhabiting fungal endophytes of *P. andina*, *P. saligna*, *D. winteri* and *N. obliqua* were isolated from surface sterilized xylem core samples. Five basidiomycetes (*Inonotus* sp., *Bjerkandera adusta* and three unknown strains), two ascomycetes (*Xylaria* sp., *Bipolaris* sp.) and one anamorphic strain were detected. *Xylaria* sp and *B. adusta* were the most frequent fungal isolates.

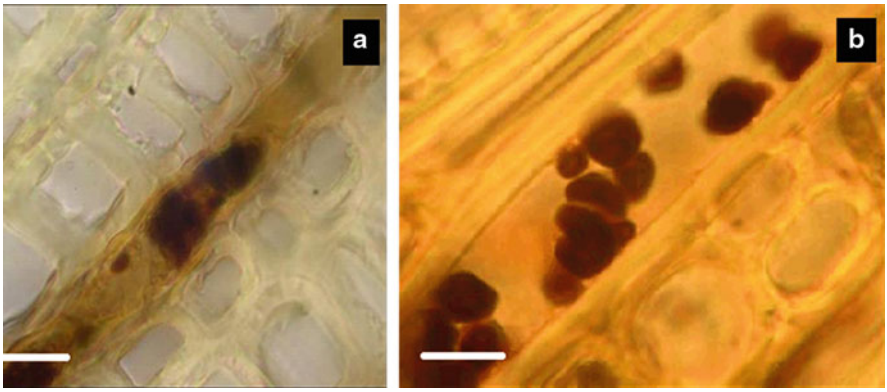

Fig. 1 Fungal propagules observed in wood core fragments of *Drimys winteri* (**a**) and *Nothofagus obliqua* (**b**) in both cases after 4 weeks of incubation avoiding external contamination (Scale bar = 5 μm, Oses et al. 2008, with permission from the editor)

Ultrastructural observations of wood cores samples by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy showed the presence of fungal hyphae attached to the inner cell surface, inhabiting xylem elements even before the induction of wood degradation. Evidence of latent infection was found mainly along the parenchyma rays, indicating fungal colonization and distribution. Results showed simultaneous decay of all wood components, characterized by a thinning cell-wall from the cell lumen to the middle lamella and erosive wood degradation, typical of non-selective white rot. By using SEM, TEM and light microscopy, it was possible to detect natural incidence of latent infections, to visualize spreading of colonization and the ability to promote wood degradation under suitable conditions.

Tangential and transverse sections of xylem samples of healthy *D.winteri* and *N. obliqua* cores were stained directly with Melzer's reagent and then analysed by light microscopy. The wood fragments had clear signs of fungal colonization and propagation after 4 weeks of incubation, present mainly along the xylem parenchyma (Fig. 1). Examination of slides from freshly cut wood did not show such signs of decay (Oses et al. 2008).

Using specific primers and a nested-Polymerase Chain Reaction (PCR) approach, Parfitt et al. (2010) successfully surveyed for the presence of 11 fungal species in the sapwood of 11 tree species. This method was superior to the previously used approaches of direct isolation from freshly felled wood, or slow drying of wood followed by isolation onto agar media. These methods are limited in the detection of species diversity under any particular set of abiotic conditions, and do not allow small-scale resolution of the location of propagules from which decay columns develop. Furthermore the PCR approach does not generate cultures for further study. By this technique it was possible to detect (1) wood decaying fungal species present in the functional xylem of branches and trunks of a wide range of tree taxa, including species in which they are less frequently detected; (2) fungal species that

had not hitherto been suspected as being latent, namely the rare (based on fruit body occurrence) *Creolophus cirrhatus* and *Hericium spp.*; (3) fungal species hitherto only suspected as being latently present (*E. spinosa* in beech); (4) fungal species known as leaf endophytes latently present in sapwood (e.g. *Epicoccum nigrum*). Also, since fungal DNA can be detected in small ($2.0 \times 0.4 \times 0.4$ cm) samples, this is a promising tool for analysis of small-scale distribution of fungi latently present within different locations of standing trees (Parfitt et al. 2010).

Some fungal endophytes might become activated and display wood decay machinery under suitable conditions. Studies have shown that endophytes are capable of promoting wood decay and producing wood degrading enzymes (Oses et al. 2006 and references therein). New approaches could help to determine the time and the mode of entry of fungal propagules, their survival in the sapwood and the cues that trigger their development. Furthermore, by these methods it can be determined when, where, and how these fungi enter their host (Parfitt et al. 2010). A deeper understanding of the processes by which fungal endophytes colonize and degrade wood, could yield important insights into carbon cycling in forests, and lead to new applications in biopulping and wood pretreatment for bioethanol production, among others.

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Diversity of Endophytic Bacteria in Forest Trees

Hironari Izumi

Abstract A group of studies on the diversity of endophytic bacteria in forest trees is presented in terms of host plant species variety and the number of reports. Many host tree species are underrepresented in these studies: Trees in the tropics as well as some important temperate tree species, such as those belonging to the genera of *Alnus* and *Fagus*, have not yet been investigated.

Endophytic bacterial diversity, on the other hand, covers a wide range of bacterial phyla including Proteobacteria, Actinobacteria and Firmicutes. The endophytic bacteria related to the genus *Acinetobacter* occur more frequently in forest trees than in agricultural crops.

Population densities of endophytic bacteria in trees vary from 10^1 to 10^6 per gram of sample. The genera *Pseudomonas* and *Bacillus* comprise the major groups of the endophytic bacterial community, and the genera *Actinobacteria, Acinetobacter* and *Sphingomonas* make up a significant proportion of the community in many trees.

More studies are required, particularly through cultivation-independent approaches, to obtain a better picture of the diversity of endophytic bacteria in forest trees.

1 Introduction

Today it is a well-known fact that plants harbour bacteria inside their tissues without evident disease symptoms, ever since the first discovery of bacteria in potato plants (Hollis 1951). Since then, studies on the diversity and isolation of endophytic bacteria have been conducted mainly on agricultural and horticultural plants due

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to their applied purposes (Hallmann et al. 1997; Sturz et al. 2000) whereas studies on woody plants are rather limited. Forest trees are, in general, the least investigated host plants even among the woody plants, as the most studied woody species are related to horticultural crop production, such as grapevine (Bell et al. 1995; Bulgari et al. 2009; West et al. 2010) citrus (Gardner et al. 1982; Araujo et al. 2002) and coffee (Vega et al. 2005).

Nevertheless, the significance of studies on bacterial endophytes in forest trees should not be underestimated. Because forest trees have different biological traits and distributions compared to most agricultural herbaceous plants, i.e., representing large biomass and long-term existence in terrestrial ecosystems, they can provide unique ecological circumstances for endophytic bacteria, which may not be seen in crop plants that are mostly annual and grown in rather restricted areas. Additionally, endophytic bacteria have positive effects on the host trees (Anand et al. 2006 and references therein), which opens up the possibility of finding useful strains for forest practices to increase timber yields or to improve environmental quality through enhancement of tree health.

In the following sections, I first address points such as how many of the forest tree hosts have been studied with respect to diversity of endophytic bacteria, followed by a section describing a couple of issues on methodology. Then I move on to describing diversity of endophytic bacteria associated with forest trees in terms of phylogenetic variety and relative abundance. Finally, I discuss the future prospects of the research on endophytic bacterial diversity in forest trees.

2 Host Tree Diversity

The reported host tree species include three angiosperms and nine gymnosperms (Table 1), including important taxonomic groups for forestry such as *Pinus, Picea, Betula* and *Quercus*. The number of published research articles varies with respect to the tree species. For example, in terms of endophyte diversity and occurrence, *Populus* spp., and *Eucalyptus* spp. are the most extensively studied tree species, reflecting the fact that these trees are important species for e.g. bioenergy and bioremediation. On the other hand, it is surprising that the major forest tree species of temperate and boreal forests such as *Pinus, Picea* and *Quercus* are not well-represented despite their ecological and economical importance for the terrestrial ecosystems. Additionally, only one study is found on endophytic bacteria in tropical trees (Wang et al. 2006). Considering that the number of world native tree species has been estimated to 7,880 (FAO 2006), we only see the endophytic bacterial diversity through a very narrow window of host tree species. Particularly, some species of the most common forest tree genera in the world such as *Abies, Fagus, Shorea, Fraxinus, Carpinus, Alnus* and *Acacia* (FAO 2006), are not represented at all. It is known from the experience gained with agronomic hosts that different host plant species are colonized by a variety of endophytic bacterial species (Rosenblueth and Martinez-Romero 2006). Thus there is a vast unknown field for

Table 1 Main genera of endophytic bacteria and their host tree

Host plant	Isolated tissue	Main bacterial genera	Reference	Note
Acer	Sap	*Pseudomonas* *Rahnella* *Janthiinobacterium* *Chryseobacterium* *Epilithonimonas* *Shingomonas* *Leuconostoc* *Lactococcus* *Weissella*	Filteau et al. (2010)	Cultivation-independent analysis
Betula	Leaf	*Bacillus*	Izumi et al. (2008)	Predominant bacteria
	Stem	*Acinetobacter* *Bacillus*		
	Root	*Renibacterium*		
	Root	*Streptomyces*	Sardi et al. (1992)	
Conzattia	Nodule	*Salmonella* *Enterobacter* *Pantoea* *Erwinia* *Klebsiella* *Citrobacter*	Wang et al. (2006)	Leguminous host
Eucalyptus	Seeds and seedling	*Bacillus* *Paenibacillus* *Enterococcus* *Methylobacterium* *Sphingomonas* *Paracoccus* *Frankiaceae*	Ferreira et al. (2008)	
	Not specified	*Enterobacter* *Pantoea* *Hafnia*	Torres et al. (2008)	
	Stem	*Erwinia/Pantoea* *Agrobacterium* *Curtobacterium* *Bacillus* *Pseudomonas* *Acinetobacter* *Burkholderia* *Lactococcus*	Procopio et al. (2009)	
Juglans	Leaf, fruit, wooded twig	*Xanthmonas* *Bacillus*	Pardatscher and Schweigkofler (2009)	

(continued)

Table 1 (continued)

Host plant	Isolated tissue	Main bacterial genera	Reference	Note
Larix	Tissue culture	*Paenibacillus*	Ulrich et al. (2008b)	
Picea	Root	*Pseudomonas* *Staphylococcus*	O'Neill et al. (1992)	
	Seed	*Pseudomonas* *Rahnella*	Cankar et al. (2005)	Both cultivation-independent and cultivation analysis
	Tissue culture	*Paenibacillus*	Ulrich et al. (2008b)	
Pinus	Root	*Paenibacillus*	Shishido et al. (1995)	
	Bud	*Methylobacterium*	Pirttilä et al. (2002)	*P. sylvestris*
	Root	*Pseudomonas*	Strzelczyk and Li (2000)	*P. sylvestris*
	Stem	*Bacillus* *Paenibacillus*	Bal (2003)	*P. contrata* ver. *latifolia*
	Root	*Bacillus* *Paenibacillus*	Izumi et al. (2008)	*P.sylvestris* Predominant bacteria
Populus	Shoot	*Arthrobacter* *Bacillus* *Paenibacillus* *Pseudomonas*	Moore et al. (2006)	
	Leaf	*Duganella* *Pseudomonas* *Sphingomonas* *Xanthomonas* *Xylophilus*	Moore et al. (2006)	
	Root	*Bacillus* *Burkholderia* *Acinetobacter* *Arthrobacter* *Enterobacter* *Herbaspirillum* *Klebsiella* *Pseudomonas*	Moore et al. (2006)	
	Leaf and branch	*Acinetobacteria* *Curtobacterium* *Methylobacterium* *Paenibacillus* *Pedobacter* *Plantibacter*	Ulrich et al. (2008a)	

(continued)

Host plant	Isolated tissue	Main bacterial genera	Reference	Note
		Pseudomonas		
		Sphingomonas		
		Xanthmonas		
		Sphingomonas	Ulrich et al. (2008a)	Cultivation-independent analysis
		Oxalobacter		
	Tissue culture	*Bacillus*	Ulrich et al. (2008b)	
		Paenibacillus		
		Methylobacterium		
	Cutting	*Acinetobacter*	Doty et al. (2009)	Possible nitrogen fixing activity
		Burkholderia		
		Enterobacter		
		Pantoea		
		Pseudomonas		
		Rahnella		
	Root and shoot	*Acinetobacteria*	Taghavi et al. (2009)	
		Enterobacter		
		Serratia		
		Stenotrophomonas		
		Rahnella		
		Rhodococcus		
		Pseudomonas		
	Stem and root	*Methylobacterium*	Yrjälä et al. (2010)	
		Burkholderia		
Quercus	Root	*Streptomyces*	Sardi et al. (1992)	
	Stem	*Bacillus*	Brooks et al. (1994)	
		Erwinia		
		Pseudomonas		
		Xanthomonas		
Robinia	Tissue culture	*Paenibacillus*	Ulrich et al. (2008b)	
Salix	Stem	*Bacillus*	Cambours et al. (2005)	Host tree was affected by frost-related dieback.
		Erwinia		
		Pseudomonas		
		Sphingomonas		
		Xanthmonas		
	Cutting	*Acinetobacter*	Doty et al. (2009)	Possible nitrogen fixing activity
		Herbaspirillum		
		Stenotrophomonas		
		Sphingomonas		
		Pseudomonas		
	Leaf and branch	*Acinetobacteria*	Kuffner et al. (2010)	Host tree was affected by heavy metals
		Methylobacterium		
		sphingomonas		

(continued)

Table 1 (continued)

Host plant	Isolated tissue	Main bacterial genera	Reference	Note
Sorbus	Root	*Bacillus* *Paenibacillus*	Izumi et al. (2008)	Predominant bacteria
	Stem	*Acinetobacter*	Izumi et al. (2008)	
	Leaf	*Bacillus*	Izumi et al. (2008)	
Ulms	Stem and root	*Bacillus* *Curtobacterium* *Enterobacter* *Pseudomonas* *Sphingomonas* *Staphylococcus* *Stenothrophomonas*	Mocali et al. (2003)	
Thuja	Stem	*Bacillus* *Paenibacillus*	Bal (2003)	

bacterial ecologists to study species richness of endophytic bacteria in different forest tree species and address interesting ecological questions such as the nature of bacteria-tree interactions and adaptation to the host environment by the endophytic bacteria.

3 Methodology for Studying Diversity of Endophytic Bacteria in Forest Trees

Isolation and identification of endophytic bacteria usually involve two main steps; surface-sterilisation of plant host tissue and cultivation of bacteria and/or obtaining clones or PCR amplified fragments (commonly 16 S rRNA gene) for sequencing. Surface-sterilisation methods to remove the bacteria residing on the surface of the plant materials can consist of chemical disinfestations and/or physical removal of outer tissues under aseptic conditions. Although chemical agents such as sodium hypochlorite are commonly used (Rosenblueth and Martinez-Romero 2006) and have been shown effective, cautions are required in interpreting the results, as suggested by Bent and Chanway (2002) based on their study of lodgepole pine root endophytes. They pointed out that endospores of a strain of Firmicutes bacteria (*Paenibacillus polymyxa*, strain Pw-2) could survive the chemical sterilisation step by producing a higher number of more resistant endospores compared to another strain, L6 (Bent and Chanway 2002). Of course, this does not imply all the spore-forming bacteria recovered from surface-sterilised material come from residual spores on the surface. But additional studies, such as direct observation of the bacterial cells in plant tissues, may be needed to provide definitive evidence of their endophytic nature. With respect to the diversity study, such microscopic

observations are shown to be very informative when probes specific for a certain group of bacteria are used to obtain taxonomic data as well as localisation of the cells (Mogge et al. 2000).

While cultivation of bacterial biomass from surface-sterilised material is very commonly used to investigate endophytic bacterial diversity in forest trees, it is known that cultivation methods are biased and the proportion of cultivable bacteria in the environment, such as soil, is estimated to be less than 1% (e.g. Torsvik and Oveås 2002). So far, only three studies have employed cultivation-independent methods to investigate the diversity of forest tree endophytic bacteria (Table 1). These studies reported rather uncommon bacterial genera such as *Weissella* and *Oxalobacter* alongside the well-known endophytic genera, *Pseudomonas* and *Acinetobacter* (Table 1). This suggests that, like in soil, only a fraction of bacteria residing inside plant tissue can be cultured. Therefore more studies using cultivation-independent methods are required to expand our knowledge about the diversity of endophytic bacteria in association with forest trees.

4 Genetic Richness of Endophytic Bacteria in Trees

Bacterial genera found to be endophytic in trees cover a broad range of phyla including alpha-, beta- and gamma-Proteobacteria, Actinobacteria and Firmicutes, comprising of 39 genera (Table 1). *Bacillus/Paenibacillus* and *Pseudomonas* are most frequently encountered genera in the listed literature, followed by *Acinetobacter* and *Sphingomonas*. Interestingly, a very low proportion of *Acinetobacter* has been reported present among soil isolates (less than 0.001%) (Baumann 1968) and in most crop plants investigated (Hallmann et al. 1997; Rosenblueth and Martinez-Romero 2006) except for one report from rice (Mano and Morisaki 2008). This may suggest a specific association between *Acinetobacter* and forest trees whereas other habitats reported for *Acinetobacter* are mainly clinical samples (Tower 2006). On the other hand, less frequently (i.e. only once) reported genera of bacterial endophytes in forest trees include *Chryseobacterium, Citrobacter, Duganella, Epilithonimonas, Leuconostoc* etc. that were found through cultivation-independent approaches (Table 1). Because the data from cultivation-independent studies are very limited, these genera cannot be considered rare.

There are difficulties in comparing the richness of bacteria between different host tree tissues reported in literature due to the ambiguity of describing the source of the tissues materials (e.g. combination of different tissues for isolation) and differences between the isolation methods used. However, the number of genera reported from different tissues as shown in Table 1 provides some indication on how the diversity of endophytic bacteria can vary between the tissues, taking into account only the number of genera clearly originating from plant tissues. The number of genera in different tissues does not vary so much (8, 11 and 11 genera for leaf, stem and root, respectively), suggesting the richness of the endophytic bacteria is the same in the

three tissues. However, one previous study showed that root endophytic bacterial communities were different from those of the stem and leaf (Izumi et al. 2008). This may indicate that a unique endophytic community in roots would rather consist of many "minor" members of the community rather than of a "major" genus, as shown in Table 1. Therefore it is important to study the diversity of such minor groups for understanding the community structure and ecology of the endophytic bacteria in trees.

5 Abundance of Endopytic Bacteria in Trees

Population sizes of the cultivated endophytic bacteria range from 10^1 (Mocali et al. 2003) to 10^6 g^{-1} CFU (Filteau et al. 2010). Although not much information is available regarding the difference of the population sizes between tissues, one example suggests that there is no difference between roots and branches (Mocali et al. 2003). Since most cultivation methods used are for general bacterial isolation, it is difficult to know the proportions or relative abundance of particular bacteria in a given community. However, a couple of studies provide some indications. Filteau et al. (2010) demonstrated that *Pseudomonas* accounted for approximately 1/10 of aerobic bacterial counts in maple sup using a selective medium and Izumi et al. (2008) demonstrated the dominant bacteria, such as *Bacillus*/*Paenibacillus* and *Acinetobacter* in leaf, root and stem populations, by finding the end points through serial dilutions of sample suspension (Izumi et al. 2008). Additionally, a determination of frequencies of bacterial groups in the total of 513 isolates demonstrated that *Actinobacteria*, such as *Microbacteriaceae* and *Sanguibacteriaceae*, was the dominant group comprising 49% of the total number of isolates (Ulrich et al. 2008a). The second most abundant group was gamma-Proteobacteria (28% of total number of isolates) including *Pseudomonas* (19%) and *Xanthomonas* (7%) (Ulrich et al. 2008a).

Cultivation-independent approaches, such as extensive sequencing of clone libraries, also provide information about relative abundance of the endophytic bacteria. Filteau et al. (2010) demonstrated that gamma-Proteobacteria such as *Pseudomonas* represent 64% of 2,239 total clone sequences in maple sap whereas alpha-Proteobacteria was the dominant group in poplar aerial parts, consisting of 51% of the total of 741 clone sequences, *Sphingomonas* being the most frequent bacterial genus (36% of total sequences) (Ulrich et al. 2008a).

Comparison between cultivation and direct molecular methods provides a different picture on the dominant bacterial groups; in the study by Ulrich et al. (2008a), *Microbateriaceae* dominated cultured bacteria whereas *Sphingomonas* dominated the clone libraries (Ulrich et al. 2008a). This illustrates the fact that these two methods present bacterial community structure quite differently. A complete picture of endophytic bacterial relative abundance may require the use of both methods.

6 Concluding Remarks

Since the time when Chanway pointed out the very limited number of reports on bacterial endophytes over fungal studies and emphasised the importance of bacteria as endophytes (Chanway 1996), 14 years have passed and still bacterial endophytes are rather under represented in scientific reports. Literature search with the key word "endophytic bacteria" returned 364 hits while "endophytic fungi" gave 672 hits (ISI Web of Knowledge, retrieved on 3 June 2010). This is certainly reflected in the research on endophytic bacteria in forest trees, of which there are a handful of papers available at the moment. Consequently, interesting ecological questions on endophytic bacteria in forest trees remain unaddressed. I bring up some examples, which I think are important and fascinating.

As forest trees cover a vast area of land mass, biogeographical aspects will provide intriguing data on bacterial endophyte distribution. Additionally on a more applied side, it would give us fundamental information to tackle the problem of global migration of more hazardous microorganisms such as plant pathogens (Hunter et al. 2008). Other open research questions concern the nature of the association between tree species and their endophytic bacteria and the degree of tree-bacteria specificity. Because the habitat inside tree tissues provides a special niche to the colonising bacteria, and because initial results suggest that trees select specific bacteria (Izumi et al. 2008), it is likely that different host tree species harbour distinctive endophytic bacterial communities. Indeed, this was partly proved by showing differences in cultivated bacterial populations between different cultivars of popular (Moore et al. 2006). However, no study has been carried out by cultivation-independent study to examine this possibility. Additionally, it would be worthwhile to look at functional diversity of endophytic bacteria through identification of genes involved in interesting ecological processes. Although there is a growing interest to use endophytic bacteria for environmental biotechnology purposes, such as phytoremediation of contaminated land (van der Lelie et al. 2009), there is not much data published on functional gene diversity in endophytic bacteria in trees. Such data would give further information about the possible use of the tree endophytic bacteria for human benefit.

Regardless of which way to proceed on research on endophytic bacteria in forest trees, there will be plenty of opportunities to contribute to expanding our knowledge on bacterial diversity and ecology.

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The Genomes of Endophytic Bacteria

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Abstract Genome sequencing and comparative genomics has had major impact on our understanding of the genetic potential, ecology, and evolution of microorganisms. Analysis of a dozen bacterial endophyte genomes have recently contributed insights into the molecular mechanisms that enable bacterial exploration of the plant interior, including genes for motility, colonization and synergistic interactions with the host. Known host-interaction systems include type IV pili, flagella, diverse dedicated secretion systems, genes for phytohormone synthesis and inhibition, bacterial volatiles, and antimicrobials. Different endophytes use different sets of known host interaction systems, suggesting that there are multiple strategies to colonize and persist within plants, and that there are different ways in which endophytes can interact with their host. The majority of host-interaction systems are shared with other bacteria, including plant- and animal pathogens. Functional exploration of the large sets of endophyte genes encoding hypothetical proteins (especially those shared with other phytobacteria) promises to further elucidate bacterial adaptation to life in plant tissue, especially in regards to plant colonization, defense evasion and plant growth promotion.

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
GI	genomic islands
MGEs	mobile genetic elements
IS	insertion sequence

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TAM	tryptamine pathway
IAN	indole-3-acetonitrile pathway
ISR	induced systemic resistance
GABA	Gamma-aminobutyrate
IAM	indole-3-acetamide pathway
IPA	Indole-3-pyruvate pathway
TSO	the tryptophan side-chain oxidase pathway
IAA	indole acetic acid
LPS	lipopolysaccharide

1 Introduction

The knowledge of how bacterial endophytes interact with plants is essential both to those who wish to develop effective and environmentally sound endophyte biotechnology applications, and to those who wish to better understand the role of this ubiquitous and diverse set of organisms in natural ecosystems. In the last decade, genome-enabled studies have become a popular and effective means of deepening our understanding of life on earth. Genome sequencing of bacterial isolates began in the early 1990s and is now, thanks to recent development in sequence technologies and advances in bioinformatics, almost routine. Other genomics techniques include gene expression analysis, which can be performed using microarrays if a reference genome is available, or with next-generation sequencing technologies (Simon and Daniel 2011), an approach that does not require a genome sequence. Sequencing the metagenome, i.e. the collective genomes of all organisms in an environment, is an increasingly popular way to study the uncultured majority of bacteria (Wooley et al. 2010), as is single-cell genomics, genome sequencing that does not require bacterial isolation (Woyke et al. 2010). These genomics methods, including isolate sequencing, have been underused as a means of understanding bacterial endophyte biology. Only a dozen of the thousands of bacterial genomes sequenced so far are from endophytes. However, analysis of a handful of endophyte genomes sequenced in the last few years demonstrates the effectiveness of this approach for developing a better understanding of the endophytic niche.

This review summarizes the findings from endophyte isolate sequencing and analysis, with an emphasis on the correlation between life style and genome content/structure. Although these genome sequences only represent a snapshot of the rich diversity of endophytes that exists, they provide major insights into the ecology and evolution of bacterial endophytes. As most genomes are from endophytes of grasses, a review of forest tree-endophyte genomes is unfortunately premature. A rice endophyte metagenome has been sequenced, but is not yet published, and so will not be covered here.

1.1 Bacterial Genome Evolution

An understanding of the ecology, role and host-interaction of a particular bacterial isolate through analysis of its genome requires knowledge on how bacterial genomes evolve. Compared to eukaryotes (though there are exceptions) bacterial genomes are remarkably plastic, experiencing gene loss and gain at evolutionary – and even ecological – timescales. Horizontal gene transfer, the non-vertical transfer of genetic material between organisms, is an important source of novelty in bacterial evolution and adaptation to new environments (Ochman and Moran 2001), including the adaptation to hosts. Though the mechanisms of gene transfer (transduction, conjugation and transformation) have been known for decades, the era of genome sequencing brought some surprises as horizontal gene transfer turned out to be more rampant than previously thought. Initially a subject of heated debate, it is now generally accepted that horizontal gene transfer in bacteria is common. Still, there must also be ecological barriers to horizontal gene transfer, given that many high taxonomic levels (e.g. phyla) of bacteria share general life strategies or traits that distinguish them from members of other groups (Philippot et al. 2009, 2010).

Compared to eukaryotes, bacterial genomes are small. However, although a given bacterial cell will have a genome in the order of a few million nucleotides, the full functional spectrum of genes available to a given population – termed the 'pan-genome' – can be quite large. The genes in a bacterial population (for example a species or a genus) can usually be classified into the 'core' and 'variable' genome components, which together make up the pan-genome (Tettelin et al. 2008). The core genome is made up of the genes that are common to all but a few individuals of a species; a backbone of essential components. The 'variable', or accessory genome, is composed of genes not found in all strains, because they were differentially gained or lost. Variable genes contribute to the diversity within bacterial species, and may confer selective advantages in specific niches. It was recently demonstrated that in *Escherichia coli*, the variable genes make up more than 90% of the pan-genome and about 80% of a typical genome (Lukjancenko et al. 2010). Variable genes can further be divided into the categories 'character' and 'accessory', where the character genes represent genes essential for colonization and survival in particular environmental niches (e.g. symbiosis or photosynthesis), and the accessory genes are mostly unknown genes that can be used to distinguish strains and serotypes (Lapierre and Gogarten 2009). Generally, the function of most genes in the latter category is unknown.

Genome analysis goes beyond merely providing a 'parts list' of the capabilities of an organism. For example, comparative genomics – the process of identifying similarities and differences between strains or species – can improve our understanding of the evolutionary context of host–bacteria interactions. Powerful comparative analysis requires adequate datasets of several relatively closely related genomes, something that is not yet available for bacterial endophytes. However, analysis of the endophyte genomes sequenced so far has significantly advanced our understanding of life in the endosphere.

2 Significance of Sequenced Endophytes

To date, genomes from nine strains of bacterial endophytes have been sequenced and published (Table 1). Two are Alphaproteobacteria; the rice endophyte *Azospirillum* sp. B510 (Kaneko et al. 2010) and the sugarcane endophyte *Gluconacetobacter diazotrophicus* Pal5 (Bertalan et al. 2009). Five are Gammaproteobacteria; *Klebsiella pneumoniae* 342, isolated from maize stem (Fouts et al. 2008), and the poplar endophytes *Pseudomonas putida* W619, *Serratia proteamaculans* 568, *Stenotrophomonas maltophilia* R551-3 (Taghavi et al. 2009) and *Enterobacter* sp. strain 638 (Taghavi et al. 2009, 2010). Two betaproteobacterial endophytes have been sequenced and published; the rice endophyte, *Azoarcus* sp. BH72 (Krause et al. 2006), and *Variovorax paradoxus* S110, a strain that has been detected in the interior of various plants (Han et al. 2011). Finally, a cyanobacterial water fern endophyte, *Nostoc azollae* 0708 has been sequenced and published (Ran et al. 2010). In addition, several endophyte genomes are on the way, some of which are completed and present in the NCBI database, though not yet published. Table 1 compiles the 14 endophyte strains that have been sequenced so far, including the source of isolation (if indicated in the publication), along with their genome size and structure. Here, designation of replicons as chromosomes or plasmids follows that of the authors, although some of these replicons could probably be classified as so called 'chromids' a recently coined term for a replicon that is neither a chromosome, nor a plasmid (Harrison et al. 2010).

With the exception of the water fern endophyte, these organisms have all been chosen for genome sequencing because of their beneficial effects on plants and their potential in agrobiotechnological applications. More specifically, published endophyte genomes were sequenced for the following reasons: *Azospirillum* B510 increases rice stem number and seed yield, and enhances host resistance to rice blast fungus and to the bacterial pathogen *Xanthomonas oryzae* (Kaneko et al. 2010). *P. putida* W619, and *S. proteamaculans* 568 are both commonly isolated endophytes of poplar, and promote shoot and root development in their host (Taghavi et al. 2009). In the same study, *S. maltophilia* R551-3 was also isolated from poplar (although not as commonly) but it has no direct plant growth promoting effects on its host. *S. maltophilia* is of interest as an emergent opportunistic human pathogen, which means that though not necessarily adapted to humans, it can take advantage of immunocompromised individuals (Looney et al. 2009). *S. maltophilia* and is therefore a potential agent of phytonotic disease (of plant origin). *Enterobacter* sp. 638, also isolated from poplar, can increase poplar growth by up to 40%, and provides systemic drought resistance to poplar (Taghavi et al. 2009). *G. diazotrophicus* Pal5, *K. pneumoniae* 342, *Azoarcus* sp. BH72, and *N. azollae* 0708 are all diazotrophic endophytes (they fix nitrogen inside the plant). *G. diazotrophicus* Pal5 can, in addition to this, promote growth, particularly in roots, inhibit the growth of the sugarcane pathogen *Xanthomonas albilineans* (Blanco et al. 2005) protect against fungal pathogens (Mehnaz and Lazarovits 2006), and promote an increase in the solubility of phosphate and zinc (Saravanan et al. 2007). *K. pneumoniae* 342

Table 1 Endophytic bacterial strains with sequenced genomes

Species	Publication	Isolated from	Class; order	Chromosome size (bp)	Plasmid size (bp)
Azoarcus sp. BH72	(Krause et al. 2006)	Kallar grass roots	Betaproteobacteria; Rhodocyclales	4,376,040	
Azospirillum sp. B510	(Kaneko et al. 2010)	Rice stem	Alphaproteobacteria; Rhodospirillales	3,311,395	1, 455, 109 723, 779 681, 723 628, 837 537, 299 261, 596
B. phytofirmans PsJN	Unpublished	Onion root	Betaproteobacteria; Burkholderiales	4,467,537 3,625,999	121, 122
Enterobacter sp. strain 638	(Taghavi et al. 2010)	Poplar stem	Gammaproteobacteria; Enterobacteriales	4,518,712	157, 749
G. diazotrophicus Pal5 A	(Bertalan et al. 2009)	Sugarcane root	Alphaproteobacteria; Rhodospirillales	3,944,163	38, 818 16, 610
G. diazotrophicus Pal5 B	Unpublished	Unknown	Alphaproteobacteria; Rhodospirillales	3,887,492	27, 455
H. seropedicae SmR1	Unpublished	Unknown	Betaproteobacteria; Burkholderiales	5,513,887	
K. pneumoniae 342	(Fouts et al. 2008)	Maize stem	Gammaproteobacteria; Enterobacteriales	5,641,239	187, 922 91, 096

(continued)

Table 1 (continued)

Species	Publication	Isolated from	Class; order	Chromosome size (bp)	Plasmid size (bp)
M. populi BJ001	Unpublished	Poplar plantlets and tissue cultures	Alphaproteobacteria; Rhizobiales	5,800,441	25, 164 23, 392
N. azollae 0708	(Ran et al. 2010)	The water fern Azolla filiculoides	Cyanobacteria; Nostocales;	5,354,700	109, 570 21, 875
P. putida W619	(Taghavi et al. 2009)	Poplar root and stem	Gammaproteobacteria; Pseudomonadales	5,774,330	
S. proteamaculans 568	(Taghavi et al. 2009)	Poplar root	Gammaproteobacteria; Enterobacteriales	5,448,853	46, 804
S. maltophilia R551-3	(Taghavi et al. 2009) 90,435	Poplar rhizosphere, root and stem	Gammaproteobacteria; Xanthomonadales	4,573,969	
V. paradoxus S110		Potato plant	Betaproteobacteria; Burkholderiales;	5,626,353 1,128,644	

is of interest because of its ability to colonize the interior of a wide range of host plants with a very small inoculum dose (Dong et al. 2003). Moreover, it is similar to *K. pneumoniae* strains that are opportunistic human pathogens. *Azoarcus* sp. BH72 colonizes not only its native host Kallar grass (Hurek et al. 1994), but also rice roots in high numbers and spreads systematically into rice shoots (Reinhold-Hurek et al. 2006). It is able to fix nitrogen in both plants. *V. paradoxus* S110 was isolated from potato plant leaves. Other *V. paradoxus* strains have been shown to stimulate root elongation in indian mustard (Belimov et al. 2005), biodegrade contaminants (Han et al. 2011), and engage in mutualistic beneficial interactions with both plants and other bacteria (Kanzler et al. 2005). Finally, *N. azollae* 0708 is a vertically transmitted extracellular cyanobacterial endophyte, which was sequenced to provide insight into the evolution of chloroplasts from a cyanobacterial ancestor (Ran et al. 2010).

3 Genome Architecture, Genome Content and Life Style

Broadly defined as bacteria inside healthy plant tissue, endophytes can display a range of different life styles, differing in the time spent free-living in the soil, as well as in their transmission dynamics, colonization strategies, and competence (Hardoim et al. 2008). Such differences should be reflected in the genomes of different endophytic bacteria. Genome size and structure usually correlates with bacterial life style. For example, host-restricted bacteria often have smaller genomes than free living relatives (Toft and Andersson 2010), whereas a large and complex genome with multiple replicons might indicate ability to survive in a variable environment (Ettema and Andersson 2009), as an increase in gene content increases robustness against environmental perturbation (Kitano 2007), promoting survival in multiple or variable niches. Large, versatile genomes are typical for e.g. nodule symbionts (Kaneko et al. 2000, 2002), which experience selection at two levels; as a result of adaptation to the stressful and variable environment in the soil, and as a result of adaptation to the plant host. Whereas nodule symbionts use soil as an alternative habitat in their life cycle, some endophytes of grasses are typically not isolated from soil (Reinhold-Hurek and Hurek 1998b). Poplar endophytes on the other hand, are believed to originate from soil and colonize the host via the roots (van der Lelie et al. 2009). Poplar is propagated by cuttings, and because these typically contain only a low number of endophytes, it is assumed that many species of poplar bacterial endophytes have an alternative life stage in soil (Taghavi et al. 2010).

Many of the endophyte genomes bear the same signatures of adaptation to a stressful and variable soil/rhizosphere environment as nodule symbiont genomes. The genomes of endophytes sequenced to date are relatively large and versatile, often comprising more than one chromosome and/or multiple plasmids or chromids. For example, *Azospirillum* sp. B510, which has the smallest chromosome of the endophyte genomes, has a remarkable set of six large plasmids. This genomic

versatility is consistent with the fact that *Azospirillum* is commonly isolated from both soil and plant interior (Hurek et al. 1994). The types of functions encoded by a genome can also indicate if an organism spends time in the soil or rhizosphere environment. For example, based on the number of transporters involved in carbohydrate, amino-acids and iron uptake, as well as some heavy metal resistance genes, Taghavi et al. (2010) suggest that *Enterobacter* sp. 638 is well adapted to survive in the plant rhizosphere.

Only three species; *Azoarcus* sp. BH72, *P. putida* W617 and *H. seropedicae* SmR1 have genomes that consist of a single chromosome without any chromids or plasmid (Table 1). Interestingly, both *Azoarcus* sp. BH72 and *H. seropedicae* SmR1 are candidates for tight association with the plant as attempts to isolate them from root-free soil have failed (Reinhold-Hurek and Hurek 1998a). Similarly, the sugarcane endophyte *G. diazotrophicus* is believed to survive poorly in soil (Kaneko et al. 2010). Though the published *G. diazotrophicus* strain does have two plasmids (of size 39 and 17 kb), they are not large enough to make a substantial contribution to the accessory genome and play an important role in adaptation to the stressful soil environment. Finally, *P. putida* W619 does not carry any plasmids, large or small. The paucity of plasmids in this strain may reflect its phylogeny rather than life style since most *Pseudomonas* genomes sequenced to date consist of a single chromosome. It is interesting to note however, that the *P. putida* endophyte strain has a smaller genome compared to non-endophytes *Pseudomonas*, possibly a result of host restriction.

4 The Plant-Associated Life Style

4.1 Transporters

Endophyte genomes are expected to encode a large diversity of transporters for the uptake of plant-produced nutrients. For example, *K. pneumoniae* 342 contains one of the highest percentage of transporters found in a bacterial genome, 15.4%, which is similar to plant/soil associated microbes like *Bradyrhizobium japonicum* and *Mesorhizobium loti* (Fouts et al. 2008). *Enterobacter* sp. 638 contains over 600 coding sequences for putative transporter proteins (Taghavi et al. 2010). The number of predicted transporters is however smaller for *Azospirillum* sp. B510 (∼300) (Kaneko et al. 2010).

4.2 Motility and Colonization

Motility is an important feature of endophytes, used both to move towards the site of infection, and to systematically spread within the plant (Hardoim et al. 2008).

Endophytic host colonization depends on a variety of surface adhesion factors that allows attachment to the host outer or cell surface.

Type IV pili mediate twitching motility, which is essential e.g. for endophytic rice colonization by *Azoarcus* sp. BH72 (Bohm et al. 2007). The BH72 genome encodes 41 putative genes for pilus assembly and regulation (compared to 30 such genes in the closely related *Azoarcus* strain EbN1) (Krause et al. 2006). In fact, all endophyte genomes carry genes for type IV pili. Therefore, based on the limited set of bacterial endophyte genomes available, the presence of type IV pili genes appears to be a universal feature of endophytes.

Flagella and chemotaxis All the sequenced endophyte genomes (including the unpublished, as revealed by a simple Blast search), encode proteins for chemotaxis and almost all encode proteins for flagellar biosynthesis. The two exceptions are the water-fern endophyte *Nostoc azollae* 0708, which is not surprising given that cyanobacteria are known to lack flagella, and *K. pneumoniae* 342. Fouts et al. (2008) speculate that the lack of flagella in the *K. pneumoniae* 342 genome may contribute to its ability to colonize the host in high numbers, given that flagella are known to induce plant defense (Felix et al. 1999). Lack of flagella might indicate a higher level of adaptation to the endophytic niche, however it also raises questions about the organisms' ability to move towards the plant. *Azoarcus* sp. BH72 contains 48 genes for flagella and chemotaxis, whereas the soil-dwelling strain, *Azoarcus* sp EbN1 does not contain any complete flagellar operons (Krause et al. 2006). The *P. putida* W619 genome contains a large cluster of 52 genes involved in flagellar biosynthesis (Wu et al. 2011). Gene expression results obtained with *P. putida* W619 showed that the transcription of some of those genes is induced in the presence of poplar roots, (Wu et al. 2011), supporting the importance of flagella in endophyte-host interactions. In the genome of *G. diazotropicus* Pal5, the >40 motility genes are clustered in a region that is absent from the genomes of other *Gluconobacter* species (Bertalan et al. 2009). *Enterobacter* sp. 638 contains many genes for motility, including three flagellar biosynthesis operons (Taghavi et al. 2010). *Azospirillum* sp. B510 encodes an impressive number of around 100 putative chemotaxis/flagella genes, most of which are located on plasmids (Kaneko et al. 2010).

Curli fibers belong to a class of fibers known as amyloids. They are involved in surface adhesion, and promote colonization (Barnhart and Chapman 2006). Similar to so many other bacterial adhesion molecules, they are implicated in pathogenesis, but their presence in endophytes demonstrates that they likely are involved in commensal/mutualistic host colonization as well. The *P. putid*a W619 genome is the only additional genome that harbors genes for curli fiber biogenesis, a feature that this strain shares with other sequenced *P. putida* strains (Wu et al. 2011).

Hemagglutinins Important for colonization in a number of plant and animal pathogens (Gottig et al. 2009; Balder et al. 2007), hemagglutinins are often described as 'pathogenicity factors', but their presence in *K. pneumoniae* 342 (Fouts et al. 2008) and *Enterobacter* sp. 638 (Taghavi et al. 2010) suggests that they could also be involved in endophyte colonization.

Cellulases Other factors involved in endophyte establishment within the plant include hydrolytic enzymes that macerate plant cell wall polymers. Whereas plant-pathogens are known to use e.g. glucoside hydrolases to degrade the host cell wall (Herron et al. 2000), the endophyte genomes carry few such genes. One exception is the *K. pneumoniae* 342 genome, with at least 38 genes encoding glycosyl hydrolases (Fouts et al. 2008). It is important to point out that a low production of macerating enzymes may be expected as these can assist in endophytic colonization, as has been shown for an *Azoarcus* sp. BH72 endoglucanase (Reinhold-Hurek et al. 2006).

Celluloses Bacteria can produce their own celluloses for attachment to the host surface. *P. putida* W619 encodes a protein involved in the production of beta-(1,2)-glucan, which in *Agrobacterium tumefaciens* is involved in attachment to plant cells (Rodriguez-Navarro et al. 2007). Interestingly, homologs are not present in two non-endophytic *P. putida* strains, but are present in other nonrelated endophytes (*Enterobacter* sp 638 and *S. proteamaculans*), pointing to a more general importance of this protein in endophyte colonization. *K. pneumoniae* 342 also encodes a beta-(1,2)-glucan, similar to *ndvB*, a gene involved nodule invasion in *Rhizobium meliloti* (Fouts et al. 2008).

4.3 Protection Against Plant Defense

To survive inside plants, endophytes need protection against non-specific plant defense compounds such as reactive oxygen species (ROS), nitric oxide, and phytoalexins (Zeidler et al. 2004). The endophyte genomes are well equipped with genes that provide protection against all three; superoxide dismutases, catalases, peroxidases, hydroperoxide reductases and glutathione-S-transferases.

To colonize the host, bacterial endophytes must overcome the strong plant defense system. Genes involved in plant defense evasion and suppression were mostly not identified in the endophyte genomes, however, genome sequences provide an excellent starting point for elucidating the factors that enable endophytes to escape plant rejection.

4.4 Quorum Sensing

Some bacteria engage in cooperative group behavior through signal molecules termed autoinducers, which can trigger specific functions in a cell-density dependent manner (Camilli and Bassler 2006). The name ‘quorum sensing‘ comes from the fact that to regulate processes via autoinducers, bacteria need to reach a critical mass (Miller and Bassler 2001). Though in many bacteria quorum sensing regulates the expression of virulence genes (Antunes et al. 2010), it is not restricted to pathogenic interactions (Sanchez-Contreras et al. 2007). In rhizobia for

example, quorum sensing can play an important part in the regulation of transfer or plasmids and Integrated Conjugative Elements (ICE, a form of mobile genetic element that can carry genes important in symbiosis) (Ramsay et al. 2006). As pointed out by Rosenblueth and Martinez-Romero (2006), it would be interesting to determine if endophytes use quorum sensing to communicate inside plants. Despite (unpublished) evidence of quorum sensing in *Azoarcus* sp. BH72, the genes for the common autoinducer (N-acyl homoserine lactone, AHL), the autoinducer synthetase (LuxI) or the autoinducer receptor (LuxR) were not found in the genome (Krause et al. 2006), indicating that this strain uses a novel quorum sensing system.

Interestingly, some of the other endophyte genomes possess these well-studied quorum sensing genes. For example, *V. paradoxu*s S110 encodes AHL synthase and its transcription regulator (Han et al. 2011). Three quorum sensing genes were found in the *G. diazotrophicus* Pal5 genome: one *luxI* autoinducer synthase gene and two *luxR*-type transcriptional regulator genes (Bertalan et al. 2009). The *Azospirillum* genome carries an AHL synthase gene and 22 *luxR* family transcriptional regulators (Kaneko et al. 2010). In the related free-living diazotroph *Azospirillum lipoferum*, quorum sensing regulates a range of activities important to plant-host interaction, such as pectinase activity, siderophore synthesis, and phytohormone production (Vial et al. 2006).

4.5 Plant Growth-Promoting Traits

Like rhizosphere bacteria, endophytic bacteria can promote plant growth in direct (e.g. through phytohormone synthesis) and indirect ways (e.g. though nitrogen fixation or defense). Many of the endophytes that have been sequenced are of interest because of their role in plant growth promotion, and consequently they encode proteins involved in such processes.

4.5.1 Phytohormone Production, Volatiles and ACC Deaminase

A wide variety of plant-associated bacteria – including both pathogens and mutualists – can affect plant physiology through the synthesis of compounds that alter plant hormone balance. These can be phytohormones, modulators of phytohormones, or compounds that mimic the action of phytohormones (Ping and Boland 2004).

IAA synthesis Auxin is produced by the plant to regulate various developmental processes. Some bacteria can interfere with plant growth and development by producing indole-3-acetic acid (IAA), the major naturally occurring auxin. IAA producers are found across the range of phytobacterial life styles and include phytopathogens (both gall-inducing- and other pathogens) (Spaepen and Vanderleyden 2010), nodule-forming bacteria (Perrine et al. 2005), free-living plant

growth-promoting bacteria in the rhizosphere (Ahmad et al. 2008), and endophytes (Madmony et al. 2005). In the context of endophytes, IAA production is generally considered a plant-beneficial characteristic (Hardoim et al. 2008).

Several different IAA pathways have been described for bacteria, including the indole-3-acetamide pathway (IAM), the Indole-3-pyruvate pathway (IPA), the tryptamine pathway (TAM), the tryptophan side-chain oxidase pathway (TSO), the Indole-3-acetonitrile pathway (IAN), and the tryptophan-independent pathway (Spaepen and Vanderleyden 2010). It has been suggested that the result of bacterial IAA production from the perspective of the plant may depend on the specific pathway used to synthesize IAA, as pathogens tend to use the IAM pathway, whereas IPA is observed in pathogenic as well as in nonpathogenic bacteria, and is the pathway used by most beneficial bacteria (Spaepen et al. 2007). Moreover, different IAA pathways may have different roles at different stages of colonization (Brandl et al. 2001; Manulis et al. 1998).

Why do bacteria synthesize IAA? Given that IAA can increase the efficiency of colonization (Suzuki et al. 2003), its production may be a colonization strategy; the stimulation of plant proliferation promotes the bacterial niche (whether plant surface or interior). However, IAA biosynthesis may also play an important role in colonization through circumvention of the host defense (Spaepen et al. 2007). Down-regulation of auxin signaling is part of a plant-induced immune response, and auxin promotes susceptibility to bacterial disease (Navarro et al. 2006). In either case, although bacterial IAA synthesis may be a desired quality of endophytes for use in agriculture and forestry, it is unclear whether bacterial phytohormone production actually promotes plant fitness.

In *Agrobacterium tumefaciens*, the genes *iaaM* and *iaaH* are involved in the IAM pathway. Two of the sequenced endophyte genomes, *Azospirillum* sp. B510 and *P. putida* W619, were reported to encode proteins involved in the IAM pathway (Kaneko et al. 2010; Wu et al. 2011), however the identified proteins do not appear to be orthologs of *A. tumefaciens iaaM* and *iaaH*, showing only relatively weak sequence similarity to those (data not shown). *P. putida* W619 is an efficient in vitro producer of IAA in comparison with other endophytic bacteria (Taghavi et al. 2009). One reason for *P. putida*'s effecient IAA production might be the presence of the IPA pathway in addition to the putative IAM pathway. Also consistent with the high level of IAA produced by *P. putida* W619 is the presence of three genes encoding putative auxin carriers (Wu et al. 2011).

IPA is common in bacteria, including those not associated with plants, probably because the genes required for the IPA pathway are also part of the Ehrlich pathway (Spaepen and Vanderleyden 2010). Though present in the close relative *Azospirillum brasilense*, no IPA genes were detected in *Azospirillum* B510. Some IPA genes were also detected in *G. diazotrophicus* Pal5, confirming the results of experiments that demonstrated IAA is mostly synthesized through this pathway (Lee et al. 2004). However, one of the required genes for IPA biosynthesis is lacking from the *G. diazotrophicus* Pal5 genome, suggesting more remains to be discovered regarding bacterial IAA production. The authors suggest that the biochemical activity could be executed by one of the many putative decarboxylases identified in the genome

(Bertalan et al. 2009). Also, the presence of genes encoding enzymes such as aromatic-L-amino-acid decarboxylase, amine oxidase and aldehyde dehydrogenases suggests that *G. diazotrophicus* Pal5 might synthesize IAA via the tryptamine pathway (TAM). Finally, the presence of two genes coding for putative nitrilases suggests that IAA might be produced by the indole-3-acetonitrile pathway (IAN), which is poorly characterized in bacteria.

Judging from the genomes sequenced so far, IAA production is not a universal feature of endophytes. The relative paucity of IAA genes in endophyte genomes might also reflect incomplete understanding of bacterial IAA pathways.

ACC deaminase Ethylene is a stress-induced plant hormone that can inhibit plant growth (Morgan and Drew 1997). Some bacteria can lower the level of ethylene in the plant by cleaving the plant-produced ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (Glick et al. 1998). Inoculation of such bacteria can mitigate the effect of various stressors by sustaining plant growth in the face of ethylene (Belimov et al. 2009; Siddikee et al. 2011). This raises the question as to why bacteria produce ACC-deaminase. According to one hypothesis, plants have evolved to select bacteria with high ACC-deaminase activity to attenuate the stress caused by high levels of ethylene (Hardoim et al. 2008). If so, ACC-deaminase activity would be a highly competitive trait in the endosphere. On the other hand, if the growth-inhibiting action of ethylene is an adaptation to stress that ultimately enhance plant survival under adverse conditions (Morgan and Drew 1997), bacterial interference may not be beneficial or even desirable to the plant. Ethylene is also required for normal plant development, and is being produced in bursts to e.g. help break seed dormancy. ACC-deaminase producing bacteria may play a role in regulating ethylene levels after such bursts, ensuring that ethylene levels stay below the point where growth is impaired (Glick 1995; Hardoim et al. 2008). Finally, there is evidence suggesting that ethylene is a key regulator of the colonization of plant tissue by bacteria (Iniguez et al. 2005), which in turn suggests that the ethylene inhibiting effects of ACC-deaminase may be a bacterial colonization strategy. Regardless of why plant-associated bacteria produce ACC-deaminase, their application can clearly be a very useful strategy to mitigate the effects of various stressors on cultivated plants.

Only a few of the genomes carry *acdS*, the gene for ACC deaminase, including *V. paradoxus* S110, which can use ACC as a carbon and nitrogen source (Han et al. 2011), and *Azospirillum* sp. B510, although it is unknown whether this bacterium has ACC deaminase activity (Kaneko et al. 2010). Putative ACC deaminase genes were found in *P. putida* W619, *Enterobacter sp*. 638, and *S. proteamaculans* 568, but all three lack the conserved amino acid signature characteristic for a genuine ACC deaminase, and consequently do not grow on ACC as their sole nitrogen source (Taghavi et al. 2009). The unpublished but completed genome of *B. phytofirmans* PsJN carries an *acdS* homolog. Inactivation of this gene leads to lost ACC deaminase activity and lost ability to promote the elongation canola seedling roots (Sun et al. 2009). Finally, the unpublished genome of *H. seropedicae* SmR1 encodes an *acdS* gene, probably functional, given that other strains of this species

have ACC-deaminase activity (Rothballer et al. 2008). Thus, only three or four of the complete endophyte genomes encode a functional AcdS protein.

Bacterial volatiles Bacterial volatile compounds, especially acetoin and 2,3-butanediol, trigger plant defense responses, effectively stimulate plant growth, and mediate drought resistance (Ping and Boland 2004; Cho et al. 2008; Ryu et al. 2003, 2004). The genes involved in the pathway, *budA, budB and budC*, were discovered in *Klebsiella terrigena* and and *Enterobacter aerogenes* (Blomqvist et al. 1993). BudB, the acetolactate synthase, converts pyruvate to acetolactate, which is subsequently converted by the acetoin decarboxylase BudA into acetoin. Acetoin is released by the bacteria or subsequently converted into 2,3-butanediol by the acetoin reductase BudC.

The *budB* gene is present in all endophyte genomes, which is not surprising, given that the protein product is involved in multiple metabolic pathways. The *budA* gene is present in *Azospirillum* sp. B510, *Enterobacter* sp. 638, *K. pneumoniae* 342, and *S. proteamaculans* 568. *Enterobacter* sp. 638 carries the *budC* gene, and so does *G. diazotrophicus* Pal5 despite lacking a *budA* homolog. Interestingly, both bacteria possessing all three *bud* genes – *Enterobacter* sp. 638 and *K. pneumoniae* 342 – each carry two more genes whose products contribute to the production of acetoin; an acetoin dehydrogenase, which can convert diacetyl (formed spontaneously from acetolactate under aerobic conditions) into acetoin, and PoxB, a pyruvate dehydrogenase (Fouts et al. 2008; Taghavi et al. 2010), which, although its main function is to convert pyruvate into acetaldehyde, converts some of the pyruvate to acetoin.

In *Enterobacter* sp. 638, the *budABC* operon is located in a genomic region next to the operon for sucrose uptake and metabolism, further indicating that acetoin production is important in the interaction between this bacterium and its poplar host. This region is discussed in more detail in Sect. 6.

The *P. putida* W619 genome also carries *poxB* (but not *budABC*). *P. putida* W619 also lacks the genes involved in the catabolic conversion of acetoin and 2,3-butanediol to central metabolites, whereas the non-endophytic strains *P. putida* F1 and *P. putida* KT2440 have them (Wu et al. 2011). Therefore, although the production of acetoin via PoxB in the *P. putida* endophyte may be very low, it can potentially be converted into 2,3-butanediol by the poplar tree, and promote plant growth that way. Thus, gene loss in *P. putida* W619 may have been selected to increase the production of acetoin available to the host.

Polyamines Polyamines (putrescine, spermidine and spermine) are a group of phytohormone-like natural compounds present in almost all living organisms, which have been shown to increase plant tolerance to a variety of abiotic stressors, including high and low temperatures, salinity, hyperosmosis, hypoxia and atmospheric pollutants (Gill and Tuteja 2010). Given that exogenous application of polyamines protects plants from damage under stress conditions, bacterially produced polyamines could potentially sustain plant growth in the face of abiotic

stress. *Azospirillum brasilense* can produce and secrete polyamines, and has the potential capacity to promote plant growth through these compounds (Perrig et al. 2007). Though not discussed in detail, a few authors note the existence of genes involved in polyamine synthesis (Bertalan et al. 2009; Han et al. 2011; Taghavi et al. 2010). For example Bertalan et al. (2009) suggest that the genes for synthesis and secretion of spermidine in the *G. diazotrophicus* Pal5 may contribute to the plant growth-promoting effects of this organism.

Although bacteria are known to produce other compounds with the potential to alter plant hormone balance such as gibberellins (Bottini et al. 1989), cytokinins (Guo et al. 2001), and salicylic acid (De Meyer et al. 1999), no search for genes involved in the biosynthesis those compounds were reported in the endophyte genome analyses. Gamma-aminobutyrate (GABA) might be involved in plant defense against invertebrate pests (Bown et al. 2006). Taghavi et al. (2009) reported the presence of genes required for GABA synthesis in *Enterobacter* sp. 638. However, because the genome lacks the gene for GABA permease, the bacterium probably does not export GABA as a protecting agent (Taghavi et al. 2010).

A better understanding of the patterns of gene retention, gain and loss that lead to the current constellation of phytohormone-related genes in the endophyte genome collection, and the significance of the presence/absence of such genes, requires a phylogenomic analysis that includes bacteria with various life styles. In addition, the relatively low frequency of known phytostimulation-related genes in the genomes of growth-promoting endophytes suggests that more endophytic phytostimulation pathways remain to be discovered.

4.5.2 Disease Protection and Induced Systemic Resistance

Endophytes can also stimulate plant growth indirectly by inhibiting phytopathogens (that would otherwise inhibit plant growth). They can do this through direct antagonism of microbial pathogens (fungal or bacterial), through competition for space and nutrients, or by activating the plant's natural defense mechanisms, called induced systemic resistance (ISR) (Bordiec et al. 2011).

ISR Bacterial determinants that are thought to produce ISRs include the cell wall outer membrane lipopolysaccharide (LPS), siderophores, flagella, antibiotics, quorum sensing N-acylhomoserine lactones, salicylic acid, and volatile compounds, such as acetoin and 2,3-butanediolsiderophores (Bordiec et al. 2011; van Loon et al. 2008). Many of these are present in the endophyte genomes as discussed above (N-acylhomoserine lactones, volatiles, flagella) or below (siderophores). Moreover, LPS genes were detected in *Enterobacter* sp. 638 (on the plasmid), in *Azoarcus* sp. BH72 (genes are clustered and most similar to LPS genes of gamma-proteobacteria), and *G. diazotrophicus* Pal5 (Taghavi et al. 2010; Krause et al. 2006; Bertalan et al. 2009).

Sideropores and iron uptake Siderophores may serve a dual purpose in plant disease protection, as elicitors of ISR, and through their role in competition for iron. Because the availability of iron often limits microbial growth, an efficient iron uptake system can outcompete pathogens, which has been demonstrated in the rhizosphere (Schippers et al. 1987; O'Sullivan and O'Gara 1992). Endophytic siderophore production has been associated with plant health (Sessitsch et al. 2004), and may contribute to protect the host plant against pathogenic infections, however the role of siderophores *in planta* is unknown, and it is also possible that endophytic siderophore production has a negative effect on the host plant. Bacteria have developed several distinct mechanisms to compete for iron, including (i) specific iron uptake transporters, (ii) the secretion of large numbers of diverse siderophores, and (iii) the synthesis of siderophore receptors to utilize siderophores released from other microorganisms.

Genome analysis suggest that *Enterobacter* sp. 638 is well-equipped to compete for iron; the genome contains genes for synthesis and secretion of the siderophore enterobactin, recovery of the iron-enterobactin complex, and extraction of the iron using an enterobactin esterase (Taghavi et al. 2010). The genes are located in a large cluster adjacent to genes encoding two ABC transporters involved in iron uptake (out of a total of nine). Furthermore, *Enterobacter* sp. 638 possesses 12 outer membrane ferric and ferric-related siderophore receptors, almost twice as many as *Escherichia coli* K12. Likewise, The *V. varivorax* S110 genome has 24 genes encoding siderophore receptors, 16 genes for siderophore biosynthesis and additional 12 genes potentially involved in iron transport and homeostasis (Han et al. 2011).

In contrast, *Azoarcus* sp. BH72 lacks biosynthetic pathways for known siderophores, and production of siderophores was not detected experimentally (Krause et al. 2006). However, *Azoarcus* sp. BH72 possesses 22 genes encoding proteins related to iron transport (more than other nitrogen fixing endosymbionts), including two genes not even present in the genome of *P. fluorescens* Pf5, which is known for its capacity to produce and take up a wide range of siderophores. The authors suggest that the strain is highly adapted to obtaining chelated iron from other microbes, possibly with an antagonizing effect on fungi and bacteria. Kaneko et al. (2010) noted that the number of iron transport proteins in the *Azospirillum* sp. B510 genome was lower than in other N2-fixing endophytes, including *Azoarcus* sp. BH72 (22 genes). Finally, Wu et al. (2011) noted that *P. putida* W619 has a smaller number of siderophore receptors than its close relatives *P. putida* F1 and *P. putida* KT2440. Iron transport and siderophores were not discussed in the other endophyte genome publications.

Antimicrobial compounds *Enterobacter* sp. 638 has been shown to produce 2-phenylethanol, and carries two genes putatively involved in its synthesis, located in regions not syntenic with other closely related strains (Taghavi et al. 2010). *Enterobacter* sp. 638 also possesses the gene *ubiC*, putatively encoding an enzyme that degrades chorismate into the antimicrobial 4-hydroxybenzoate. The gene *ubiC* is also annotated in the genomes of *Azoarcus* sp. BH72, *H. seropedicae* SmR1, *K. pneumoniae* 342 and *S. maltophilia* R551-3.

P. putida W619 carries a gene encoding mannitol dehydrogenase, which might protect against fungal pathogens (Wu et al. 2011; Jennings et al. 2002). *S. proteamaculans* 568 and *K. pmeumoniae* 342 also contain genes annotated as mannitol dehydrogenases.

Bacterial chitinases might protect the plant against fungal pathogens by lysing fungal cell walls, but could also have a role in triggering plant defense mechanisms (Ryan et al. 2008). A chitinase from *S. maltophilia* strain C5 suppresses fungal disease in Kentucky bluegrass by activating plant resistance genes (Kobayashi et al. 2002). *S. maltophila* R551-3 encodes a homolog of this gene, as well as two other chitinases, and *S. proteamaculans* 568 encodes two chitinases, with possible role in fungal antagonism.

4.5.3 Nitrogen Fixation

Half of the endophytes whose genomes have been sequenced are able to fix nitrogen. Genome analyses confirm the presence of the structural and regulatory genes for the nitrogenase complex in *Azoarcus* sp. BH72, *Azospirillum* sp. B510, *G. diazotrophicus* Pal5, *H. seropedicae* SmR1, *K. pneumoniae* 342, *N. azollae* 0708, and in several cases, the absence of those same genes in close relatives. Although none of the tree-endophytes sequenced are nitrogen fixers, diazotrophic endophytes have been detected in e.g. poplar (Doty et al. 2009).

5 Secretion Systems in Endophytes

Many plant- and animal associated bacteria, both pathogenic and mutualistic, encode specific secretion systems used to translocate so called ‘effector’ molecules (usually proteins but sometimes DNA) that modulate interactions with the host. Effectors are secreted across the cell membrane(s), from the bacterial cell cytoplasm into the extracellular milieu, or directly injected into the cells of the eukaryote host. There are currently seven known distinct, non-general secretion systems (designated type I through type VII). While some of these (e.g. the type II secretion system), depend on the so called *Sec* pathway to have proteins exported across the inner membrane to the periplasm before translocation across the outer membrane, others use complex multi-component protein assemblies that directly translocate proteins from the cytoplasm to the extracellular environment. Although specialized secretion systems such as types III and IV are often associated with pathogenicity, it is the effector proteins they deliver, not the systems themselves that are harmful (Dale and Moran 2006). In fact, secretion systems are commonly used in mutualistic interactions between bacteria and both plants and animals (Deakin and Broughton 2009). For example, a type III secretion system is involved in the establishment of symbiosis by the tsetse fly endosymbiont *Sodalis glossinidius* (Dale et al. 2002), and a type IV secretion system is used by the nodule-forming plant symbiont

Mesorhizobium loti (Hubber et al. 2007). Collectively, the endophyte genomes encode the entire range of secretion systems.

Type I secretion systems are simple, composed of an ABC transporter, a multimeric Membrane Fusion Protein (MFP), and a specific outer membrane protein (OMP) (Delepelaire 2004). In rhizobia, proteins secreted via Type I secretion systems play indirect roles in symbiosis (Finnie et al. 1998). This secretion system is present in the genomes of *Azoarcus* sp. BH72, *K. pneumoniae* 342 and *P. putida* W619. The latter is homologous to the type I secretion system in *Pseudomonas fluorescens* WCS365 that exports a protein called LapA involved in biofilm formation (Hinsa et al. 2003). *P. putida* W619 contains an additional gene coding for an OMP component, and next to it, a putative adhesin and a surface-adhesion outer membrane-like protein with weak similarity to LapA (Wu et al. 2011). These two proteins are candidates for secretion by the Type I system, with possible roles in host colonization.

Type II secretion systems are often used by pathogens to export toxins and hydrolytic enzymes proteins from the periplasm across the outer membrane (Sandkvist 2001). The sugar-cane endophyte *G. diazotrophicus* grows on sucrose but does not directly metabolize it. Instead, the bacterium use a Type II secretion system to secrete levansucrase, which converts sucrose to beta-1,2-oligofructans and levan (Hernandez et al. 1995). Type II secretion systems were also annotated in the genomes of *K. pneumoniae* 342 and *Azoarcus* sp. BH72.

Type III secretion systems are related to bacterial flagella (Blocker et al. 2003) and present in many pathogenic bacteria of animals and plants (McCann and Guttman 2008), but also in endosymbionts as discussed above. Type III secretion systems are used to directly inject effectors into the cytoplasm of host cells (Mota et al. 2005). Phytopathogens use them to inject effector proteins that suppress plant innate immunity and enable colonization of plant tissue (Abramovitch et al. 2006; de Torres et al. 2006). Legume symbionts use them to inject so called 'nodulation outer proteins' involved in host range determination and nodulation (Deakin and Broughton 2009). Only one of the published endophyte genomes (that of *V. paradoxus* S110) encodes a putative Type III secretion system (Han et al. 2011). The paucity of this specific host-interaction system in e.g. *Azoarcus* sp. BH52 was interpreted as adaptive; the authors suggested that the absence of type III secretion systems may prevent the export of toxic proteins to the host (Krause et al. 2006). Similarly, Wu et al. (2011) noted that *P. putida* W619 and other non-pathogenic *P. putida* strains lack the type III secretion system present in the plant pathogen *P. syringae* (Wu et al. 2011). However, Type III secretion systems may have a significant role in the biology of beneficial *Pseudomonas* sp. as DNA hybridization analysis indicates their presence in many plant-colonizing, growth-promoting strains of this species (Preston et al. 2001). Interestingly, two of the unpublished genomes (those of *B. phytofirmans* PsJN and *H. seropedicae* SmR1) encode putative Type III secretion systems, suggesting a possible role of type III secretion in the interaction between these endophytes and their host plants.

Type IV secretion systems are used to translocate both DNA and protein substrates across bacterial membranes, and generally require direct contact with the target cell (Alvarez-Martinez and Christie 2009). There are three types of Type IV secretion systems with different functions; conjugation systems, effector translocator systems, and DNA release/uptake systems. In addition, Type IV secretion systems have been shown to be essential for adhesion to erythrocytes and to determine host range in the mammal-associated bacterium *Bartonella* (Vayssier-Taussat et al. 2010; Nystedt et al. 2008). The effector translocator system is important in bacterium-host interactions as they deliver proteins or DNA to eukaryote cells. However, because Type IV effector translocators have evolved from conjugation systems multiple times during evolution, and can do so in a relatively short time (Frank et al. 2005), the role of a newly discovered, uncharacterized Type IV secretion (conjugation vs. effector translocation) is difficult to predict. Type IV secretion systems are found in *Azospirillum* sp. B510, *G. diazotrophicus* Pal5 (four, all in accessory regions), *K. pneumoniae* 342 (present on integrated element and potentially part of a conjugal transfer system), *V. paradoxus* S110, and in the unpublished genome of *M. populi* BJ001.

The type V secretion pathway is often involved in pathogenesis, and encompasses encompasses the autotransporter proteins, the two-partner secretion system, and the Vc or AT-2 family of proteins (Henderson et al. 2004). The autotransporters are large proteins equipped with two translocator domains that enable their export across bacterial membranes. Autotransporters are present in *Azoarcus* sp. BH72, *Enterobacter* sp. 638 (on the plasmid), in *K. pneumoniae* 342, and in *P. putida* W619 (with a pectin/lyase/pertactin domain).

Type VI secretion systems are Sec-independent and related to bacteriophage DNA injection machines (Leiman et al. 2009). Although they can be used to deliver effectors into eukaryote cells (Schwarz et al. 2010b), for example by the pathogen *Vibrio cholerae* (Bingle et al. 2008), it appears as if most bacteria use them to defend against simple eukaryotic cells and other bacteria in the environment (Schwarz et al. 2010a). It has also been demonstrated that the Type VI secretion system is a determining factor for host-specificity in the symbiont *Rhizobium leguminosarum* (Van Brussel et al. 1986). Therefore, endophytic type VI secretion systems could have two possible 'beneficial' roles; in host interaction, or in plant defense by antagonizing pathogenic microbes in the endosphere. The type VI secretion system is annotated in *K. pneumoniae* 342, *P. putida* W619, *B. phytofirmans* PsJN, and *V. paradoxus* S110.

Thus, every type of non-general secretion system, except the Type VII secretion system recently discovered in *Mycobacteria* (Abdallah et al. 2007), is annotated in several of the endophyte genomes. Notably, *K. pneumoniae* 342, which can colonize the interior of a wide range of host plants with a very small inoculum dose, encodes all of them except a type III secretion system (Fouts et al. 2008). Endophytic secretion systems are prime candidates for involvement in various host-interaction processes including attachment, colonization, immune evasion and plant defense.

6 Horizontal Gene Transfer and Genomic Islands

In bacteria, horizontal gene transfer plays a key role in the adaptation to specific lifestyles and environmental niches. Horizontally transferred genes are often clustered together on chromosomes on clearly defined 'genomic islands' (GI) of genes that either are transferred as a group through transformation, conjugation or transduction, or that are hot spots of horizontal gene transfer (Juhas et al. 2009). Genomic islands are easily detected e.g. through comparative genomics due to the tendency of these regions to differ between otherwise closely related strains, or by the presence of typical features, such as duplicated portions of tRNA genes at their boundaries (created by integration into the tRNA gene), the presence of an integrase gene, or a GC content that is different from the rest of the chromosome (Juhas et al. 2009). Most sequenced bacterial genomes, except those of obligate symbionts, for which gene loss dominates over gain (Moran 2003) have genomic islands, and the expectation is therefore that all endophytes, unless in an obligate association with the host plant, have them. Several of the published endophyte genome papers include an analysis of genomic islands, however, the results are not directly comparable since different methods were used to detect them. Detected genomic island typically encode proteins involved in adaptation to the endophytic lifestyle.

For example, 31 putative genomic islands were identified for *P. putida* W619 (Wu et al. 2011). This species was isolated from a tree growing on a site with contaminated groundwater, and was therefore expected to possess the capacity to deal with heavy metals. Putative heavy metal-responsive genes, many of which are absent in other *P. putida* strains were found on genomic islands on the W619 chromosome. Interestingly, the mannitol dehydrogenase, putatively involved in defense against fungi, is located on a putative genomic island and is lacking from other *P. putida* strains. This gene is perhaps a recent addition to the genome, giving *P. putida* W619 a competetive edge in the endosphere.

In contrast, *Azoarcus* sp. BH72 contains few GIs compared to its soil-borne relative (Krause et al. 2006), and only eight islands of size 6–70 kb were located in *Azospirillum* sp. B510 genome (Kaneko et al. 2010). Twenty-eight genomic islands, altogether encoding >800 proteins, were predicted in the *G. diazotrophicus Pal5* genome (Bertalan et al. 2009). Two of them appear to be important for adaptation to the endophytic niche, carrying genes involved in oxidative stress, proteases, biosynthesis of antimicrobial agents, amino acid metabolism and secondary metabolites, transport systems and transcriptional regulators.

Eighteen genomic islands were predicted in the *Enterobacter* sp. 638 genome, harboring genes related to sugar transport, adhesion, pectate utilization, iron uptake through siderophore receptors, nitrate reduction, pilus biosynthesis, transporters and regulators (Taghavi et al. 2010). One of the genomic islands is extraordinarily interesting, encoding genes involved sucrose transport and utilization next to genes for synthesis of the volatiles acetoin and 2,3-butanediol, suggesting a coupling in the expression of these two gene clusters. Indeed, quantitative RT–PCR demonstrated

that the production of acetoin and 2,3-butanediol is induced by the presence of sucrose in the growth medium (Taghavi et al. 2010). This result illustrates that genomic location can give clues to function. It also suggests a significant role of endophytic volatiles *in planta,* which is interesting given that several of the other endophyte genomes also encode them.

Eleven 'site-specific integrated elements' were identified in the genome of Kp342, including two putatively integrated plasmids (Fouts et al. 2008). One integrated element encodes a beta-(1,2)-glucan, similar to *ndvB*, a gene involved in nodule invasion in *Rhizobium meliloti*.

7 Mobile Genetic Elements

Mobile genetic elements (MGEs) such as insertion sequence (IS) elements, prophages, and plasmids tend to reflect the degree of plasticity of genomes (Frost et al. 2005). In other words, a genome with many mobile genetic elements is usually plastic and more amenable to genetic change. The *Azoarcus* sp. BH72 genome contains only eight MGE loci, in stark contrast to the closely related soil strain, *Azoarcus* sp. EbN1, which contains over 200 genes encoding transposases. Krause et al. (2006) suggest that the lack of MGEs in *Azoarcus* reflects adaptation to the stable, low-stress endophytic niche, whereas the higher number of MGEs in the soil-dwelling relative, and in rhizosphere bacteria in general, reflect a need for ongoing adaptation to a variable niche. Interestingly, the sugarcane endophyte *G. diazotrophicus* Pal5, which, like *Azoarcus* BH72, seems poorly adapted to the soil environment (similar to *Azoarcus*, it is rarely isolated from soil), contains 190 transposases, more than any other endophyte (Bertalan et al. 2009). Thus, the number of transposases in a genome might not reflect the need for continuous adaptation, and a low number of transposases may not necessarily be a result of adaptation to a stable niche. As pointed out by Bertalan et al. (2009), expansion of IS elements can be a non-adaptive consequence of host restriction (Moran and Plague 2004): when bacteria lose the ability to live in the environment, their effective population size is reduced, which in turn decreases the efficiency of purifying selection (the selective removal of alleles that are deleterious). However, the presence of other types of MGEs such as plasmids and prophages may still correlate with life style factors.

7.1 Prophages

Phage transduction is a major mechanism of horizontal gene transfer in bacteria, and genes transferred this way are often located adjacent to prophages (integrated bacteriophages) in bacterial genomes. The *Enterobacter* sp. 638 genome carries eight prophages, an unusually high number, and over 300 phage proteins (Taghavi et al.

2010). Six of the prophages are flanked by regions not present in closely related bacteria. Probably acquired through phage transduction, these regions contain genes important in bacterium-plant interactions such as amino-acid and iron transporters, and a hemagglutinin. Two prophages were detected in the *Azospirillum* sp. B510 chromosome, two in *K. pneumoniae* 342, and three in *P. putida* W619. The other genomes lacked prophages or were not investigated for their presence. Kaneko et al. (2010) were able to demonstrate release of phage particles from *Azospirillum* sp. B510 cells is induced by mitomycin C, and estimated the DNA in the released phage was to be10 kb in size. Because none of the *Azospirillum* sp. B510 prophages are 10 kb long, the authors suggest that they may have overlooked some other prophages. Alternatively, the *Azospirillum* genome might encode a gene transfer agent or defective prophage that randomly packages 10 kb pieces of the host genome, as has been demonstrated in other Alphaproteobacteria, such as for example *Bartonella grahamii* (Berglund et al. 2009).

7.2 Plasmids

Plasmids extend the accessory gene content of bacteria, and can sometimes encode proteins that give a competitive edge in a particular niche. The *Enterobacter* sp. 638 genome includes one plasmid, which based on the analysis of the 'backbone' genes (those involved in plasmid replication and maintenance), belongs to a family of plasmids generally involved in host interaction and virulence (Taghavi et al. 2010). The *Enterobacter* sp. 638 plasmid carries many genes related to plant adhesion and colonization, suggesting a role in host-endophyte interaction. Other plasmid-encoded genes with relevance for the endophytic life style include genes for flagella and nitrogen fixation on *Azospirillum* sp. B510 plasmids, and Type IV secretion on a *G. diazotrophiucs* Pal5 plasmid (Kaneko et al. 2010; Bertalan et al. 2009).

8 Comparative Genomics

8.1 Comparisons with Close Relatives

Contrasting genomes of close relatives with different life styles is an efficient way to associate presence and absence of genes with life style factors such as niche specificity or host range. However, the result of such comparisons must be interpreted with caution; first it is impossible to know if differences have adaptive meaning or if they simply reflect neutral gains and losses of genes. Second, with only a few data points (e.g. a few genomes) we rarely have sample sizes big enough to draw statistically significant conclusions about the presence and absence of genes. Still this is a common and valid approach to detecting genes putatively responsible

for life in a particular niche. For example, the endophytic *K. pneumoniae* 343 was compared to a clinical strain and potential pathogen isolated from the human respiratory tract, *K. pneumoniae* MGH78578 (Fouts et al. 2008). This strain can colonize plants, but at a much lower efficiency than endophytic *K. pneumoniae* strains. Apart from the nitrogen fixation genes (present in the endophyte only), the two strains show differences in content of genes encoding transcription factors (more in the endophyte), signal transduction (more in the endophyte), surface-associated structures (different, more in the clinical isolate), and secretion systems (two type IV secretion systems present in the endophyte but absent from the clinical isolate). It is possible that the larger number of transcription factors in the endophyte is advantageous, if their purpose is to downregulate genes whose expression would otherwise interfere with plant colonization by inducing plant defense. Furthermore, the smaller number of surface-structures in the endophyte relative to the clinical isolate may reflect an adaptation to avoid the plant immune system, and the two Type IV systems present only in the endophyte may play a role in interaction with the host.

A similar approach was taken for analysis of the *G. diazotrophicus* Pal5 genome, but using the three phylogenetically closest genomes (Bertalan et al. 2009). Regions exclusive to the endophyte included genes for type IV secretion and flagella. Genome comparison between *Azoarcus* sp. BH72 and the related soil bacterium strain EbN1 revealed an unusually low degree of conserved gene order and content (Krause et al. 2006). Genes exclusive to or more common in the endophyte included those for pilus assembly and for other cell surface components potentially important for plant-microbe interactions. The latter were more closely related to those of plant-associated bacteria than to the *Azoarcus* relative, suggesting gene transfer. Moreover, the endophyte encoded more proteins related to iron-transport, and of course, to nitrogen fixation.

8.2 Comparative Endophyte Genomics

Attempts to identify endophyte-specific genes were not particularly successful. Bertalan et al. (2009) searched for common and exclusive coding sequences among nine endophytic genomes but found very few. Similarly, no uniquely shared genes were found between *K. pneumoniae* 342 and *Azoarcus* sp. (Fouts et al. 2008). The presence of unique ‘endophyte genes’ shared only among current endophyte genomes seems unlikely, given both the diversity of organisms occupying this broadly defined niche, and the diversity of hosts from which they were isolated.

8.3 Comparisons with Other Plant-Associated Bacteria

When the *K. pneumoniae* 342 genome was sequenced, only one other endophyte genome (that of *Azoarcus* sp. BH72) had been described. Therefore, to search for

proteins unique to phytobacteria and identify putative genes important for a plant-associated lifestyle, protein sequences of *K. pneumoniae* 342 were compared to those of 28 genomes of other types of bacteria that interact with plants, including plant pathogens and rhizobia (Fouts et al. 2008). Twenty-three proteins were detected, the majority of which were classified as "hypothetical proteins" with unknown function. The fact that most of these proteins have unknown functions likely reflect a gap in our knowledge of plant-bacteria relationships, and make them interesting targets for further functional studies.

Fouts et al. (2008) did another creative comparison, between the *K. pneumoniae* 342 gene complement and 'plant-induced' genes from other organisms. These were genes that in earlier studies had been shown to be turned on specifically during colonization or growth associated with plants. Homologs of over 200 such plant-induced proteins were searched for in the *K. pneumoniae* 342 genome. Genes appearing to be involved in adaptation to life in the endosphere were detected, including amino acid nucleotide biosynthesis (amino acids are limited within the plant), stress response genes (to evade plant defense), and plant attachment (e.g. hemagglutinins).

9 Conclusion

Genome analysis of currently sequenced endophyte genomes demonstrates that diverse genome characteristics and diverse sets of host interaction genes are used for colonization of the endophytic niche. Host interaction systems are shared with other phytobacteria as well as with bacteria infecting animals. This diversity likely reflects the fact that bacterial endophytes are broadly defined (as microbes living inside healthy plants), that sequenced endophyte genomes come from a diverse set of bacteria (alpha-, gamma- and beta-proteobacteia, and cyanobacteria) and that the isolates were obtained from a diverse set of hosts (from grasses to trees) and from a relatively diverse set of tissues (roots, stems and cuttings).

This diversity in strategies used to explore the niche inside plants demonstrates the existence of various evolutionary routes to becoming an endophyte. Moreover, the evolutionary routes taken by the bacteria sequenced so far are independent; they are all more closely related to non-endophytes than to each other, and there are significant differences in the type and number of genes used to interact with the plant host. Consequently, attempts to identify shared and unique genes among endophyte genomes have been unsuccessful, and may continue to be so as the number of sequenced endophyte genomes increase. Instead, identification and analysis of known host-interaction systems in endophytes suggest that among the many genes encoding hypothetical proteins, candidates for relevance to the endophytic niche (i.e. with roles in colonization, plant defense evasion and growth promotion) will sometimes be present in all endophyte genomes (e.g. motility genes), but more often be present only in a subset of all endophyte genomes (e.g. bacterial volatiles or type III secretion systems). Moreover, such genes are likely to be shared with other

phytobacteria (e.g. ACC deaminase and IAA biosynthesis genes), and sometimes other host-associated bacteria (e.g. secretion systems and hemagglutinins).

As more endophyte genomes are sequenced, hopefully from understudied hosts such as forest trees, and representing different transmission routes and degrees of interaction with the host (including vertically transmitted, obligate and intracellular endophytes), the known repertoir of genes underlying the adaptation to life within plants is likely to expand.

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Part II
Endophytes in Tree Growth Promotion and Stress Tolerance

Endophytic Bacteria in Tree Shoot Tissues and Their Effects on Host

Anna Maria Pirttilä

Abstract The interactions between the plant and endophytic bacteria in the shoots likely differ to some extent from those in the roots. Shoot endophytic bacteria are typically isolated during plant tissue culture started from shoot tips (buds) or embryos. With methods such as in situ hybridization and transmission electron microscopy, endophytic bacteria have been localized in buds, seeds, and flowers of forest trees, and GFP tagging has been used to observe colonization of seedlings by endophytic bacteria. Vertical transmission of endophytic bacteria has been suggested. Shoot endophytic bacteria share many plant growth-promoting effects with the root endophytes, the ability of producing plant growth hormones and nitrogen fixation being the most common ones. In addition, some shoot endophytes may affect plant growth through production of adenine derivatives and vitamin B_{12}. Many more likely remain to be determined by powerful methods such as genomics and metabolomics, which will be valuable tools for describing the significance of endophytic bacteria for forest trees in the future.

Abbreviations

(TEM)	transmission electron microscopy
(PHB)	polyhydroxybutyrate
(GFP)	green fluorescent protein
(IAA)	Indole-acetic acid

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1 Introduction

Whereas the majority of endophyte studies of forest trees have concentrated on the diversity of fungi, very little is known about endophytic bacteria and especially their function in tree tissues. Endophytic bacteria mainly in the genera *Pseudomonas, Bacillus, Paenibacillus, Erwinia* and *Burkholderia* are found almost in every tissue of a tree (for details, see chapter by Izumi in this volume). Most studies have been performed on root-associated endophytic bacteria, which may however differ from the shoot-associated endophytes on their diversity and function (Moore et al. 2006; Yrjälä et al. 2010). Plant shoot tissues are exposed to UV radiation, rapidly fluctuating temperatures, alternations in relative humidity and limited nutrient resources compared to roots. The shoot tissues are dominated by pigmented bacteria that are more rarely found in the roots, and the ability to utilize methanol is typical for shoot-associated bacteria (Fall 1996; Pirttilä et al. 2008; Yrjälä et al. 2010).

In this chapter tree shoot tissues, especially shoot tips (buds), flowers, seeds, and seedlings, are discussed with respect to endophytic bacteria and their role in tree development and growth. Because the research on epiphytic bacteria present on leaves has much elucidated the role of bacterial life in shoot tissues, some references to those studies are made as examples.

2 Tissue Cultures and Shoot Tips

Typically endophytic bacteria of tree shoot tips are found during tissue culture, because the shoot tip meristems or embryos are often used as the starting material. For example, endophytic bacteria have been detected in the tissue cultures of hazelnut (*Corylus avellana* L., *C. contorta* C.) (Reed et al. 1998), sour cherry (*Prunus cerasus* L.) (Kamoun et al. 1998), various species of poplar, larch, black locust (*Robinia pseudoacacia* L.), Norway spruce (*Picea abies* Karst.) (Ulrich et al. 2008; Van Aken et al. 2004), and Scots pine (*Pinus sylvestris* L.) (Laukkanen et al. 2000; Pirttilä et al. 2000). In the study by Ulrich et al. (2008), the majority of endophytes were identified as members of *Paenibacillus* in 5-year old cultures initiated from shoot tips or immature or mature zygotic embryos of poplar, larch, black locust and spruce. Other genera such as *Methylobacterium*, *Stenotrophomonas* and *Bacillus* were occasionally detected in these cultures (Ulrich et al. 2008). The *Paenibacillus* spp. had no visible negative effect on the plant development, and one strain isolated from poplar cultures had a growth-promoting effect on seedlings (Ulrich et al. 2008).

Plant tissue culture as a propagation tool is selective on endophytes, as some species can thrive in the cultured tissues, being enriched through generations, and others can vanish or die during the procedures (Koskimäki et al. 2010). Even if plant tissue culture is the condition where many endophytic bacteria are encountered, it is likely that the majority of endophytes can never be seen in vitro (Koskimäki et al.

2010). Therefore culture-independent techniques, such as in situ hybridization, should be applied and developed further for the research on endophytes.

In Scots pine, endophytic bacteria *Methylobacterium extorquens*, *Pseudomonas synxantha*, *Mycobacterium* sp. and the yeast *Rhodotorula minuta* were isolated from callus cultures originating from shoot tips (Laukkanen et al. 2000; Pirttilä et al. 2000, 2003). When oligonucleotide probes were developed to detect the endophytes in the pine tissue by in situ hybridization, they were found in the cells of scale primordia, the meristems, and around the resin ducts of the intact buds (Pirttilä et al. 2000, 2003). In the tissue cultures, they were localized in the cells of the growing callus of Scots pine whereas no endophytes were detected in embryogenic tissue of European black pine (*Pinus nigra* Arn.) (Pirttilä et al. 2002). Furthermore, the endophytes formed biofilms in the Scots pine calli (Pirttilä et al. 2002). Endophytes have also been found as biofilms in plantlets of in vitro-grown potato, and the biofilm might be a common form for endophytic bacteria living inside plant tissue (Bandara et al. 2006; Podolich et al. 2009).

Besides localizing the microbes, the in situ hybridization method can reflect changes in the metabolic activity of the microbes as it measures ribosomal RNA (DeLong et al. 1989). When the presence or metabolic activity of endophytes in the Scots pine shoot tips was studied by in situ hybridization throughout the year, it depended on the growth season. The endophytes were undetectable during the winter season and infrequently found in the vigorously growing shoot tips of the mid-summer, whereas the highest quantities (or metabolic rates) were detected in tissues of spring and autumn, prior to growth or differentiation of the bud. All endophytes studied (*Methylobacterium* spp., *P. fluorescens* subgroup, *Mycobacterium* sp. and *R. minuta*) were present in the shoot tips, *Methylobacterium* spp. being the most common microbe throughout the year (Pirttilä et al. 2005).

Fluorescent in situ hybridization has been used to study the location of the poplar endophyte, *Methylobacterium populi* (Tanaka et al. 2008, and see chapter by Van Aken et al. in this volume). Another method for studying endophytes without culturing is transmission electron microscopy (TEM), which enables very high magnification of the plant tissue and study of the location of bacteria in the cellular compartments. However, specific expertise is needed to identify the bacterial cells and no information can be obtained on the species. By TEM, endophytic bacteria have been detected in linden (*Tilia cordata* L.) and blue spruce (*Picea pungens* var. *glauca* L.) (Doronina et al. 2004; Pirttilä et al. 2008). Bacterial cells were found on ultrathin sections of buds of linden and needles of blue spruce (Doronina et al. 2004; Pirttilä et al. 2008). Some of the bacteria contained polyhydroxybutyrate (PHB) granules that are characteristic of methylotrophic bacteria.

3 Flowers, Seeds and Seedlings

Besides bud tissues, endophytic bacteria are found within the reproductive organs of conifers. The bacterium *Enterobacter cloacae* has been isolated from pollen of *Pinus halepensis* and *P. pinea* and from fertilized ovules of *Pinus brutia*

(Madmony et al. 2005). In Scots pine, endophytes were not detected in pollen grains but the sporogenous cells of the male flowers contained endophytes as biofilm-like structures based on in situ hybridization studies (Pirttilä 2010). Less endophytes were found in the female inflorescences of Scots pine, but seed embryos were heavily colonized (Pirttilä 2010). In the seeds of Norway spruce, bacterial endophytes belonging to the genus *Rahnella* have been isolated from the embryo and endosperm (Cankar et al. 2005). The quantity or culturability of endophytes in the spruce seeds decreased with time (Cankar et al. 2005).

The pollen or seeds of other forest tree species have rarely been studied. In *Eucalyptus*, endophytes such as *Bacillus, Enterococcus, Paenibacillus* and *Methylobacterium* were isolated from seeds and seedlings (Ferreira et al. 2008). Specific strains of *Bacillus*, *Paenibacillus*, and *Enterococcus* were present both in seeds and seedlings grown from the seeds, suggesting a vertical transmission of the endophytes. In the same study, an endophytic strain of *Pantoea agglomerans*, isolated originally from *E. grandis*, was tagged with GFP and inoculated into the seeds of two species and one hybrid of *Eucalyptus*, and the colonization was observed by fluorescence microscopy. Colonization of the seedlings was confirmed for *E. grandis* and the hybrid *E. grandis* × *E. globulus*, but not for *E. urophylla*. The strain was detected colonizing the seedling roots mainly in intercellular spaces, as well as in xylem vessels of the stem. No leaves of any seedling were colonized by the GFP-tagged strain which, on the other hand, indicates a horizontal transmission.

4 Effect of Shoot-Associated Bacteria on Plant Host

In general, many studies have outlined the positive effects of shoot epiphytic and endophytic bacteria, specifically in the genus *Methylobacterium*, on tissue organogenesis and embryogenesis (Holland and Polacco 1994; Visser et al. 1994; Murthy et al. 1999; Kalyaeva et al. 2001). Methylotrophs have the unique ability to utilize methanol and methane as the energy source and are ecologically important organisms as they minimize the emission of methanol and methane from plants to the atmosphere (Fall 1996; Fall and Benson 1996; Keppler et al. 2006).

Methylotrophic bacteria typically have a positive effect on plant growth, demonstrated by several studies. In 1991 the first observation on the positive effect of methylotrophs on plants was made by Nonomura and Benson when they sprayed corn, spinach, beet, and soybean plants with 20% methanol. This increased the colonization of the plants by methylotrophs and eventually resulted in higher crop yield (Nonomura and Benson 1991). The following year, Holland and Polacco made the discovery that heat-treated soybean seeds had a decreased germination rate and a fall of 95–97% in the epiphytic methylotroph populations. When the heat-treated soybeans were inoculated with methylotrophs, the germination rates were restored. Therefore, they made the conclusion that the germination rate was dependent on the number of methylotrophs on the seeds. They also noted that the presence of methylotrophs improved root formation and increased biomass of the

soybean seedlings (Holland and Polacco 1992). Holland (1997) explained these observations by the ability of the bacteria to produce the phytohormone cytokinin.

Since then, several findings on the positive effect of methylotrophs on plant growth and development have been made. Epiphytic methylotrophic bacteria stimulate seed germination of soybean (Holland and Polacco 1994; Freyermuth et al. 1996; Holland 1997; Koenig et al. 2002), induce formation of morphogenic calli and shoots and promote the development of the regenerated plants of *Triticum aestivum* L., *Nicotiana tabacum* L., *Solanum tuberosum* L., and *Linum usitatissimum* L. (Kalyaeva et al. 2001). Some *Methylobacterium* strains co-synthesize compounds commonly known as plant products (Zabetakis 1997; Koutsompogeras et al. 2007) and some strains produce plant growth hormones (Ivanova et al. 2000, 2001; Koenig et al. 2002). Additional mechanisms for plant growth promotion have been suggested to exist (Koenig et al. 2002). It is likely that endophytic bacteria of the shoots have several ways of affecting the development and the growth of tree host. Below, some generally known growth-promoting effects, especially with respect to methylotrophs, are described in more detail.

4.1 Phytohormone Production

Plant-associated bacteria typically produce plant growth hormones such as cytokinins, auxins and gibberellins. Whereas gibberellin production is most typical for the root-associated bacteria, cytokinins have been identified in some leaf isolates, and auxin production is common to all plant-associated microbes (Bottini et al. 2004; Ivanova et al. 2008). It should be noted however, that a study on endophytic bacteria of *Solanum nigrum* suggests that the growth promotion effects cannot be generalized to all host plants, even if the underlying mechanisms were general, such as phytohormone production (Long et al. 2008).

Auxins are a group of indole derivatives that have various growth-promoting functions in plants, such as promotion of root formation, regulation of fruit ripening, and stimulation of cell division, extension, and differentiation. Indole-acetic acid (IAA) is the most-well known auxin. Epiphytic and root endophytic bacteria most typically synthesize and secrete auxins (Brandl and Lindow 1996; Bastián et al. 1998; Costacurta et al. 1998; Doronina et al. 2002; Gamalero et al. 2003; Ivanova et al. 2001, 2008; Merzaeva and Shirokikh 2010). In graminaceous plants, a shoot endophytic *Pseudomonas stutzeri* strain was identified from *Echinacea* tissue culture capable of producing IAA (Lata et al. 2006). In poplar, the endophytes *Enterobacter* str. 638, *Stenotrophomonas maltophilia* str. R551-3, *Serratia proteamaculans* and *Pseudomonas putida* str. W619 have been identified as IAA-producing bacteria (Taghavi et al. 2009). Another interesting finding is that the endophytic bacterium *Enterobacter cloacae*, isolated from pollen grains of *Pinus* spp., produces IAA and promotes growth of mung bean cuttings (Madmony et al. 2005).

Cytokinins are a group of compounds with the backbone of adenine having a substitution at the N-6 atom of the purine ring. These compounds are important

in many steps of plant development, as they stimulate plant cell division, induce germination of seeds, activate dormant buds and play a role in apical dominance. Cytokinins also induce the biosynthesis of chlorophyll, nucleic acids, and chloroplast proteins at the early stages of leaf development (Skoog and Armstrong 1970). Both pathogenic and beneficial plant-associated bacterial species are capable of synthesizing cytokinins (Akiyoshi et al. 1987; Timmusk et al. 1999; Garcia de Salamone et al. 2001). Among plant-associated methylotrophs, species such as *Methylovorus mays*, and *Methylobacterium mesophilicum* JCM 2829 synthesize and excrete cytokinins (Ivanova et al. 2000; Ivanova et al. 2008). A study on the type strain of *Methylobacterium extorquens*, which is a soil isolate, and several epiphytic *Methylobacterium* strains isolated from Arabidopsis, barley, maize, and soybean revealed that all strains produced trans-zeatin, which was tRNA-derived rather than synthesized de novo (Koenig et al. 2002). When heat-treated soybean seeds were germinated with the strains, cytokinin-null (*miaA*) mutants incapable of cytokinin synthesis stimulated germination at the same level as the wild-type bacteria. The authors concluded that although cytokinin production by the methylotrophs could play a role in the interaction, the bacteria-produced cytokinins might not be responsible for the stimulation of seed germination (Koenig et al. 2002).

4.2 Other Modes of Growth Promotion

Although many plant-associated bacteria produce plant growth hormones, there may be great variations in the quantities between the strains within a species (Ivanova et al. 2008). Some strains of a species may produce high quantities of a phytohormone and others zero (Ivanova et al. 2008). The bud endophytes of Scots pine, *Methylobacterium extorquens* str. F and *Pseudomonas synxantha* str. G, produce compounds that extend the viability and affect morphology of callus tissues in vitro (Pirttilä et al. 2004). However, these compounds are not the most common phytohormones, such as cytokinins, gibberellins, or auxins. Instead, *M. extorquens* str. F excretes adenine and adenine ribosides in the culture medium (Pirttilä et al. 2004). Adenine can be used in plant meristem cultures to induce growth. Whereas the mode of action of adenine on plant growth promotion is unknown, it is most effective when applied together with ammonium nitrate and cytokinins (George and Sherrington 1984). Furthermore, in feeding experiments with *Coffea arabica*, adenine riboside has been detected as the metabolite of adenine (Baumann et al. 1994). Adenine riboside is also abundant in the vascular cambial region of *Pinus sylvestris*, which is uncommon for other plants (Moritz and Sundberg 1996, Pirttilä et al. 2004).

The epiphytic methylotrophs are able to synthesize other bioactive compounds, such as vitamin B_{12} (Nishio et al. 1977; Ivanova et al. 2006, 2008). Vitamin B_{12} is a group of compounds with trivalent cobalt as a cofactor. These compounds function as the coenzyme in isomerization and transmethylation reactions in the biosynthesis of compounds containing methyl groups. The enzymes with the coenzyme form of

vitamin B_{12} are found in many flowering plants that cannot synthesize vitamin B_{12} themselves (Holland and Polacco 1994). Furthermore, exogenously applied vitamin B_{12} increases the biomass, amount, length, and the degree of branching of moss gametophytes (Basile et al. 1985), the same effects which are induced by epiphytic methylotrophs (Koopman and Kutschera 2005). Whether vitamin B_{12} produced by shoot endophytes plays a role in the development of forest trees remains to be determined.

Nitrogen fixation is a well-studied function in the rhizobial and actinorhizal symbioses, and almost all root endophytes fix nitrogen (Baldani et al. 1997). However, the agricultural significance of the endophytic nitrogen fixation may be low (Dalla Santa et al. 2004). Some diazotrophic (nitrogen-fixing) strains have been isolated as endophytes from tree tissues, such as *Paenibacillus* str. P22 from poplar (Ulrich et al. 2008). When in vitro-grown poplar plants were grown with the *Paenibacillus* str. P22, asparagine and urea were increased, and sugars and organic acids were decreased in the metabolite profile of the inoculated plants, which points to nitrogen being fixed by the bacterium and assimilated by the plant (Scherling et al. 2009). Diazotrophic strains belonging to *Burkholderia*, *Rahnella*, *Sphingomonas* and *Acinetobacter* have as well been isolated from the stems of poplar and willow (Doty et al. 2009). Whereas not all strains isolated from the shoot tissues fix nitrogen, the *Methylobacterium mesophilicum* and epiphytes in general can affect plant nitrogen metabolism through the urease enzyme (Holland and Polacco 1992, 1994). Holland and Polacco (1992) observed that urea accumulates in tissues of soybean double mutants with impaired urease activity. When the mutants were colonized by the epiphytes, the urease activity was restored to a level of 20–40% of that of the wildtype plants due to the activity of the bacterial urease enzyme (Holland and Polacco 1992). Similar or other means of affecting the nitrogen balance by endophytes in the tree shoot tissues may exist.

5 Conclusions

Although many endophytes probably enter the plant from the soil through the roots and are able to colonize the entire plant through the vascular tissues, tree shoot tissues and reproductive organs provide different ecological niches for endophytic bacteria compared to roots (Moore et al. 2006; Yrjälä et al. 2010). Because the growth-promoting effects of endophytes can be strain- and host-specific (Long et al. 2008), the term “endophyte” cannot be generalized with respect to function and significance, and each case should be studied separately. We have found that the endophytes isolated from buds of Scots pine can increase growth of pine seedlings to the same extent as mycorrhizal fungi (Metsometsä et al., unpublished). The mechanisms described in this chapter can be responsible for the increased plant growth, in addition to many mechanisms that still remain to be discovered. As mycorrhizal fungi are today acknowledged significant organisms for health and growth of forest trees, endophytic bacteria can provide a number of benefits for

forestry in the future. New methodologies such as genomics and metabolomics will be valuable tools for describing the significance of endophytic bacteria for forest trees.

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Growth-Promoting Endophytic Fungi of Forest Trees

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Abstract This chapter focuses on the relatively new field of research on endophytic fungi of trees that directly improve plant growth. Endophytic fungi of Scots pine and Norway spruce enhance root development of seedlings and cuttings. Endophytic yeast strains isolated from poplar trees can increase overall growth of not only poplar but also of crop plants. Both groups of endophytic fungi were shown to produce plant hormones. This chapter provides a brief review of these few cases in which endophytic fungi of trees were shown to directly increase plant growth and it serves as a call for more research into this important area.

Abbreviations

(IBA) indole butyric acid
(BnR) binucleate *Rhizoctonia*
(IAA) indole-3-acetic acid

1 Introduction

Most research on fungal endophytes of forest trees indicated an improvement of plant health by protection from pathogens or by increasing stress tolerance. These indirect growth-promotion effects are discussed in other chapters of this volume, e.g. chapter by Miller. Mycorrhizae have long been known to improve tree growth through increasing nutrient and water availability. However, since mycorrhizae are

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generally not considered as endophytes since they do not reside entirely within the plant (Rodriguez et al. 2009), contributions of this important group of fungi are not discussed here. Research on endophytic fungi of non-woody plants has clearly demonstrated the importance of these fungi to increase plant growth as well as stress tolerance (Gasoni and deGurfinkel 1997; Ernst et al. 2003; Mucciarelli et al. 2003; Rodriguez et al. 2009). However, currently only two groups of endophytic fungi isolated from woody plants have been shown to have direct plant growth-promoting effects.

2 Fungal Endophytes of Conifers

Conifer root growth-promoting fungal endophytes were isolated from Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L.) in Norway and Finland (Hietala et al. 1994; Gronberg et al. 2006). The endophytes were identified as non-mycorrhizal binucleate *Rhizoctania* (*Ceratorhiza* sp.), a species that can be a mild pathogen implicated in conifer root rot diseases. However it was determined that it is primarily the uninucleate strains, not the binucleate strains, that are the causal agents of damping off and root dieback (Lilja 1994) although both strains can co-exist on the same seedlings (Hietala 1995). Since the binucleate endophyte strains are related to orchid mycorrhizae, it was hypothesized that they may enter into potentially beneficial, but non-mycorrhizal relationships with the conifer species from which they were isolated (Hietala and Sen 2006).

The endophytic binucleate fungus has several positive effects on the roots of both Scots pine and Norway spruce (Gronberg et al. 2006). When seedlings were inoculated with the binucleate *Rhizoctonia* (BnR) strains, root growth was enhanced despite a very low infection rate in soil deficient in N and P (Gronberg et al. 2006). The root length increased significantly in the first few months post infection, and an increase in the number of short roots was observed after 240 days. This increase in number had important implications for the colonization by ectomycorrhizal fungi. By inducing more short roots in conifer seedlings using the BnR strains, the plants were more heavily colonized by beneficial mycorrhizal fungi, leading to more effective acclimation and survival following transplantation (Gronberg et al. 2006).

In a closely-related study, it was found that BnR strains also induced adventitious root formation in cuttings of Scots pine (Kaparakis and Sen 2006). Non-inoculated cuttings had an average rooting rate of approximately 23% while cuttings inoculated with different BnR isolates had rates from 57% up to 97%. Some of the isolates also induced a significantly higher number of roots per cutting. The root initiation occurred in the absence of exogenenous auxin (IBA); however, the response was twice as fast with an IBA pulse. It was hypothesized that a threshhold level of the hormone is required to initiate the adventitious organogenesis, and this level was achieved faster with the IBA pre-treatment.

The mechanism for the changes in root growth and architecture in the conifer seedlings and cuttings are likely due to the production of plant growth regulators

(Gronberg et al. 2006). Production of the phytohormone, indole-3-acetic acid (IAA), from L-tryptophan was shown to occur by some species of *Rhizoctonia* (Furukawa et al. 1996). A low-molecular weight protein signal is thought to be the trigger of the hormone synthesis. Overall, the studies with binucleate *Rhizoctonia* strains provide evidence that endophytic fungi can be used to improve the rooting rate of cuttings, the magnitude of mycorrhizal colonization, and the acclimation ability following transplanting, all of which will help in the development of more sustainable conifer propagation in forest nurseries.

3 Fungal Endophytes of *Populus*

Beneficial fungal endophytes have also been isolated from the angiosperm trees, *Populus*. Cottonwood (*P. trichocarpa* and hybrids) was found to harbor endophytic yeasts within the stems. These yeasts were identified as *Rhodotorula graminis* (strain WP1) from a riparian *P. trichocarpa* and *R. mucilaginosa* (strains PTD2 and PTD3) from *P. trichocarpa* × *deltoides* hybrids grown in greenhouses in two different regions (Xin et al. 2009a). The yeasts are budding, of a size range of 5–7 × 3–4 μm, and are pink-pigmented (Fig. 1). Strain WP1 from the riparian poplar was isolated along with an abundance of nitrogen-fixing bacterial endophytes (Doty et al. 2009; Xin et al. 2009b). Diazotrophic endophytes can enhance crop growth both by providing essential nitrogen and through the production of phytohormones (Doty 2011). To determine if the endophytic yeasts of poplar synthesized hormones, as did many of the other endophytes of poplar, cultures were assayed for IAA production. As with the *Rhizoctonia* strains (Furukawa et al. 1996), the fungi efficiently converted L-tryptophan to phytoactive IAA. The hormone is not produced *de novo*, but is made using plant-derived L-tryptophan as the substrate, a more metabolically efficient pathway for endophytes (Xin et al. 2009a). Of the three endophytic yeast

Fig. 1 *Rhodotorula graminis* strain WP1 is an endophytic yeast of *Populus trichocarpa*

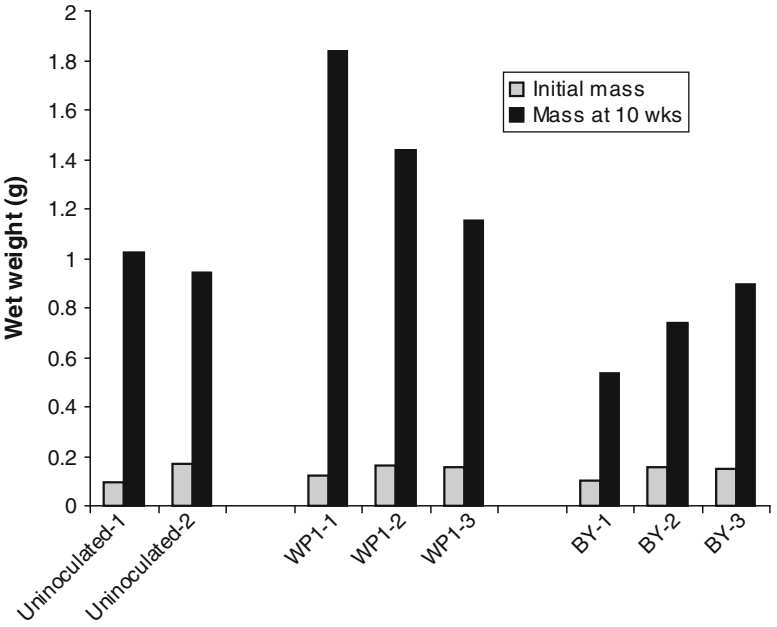

Fig. 2 Endophytic yeast enhanced the growth of poplar. *Populus trichocarpa* Nisqually-1 plants were weighed and then planted in autoclaved sand moistened with minimal nutrient solution. Three were inoculated with the poplar endophytic yeast strain, WP1, three with a baker's yeast control, and two were left uninoculated. After 10 weeks, the roots were rinsed and the plants were weighed again. The plants inoculated with WP1 gained an average of 1.33 g while the uninoculated and baker's yeast controls gained an average of 0.85 g and 0.59 g, respectively

strains isolated from poplar, strain WP1 produced the most IAA, over twice as much as the strains isolated from greenhouse-grown poplar. Rapid root growth is essential in riparian poplar where the seedlings must keep up with the receding water table following seasonal flooding of the river. Roots of poplar seedlings grow at an amazing rate of one cm per day immediately following germination (Stettler 2009). Since the substrate along the river is often rocky, long roots would also be an advantage to serve as an anchor for the next flooding event. Therefore, endophytes that enhance root growth could be key to the survival of riparian poplar.

Experiments are underway to determine if the endophytic yeasts from poplar stimulate plant growth. In a preliminary study, cuttings of *Populus trichocarpa* clone Nisqually-1 were inoculated with strain WP1, with a baker's yeast control, or were left uninoculated. As shown in Fig. 2, the WP1-inoculated poplar had the greatest growth rate, a 64% higher weight gain compared to the uninoculated poplar over the 10-week experiment (Doty, unpublished). With funding from the NSF Energy for Sustainability program, large-scale experiments have been initiated to study the impact of endophytes on poplar growth at the greenhouse and field levels.

Experiments with the endophytic yeast strains on crop plants have demonstrated that WP1 can dramatically enhance the growth of corn and rice (Redman and Doty, unpublished). The genomic sequence of the strain WP1 has recently been determined by the Joint Genome Institute. Identification of the genes involved in plant growth enhancement and in plant-microbe interactions in general will help us to better understand this important symbiosis.

4 Conclusions

In summary, the study of fungal endophytes that directly promote the growth of trees is a new area of research with publications in only the last several years and with much of the work still on-going. With results indicating pronounced effects on rooting of conifer seedlings and cuttings, and of increased growth rates of poplar as well as of agricultural crops using the poplar yeast endophyte, the field of research in tree-fungal symbiosis is poised for profound new developments.

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The Endophytic *Trichoderma hamatum* Isolate DIS 219b Enhances Seedling Growth and Delays the Onset of Drought Stress in *Theobroma cacao**

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Abstract *Theobroma cacao* (cacao) is a tropical understory tree with sensitivity to drought. Cacao responds to drought by decreases in net photosynthesis, PS II efficiency, stomatal conductance, water potential and changes in leaf florescence. Drought also alters cacao gene expression as well as leaf glucose and free amino acid content. In recent years an incredible diversity of fungal endophytes has been identified in association with cacao. These endophytes are being studied for the benefits they provide to cacao including tolerance to biotic and abiotic stresses. During establishment of the endophytic association between cacao and fungal endophytes both plant and fungal gene expression are altered. The endophytic *Trichoderma hamatum* isolate DIS 219b delays the onset of drought stress in cacao. This delay manifests itself through enhanced root growth, maintenance of stomatal conductance, water potential, net photosynthesis, and PSII efficiency, changes in free amino acid concentrations, and a delay in drought-induced changes in leaf gene expression. The cacao plant and DIS 219b adapt to each other and this adaptation may contribute to the observed plant growth promotion and the delay in onset of drought stress. The increase in root growth is thought to increase water uptake and availability, delaying the time point where the water supply becomes limiting and drought stress occurs.

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Abbreviations

MIP	major intrinsic protein
BGF	blue-green fluorescence
PA	polyamine
EST	expression sequence tag
TcODC	ornithine decarboxylase
TcADC	arginine decarboxylase
TcSAMDC	S-adenosylmethionine decarboxylase
TcTPP	trehalose-6-phosphatase
TcSOT	putative sorbitol transporter
TcPR5	osmotin-like protein
TcNI	putative alkaline/neutral invertase
TcCESA3	putatively encoding a cellulose synthase
TcLOX	13-lipoxygenase
AOC	allene oxide cyclase
TcTIP	a tonoplast intrinsic protein
TcRPK	receptor-like protein kinase
TcMKK4	putative mitogen-activated protein
TcSTK	serine/threonine protein kinase
NR	nitrate reductase
HK	histidine kinase
TcHK	sensor type histidine kinase
TcMAPK3	mitogen-activated protein kinase
TcPP2C	protein phosphatase
TcZFP	C2H2 zinc finger protein
ABA	abscisic acid
VAM	vesicular-arbuscular mycorrhiza
ASP	asparagine
HIS	histidine
ARG	arginine
PRO	proline
GABA	gamma-aminobutyric acid
VAL	valine
LEU	leucine

1 Cacao and Drought

Theobroma cacao (cacao), the source of chocolate, is an understory tree grown in tropical areas where rainfall is sufficient (Wood and Lass 2001). Cacao production is severely affected by drought (Keil et al. 2008 , Moser et al. 2010). Seasonal rainfall

patterns also exert major control on harvest times (Mohd Razi et al. 1992 , Belsky and Siebert 2003). Extended dry seasons, possibly associated with global warming, can weaken mature trees and may lead to premature death, despite surviving initial drought cycles (Personal Communication, Freddy Amores, INIAP, Ecuador). As recently shown, the crop management system used can influence the response of cacao to drought (Belsky and Siebert 2003). There is interest in the development of drought tolerance in cacao through plant breeding and crop management practices, but progress towards reaching this goal has been slow (Belsky and Siebert 2003).

2 *Trichoderma* and Other Microbes Alter Plant Responses to Stress

Trichoderma species, commonly considered soil saprophytes, have also been described as opportunistic, avirulent plant symbionts (Harman et al. 2004a) or more simply, endophytes (Evans et al. 2003). Beneficial activities attributed to *Trichoderma*-plant interactions include induced disease resistance (Harman et al. 2004a), plant growth promotion (Bailey and Lumsden 1998; Harman et al. 2004a; Bae et al. 2009), and tolerance to abiotic stresses (Harman et al. 2004a; Bae et al. 2009). Several classes of endophytes are known to alter the response of plants to abiotic stresses. Endophytes of cool-season grasses induce tolerance to multiple biotic and abiotic stresses (Malinowski and Belesky 2000). Some of the mechanisms used by cool-season grass endophytes to alter the drought response include avoidance through morphological adaptations, tolerance through physiological and biochemical adaptations, and enhanced drought recovery (Malinowski and Belesky 2000). Mycorrhiza alter the response of plants to abiotic stresses (Augé 2001). The most studied mechanism employed by mycorrhiza to enhance drought tolerance relates to enhanced growth (Augé 2001).

3 Cacao Endophytes

Cacao supports a diverse microbial community including many endophytic fungi (Arnold et al. 2003; Evans et al. 2003; Rubini et al. 2005). Unique associations have been detailed for endophytes and cacao leaves, stems, branches, flower cushions, and fruits in efforts principally targeting disease control. Arnold et al. (2003) collected 1,172 fungal isolates (344 morphotaxa) from cacao leaves. Rubini et al. (2005) isolated many genera of fungal endophytes from cacao branches. Evans et al. (2003) identified biocontrol fungi for use against *Moniliophthora roreri*, causal agent of frosty pod on cacao. *Theobroma gileri* in Ecuador was sampled for endophytes for use in *Theobroma cacao*. Fungi were isolated from trunks and pods after removal of surface tissues. More than 40 genera were recorded,

including many *Trichoderma* species. Most of the fungi caused no symptoms on cacao and could be re-isolated from inoculated seedlings. *Trichoderma hamatum* isolate DIS 219b is of particular importance in this text (Bailey et al. 2006, 2008; Bae et al. 2009). *Trichoderma hamatum* isolate DIS 219b was isolated from a *Theobroma gileri* pod in Guadual, Lita, Esmeraldas Province, Ecuador (Evans et al. 2003).

Many *Trichoderma* isolates have been identified as cacao endophytes (Evans et al. 2003; Holmes et al. 2004; Bailey et al. 2008). Newly identified *Trichoderma* species include *Trichoderma ovalisporum* (Holmes et al. 2004), *Trichoderma martiale* (Hanada et al. 2008), *Trichoderma stromaticum* (Samuels et al. 2000), *Trichoderma theobromicola* and *Trichoderma paucisporum* (Samuels et al. 2006b), *Trichoderma koningiopsis* (Samuels et al. 2006a), and *Trichoderma evansii* (Samuels and Ismaiel 2009). The large number of endophytes found in cacao leads to the following questions. Are the endophytes unique in their abilities to colonize cacao and/or is cacao unique in its ability to promote endophytic colonization by many different microbes? So far, attempts to induce defense responses in cacao have given muted molecular responses. Induced systemic resistance against disease (Resende et al. 2002; Arnold et al. 2003; Melnick et al. 2008) does occur in cacao. Resende et al. (2002) noted that the time course required for induction of resistance in cacao (around 30 days) in response to acibenzolar-S-methyl treatment was longer than that observed in annual crops (6 or 7 days). Melnick et al. (2008) noted a similar time course in cacao for resistance to *Phytophthora capsici* induced by treatment with *Bacillus*. Arnold et al. (2003) detected induced resistance to a *Phytophthora* sp. in a shorter time frame (18 days) after treatment with a consortium of endophytic fungi. It is unclear how the slow induced resistance response might influence endophytic colonization of cacao.

4 The Interaction Between *Trichoderma* and Cacao

4.1 The Physical Association Between Trichoderma and Cacao

A group of endophytic *Trichoderma* isolates were applied to cacao seedlings to characterize their colonization abilities (Bailey et al. 2008). Cacao roots, stems, and cotyledons were heavily colonized by all the *Trichoderma* isolates studied. Plumules were less heavily colonized by many isolates and leaves were poorly colonized regardless of the isolate. The xylem was heavily colonized by only *Trichoderma hamatum* isolate DIS 219b and *Trichoderma harzianum* isolate DIS 219f.

Trichoderma species have been shown to penetrate roots directly (Yedidia et al. 2000), although penetration was restricted to the first few cells encountered. Root hairs are heavily colonized by some *Trichoderma* isolates (Yedidia et al. 2000; Harman et al. 2004b). The endophytic colonization of above-ground tissues forces the consideration of other pathways for penetration. *Trichoderma* penetrates the

woody tissues of cacao to a greater depth than a few cells (Evans et al. 2003; Bailey et al. 2008). One possible pathway for penetration is through the glandular trichomes (Bailey et al. 2009). Trichomes, like root hairs, also differentiate from epidermal cells (Ishida et al. 2008). Similar to observations in roots (Yedidia et al. 2000; Harman et al. 2004a), *Trichoderma* mycelia form a close association with the stem epidermis and the trichome stalk in many areas (Bailey et al. 2009) and penetration could result from these associations. *Trichoderma* isolates, including DIS 219b, penetrate and survive inside the head of cacao glandular trichomes. *Trichoderma* can move through the head, into, and through the stalk cells.

Trichoderma isolates with specific abilities to colonize roots and proliferate with root growth have been described (Harman et al. 2004a, b). These studies have not been carried out in perennial plant species over multiple years. A significant observation from the initial studies with *Trichoderma* on cacao was the failure of any isolate to completely colonize meristematic tissues (Bailey et al. 2008). These studies were carried out under conditions that limited competition from the greater diversity of microbes present under cacao field situations. The implication is that a single *Trichoderma* application should not be expected to completely colonize a cacao tree over the long term. This implication is supported by the previously described endophyte surveys indicating that multiple endophytes inhabit individual trees. Perhaps a more favorable outlook based on this observation is that we may be able to apply multiple endophytes to a single tree.

4.2 The Molecular Response of Cacao to Trichoderma

The interactions between four *Trichoderma* species, including DIS 219b, and cacao were characterized at the molecular level (Bailey et al. 2006). Several cacao ESTs (expressed sequence tags) induced in response to *Trichoderma* colonization share homology with genes induced in response to environmental stresses. Examples are as follows. Ornithine decarboxylase (EST *P1*) is a primary control point in polyamine biosynthesis. Changes in ornithine decarboxylase and polyamines have been associated with abiotic stresses such as drought (Capell et al. 2004). Zinc finger proteins (EST P*13*) are commonly associated with responses to biotic and abiotic stresses (Kim et al. 2004). Glutathione-S-transferase-like proteins (EST *P4*) have a role in protecting cells from oxidative injury by detoxifying compounds that damage cells, contributing to resistance of plants to biotic and abiotic stresses (Dixon et al. 2002). EF-hand, calcium-binding motifs (EST *P29*) are found in a super-family of proteins involved in calcium signaling leading to the activation or inactivation of proteins and are involved in developmental processes and responses to stress (McCormack et al. 2005). One of the ESTs more highly induced in cacao by *Trichoderma* colonization, EST *P59*, shares homology with carbohydrate-oxidase encoding genes (Carter and Thornburg 2004). Carbohydrate oxidases produce hydrogen peroxide as a product and are important in plant defense against microbes (Carter and Thornburg 2004). Expression of EST *P31* was repressed in cacao by

colonization with the *Trichoderma*. EST *P31* is related to the major intrinsic protein (MIP) superfamily. MIPs function as membrane channels that selectively transport water, small neutral molecules, and ions out of and between cells. The repression of MIP gene expression may reduce membrane water permeability encouraging water conservation (Smart et al. 2001). In a follow-up study, additional stress-related ESTs were induced during initial colonization of cacao seedlings by DIS 219b (Bae et al. 2009).

4.3 The Molecular Response of Trichoderma to Cacao

While cacao is responding to colonization by *Trichoderma*, gene expression in *Trichoderma* is being altered in response to cacao (Bailey et al. 2006). Although many *Trichoderma* ESTs studied by Bailey et al. (2006) were detected in multiple *Trichoderma*/cacao interactions, most of the *Trichoderma* ESTs were initially identified in tissues colonized by DIS 219b. Several ESTs putatively encoding enzymes associated with carbohydrate hydrolysis, protein digestion, and lipid metabolism were identified. These enzymes are of obvious potential importance in the establishment of endophytic associations functioning in acquisition of nutrients and possibly aiding in the colonization process itself. ESTs with potentially diverse hydrolytic activities were identified including a glucosyl hydrolase-family 2 protein (F3) and a xyloglucan hydrolase-family protein (F7). Glucosyl hydrolase-family 2 protein activities include β-galactosidase, β-mannosidase and β-glucuronidase activities. Xyloglucan hydrolase family proteins are thought to function in the digestion of cell wall carbohydrates (Grishutin et al. 2004). Hydrolytic enzymes are also important in the parasitism of other fungi by *Trichoderma* species facilitating the digestion of cell walls (Harman et al. 2004a). A serine protease (F11) was identified. Serine proteases have been shown to function in mycoparasitism by *Trichoderma* species (Steyaert et al. 2004) and in the endophytic association between *Acremonium typhinum* and the grass, *Poa ampla* (Lindstrom et al. 1993). An EST (EST F12) putatively encoding a Δ3, Δ2-enoyl-CoA isomerase was also identified.Δ3, Δ2-enoyl-CoA isomerase is essential for the beta-oxidation of unsaturated fatty acids (Geisbrecht et al. 1998).

5 The Drought Response of Cacao

5.1 Drought Alters Cacao Physiology

During drought, plants can show the following symptoms: cessation of root and shoot growth, decreased stomatal conductance, reduced net CO_2 assimilation, impaired photosynthesis, accumulation of solutes, and leaf senescence

(Passioura 1996). In addition, the blue-green fluorescence of plants often increases (Lichtenthaler and Miehé 1997). In our experiments, cacao responded to drought, as expected. Cacao seed were planted in pre-sterilized soilless mix and grown in sealed double magenta box. Once the seedlings had emerged, the magenta box tops were removed, and the seedlings were grown for 32 days after planting before the water regimens were altered. The earliest responses to drought in cacao leaves were observed after 7 days of withholding water as decreases in net photosynthesis, PS II efficiency, and stomatal conductance (Bae et al. 2008). An increase in the blue-green fluorescence (BGF) emission and a decrease in leaf water potential were noted after 10 days of withholding water, indicating severe drought stress (Bae et al. 2008; Bae et al. 2009). Biomass production was also reduced (Bae et al. 2009).

5.2 Drought Alters Cacao Gene Expression

Although the genes responding to *Trichoderma* colonization and drought overlap, they sometimes respond in opposite directions. One of the first research efforts targeting drought focused on ESTs putatively involved in polyamine biosynthesis (Bae et al. 2008). ESTs putatively encoding an ornithine decarboxylase (*TcODC*), an arginine decarboxylase (*TcADC*), and an S-adenosylmethionine decarboxylase (*TcSAMDC*) were induced in leaves after withholding water for 10 days. *TcADC* and *TcSAMDC* were more sensitive to drought in the roots showing induction at 7 days of withholding water. *TcODC* (Bailey et al. 2006) and *TcADC* (unpublished data) were also induced during the initial interaction between DIS 219b and 9-day-old cacao seedlings. It is possible that the induction of PA biosynthesis genes in roots participates in altering the root architecture in response to stress (Hummel et al. 2002).

Nineteen additional drought-responsive ESTs were identified in cacao (Bae et al. 2009), including ESTs putatively involved in the production of osmoprotectants and/or regulatory metabolites. Osmolytes accumulate under drought stress in order to maintain cell turgor and stabilize cell proteins and structures (Valliyodan and Nguyen 2006). *TcTPP*, induced by drought, putatively encodes the enzyme trehalose-6-phosphatase. Trehalose confers drought tolerance to transformants engineered to overexpress enzymes in the trehalose pathway, including TPP (Garg et al. 2002). A putative sorbitol transporter (*TcSOT*), the major phloem-translocated carbohydrate in some plants (Watari et al. 2004), was induced in roots and leaves. An osmotin-like protein (*TcPR5*) was induced by drought. Osmotin-like proteins, members of the PR-5 protein family, are commonly associated with tolerance to osmotic stress and in plant defense (D'Angeli and Altamura 2007). *TcNI*, a putative alkaline/neutral invertase, was also induced by drought. Alkaline/neutral invertases participate in the hydrolysis of sucrose, providing a source of carbon for the biosynthesis of other osmoprotective substances and/or as a source of energy (Sturm 1999). Lastly, *TcCESA3*, putatively encoding a cellulose synthase, was repressed in

the roots by drought. Foyer et al. (1998) suggested that drought stress can increase sugar accumulation through inhibition of cellulose synthesis.

TcLOX and *TcAOC* were studied for their potential involvement in jasmonic acid biosynthesis. *TcLOX*, induced by drought in roots, putatively encodes a 13-lipoxygenase involved in jasmonic acid biosynthesis through a pathway that includes allene oxide cyclase (AOC). *TcAOC* was repressed by drought in roots. Although also localized in chloroplasts, Hause et al. (2003) verified that AOC in tomato is associated with companion cells and plastids within sieve elements of vascular bundles, allowing for its possible involvement in the drought response.

Drought-induced changes in expression of ESTs *TcTIP*, *TcABC-T*, and *TcSOT* indicate that drought alters the movement of molecules across membranes in cacao (Bae et al. 2009). EST *TcTIP*, putatively encoding a tonoplast intrinsic protein, was repressed in the roots by drought. The initial colonization of cacao seedlings by DIS 219b also strongly repressed *TcTIP* (*P31*) expression (Bailey et al. 2006). The repression of aquaporin gene expression may result in reduced membrane water permeability and encourage water conservation during drought (Smart et al. 2001). Expression of *TcABC-T* was also repressed in roots by drought. ABC transporters function in the movement of molecules across membranes (Jasinski et al. 2003).

Orthologues of *TcSEN1* are found in many plant species and are induced by darkness and during senescence (Shimada et al. 1998). A recent study by Yang et al. (2003) suggested that the tobacco orthologue, Ntdin, plays a role in N- and S metabolism. As was observed with EST *TcNR* in cacao roots, NR enzyme activity and transcript abundance are known to be sensitive to repression by drought (Ferrario-Méry et al. 1998). The influence of drought (repression) on *TcNR* expression was opposite to that observed in cacao seedlings responding to initial colonization by DIS 219b.

Drought stress triggers signal transduction-activating components of the drought response (Beck et al. 2007). In cacao roots, *TcRPK* (putative receptor-like protein kinase), *TcMKK4* (putative mitogen-activated protein), and *TcSTK* (putative serine/threonine protein kinase) were down-regulated in response to drought. *TcMKK4* and *TcSTK* were induced in cacao seedlings responding to colonization by DIS 219b (Bae et al. 2009). *TcHK* (putative sensor type histidine kinase), *TcMAPK3* (putative mitogen-activated protein kinase), *TcPP2C* (putative protein phosphatase 2 C), and *TcZFP* (P13, a putative C2H2 zinc finger protein) were up-regulated in response to drought and in cacao seedlings responding to colonization by DIS 219b. Histidine kinases are membrane-associated proteins that bind ligands and serve as sensory preceptors, transmitting signals that participate in regulatory cascades. HK can activate MAPK pathways (Zhu 2002). MAP kinases are inducible by various effectors including wounding, drought, cold, and salt, in addition to several plant hormones (Morris 2001). PP2Cs are ubiquitous protein phosphatases, some of which are involved in ABA responses (Saez et al. 2004). C2H2 zinc finger proteins may function as key transcriptional repressors that are involved in the responses of plants to stresses (Ciftci-Yilmaz and Mittler 2008).

6 DIS 219b Delays the Drought Response of Cacao

6.1 DIS 219b Delays Physiological Drought Responses

The drought-induced changes in net photosynthesis and stomatal conductance observed in non-colonized seedlings were delayed and/or reduced in DIS 219b-colonized seedlings, and changes in fluorescence emissions of cacao leaves was greater for non-colonized seedlings than colonized seedlings (Bae et al. 2009). A delay in the drooping of leaves in response to drought was also associated with DIS 219b colonization (Bae et al. 2009). The impact of endophytes on the plant drought response has been intensely studied in cool-season grasses (Malinowski and Belesky 2000). Endophyte-infected tall fescue showed improved tolerance to drought and faster recovery after drought. The cool-season grass endophytes often cause plants to respond to drought earlier, resulting in accelerated stomatal closure and reduced water loss (Malinowski and Belesky 2000). Plants colonized by vesicular-arbuscular mycorrhiza (VAM) under drought conditions can maintain stomatal conductance and leaf water potential (Augé 2001), resembling the responses of DIS 219b-colonized cacao seedlings to drought. The primary impact of VAM on plant–water relationships has been attributed to changes in plant growth and development and is commonly associated with enhanced phosphorus acquisition (Augé 2001).

6.2 DIS 219b Delays the Molecular Drought Response of Cacao

The delay in the drought response due to DIS 219b colonization as measured using physiological parameters was further evidenced by delays in drought-induced changes in gene expression (Bae et al. 2009). The drought-induced changes in gene expression were delayed in leaves of colonized seedlings, whereas many of the drought-induced changes in gene expression in roots were not delayed. ESTs *TcODC*, *TcADC*, and *TcSAMDC*, putatively involved in polyamine biosynthesis (Bae et al. 2008) show a delayed response to drought in DIS 219b-colonized seedlings (Fig. 1). Of the tissue/EST combinations studied, only *TcADC* in leaves failed to show delayed induction by drought in DIS 219b-colonized seedlings. In leaves, the drought-altered expression of ESTs *TcrbcS*, *TcTPP*, and *TcMAPK3* was also delayed in the colonized seedlings (Bae et al. 2009).

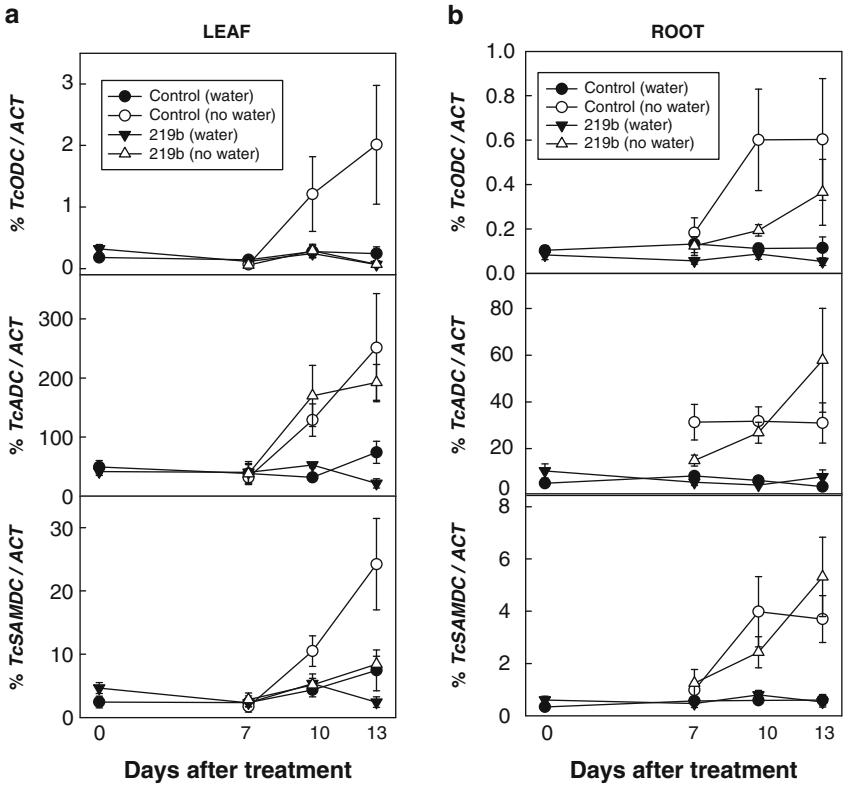

Fig. 1 Expression pattern for cacao ESTs *TcODC* (putative ornithine decarboxylase), *TcADC* (putative arginine decarboxylase), and *TcSAMDC* (putative S-adenosylmethionine decarboxylase). Cacao seeds were planted in soil inoculated with *Trichoderma hamatum* isolate DIS 219b or uninoculated and grown using the double magenta box system as described in Bae et al. (2009). Seedlings were watered every other day for 32 day of growth before drought treatment. Total RNA was isolated from leaves (**a**) and roots (**b**) harvested 0, 7, 10, and 13 day after the last watering. Treatments: Control (water), non-colonized seedlings watered every other day, Control (no water), non-colonized seedlings with water withheld 13 day , 219b (water), colonized seedlings watered every 2 day, 219b (no water), colonized seedlings with water withheld 13 day. Relative mRNA levels were calculated for qPCR results with respect to ACTIN transcripts (% of ACTIN). Bars show means ± standard error (n = 6)

7 Changes in Metabolites in Response to Drought and DIS 219b

The glucose content of cacao leaves increased during drought stress (Bae et al. 2009). During drought stress, soluble carbohydrates (glucose, fructose, sucrose, stachyose, mannitol, and pinitol) can accumulate in leaves (Valliyodan and Nguyen 2006). Soluble carbohydrates serve as protectants, nutrients, and metabolite-signaling

molecules that modulate the transcription of genes involved in sugar-sensing mechanisms (Ho et al. 2001; Li et al. 2006).

Concentrations of seven soluble amino acids (ASP, HIS, ARG, PRO, GABA, VAL, and LEU) increased in cacao in response to water stress (Bae et al. 2009). Concentrations of four soluble amino acids were altered by DIS 219b colonization. Concentrations of ASP and GLU decreased in response to DIS219b colonization while ALA and GABA concentrations increased. A positive relationship between PRO accumulation and drought tolerance has been reported in many plant species (Simon-Sarkadi et al. 2006). GABA concentrations in cacao leaves increased in response to drought and DIS 219b colonization. ALA concentrations also increased in response to DIS 219b colonization. The ALA response may be a by-product of the GABA shunt, as a result of the activity of GABA transaminase using pyruvate as the amino group acceptor (Shelp et al. 1999). GABA is produced in response to various abiotic stresses including water stress (Fait et al. 2005; Mazzucotelli et al. 2006).

8 *Trichoderma hamatum* Isolate DIS 219b Promotes Cacao Growth

In biomass studies, colonization of cacao seedlings by DIS 219b enhanced growth, resulting in an increase in root fresh weight, root dry weight, total fresh weight, root water weight, total water weight, and the root dry weight/root fresh weight ratio (Bae et al. 2009). DIS 219b-induced plant growth promotion and the response to drought sometimes leads to striking differences in the leaf flushing in cacao seedlings (Fig. 2). Plant growth promotion is often observed in response to *Trichoderma* colonization but the mechanisms have not been fully explained (Harman et al. 2004a; Adams et al. 2007). Enhanced nutrient availability and increased nutrient uptake efficiency, among others, are proposed mechanisms involved in *Trichoderma*-induced plant growth promotion (Harman et al. 2004a). The impact of endophyte-induced plant growth promotion on drought tolerance has been extensively studied for VAM/plant associations and endophytes of cool-season grasses (Malinowski and Belesky 2000; Augé 2001). The abilities of larger plant tissues to store water are commonly cited mechanisms for enhancing drought tolerance in plants (Malinowski and Belesky 2000; Augé 2001). It bears mentioning here that EST *TcNR* (a putative nitrate reductase) was dramatically induced during the initial interaction between DIS 219b and 9-d-old cacao seedlings (Bae et al. 2009). The endophytic fungus *Piriformospora indica* promoted growth of Arabidopsis and tobacco seedlings and stimulated nitrogen accumulation and the expression of a gene encoding NR in roots (Sherameti et al. 2005). Sherameti et al. (2005) noted that recruitment of nitrogen in endophytic interactions differs from mycorrhizal interactions in which the fungus preferentially recruits ammonium rather than nitrate.

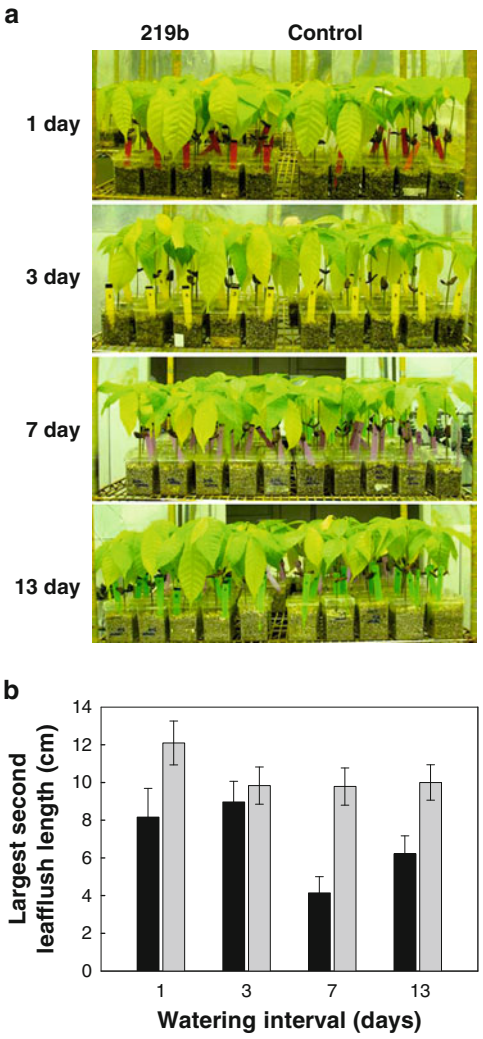

Fig. 2 Cacao seed were planted in soil inoculated with *Trichoderma hamatum* isolate DIS 219b or uninoculated and grown using the double magenta box system as described in Bae et al. (2009). Seedlings were watered every other day for 32 day before altering the watering cycles. The watering cycle was then changed to 1, 3, 7, or 13 day and maintained for 13 day. Leaf production by cacao seedlings proceeds in flushes of two to four new leaves at a time. The treatments were grouped and photographed (**a**) and the length of the largest second flush leaf was measured (**b**). Black bars represent uninoculated seedlings and grey bars represent DIS 219b treated seedlings. Bars show means ± standard error (n = 6)

9 Conclusions

Drought-induced physiological changes in cacao leaves and changes in root gene expression preceded changes in leaf gene expression. Much of the drought-altered expression of ESTs in roots was not influenced by DIS 219b colonization, whereas ESTs responding to drought in leaves demonstrated a delayed drought response when the seedlings were colonized by DIS 219b. Most of the physiological measurements showed a similar delay in response to drought when seedlings were colonized by DIS 219b. The simplest explanation is that DIS 219b colonization enhanced root growth, resulting in improved water acquisition and an increase in water content. The roots of colonized seedlings perceived the dry soils and responded, while the leaves took advantage of the increased water availability through the roots, resulting in a delayed drought response. The changes in plant growth may be mediated through the direct effects of initial DIS 219b colonization on cacao gene expression. The concentrations of several amino acids, notably GABA, were also directly responsive to DIS 219b colonization. These responses leave open the possibility that colonization pre-adapts cacao to drought as a component of the altered signal transduction pathways.

"How *Trichoderma* isolates induce plant growth promotion?" is a difficult question to answer. *Trichoderma* isolates cause mild stress to the cacao seedlings during colonization. Almost all of the genes induced in seedlings in response to colonization point to a stress response. Under conditions, which promoted intensive *Trichoderma* growth, the roots became noticeably discolored (Bailey et al. 2008). As observed with other plant-*Trichoderma* interactions (Bailey and Lumsden 1998), only under the most extreme of conditions does *Trichoderma* colonization cause sufficient damage to limit seedling growth. The observed plant growth enhancement may be attributable to the acclimation of the seedlings to mild stress induced by *Trichoderma*. Acclimation to a stress can leave plants pre-adapted to additional stress cycles and additional stresses (Selote and Khanna-Chopra 2006; Patade et al. 2009).

Another obvious question is, "will pre-colonizing of cacao seedlings with DIS 219b enhance root growth once the plants are transplanted to the field?" In a preliminary study, cacao seedlings pre-colonized with 14 tropical endophytic *Trichoderma* isolates were transplanted into the field in Ecuador. Many of the isolates, including DIS 219b enhanced root growth after 9 months in the field (unpublished data). By continued study of the interaction between *Trichoderma* and cacao and impact of biotic and abiotic stresses on cacao, it should be possible to understand and exploit the stress responses of cacao in the field to the benefit of farmers.

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Part III
Abiotic Factors Affecting Endophyte Diversity

Responses of Foliar Endophytes to Pollution

Marjo Helander, Salla-Riikka Vesterlund, and Kari Saikkonen

Abstract In addition to natural environmental variation, plants and their associated microorganisms may encounter a series of anthropogenic environmental changes. Direct effects of pollutants on organisms are feasible to study, but impacts of air pollution on more complicated ecosystem functions are often difficult to interpret. Effects of pollutants on various parts of the ecosystem were intensively studied in 1980s and 1990s, and several studies revealed that plant-associated fungi, especially epiphytic fungi on the phyllosphere, were sensitive to air pollutants. Furthermore, endophytic fungi living asymptomatically in the leaf tissues were observed to respond negatively to sulphuric acid and heavy metal deposition. Some fungal endophyte species and strains in tree foliage have been observed to adapt to the toxic environment during long continuous pollution.

Abbreviations

DC	dry control
IC	irrigation control
MA	malt extract agar
E−	uninfected seeds
E+	endophyte infected seeds
ME−	manipulatively uninfected seeds
ME+	manipulatively endophyte-infected seeds

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1 Introduction

In addition to natural stresses limiting their growth and survival, plants and their associated microorganisms are facing a wide range of anthropogenic environmental changes. These include a wide array of chemicals deposited through air, soil, and water, having an in immediate contact with organisms in nature. Pollutants are defined as any material present in the environment in such abnormally high concentration, that it causes alteration of the biochemical or physiological state of organisms with which it comes into contact (Hughes 1988). The most common pollutants include ozone, sulfur dioxide, nitrogen oxides, fluorides and heavy metals. Of these, e.g. heavy metals can be detrimental for living organisms in relatively low concentrations, even if some of them are necessary as trace elements (Barcelo and Poschenrieder 1990). Unlike many other abiotic stresses that living organisms have to deal with, such as occasional drought or anomalous weather, pollution often represents a continuous source of strain. Because pollution is often such a long-lasting abiotic factor, certain adaptations are likely to develop in the exposed organisms.

The long-term effects of air pollutants to plants can comprise either morphological or physiological responses, which differ between species and populations depending on the environment (Grime and Campbell 1991; Cape 1993). In some areas of evident damage caused by pollutants, e.g. in the surroundings of metal smelters, damage in the vegetation can clearly be seen, for example when trees have lost needles, leaves and branches, or the frequency of certain plant species has decreased, or some species have disappeared. However, such direct effects of pollutants on plant performance are unlikely to be of major importance in large areas that experience low or moderate levels of anthropogenic emissions. Instead, the effects of air pollutants may rather take place indirectly e.g. through soil processes, such as disturbances in decomposition and nutrient cycling. The most complicated and elusive effects of anthropogenic pollutants may be realized through changes in multitrophic interactions of organisms. For example, environmental pollution may decrease the number of susceptible species and open new windows for resistant species, leading to imbalance in interactions among plants and associated organisms. Thus, changes caused by pollutants in assemblages and interactions among plant associated microorganisms in roots (mycorrhizal) or foliage (epiphytic, endophytic and pathogenic foliar fungi) may lead to herbivore and plant pathogen outbreaks in large areas. The balance between epiphytic, endophytic and pathogenic microfungi in plant foliage may be of crucial importance when causes of plant pathogen epidemics are investigated. A host-endophyte system exposed to pollution is affected simultaneously through numerous pathways, and their relative importance is difficult to identify precisely. Various pollutants have the potential to modify fungal interactions in plant foliage (Ranta et al. 1995), and thus to contribute to pathogen epidemics in host plant populations, which in turn may alter whole plant communities by altering the competitiveness of diseased plant communities. In agriculture and forestry, these epidemics, in turn, may cause huge economic costs.

Although environmental pollution impacts the structure, function and interactions on all levels of the ecosystem, research has mainly focused on the direct effects of pollution on economically important species. Direct effects of pollutants on organisms are feasible to study, but impacts of air pollution on more complicated ecosystem functions are often difficult to interpret. Many plant associated microorganisms, e.g. endophytic fungi, potentially modify all types of plant-plant, plant-herbivore and plant-pathogen interactions (Ahlholm et al. 2002; Saikkonen et al. 1998, 2004). Endophytic fungi are here referred to as fungi that live the entire or at least a significant part of their life cycle asymptomatically inside plant tissues (Saikkonen et al. 1998, 2004). The endophytic flora thus consists of organisms with variable ecological functions, some endophytes being latent pathogens, some epiphytes having an endophytic phase. Fungi living endophytically within the host tissue frequently include species that are closely related to the pathogens of the host (Sherwood-Pike et al. 1986), or species that themselves are considered important pathogens (Espinosa-Garcia and Langenheim 1990). Some asymptomatic endophytes have probably even evolved from plant pathogens (Freeman and Rodriguez 1993). Thus, the role of endophytic fungi associated with woody perennials can be complex and labile both in ecological and evolutionary time (Saikkonen 2007). Horizontally transmitted fungal endophytes of plants are dispersed by spores from plant to plant, usually causing highly restricted local infections. Successful endophyte infection depends on the exposure of plant foliage to fungal inocula. Thus, infection frequencies tend to be higher in dense and closed host stands compared to open ones (Petrini and Carroll 1981; Legault et al. 1989; Helander et al. 1993a,b, 2006). In deciduous trees the unfolded leaves are gradually infected by endophytes, in such a way that late in season the leaves may harbor an assemblage of endophytic fungi from various genotypes, species, and genera (Lappalainen and Yli-Mattila 1999).

In addition to horizontally transmitted endophytes of single-spore origin, many pooid grasses host also systemic (i.e. growing throughout the plant) endophytes belonging to the family Clavicipitaceae (genera *Epichloë/Neotyphodium*) (Clay 1990), which transmit vertically via plant seeds. In this highly integrated symbiosis, hyphae grow intercellularly and asymptomatically throughout the above-ground tissues of the host grass. While growing into the developing inflorescence and seeds, the systemically growing fungus is vertically transmitted from maternal plant to offspring. The symbiosis between endophytes and grasses is generally considered to be a classic example of microbe-plant mutualism (Clay 1990; Schardl and Clay 2002). The close link between fitness of the endophyte and its host grass is presumed to align the interests of both partners towards a mutually beneficial cooperation, a view that seems to be supported by empirical evidence, especially in nutrient-rich agronomic environments (Saikkonen et al. 1998, 2004, 2006, 2010). Endophyte-infected (E+) grasses have improved growth, reproduction and resistance/tolerance against different abiotic and biotic stresses, e.g. drought, herbivores and pathogens, compared to endophyte free (E−) plants (Marks et al. 1991; Gwinn and Gavin 1992; Elbersen and West 1996). The prevalent explanation of endophyte protection (defensive mutualism) of host plants is based on secondary metabolites (alkaloids)

with antiherbivore properties produced by the symbiotic association between the host plant and its endophytes (Clay 2009). Systemic endophytes of grasses occur frequently in variable environments from the agronomic arena (Clay 1990; Saikkonen et al. 2004, 2006) to the subarctic (Saikkonen et al. 2002; Wäli et al. 2007). Thus, the symbiosis is likely to encounter pollution pressure in some environments.

2 Acid Rain Decreases Foliar Fungi in Woody Plants

Sulphuric and nitric acid, the main acidifying agents in precipitation, are formed by the oxidation of both natural and manmade emissions, the latter originating mainly from the combustion of fossil fuels. The acidic pollutants are readily transported by air masses, and their effects can be seen over extensive rural areas, in addition to areas in close vicinity of the sources (Irwin and Williams 1988). During the last decades, however, the overall sulfur emission in the world has significantly decreased (Lehmann et al. 2008).

Abundantly occurring epiphytic microfungi inhabit all surfaces of the above ground parts of plants and are directly exposed to abiotic factors including acid rain. Since the epiphytic life stage of the fungus is extremely vulnerable to the direct effects of air pollution, responses to pollutants as well as to other abiotic changes are rapidly detected in epiphytic fungal communities (Helander and Rantio-Lehtimäki 1990; Helander et al. 1993b). Occasionally some endophytes have a prolonged epiphytic life stage or they may antagonistically interact with phyllosphere epiphytes before entering the plant foliage. This happens when the spores of horizontally transmitted endophytes are released e.g. from fallen leaves, needles, or other plant parts and are further dispersed by the wind, rain splashes or insects onto the living surfaces of suitable host plants. There, the spores can germinate and enter the host plant. In addition, some commonly occurring epiphyllous microfungi, e.g. *Aureobasidium pullulans*, are occasionally observed to grow endophytically (Albrectsen et al. 2010). Acid rain is likely to effect endophytes in the sporulating, dispersal and germinating phases when the fungus is not protected by plant cells and is potentially fully exposed to pollutants.

Epiphytic microfungi respond to abiotic changes within days or possibly already within hours (Helander and Rantio-Lehtimäki 1990), because they are directly exposed to pollutants. Their use as bioindicators has been discussed (Richardson et al. 1985), but unfortunately they also respond readily to all other abiotic factors such as the microclimate and weather changes. Careful sampling and an enormous number of samples would be required to obtain reliable results about the effects of pollutants on epiphytic microfungi under natural conditions.

Several studies have revealed that acid rain decreases the frequency of horizontally transmitted fungal endophytes in tree foliage (Helander et al. 1993a,b, 1994; Helander 1995; Asai et al. 1998). Fungal endophyte infections in plant foliage tend to decrease if the plant is exposed to sulfuric and combined sulfuric and nitric acid treatments, while nitric acid alone does not seem to affect endophyte infections

(Helander et al. 1993a, b, 1994). In an experimental field study the effects of the sulfuric acid treatment were evident when trees had been exposed to moderate levels of acid rain for seven to eight growing seasons (Helander et al. 1993a,b, 1994) while shorter pollution history (3 years) did not significantly affect foliar endophyte frequencies (Helander 1995).

The surface morphology of plants may also be modified by acid rain, facilitating the entry of fungal infections into the plant tissue. Many fungal species use topographic signals on the plant surface in entering their host plant (Allen et al. 1991). Although morphological changes in the phyllosphere caused by air pollutants have not been observed in broad leaved species (Adams et al. 1984), in coniferous trees they are very well documented (Barnes and Brown 1990; Bäck et al. 1994). Due to their longevity, the needles of boreal conifers encounter exposure to pollutants over several years. In subarctic northern Scandinavia the needles of Scots pine (*Pinus sylvestris* L.) commonly have 5–7 year classes (Helander et al. 1994). The surface of needles is the interface between the plant and the atmosphere and exposed directly to the solutes in precipitation. Unlike pollutant gases, which enter plant tissues through the stomata, ionic pollutants are generally excluded from entering the leaves of higher plants by a water-proof waxy cuticle (Cape 1993). If the protective leaf surface is damaged, e.g. when acid deposition degrades the wax layer (Bäck et al. 1994), this could either facilitate the penetration of the endophytic fungus into the needle or prevent the ability of the fungus to recognize the host plant.

The indirect effects of acid rain on endophytic foliar fungi are presumably seen through changes in the host plant when, for example, acid rain changes the nutrition content of the soil and leads to the accumulation of hazardous ions. Pollutants may also decrease the decomposition rate of plant litter and thus affect soil nutrient cycling (Vorobeichik 2002). Detrimental effects of acid deposition on plant growth have been detected especially when sulfuric acid has been involved as a pollutant (Sheppard et al. 1993), whereas the fertilizing effect of N deposition is mentioned as a possible cause of increased plant growth (Kauppi et al. 1992). These indirect processes of pollutants can lead to physiological changes in the host plant, modify plant resistance to endophytic and pathogenic fungi, and affect the plant-microbe interactions.

3 Heavy Metal Pollution and Fungal Endophytes

Toxic metals in nature affect fungal populations by reducing species abundance and diversity and by selecting resistant/tolerant strains (Gadd 1993; Helander 1995; Ruotsalainen and Kozlov 2006). Heavy metal pollution has been shown to decrease the number of endophyte infections and change diversity and species composition of fungi in plants (Bewley 1980; Bewley and Campbell 1980; Helander 1995; Ruotsalainen et al. 2007). Long-term heavy metal pollution along gradients near metal smelters in Russia and Finland showed a clear positive correlation between

increased distance from the pollution source and the endophyte infection frequency of Scots pine needles (Helander 1995).

Different endophyte species may also respond differently to heavy metal pollution. Whereas the total number of endophyte-infected pine needles was not affected by simulated copper and nickel treatments in a field experiment (Helander 1995), the number of needles infected by the common pine needle endophyte, *Hormonema* sp., was reduced by 80%. Especially needle nickel content was strongly negatively correlated with the number of *Hormonema* infections. Interestingly, the infection rates of *Hormonema* sp. endophyte were not significantly correlated with the distance from a copper-nickel smelter in southern Finland (Helander 1995), but in laboratory experiments *Hormonema* strains isolated from samples near the smelter had higher tolerance to copper and nickel compared to strains isolated from less polluted areas. This indicates that the *Hormonema* strains close to the smelter have adapted to their toxic environment during the 50 years of continuous pollution (Helander 1995). Thus, if heavy metal pollution is an occasional and unpredictable phenomenon in a given environment, the endophyte community is sensitive to subsequent stress caused by pollution. However, if heavy metal pollution continues over several endophyte generations, selection towards heavy metal tolerant strains may take place in that environment.

4 Empirical Studies and Sampling

The effects of anthropogenic activities and associated pollution on micro- and macro-organisms are frequently compared between urban and rural environments (Jumpponen and Jones 2010). However, interpretation of causal links in correlative studies is often difficult, e.g. urban communities are usually distinct from the surrounding area and characterized by a high frequency of exotic species (Kareiva et al. 2007). Furthermore, the observed contrasts in the frequency and diversity of organisms between rural and urban environments are caused by a combination of interlinked and correlated drivers including land management and the selection of plants and animals (McKinney 2002; Jumpponen and Jones 2010). Thus, urban microbial communities are directly and indirectly affected by human land management by e.g. the removal of plant litter, which inhibits the inocula of endophytes from entering their host plants (Jumpponen and Jones 2010).

Point polluters, including smelters, power plants and other industrial constructions, have served as models for studying the effects of air pollution on the biota. In these isolated pollution sources the effects pollution can more easily be associated with either the distance from the polluter, or deposition levels, compared with large-scale gradients or urban areas (Ruotsalainen and Kozlov 2006). However, the gradient approach analysis of pollution effects on the biota has to be carefully planned and replicated, and the interpretation of the results should reflect other possible environmental variables besides the study organism that might be correlated with and affected by the pollutants. For example, the plant composition

of the pollution gradient may change and thus restrict the available fungal inocula of horizontally transmitted endophytes. In addition, forests are often exposed to a wide array of co-occurring pollutants derived from different sources, which makes it difficult to differentiate among particular types of pollutants and associated effects.

Controlled, well-replicated, long-term field experiments would be an ideal set-up to study the effects of pollution on plant, animal and fungal communities and their interactions with other species. Changes associated with air pollution are often slow or may take place gradually according to the different phases of pollution. This poses a major challenge on the experimental approach with respect to optimization of realistic and biologically rational manipulations with a sufficient duration, to test the proposed research question. The appropriate length of experimental manipulation obviously depends on the system and on the hypothesis being tested.

The following case study represents one of the few long-term experimental studies designed to unravel the various effects of pollution on the ecosystem. In this particular study, both woody plants and grasses were used as host-endophyte model systems to elucidate global, anthropogenic environmental changes, which may indirectly affect ecosystem functions via ubiquitous microbial communities.

5 A Case Study: Long-Term Effects of Pollution on Endophytes

The purpose of this case study was to investigate long-term effects of simulated acid rain, heavy metal pollution and their combination on (1) the frequency and species composition of horizontally transmitted endophytes and (2) the germination and seedling survival of a vertically (via seeds) transmitted endophyte- grass combination. We chose Scots pine (*Pinus sylvestris* L.) as the model host plant species for horizontally transmitted endophytes, because the needle age of Scots pine varies between 5 and 7 years, which enables the study of cumulative effects of air pollution on needle endophytes. Red fescue (*Festuca rubra* L.) is a globally spread naturally occurring perennial bunch grass which is also cultivated in amenity lawns and road sides. We used red fescue as the model species for vertically transmitted endophytes, because it is commonly infected by systemic endophytic fungi (*Epichloë festucae* Leuchtm., Schardl & Siegel) (Wäli et al. 2007; Saikkonen et al. 2010).

5.1 Study Area

The acid rain and heavy metal simulation experiment was conducted within the northern boreal zone (Hämet-Ahti 1981) near the Kevo Subarctic Research Station in the Finnish Lapland (69° 45N, 27° 00E). Some of the northernmost pine stands in the world can be found in the local river valleys of Kevo area, although these stands

are located about 60 km north of the continuous pine forest. The soil is mostly poor podzol and the growing season lasts from June to August (Hinneri 1974). The mean annual precipitation of the study area was ca. 390 mm (30–40% as snow), and the mean annual temperature was −2°C (minimum −40°C) during the study period (1991–2009). Because of the exceptionally low background pollution values, this area is ideal for experimental air pollution studies (Laurila et al. 1991).

5.2 Experimental Setup

Experimental plots (5 m × 5 m in size) with at least one pine (*Pinus sylvestris* L.) and one mountain birch (*Betula pubescens* var. *czerepanovii* (N.I. Orlova) Hämet-Ahti) in each plot were established in the research area in 1991. Experimental plots (total 25) were grouped into five blocks and within each block the plots were randomly assigned into five treatments: dry control (DC) receiving only natural rain, irrigation control (IC) treated with lake water of pH 5, acid rain treatment (pH3) sprinkled with lake water with added sulfuric acid, heavy metal treatment (CuNi) with copper and nickel sulfates; 0.83 and 0.50 mg m^{-2} in each irrigation, respectively, and combined heavy metal and acid rain treatment (pH3 + CuNi). Sprinkler irrigation simulating acid rain was applied over the tree canopies and ground of the experimental plots three times a week during the growing seasons for 18 years, to provide the equivalent of 5 mm of rain on each treatment day. Such quantities of pollutants are deposited in the surroundings (40–50 km from the source) of the Severonickel smelter complex in Monchegorsk, Kola peninsula, Russia (Jevtjugina 1990). The treatment solutions were applied with sprinklers both to the tree canopy and to the ground. The rainfall was doubled by the water irrigations during the growing season.

5.3 Sampling

The pines were sampled in the beginning of August 2008. Twenty three-year-old needles were randomly taken from the height of 1.5 m (total 500 needles) and processed in a laboratory during the sampling day. The needles were surface sterilized from epiphytic microbes in a laminar hood with 75% ethanol (30 s), 4% Na-hypochlorite (1 min), and again with 75% ethanol (15 s). After air drying for 5 min, the needles were cut into five pieces of equal size with a sterile scalpel and placed in 90 mm Petri dishes in 2% malt extract agar (MA). Fungal colonies growing out of the needles were identified and counted both 1 month and 3 months after the sampling using morphological characteristics and ITS (internal transcribed spacer) sequencing.

Red fescue seeds were collected from wild populations in the municipality of Utsjoki close to the experimental area. The endophyte infection status of mother plants was checked by staining (Saha et al. 1988) and by microscopic examination

of three seeds per plant individual. If the plant is infected by a systemic endophyte, the infection is transmitted to practically all developing seeds of the mother plant. In June 2008, four types of seeds were sown to each of the 25 experimental plots: uninfected seeds (E−), endophyte infected seeds (E+), manipulatively uninfected seeds (ME−) from which endophyte infection had been removed by heat treatment (Saikkonen et al. 2010) and manipulatively endophyte-infected seeds (ME+) which were originally uninfected seeds and then were artificially infected by endophytes (Saikkonen et al. 2010). All vegetation was removed from an area of a 0.5 m × 0.5 m at each experimental plot, which was then divided into four equal squares. 25 seeds were randomly sown from one of the seed treatment bulks (E−, E+, ME−, ME+) to each square. The germination and survival of the seeds was analyzed in August 2008 and in June and August 2009.

5.4 Statistical Analyses

Total number of fungal infections (colony forming units/needle) and the number of *Lophodermium* sp. in pine needles were statistically analyzed. The data on other identified fungi was not sufficient for the statistical analyses. All analyses were performed with SAS statistical software (SAS Institute Inc. version 9.1). The variables were transformed (log + 1) to normalize the distributions. The analysis of variance (Proc Mixed, SAS Institute Inc. 2004) was used to test differences between the treatments in experimental field study plots. Block was used as a random variable, and pairwise comparisons were conducted with Tukey's test.

5.5 Results

5.5.1 Pine

From pine needles, *Lophodermium* sp., *Pezizales* sp., *Cenangium ferruginosum* Fr.: Fr., *Hormonema* sp., and some unidentified or sterile fungi were isolated. The needles harbored on average 0.9 endophyte infections, and 46% of the infections consisted of *Lophodermium* sp. When the total number of infections was analysed, both the acid rain treatment (pH3) and the combined heavy metal and acid rain treatment (CuNi + pH3) differed statistically from the other treatments ($df = 4$, $F = 6.31$, $p > 0.0001$), in such a way that there were significantly less infections (colony forming units/needle) in these treatments than in dry control (DC), irrigation control (IC) or heavy metal treatment (CuNi) (Fig. 1). In the analysis on *Lophodermium* sp., the needles of the dry control and heavy-metal treatments had significantly more infections than those of the other treatments ($df = 4$, $F = 3.99$, $p > 0.0034$) (Fig. 1).

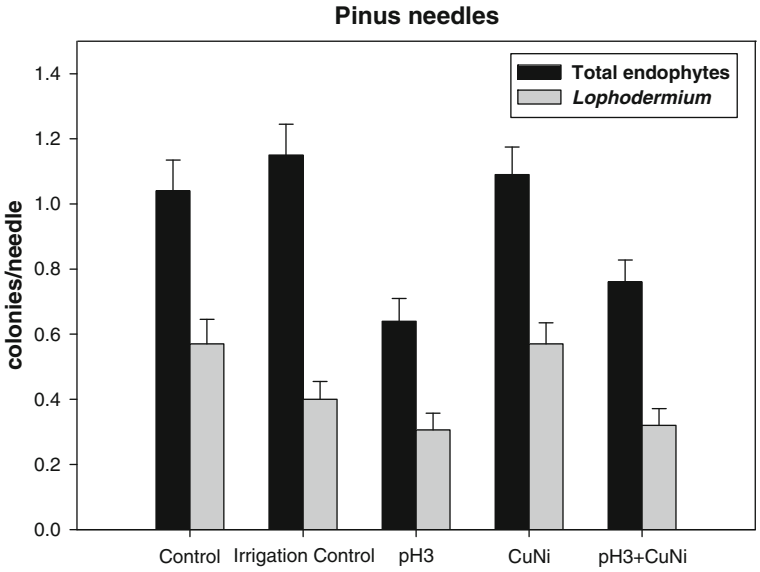

Fig. 1 Effects of pollution treatments on pine needle endophytes

5.5.2 Grasses

A month after sowing, 27% of the *Festuca rubra* seeds had germinated and 16% of the seedlings were still alive the following summer. It is noteworthy that the seedbank of *F. rubra* is practically non-existent (Wäli et al. 2009). Seed germination in June 2008 (Fig. 2) did not differ between the endophyte infection statuses ($df = 3$, $F = 0.36$, $p > 0.4055$), pollution treatments ($df = 4$, $F = 1.43$, $p > 0.2308$), or their interaction ($df = 12$, $F = 1.06$, $p > 0.4055$). However, the survival of the germinated seedlings in August 2008 and June 2009 was only 6–7% in pH3 and pH3 + CuNi treated plots, while in control and CuNi treated plots 20–25% of the seedlings survived (Fig. 2) (August 2008: $df = 4$, $F = 6.59$, $p > 0.0001$ and August 2009 $df = 4$, $F = 16.18$, $p > 0.0001$).

6 Conclusions

The present experiment in the subarctic ecosystem clearly indicated that sulphuric acid precipitation (pH3) had significant effects on the examined organisms, whereas they appeared insensitive to nearly 20 years of heavy metal exposure. Acidification decreased seed germination and seedling survival of the grasses, and also reduced the total frequency of horizontally transmitted needle endophytes in Scots pine needles. The endophyte status of grass seeds, however, had no influence on seedling

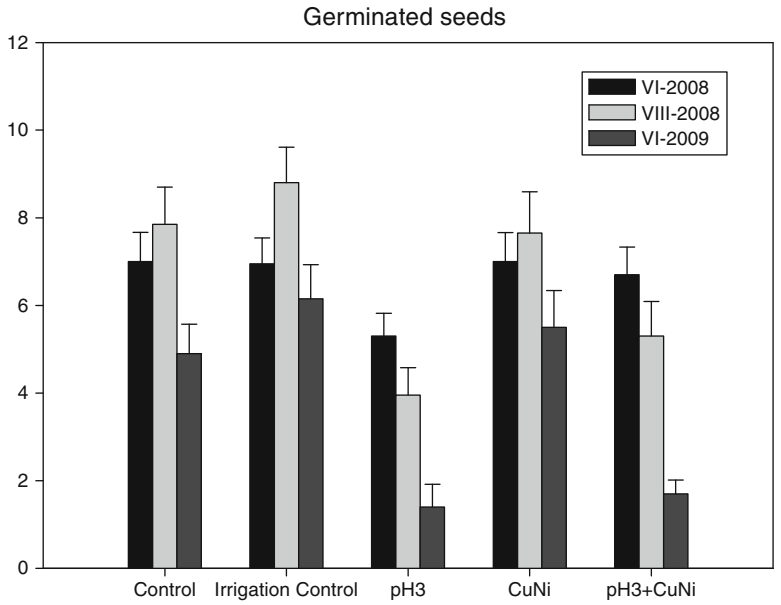

Fig. 2 Effects of pollution treatments on *Festuca rubra* seedling survival in June 2008, August 2008 and June 2009

establishment. Similar results have been obtained in previous studies (Helander et al. 1993a, b, 1994; Helander 1995; Asai et al. 1998).

The low responses of endophytic fungi and grass seeds to heavy metals might result from accumulation of heavy metals to soil in an insoluble form, or from the ability of the organisms to adapt to them. The most commonly isolated pine needle endopyte, *Lophodermium* sp., was found at equal frequency in control and heavy-metal treated needles, while the trees on acid-treatment plots had significantly less infections by *Lophodermium* sp. endophytes. This result possibly indicates that during a long-term experimental heavy metal treatment, fungal endophytes can adapt to the higher environmental copper and nickel levels. A similar adaptation of another fungal endophyte, *Hormonema* sp., has previously been described on pollution gradients around factory complexes (Helander 1995).

The *Festuca rubra* seeds used in the experiments were collected from environments with no previous exposure to acid precipitation. According to the described long-term field study, grass seedlings may encounter strong selection in an acid environment, and the surviving seedlings may be better adapted to such conditions. In an earlier study on tall fescue (*Festuca arundinacea)* the biomass of the grass was observed to decrease with increasing acidity, but this response was interactively dependent on the age of the plant and its endophyte status (Cheplick 1993). It was found that acid rain may be deleterious to tall fescue growth at specific stages of development, but biomass production in response to acid rain is not likely to be influenced by fungal endophytes.

The majority of previous studies on air pollution and endophytes have concentrated on the endophytes of woody plants, reflecting the fact that air pollution was considered to cause forest decline in the in the 80s, whereas at that time, grass endophytes were studied primarily in the context of agriculture. Starting in the early '90s' the scientific community has shifted its focus from pollution research to biodiversity and climate change studies, leaving the effect of air pollution on grass endophytes an understudied area.

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Dark Septate Endophytes and Mycorrhizal Fungi of Trees Affected by Pollution

Matevž Likar

Abstract Microorganisms are involved in metal biogeochemistry through a variety of processes that promote the bioavailability and uptake of metals and minerals by plants. Among the microorganisms that have the most intimate relationships with plants are mycorrhizal fungi and other fungal endophytes, like dark septate endophytes. These microorganisms populate the rhizosphere and plant roots. Many endophytic fungi can survive in high concentrations of toxic metals, and can adapt to metal stress, resulting in tolerant genotypes. Furthermore, fungal endophytes have been shown to ameliorate metal toxicity for their plant hosts, by restricting the uptake of toxic metals and by improving the supply of essential elements. As effective metal phytoremediation strategies depend on the ability of the plant to tolerate and accumulate metals from the environment, the wide prevalence of endophytic fungi and their potential to modulate metal speciation, toxicity and mobility make them a key to any remediation efforts. Further studies of the diversity, biochemistry and interactions of fungal endophytes with plants will help to develop powerful phytoremediation applications in the future.

Abbreviations

AM	arbuscular mycorrhiza
EM	ectomycorrhizal fungi
DSEs	dark septate endophytes

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1 Introduction

With increasing pollution due to various human activities, ecosystems are being contaminated by many and various organic and inorganic pollutants. Migration of contaminants to non-contaminated sites as dust or leachates through the soil can contribute further towards contamination of ecosystems. Heavy metals form the main group of inorganic contaminants (Alloway 1990). The toxicity of excess metals affects plant metabolism, including synthesis of photosynthetic pigments due to the metal binding to protein sulphydryl groups (Van Assche and Clijsters 1990), and the direct destruction of photosynthetic pigments through the generation of highly active oxygen radicals (Pinto 2003). The resulting changes in the photosynthetic properties of plants can lead to severe physiological stress and changes in carbon allocation, with possible impact on the below-ground fungal communities and their association with the plant roots (Smith and Read 2008).

2 Mycorrhizal Fungi and Fungal Endophytes

The majority of terrestrial ecosystems are dominated by plants that form associations with mutualistic fungi like arbuscular mycorrhiza (AM), ectomycorrhizal (EM) fungi, and dark septate endophytes (DSEs) (Smith and Read 2008). The AM fungi comprise some 150 species of glomeromycetous fungi (Schüssler et al. 2001) and they are found associated with 80% of plant species (mostly herbaceous plants, but also various woody plant families). The EM fungi include about 6,000 species of ascomycetes and basidiomycetes (Smith and Read 2008), and they are confined chiefly to a limited number of woody plant families. Mycorrhizal fungi provide plants with mineral nutrients, especially phosphorus, in exchange for carbon compounds (Johnson et al. 2002; Bücking and Shachar-Hill 2005; Li et al. 2006), and they can protect their host plants against biotic and abiotic stress (Azcon-Aguilar and Barea 1996; Ruíz-Lozano 2003; Vogel-Mikuš et al. 2006).

In contrast to the vast knowledge on EM and AM fungi, DSEs are poorly known. The DSE fungi are frequent colonisers of plant roots under extreme environmental conditions (Read and Haselwandter 1981; Barrow 2003). Despite indications of potential importance of DSEs for plant growth and development (Fernando and Currah 1996; Mullen et al. 1998; Barrow 2003), little is known about their identity and ecology and effects on host plants (Jumpponen and Trappe 1998). Several DSE fungi have been shown to use the major forms of carbon, nitrogen and phosphorus that are commonly present in plant litter and detritus (Caldwell et al. 2000), and they might thus provide plants with nutrients from plant-inaccessible pools. While DSE isolates have been shown to promote the uptake of nitrogen and phosphorus into plants (Haselwandter and Read 1982; Jumpponen and Trappe 1998), their effects on overall plant biomass appear to depend on the individual host–symbiont combination and the soil nutrient status (Fernando and Currah 1996; Jumpponen

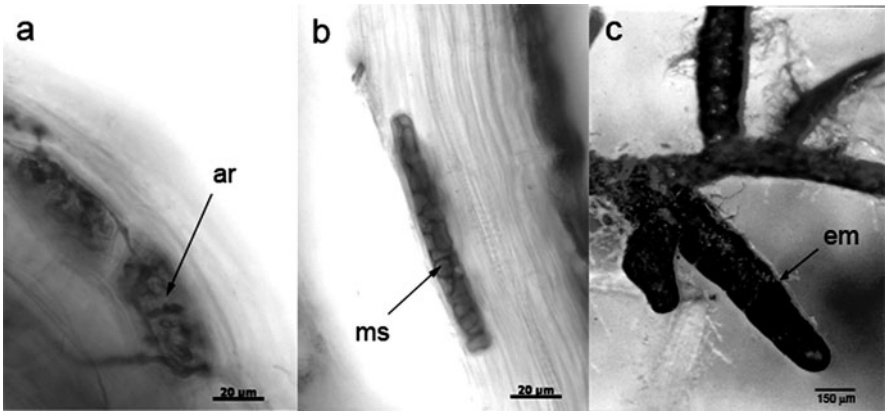

Fig. 1 Typical structures of fungal endophytes: (**a**) arbuscular mycorrhizal fungi, (**b**) dark septate endophytes, (**c**) ectomycorrhizal fungi on the roots of *S. caprea* shrubs from polluted and non-polluted sites. ar, arbuscules; ms, microsclerotia; em, ectomycorrhizal root tip

and Trappe 1998). Madyam and Jumpponen (2005) reviewed DSE abundance in different habitats and provided data on their possible roles in the ecosystems, and the diversity of DSE is discussed by Grünig and Sieber in this volume (ed. note).

2.1 Effects of Pollution on Fungal Endophytes – Colonisation

Extremely polluted sites are reported to have lower rates of mycorrhizal colonisation, fewer fungal propagules, and lower fungal species diversity (Gadd 1993; Hartley et al. 1997; Leyval et al. 1997; Markkola et al. 2002). However, distinct AM and EM structures (Fig. 1), and colonisation levels similar to *Salix* spp. growing on non-polluted sites (van der Heijden and Vosatka 1999; Trowbridge and Jumpponen 2004; Obase et al. 2007) were found on the roots of *Salix caprea* growing in heavy-metal enriched soils (Hrynkiewicz et al. 2008; Likar and Regvar 2009; Regvar et al. 2010). Similarly, some studies on AM have shown decreased colonisation of plants growing in metal enriched soils (Bi et al. 2003; Karlinski et al. 2010; Zarei et al. 2008), whereas others have reported only marginal effects of pollution on AM colonisation (Whitfield et al. 2004) and observed AM even in several metal hyperaccumator species (Hildebrandt et al. 1999; Turnau and Mesjacz-Przybylowicz 2003; Vogel-Mikuš et al. 2006). Furthermore, Hildebrandt et al. (1999) and Vogel-Mikuš et al. (2006) observed the highest levels of mycorrhization of *Viola* and *Thlaspi* species at highly contaminated sites. This would suggest little or no impact of metal pollution on the mycorrhizal colonisation of (tolerant) plants growing on metal-enriched soils.

Studies examining DSE often find good colonization by these fungi in plants growing in extreme habitats (Barrow 2003; Sonjak et al. 2009), including heavy-metal polluted sites (Ruotsalainen et al. 2007; Deram et al. 2008; Likar and Regvar 2009; Regvar et al. 2010). DSE seem to possess high tolerance to elevated metal conditions, as Ruotsalainen et al. (2007) reported that the DSE colonisation of *Deschampsia flexuosa* growing at a polluted site was not affected by the elevated metal concentrations, whereas DSE colonisation of *S. caprea* growing on a metal polluted site even showed a positive correlation with higher soil metal content (Likar and Regvar 2009). The high tolerance of DSE to metal pollution and their great abundance in stressful habitats suggest that DSEs may have an important function for host survival in these extreme ecosystems.

2.2 *Effects of Pollution on Fungal Endophytes – Diversity*

Due to increased selection pressure, a decreased diversity of fungal endophytes would be expected on metal-enriched sites (Gadd 1993; Hartley et al. 1997; Leyval et al. 1997). Indeed, some studies have shown a clear decrease in the diversity of mycorrhizal fungi with increasing pollution levels (Zarei et al. 2008; Zarei et al. 2010). In contrast, however, comparisons of different studies that have focused on *Quercus garryana*, *Pinus sylvestris* and *S. caprea* (Table 1) show very similar EM diversities between various growing sites, including those that are polluted with heavy metals. These observations suggest that the ability to tolerate conditions of metal-enriched soils is probably widespread among the EM fungi. Here, the selection pressure for higher metal tolerance would be the highest mostly for the early colonisers. Later, the build-up of litter and the biotransformation of the heavy metals will help to create microsites that are less exposed to the metal enrichment, thus allowing organisms less adapted to such extreme toxicity to become established (Ernst et al. 1990). The fast transformation of the high-metal microsites to microsites with more moderate concentrations of metals is probably one of the reasons for the absence of pronounced differences in the general diversity of fungal endophytes between polluted and non-polluted sites, observed in most studies.

Ascomycete EM communities on the roots of *S. caprea* growing on metal-enriched sites were dominated by Sordariaceae (Likar and Regvar 2009; Regvar et al. 2010), whereas EM basidiomycetes on those sites belonged mainly to Thelephoraceae and Cortinariaceae (Hrynkiewicz et al. 2008; Likar and Regvar 2009; Regvar et al. 2010). All observed fungal groups and their representatives were frequently reported to form EM associations with *Salix* species under a wide range of growing conditions (Trowbridge and Jumpponen 2004; Obase et al. 2007; Mühlmann and Peintner 2008). Similar trends were seen for other tree species colonised by a wide range of common EM basidiomycetes on metal-enriched soils (Krpata et al. 2008; Urban et al. 2008), which suggests that under the selection pressures of metal toxicity, the non-tolerant EM genotypes appear to be replaced

Table 1 Diversity of EM fungal species on the roots of *Q. garryana*, *P. sylvestris* and *S. caprea* growing on polluted and non-polluted sites

Site and plant species	No. of EM species (methods used)	Reference
Non-polluted		
Q. garryana	39 (root tips, RFLP)	Valentine et al. 2004
	42 (root tips, RFLP)	Moser et al. 2005
P. sylvestris	7 (soil DNA extraction, TRFLP)	Genney et al. 2006
	9 (morphotyping)	Markkola et al. 2002
	13 (soil DNA extraction, clonal library)	Smit et al. 2003
	11 (root tips, DGGE)	Landeweert et al. 2005
	14 (soil DNA extraction, DGGE)	
	33 (root tips, RFLP)	Jonsson et al. 1999
S. caprea	6 (root sample, TTGE)	Likar and Regvar 2009
Polluted		
Q. garryana	46 (root tips, RFLP)	Moser et al. 2005
P. sylvestris	9 (morphotyping)	Markkola et al. 2002
	18 (root tips, RFLP)	Urban et al. 2008
S. caprea	5 (root sample, TTGE)	Likar and Regvar 2009
	14 (root tips, direct sequencing)	Hrynkiewicz et al. 2008
	26 (root tips, RFLP)	Regvar et al. 2010

RFLP restriction fragment length polymorphism; *TRFLP* terminal restriction fragment length polymorphism; *DGGE* denaturing gradient gel electrophoresis; *TTGE* temporal temperature gradient electrophoresis

by the tolerant genotypes, rather than by new, and possibly more tolerant, fungal species. Furthermore, it appears that on non-polluted soils, metal-tolerant genotypes are not necessarily inferior to the non-tolerant genotypes, as has been shown for some suilloid fungi (Colpaert et al. 2004). As the tolerant genotypes may not become rapidly outcompeted by the non-tolerant genotypes or by other pioneer fungi on non-polluted soils, the metabolic burden of the fungal tolerance could be relatively low (Colpaert et al. 2004).

3 Mechanisms of Metal Tolerance in Endophytic Fungi

The various mechanisms behind the metal tolerance of fungi can be either extracellular (e.g. chelation, cell-wall binding) and/or intracellular (e.g. binding to non-protein thiols, compartmentation) detoxification mechanisms (Bellion et al. 2006). Binding to the cell wall, which is also known as biosorption (Gadd 1993), is a mechanism that does not depend on the metabolic activity of the fungus, and it results from a large number of cell-wall binding sites provided by free carboxyl, amino, hydroxyl, phosphate and mercapto groups (Strandberg et al. 1981). Binding of Cd to cell walls has been shown to represent a substantial fraction of the metal accumulation in *Paxillus involutus*, and this may also be one of the mechanisms by

which mycorrhizal fungi tolerate high levels of metals (Blaudez et al. 2000). The metal biosorption capacity and survival under such environmental stress can further be enhanced by the presence of melanins among the cell-wall components (Fogarty and Tobin 1996).

In addition to immobilisation in the cell wall, fungi have several other ways to resist toxicity when they are exposed to metals. These include immobilisation by formation of insoluble organo-metal complexes, solubilisation by heterotrophic leaching and siderophores, and production and release of metallothioneins or metallothionein-like peptides (Gadd 2004; Bellion et al. 2006). The most common form of precipitated organo-metal complexes in the soil environment are Ca oxalate crystals, and exudation of oxalate by fungi can also produce oxalate complexes with other metals, such as Cd and Al (Gadd 1999). Furthermore, heterotrophic metabolism can lead to leaching of the metals as a result of the efflux of organic acids and siderophores (Gadd 2004). Despite extracellular chelation and cell-wall binding, large quantities of metals might still enter the fungal cells. Thiol-containing metal-binding proteins known as metallothioneins are modulators of intracellular levels of metals in fungi (Howe et al. 1997). In addition, the role of glutathione as a metal chelator in fungi has clearly been established (Pocsi et al. 2004). Intracellular metallothioneins and metallothionein-like peptides are involved in homeostasis of essential trace metals and sequestration of toxic metals in fungal cells, thus limiting the progression of heavy-metal-initiated cell injury (Bellion et al. 2006).

Thus, as indicated above, although the fungal burden for tolerance to increased soil concentrations of heavy metals appears to be relatively low (Colpaert et al. 2004), some trade-offs are to be expected (Hartley et al. 1997). Indeed, Gonçalves et al. (2009) observed a trade-off between maximum specific growth rate (μ_{max}) and tolerance of *Cenococcum geophilum* to serpentine soil conditions: isolates from serpentine soils showed a significantly higher EC_{50} for Ni, although this was accompanied by a decreased μ_{max}.

4 Alleviation of Host Metal Toxicity by Symbiotic Fungi

4.1 Ectomycorrhizal Fungi

Heavy-metal toxicity provides strong selection pressure that can lead to the evolution of, or community shifts towards, specialized fungal genotypes (Markkola et al. 2002; Colpaert et al. 2004; Adriaensen et al. 2005; Gonçalves et al. 2009) that can then effectively alleviate the heavy metal toxicity for their host plants (Adriaensen et al. 2005; Adriaensen et al. 2006; Krznaric et al. 2009). This can be achieved by providing a more balanced access for the host plants to mineral elements, either by improving the supply of essential elements, or by reducing the relative uptake of the toxic elements (Marschner and Dell 1994). Evolutionary adaptation to heavy

metals is well documented for groups such as bacteria (Diels and Mergeay 1990) and animals (Levinton et al. 2003), and especially for plants (Baker et al. 1986; Schat and Verkleij 1998), but it is less studied in fungi.

There have, however, been several studies that have screened natural fungal populations for sensitive and insensitive (tolerant) genotypes. Differences in metal tolerance between populations from metal-enriched and non-polluted sites have been observed for several fungal species (Egerton-Warburton and Griffin 1995; Colpaert et al. 2004; Gonçalves et al. 2007; Gonçalves et al. 2009), but the genetic differences between the populations from metal-enriched and non-polluted sites have rarely been found (Panaccione et al. 2001; Gonçalves et al. 2007; Muller et al. 2007). Furthermore, the evolution of metal tolerance in fungi appears to be strongly specific for the metal that contaminates the soil (Colpaert et al. 2000; Adriaensen et al. 2005; Krznaric et al. 2009). Thus, Adriaensen et al. (2005) showed that *S. luteus* isolates from a Cu mine had high tolerance to Cu, but not to Zn, whereas Zn-tolerant isolates were shown to be Cu sensitive.

Knowledge of the roles of ectomycorrhizal fungi in the amelioration of heavy-metal toxicity in their hosts is still developing, but some studies on *S. luteus* provide good evidence on protective role of the EM against metal toxicity (Adriaensen et al. 2005; Krznaric et al. 2009). Still, both studies showed that only *S. luteus* isolates that exhibited tolerance to the high metal concentrations provided protection for the plant host. Similarly, the importance of metal tolerance of the plant host for the outcome of the symbiosis in conditions of elevated metal concentrations was demonstrated by Kayama et al. (2006). When *Picea glechnii*, a spruce species that is distributed widely on serpentine soils in northern Japan was inoculated and planted in metal-enriched soil, it maintained high levels of EM colonisation on this substrate, whereas EM colonisation was reduced in the non-tolerant *Picea abies* compared to the control soils (Kayama et al. 2006). This suggests that the adaptation to soils polluted with heavy metals depends on a combination of the (non)-tolerant plant host and the fungal endophyte and demonstrates the complexity of the responses of plant-fungus interactions to soil metal enrichment.

One possibility suggested is that the protection of the host is the result of the physical barrier formed by the fungal mantle (Dixon and Buschena 1988). Indeed, not only the fungal mantle, but the whole fungal biomass, including the sporophores, the extramatrical mycelium, and even the dead biomass can provide binding opportunities for the metals, and thus reduce their availability and toxicity for the host plants (Colpaert and Van Assche 1993). Comparisons of the Cd and Cu partitioning between different fractions in the rhizosphere of inoculated and non-inoculated pine seedlings showed a strong tendency for a change from loosely associated fractions to strongly associated fractions (Huang et al. 2008). In particular, the organically bound Cd fraction was significantly larger in the rhizosphere of the inoculated seedlings than in the rhizosphere of those not inoculated, thus indicating immobilization of the toxic metals by the mycorrhizal fungi.

4.2 Arbuscular Mycorrhizal Fungi

Similar to the EM fungi, induction of tolerance to increased heavy-metal content has also been seen for AM fungi. Amir et al. (2008) showed that isolates of AM fungi from ultramafic soils were clearly more tolerant to Ni than isolates from non-ultramafic soils. Several studies have reported high abundance of *Glomus mosseae* on polluted sites (Turnau et al. 2001; Vallino et al. 2006; Zarei et al. 2008), which suggests a certain adaptation of specific ecotypes of this species to a wide range of harsh conditions.

Colonisation of *Liriodendron tulipifera* by AM fungi has been shown to confer tolerance to Al, despite the increased Al uptake into the shoots and roots of the plants compared to non-mycorrhizal controls (Lux and Cumming 2001). Later on, Klugh and Cumming (2007) attributed the increased tolerance of AM-colonized *L. tulipifera* plants to facilitation of P acquisition and sustenance of high production of organic acids even at high Al concentrations, thus reducing the Al bioavailability. The down-regulation of plant genes involved in heavy-metal tolerance (Ouziad et al. 2005) and the up-regulation of stress-related AM fungal genes indicate that fungal heavy-metal tolerance mechanisms have at least a partial role in the increased heavy-metal tolerance of the mycorrhizal host (Hildebrandt et al. 2007).

Even for highly tolerant metal hyperaccumulator species, it appears that AM fungi can provide some benefits for the plant host. In mycorrhizal *Thlaspi praecox* plants, the concentrations of P were higher and those of Cd and Zn were lower than in non-mycorrhizal plants, indicating the selective advantage of the mycorrhizal plants in metal-enriched soils (Vogel-Mikuš et al. 2006).

4.3 Dark Septate Endophytes

Various reports of positive impacts of DSE colonisation on their plant hosts support the view that DSEs do indeed have a beneficial role for plant growth and survival (Fernando and Currah 1996; Mullen et al. 1998). DSE fungi have been found in great abundance on roots of plants from metal-enriched sites (Cevnik et al. 2000; Ruotsalainen et al. 2007; Likar and Regvar 2009; Regvar et al. 2010). This is probably the result of the greater heavy-metal tolerance of DSE fungi, due to the presence of melanin in their cell walls (Gadd 1993; Fogarty and Tobin 1996). Even though the interactions of DSE fungi and their hosts are often unclear (Jumpponen and Trappe 1998), enhanced host mineral nutrition has been shown for *Phialocephala fortinii* on *Salix glauca* L. (Fernando and Currah 1996). In addition, Mullen et al. (1998) proposed that nitrogen uptake can occur through DSE fungi early in the season before any root growth or AM colonisation takes place. Plant pioneer species that grow in stressful habitats can certainly benefit from symbiosis with more tolerant fungi, such as the DSEs.

5 Outlook

This chapter has focused on the recent evidence on an important role of fungal endophytes in survival of trees growing in metal-enriched soils. It appears that despite the high selective pressures exerted on trees growing in metal-enriched soils, the fungal endophyte communities of such trees do not differ dramatically from those of trees growing in non-polluted soils. This is probably the result of adaptive metal tolerance, as shown by several studies (Panaccione et al. 2001; Colpaert et al. 2004; Adriaensen et al. 2005). In particular for the mycorrhizal fungi, a potential to effectively alleviate heavy-metal toxicity with beneficial effects on the host plant has already been demonstrated (Marschner and Dell 1994), and as such, mycorrhizal fungi can be considered to have pivotal roles in any phytoremediation efforts (Gadd 2010).

While mycorrhizal functions in metal-enriched environments may be relatively well understood, very little is known about the function of DSEs. Their prevalence in extreme habitats and their alleged roles in protecting plants under abiotic stress (Haselwandter and Read 1982; Mullen et al. 1998; Ruotsalainen 2003) make studies on DSEs even more compelling. Furthermore, an interesting set of studies is emerging where a wider spectrum of interactions is being considered. Indeed, several studies have already addressed the effects of saprophytic fungi (Arriagada et al. 2009; Arriagada et al. 2010) and rhizosphere bacteria (Krupa and Kozdroj 2007; Kozdroj et al. 2007; Epelde et al. 2010) on interactions between fungal endophytes and plants growing on metalliferous soils. These studies highlight the need to consider fungus-bacteria and fungus-fungus interactions when studying mineral weathering and plant nutrition.

Further and more in-depth knowledge relating to these metal tolerance mechanisms in fungal endophytes and their potential positive effects on the survival of their plant hosts in metal-enriched soils will ultimately lead to powerful applications in bioremediation, as has already been indicated in several reviews (Khan et al. 2000; Bellion et al. 2006; Gadd 2010).

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Part IV
Bacterial Endophytes in Phytoremediation

Improved Phytoremediation of Organic Contaminants Through Engineering of Bacterial Endophytes of Trees*

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Abstract This chapter describes the possibilities of using engineered plant-associated endophytic bacteria to improve phytoremediation of organic contaminants by complementing the metabolic properties of their host plant. Analysis of the endophytic communities isolated from trees grown on groundwater contaminated with benzene, toluene, ethylbenzene, and xylene (BTEX) or trichloroethylene (TCE) revealed the presence of many strains able to degrade BTEX compounds or resist TCE. One would therefore expect that natural communities of endophytic bacteria can significantly contribute to the efficiency of the phytoremediation process. However, especially for the phytoremediation of TCE, *in situ* evapotranspiration measurements revealed that a significant amount of the contaminant and its metabolites evaporated to the atmosphere, pointing to a far from optimal situation.

An alternative proactive approach to natural enrichment is to inoculate plants with endophytic bacteria that are engineered to optimally metabolize the contaminant of interest, thereby improving the overall phytoremediation process. Examples

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of successful bioaugmentation to improve the phytoremediation of BTEX and TCE under greenhouse and field conditions are presented, and the possibilities to extend this concept to other contaminants are discussed.

Abbreviations

ACC	aminocyclopropane-1-carboxylate
TCE	trichloroethylene
BTEX	benzene, toluene, ethylbenzene and xylene
GRAS	Generally Recognized as Safe

1 Introduction

Conventional, primarily civil-engineering based physico-chemical methods to remediate contaminated soils and groundwater are frequently expensive, environmentally invasive, labor intensive, and do not make cost-effective use of existing resources. Among these techniques, the excavation and removal of contaminated soil, pump and treat of contaminated groundwater, or the *ex situ* treatment of contaminated soil are the most commonly applied. Especially for the remediation of large-scale contaminated areas biological remediation strategies can provide cost-effective, durable and validated alternatives, justifying the increased attention for their application in soil and groundwater remediation.

Generally, strategies making use of plant-microbe partnerships are classified under the general heading of phytoremediation. Phytoremediation is a low cost, *in situ*, solar powered technology that requires minimal site disturbance and maintenance, and which can be applied to clean up and/or stabilize inorganic as well as organic contaminants (Vangronsveld et al. 2009). Although very promising at first sight, phytoremediation can be limited by several factors such as depth of the contaminant, phytotoxicity caused by the contaminants, poor soil quality and inhibition of plant growth and establishment due to stress caused by the presence of contaminants. Furthermore, phytoremediation of highly water-soluble and volatile organic xenobiotic compounds, such as MtBE, benzene, toluene, ethylbenzene, and xylene (BTEX compounds) or trichloroethylene (TCE) that are rapidly taken up by plants, is often limited due to insufficient degradation of the pollutants by rhizosphere organisms, as a result of insufficient contact time, and by a lack in metabolic capacity of the plant to deal with high levels of these contaminants (Burken and Schnoor 1996; Burken and Schnoor 1999; van der Lelie et al. 2001; Schwitzguebel et al. 2002; Ma and Burken 2003; Weyens et al. 2009b; Weyens et al. 2009a). This often results in phytotoxicity, the accumulation of intermediate degradation products and the release of contaminants back into the environment via evapotranspiration, potentially resulting in new environmental problems (Trapp et al. 2000). Especially in trees

such as various cultivars of poplar, which are frequently being used for the phytoremediation of organic contaminants in groundwater, the time between uptake of molecules by the roots and arrival of the molecules in the leaves can be several hours to days as the compounds travel through the vascular system (McCrady et al. 1987). It seems reasonable to hypothesize that endophytic bacteria residing in the xylem, possessing the genetic information required for efficient degradation of a pollutant, can promote degradation as the pollutant moves through the plant vascular system.

This chapter describes the possibilities of using engineered plant-associated endophytic bacteria to improve phytoremediation of organic contaminants by complementing the metabolic properties of their host plant. Although engineered endophytic bacteria have also been tested to improve the phytoremediation of heavy metals (Lodewyckx et al. 2001) this work is still in its infancy and will not be reviewed in this chapter.

2 Commonly Found Genera of Cultivable Endophytic Bacteria

Endophytic bacteria colonize internal plant tissues of healthy plants without causing visible external signs of infection or disease (Misaghi and Donndelinger 1990; Sturz 1995). Since these endophytic bacteria can proliferate inside the plant tissue, they are likely to interact closely with their host, face less competition for nutrients, and are more protected from adverse changes in the environment than bacteria in the rhizosphere and phyllosphere (Reinhold-Hurek and Hurek 1998a, b). Furthermore, like rhizosphere bacteria, endophytic bacteria can have beneficial effects on their host plant and many efforts have been made to elucidate the direct and indirect mechanisms by which plant-associated bacteria enhance plant growth (Sharma et al. 2003; Glick 2004; Glick et al. 2007) as was the topic of various reviews (Mastretta et al. 2006; Doty 2008; van der Lelie et al. 2009; Weyens et al. 2009a, b). Direct plant growth promoting mechanisms may involve nitrogen fixation by free living endophytic bacteria (James 2000; Doty 2008), especially diazotrophs, the supply of unavailable nutrients such as phosphorus and other mineral nutrients, production of plant growth regulators such as auxins, cytokinins and gibberellines (Bent et al. 2001; de Salamone et al. 2001; Asghar et al. 2004), and suppression of ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Belimov et al. 2005; Dell'Amico et al. 2005), the latter being of special interest while growing plants on contaminated soils (Glick et al. 1998; Ma et al. 2002). Plant-associated bacteria can indirectly benefit the plant growth by preventing the growth or activity of plant pathogens through competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins and through induction of plant defence mechanisms (Kloepper et al. 2004). In general, free living bacteria usually do not rely on a single mechanism of plant growth promotion, and may involve more of the above listed individual mechanisms. The availability of both plant and endophytic genome sequences, such as those of poplar (Tuskan et al. 2006)

and the endophytic bacterium *Enterobacter* sp. 638 (Taghavi et al. 2010) allows a systems biology approach to better understand the mutual beneficial plant endophyte interactions, which can be further exploited for various applications including endophyte-enhanced phytoremediation.

Endophytic bacteria have been found in numerous plant species, and show a tremendous amount of diversity not only in plant hosts but also in bacterial taxa. Lists of host species with their associated endophytic bacterial diversity can be found in earlier reviews (Lodewyckx et al. 2002; Hallmann and Berg 2006; Bacon and Hinton 2007). Many of the bacterial isolates belong to the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Beattie 2007). In general *Pseudomonaceae*, *Burkholderiaceae*, and *Enterobacteriaceae* are among the most common genera of cultivable endophytic species found (Mastretta et al. 2006), which is important as many members of these genera have implicated in the remediation of various contaminants, and thus provide a resource for metabolic pathways that can be applied to engineer the metabolic capabilities of closely related endophytic strains. For instance, among the cultivable bacteria isolated from *P. trichocarpa* × *deltoides* trees that were used to remediate a benzene, toluene, ethylbenzene, and xylene (BTEX) contaminated groundwater plume, the gamma-Proteobacteria were found to dominate the collection, comprising 59% of the total bacteria, including 42% *Pseudomonas* species, with smaller percentage numbers of *Xanthomonas*, *Acinetobacter* and *Enterobacter* species representing the majority of the remainder of the gamma-Proteobacteria (Porteous Moore et al. 2006). The beta-Proteobacteria made up 18% of the isolates, with *Burkholderia* spp. (10%) and *Herbaspirillum* spp. (4%) representing the majority. The alpha-Proteobacteria and the Gram-positive bacteria comprised 13% and 10%, respectively, of the total number of isolates. Several of the isolated strains were able to metabolize BTEX compounds as sole carbon source, and grew in the presence of trichloroethylene (TCE), thus providing a natural resource for endophytic bacteria able to assist their host plant in the degradation of these contaminants. A dominance of the *Enterobacteriaceae*, including *Serratia* sp., *Serratia plymuthica*, *Serratia proteamaculans* and *Rahnella* sp., and *Pseudomonas* sp. was observed for endophytes isolated from hybrid poplar H11-11 (*Populus trichocarpa* × *P. deltoides*) and native willow (*Salix gooddingii*) that had been growing in the presence of carbon tetrachloride (Taghavi et al. 2009), while *Burkholderia* species were among the most frequently found endophytic bacteria among hybrid aspen (*Populus tremula* × *P. tremuloides*) seedlings (Yrjälä et al. 2010). The cultivable endophytic community isolated from English oak, grown on a TCE-contaminated site, was dominated by Actinobacteria (65.1%), with *Frigobacterium* spp. (45.0%) and *Okibacterium* spp. (13.0%) forming the majority of the group (Weyens et al. 2009c). Proteobacteria represented 23.1% and were dominated by gamma-Proteobacteria (17.9%) including *Pseudomonas* spp. (9%), *Xanthomonas* spp. (4.6%), *Enterobacter* spp. (3.4%) and *Erwinia* (0.8%). In contrast, *Pseudomonas* species were predominantly found (67.3%) in association with common ash, grown on the same TCE-contaminated site (Weyens et al. 2009c).

Based on these results one would expect that the presence of endophytic bacteria, several of which can degrade contaminants such as BTEX compounds and resist

TCE, would significantly contribute to the efficiency of the phytoremediation process. However, *in situ* evapotranspiration measurements revealed that a significant amount of TCE and its metabolites was evaporating through the leaves to the atmosphere (Weyens et al. 2009c, d), pointing to a far from optimal situation, especially for the phytoremediation of TCE.

3 Engineered Endophytes to Enhance Phytoremediation – Complementing the Plant's Metabolic Properties

Natural strains of endophytic bacteria have been identified that are capable to degrade the contaminants present in the environment of their host plant, and the presence of contaminants was found to result in a selective enrichment of endophytic bacteria naturally able to degrade them (McGuinness and Dowling 2009). For instance, Siciliano et al. (2001) were the first to describe that plants have the ability to selectively enhance the prevalence of endophyte bacteria that contain the catabolic pathways for the degradation of specific pollutants present in the soil environment. This process is both plant- and contaminant dependent. Analysis of the cultivable microbial communities associated with poplar growing on a BTEX contaminated ground water plume also demonstrated that, once the poplar roots get in contact with the BTEX contaminated groundwater, rhizosphere and endophytic bacteria able to degrade toluene are enriched (Barac et al. 2009). Interestingly, once the BTEX plume was remediated, the numbers of cultivable toluene degrading rhizosphere and endophytic bacteria decreased below the detection limit, indicating that their population increase resulted from selective enrichment due to the presence of the contaminants, and that this process was reversible once the contaminant was remediated.

An alternative proactive approach to natural enrichment is to inoculate plants with endophytic bacteria that are engineered to optimally metabolize the contaminant of interest. This approach seems to be of relevance as the presence of natural strains of degrader endophytes in the environment often seems to be insufficient for the efficient remediation of contaminants, especially BTEX compounds, TCE and other volatile and water soluble organic solvents under field conditions (Ma and Burken 2003; Weyens et al. 2009c, d). Barac et al. (2004) were the first to show proof of concept that phytoremediation of volatile and water-soluble organic contaminants could be improved by using recombinant endophytic bacteria modified to contain the appropriate degradation pathway. The endophytic bacterium *Burkholderia cepacia* VM1330, equipped with the *tom* toluene degradation pathway, was able to reduce toluene phytotoxicity and evapotranspiration from its yellow lupine host plant. It should be noted that none of the endogenous endophytes found in association with yellow lupine were able to metabolize toluene.

Inoculation of poplar – one of the "trees of choice" for phytoremediation purposes (Schnoor et al. 1995) – with the engineered endophytic bacteria *B. cepacia* VM1468 (Taghavi et al. 2005) or *Pseudomonas putida* W619-TCE (Weyens et al. 2010) resulted in improved growth of the cuttings and a reduction

in phytotoxicity and evapotranspiration of toluene and TCE, respectively. In the case of the improved TCE degradation it should be noted that after inoculation with *P. putida* W619-TCE, no dichloroacetic acid and trichloroacetic acid, which represent typical intermediates of plant based TCE metabolism, could be detected (Weyens et al. 2010).

Germaine et al. (2006) described the inoculation of pea (*Pisum sativum*) with a genetically tagged bacterial endophyte that naturally possesses the ability to degrade 2,4-dichlorophenoxyacetic acid. Inoculated plants showed a higher capacity for 2,4-dichlorophenoxyacetic acid removal from soil and showed no 2,4-dichlorophenoxyacetic acid accumulation in their aerial tissues. Inoculation of plants with the engineered, naphthalene-degrading endophytic *P. putida* strain VM1441 (pNAH7) resulted in plant protection against the phytotoxic effects of naphthalene (Germaine et al. 2009). When inoculated plants were exposed to naphthalene, both seed germination and plant transpiration rates were higher than those of the non-inoculated controls. The inoculation of plants with this strain also facilitated higher (40%) naphthalene degradation rates compared with non-inoculated plants in artificially contaminated soil.

Although application of engineered endophytic bacteria to improve phytoremediation of volatile organic contaminants has several obvious advantages over application of engineered rhizosphere bacteria or genetic engineering of the plant's metabolism, several obstacles have to be overcome before this technology can move towards full scale field application (Newman and Reynolds 2005; van der Lelie et al. 2005). The major concerns that need to be addressed include the persistence and stability of the engineered organisms and their degradation capabilities in field-grown plants, as phytoremediation projects can conceivably last for decades. As long as a selection pressure is present, there is an advantage for the endophytic community members that possess the appropriate degradation characteristics (Barac et al. 2009). However, this is no guarantee that strains of an inoculum will become an integrated part of the endogenous endophytic community.

Horizontal gene transfer has been shown to play an important role in allowing a microbial community to rapidly adapt to a new environmental stress (Dong et al. 1998; Ronchel et al. 2000), and it was speculated that it could play an important role in adapting the endogenous endophytic community (van der Lelie et al. 2005). Rather than integrating a new bacterium into a stable community, the degradation pathway is transferred among the members of the community. The importance of horizontal gene transfer in adapting the metabolic properties of the endophytic community was first observed when extending the concept of engineered BTEX degrading endophytic bacteria to poplar (Taghavi et al. 2005). Using *B. cepacia* VM1468, whose natural plant host is yellow lupine, successful protection of the inoculated host plant against toluene toxicity and a decrease in toluene evaporation by inoculated poplar were observed. However, when analyzing the microbial communities of poplar inoculated with *B. cepacia* strain VM1468, this strain was not identified among the cultivable strains, but instead it was observed that the strain had successfully transferred its toluene degradation pathway, encoded by the *tom* operon (Shields et al. 1995) on plasmid pTOM-Bu61, to members of

the endogenous plant-associated bacterial communities. Similar observations were made when the trees were inoculated with the rhizosphere strain *B. cepacia* Bu61 (pTOM-Bu61). Horizontal gene transfer of this plasmid to endogenous members of the endophytic community associated with poplar seems to be specific for plasmid pTOM-Bu61 and happened both in the presence and in the absence of toluene (Taghavi et al. 2005). Therefore it is hypothesized that this plasmid must either be highly transferable, or is providing an additional selective advantage to its recipient that is not solely linked to toluene metabolism.

4 Plasmid pTOM-BU61 Is an Important Tool for Improved BTEX and TCE Phytoremediation

Plasmid pTOM-Bu61 is a derivative of plasmid pTOM that, due to the insertion of an IS element, constitutively expresses the *tom* toluene degradation pathway (Shields and Reagin 1992), including the toluene-ortho-mono oxygenase gene *tomA*. As a result, toluene metabolism is being expressed even at concentrations that are below the induction threshold for the wild type *tom* operon. Furthermore TCE degradation, which is catalyzed by TomA (Shields et al. 1995) and whose expression normally depends on induction and co-metabolization of toluene, also becomes constitutive. So in contrast to endogenous toluene degrading endophytes, engineered endophytes that possess the pTOM-Bu61 plasmid will be able to assist their host plant with toluene degradation even when the concentration of the contaminant *in planta* it too low to induce the expression of naturally occurring toluene degradation pathways. In addition, TCE metabolism will no longer depend on the presence of other contaminants, such as toluene, which limit the application of natural endophytes for improved TCE phytoremediation.

The constitutive expression of the toluene-TCE degradation pathway might also allow its host to grow on other plant-synthesized phenolic compounds, such as precursors involved in lignin-biosynthesis, that normally do not induce the expression of this pathway, thereby providing an additional selective advantage to its recipient. This might explain why pTOM-Bu61 plasmid is successfully transferred and established among the endogenous members of the endophytic community, even in the absence of toluene selection (Taghavi et al. 2005).

5 Bioaugmentation with Engineered Endophytic Bacteria Improves Phytoremediation of TCE Under Field Conditions

As previously indicated, the presence of bacteria able to metabolize BTEX compounds and to resist elevated levels of TCE among members of the endogenous endophytic community of poplar was found in various instances to be insufficient

for efficient remediation, resulting in significant levels of contaminant release via evapotranspiration (Ma and Burken 2003; Weyens et al. 2009c). This prompted Weyens et al to introduce the pTOM-Bu61 plasmid into *P. putida* W619 and to use this engineered strain, W619-TCE, for bioaugmentation with poplar trees in order to improve TCE phytoremediation under field conditions (Weyens et al. 2009d). Since *P. putida* W619-TCE was engineered via horizontal gene transfer, its deliberate release is not restricted under European GMO regulations, paving the way for its application.

P. putida W619 was originally isolated as a root endophyte from poplar (Taghavi et al. 2005). The genome of this strain was sequenced (Taghavi et al. 2009) and found to be closely related to that of *P. putida* KT2440 (Timmis 2002; Dos Santos et al. 2004), a microorganism Generally Recognized as Safe (GRAS) certified for the cloning and expression of foreign genes. Other important properties of W619 include its resistance to various heavy metals such as nickel, copper, zinc, cadmium and arsenic, and synthesis of indole-3-acetic acid (IAA), a phytohormone that stimulates root growth and development. A detailed analysis of the *P. putida* W619 genome in function of its endophytic behaviour, and a comparison with the genome sequences of the closely related *P. putida* strains KT2440, F1 and GB-1 with emphasis on their specific niche adaptation was recently published (Wu et al. 2011).

The *in situ* rhizosphere inoculation of established poplar trees on a TCE-contaminated site with the TCE degrading strain *P. putida* W619-TCE turned out to be successful (Weyens et al. 2009d). Three months after inoculation, *P. putida* W619-TCE was successfully established and became the dominant member of the endogenous community in the roots of the inoculated trees. It is important to notice that *P. putida* W619-TCE was absent from the rhizosphere or other tree compartments. However, further horizontal gene transfer resulted in the distribution of the pTOM-Bu61 plasmid to a *Frigoribacterium* species that was present as an endogenous stem endophyte. The presence of the pTOM-Bu61 plasmid seemed to provide a selective advantage to its hosts. As compared to non-inoculated trees, *P. putida* W619-TCE successfully replaced an endogenous *P. putida* strain from the root endophytic community, while the relative abundance of the *Frigoribacterium* species significantly increased after it obtained the pTOM-Bu61 plasmid, this at the expense of the relative abundance of a *Curtobacterium* species (Weyens et al. 2009d).

Three months after inoculation, the *in situ* bioaugmentation with *P. putida* W619-TCE resulted in a 90% reduction of TCE evapotranspiration by poplar under field conditions (Weyens et al. 2009d). It was confirmed that this reduced TCE evapotranspiration was directly related to bioaugmentation with *P. putida* W619-TCE, as only the endophytic *Pseudomonas* sp. and *Frigoribacterium* sp. could constitutively degrade TCE with efficiencies similar to *P. putida* W619-TCE, and no TCE-degradation phenotype or pTOM-Bu61-like plasmids were found among the cultivable members of the different endophytic and rhizosphere communities of the non-inoculated poplar trees. However, the *Pseudomonas* sp. and *Frigoribacterium* sp. lost their TCE degradation capacity within 20 generations when cultivated

in the laboratory under non-selective conditions. Nevertheless, since the pTOM-Bu61 could also be transferred to the endogenous community present in poplar cuttings under non-selective conditions (Taghavi et al. 2005), it cannot be excluded that this plasmid will be maintained in endophytic bacteria actively colonizing their host plant, as it might provide additional selective advantages to its host bacterium.

6 Conclusions

The concept of engineered endophytes for remediation purposes is broadly applicable. Many endophytic strains are closely related to environmental strains that carry mobile DNA elements with degradation pathways for a broad spectrum of organic contaminants. Hence, it may be relatively straightforward to construct, via conjugation, non-GMO endophytic bacteria with *a la carte* degradation properties. However, it is important to understand the genetic potential of the endophytic strain, as endophytic bacteria can be closely related to opportunistic pathogenic bacteria, as was demonstrated for *Stenotrophomonas maltophilia* R551-3 (Ryan et al. 2009). Similar problems and consequently public and regulatory resistance against their deliberate release will exist against the application of *Burkholderia* species, several of which were isolated as opportunistic pathogens (Valvano 2006).

Engineered endophytic bacteria to improve the phytoremediation of organic contaminants can be applied via two strategies or a combination thereof: the successful establishment of an engineered endophytic strain among the endogenous community; or in case of plasmid encoded properties, the successful distribution of the plasmid among members of the endogenous endophytic community. In both cases the bioaugmentation strategy requires the endophytic strain to successfully enter the host plant. For instance, a comparison between the bioaugmentation potential for toluene degradation by the endophytic stain *Burkholderia cepacia* VM1468 and the rhizosphere strain *B. cepacia* Bu61, both containing pTOM-Bu61, showed a clear advantage for the endophytic strain, as it could directly deliver the conjugative plasmid to members on the endogenous endophytic community (Taghavi et al. 2005).

In general, horizontal gene transfer has been shown to play an important role in adapting microbial communities to a new environmental stress factor, including rhizosphere and endophytic communities (van Elsas et al. 1998; Ronchel et al. 2000; Devers et al. 2005; Taghavi et al. 2005; Germaine et al. 2006; Weyens et al. 2009d). In future work, this concept can be extrapolated to contaminants other than BTEX and TCE. As many catabolic pathways are found in soil bacteria where they are encoded on self-transferable plasmids or transposons, natural gene transfer offers great potential for the construction of endophytic strains with new catabolic functions. Moreover, heterologous expression of these catabolic functions might not be a major problem, especially when the donor strain and the recipient endophytic strain are closely related, as frequently is the case. In addition, this concept can not

only be applied with the purpose of remediation, but also has potential applications in the protection of food chains, as demonstrated for the organochlorine herbicide 2,4-dichlorophenoxyacetic acid (Germaine et al. 2006).

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Endophyte-Assisted Phytoremediation of Explosives in Poplar Trees by *Methylobacterium populi* BJ001^{T}*

Benoit Van Aken, Rouzbeh Tehrani, and Jerald L. Schnoor

Abstract Phytoremediation is the use of plants for the treatment of environmental pollution. For a long time, bacteria in the rhizosphere have been recognized playing a significant role in the transformation of organic contaminants by higher plants. Although plants are known to detoxify and, to some extent, metabolize organic pollutants, they are autotrophic organisms that are not capable to fully mineralize organic molecules. Plant-associated bacteria can therefore complement the biodegradation capabilities of plants. Increasing interest has been given recently to endophytic bacteria for their potential role in phytoremediation of organic pollutants. In this study, a pink-pigmented symbiotic bacterium was isolated from hybrid poplar tissues (*Populus deltoides* × *nigra* DN34) that were used for the biodegradation of the toxic explosives, TNT, RDX, and HMX. On the basis of its physiological, genotypic, and ecological characteristics, the isolate has been recognized as a novel bacterial species, *Methylobacterium populi* strain BJ001^{T}. The bacterium in pure culture was shown to degrade the explosives, TNT, RDX, and HMX. TNT was fully transformed in less than 10 days with the production of reduction metabolites including amino-dinitrotoluenes and diamino-nitrotoluenes. No significant mineralization of ^{14}C-TNT into $^{14}CO_2$ was recorded. The bacterium was also shown to transform RDX and HMX in less than 40 days. After 55 days of incubation, about 60% of initial ^{14}C-RDX and ^{14}C-HMX were mineralized into $^{14}CO_2$. The metabolites detected from RDX transformation included a mononitroso derivative and a polar compound tentatively identified as methylenedinitramine.

*To Annabelle

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These observations suggest that *Methylobacterium populi* BJ001^T may play a significant role in the metabolism of explosives in poplar plants.

Abbreviations

TNT	2,4,6-trinitrotoluene
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
USEPA	U.S. Environmental Protection Agency
PPFM	pink-pigmented facultative methylotroph
MS	Murashige and Skoog medium
2,4-D	2,4-dichlorophenoxyacetic acid
LB	Luria-Bertani
ADNTs	amino-dinitrotoluenes
DNATs	diamino-nitrotoluenes

1 Introduction

Living organisms are commonly exposed to natural or xenobiotic toxic contaminants. As a consequence, they have developed multiple detoxification mechanisms to prevent harmful effects from exposure to these compounds (Singer 2006). Bacteria, more than higher life forms, are extremely versatile organisms, which allows them to constantly develop new metabolic pathways for the degradation of a large range of xenobiotic pollutants. While provided with lower adaptation capabilities, higher organisms, such as plants and mammals, also possess detoxification mechanisms to counteract the harmful effects of toxic contaminants (Sandermann 1994).

1.1 Phytoremediation

Phytoremediation is the use of plants for the treatment of soil contaminated by toxic chemicals (Schnoor et al. 1995). The concept of using plants for remediation of organic pollutants emerged a few decades ago with the recognition that plants were capable of metabolizing toxic pesticides (Cole 1983). Since then, phytoremediation has been shown to efficiently reduce chemical hazards associated with various classes of organic pollutants, including chlorinated compounds, pesticides, hydrocarbons, and explosives (Schnoor et al. 1995; Salt et al. 1998; Meagher 2000; Pilon-Smits 2005; Van Aken et al. 2010). Phytoremediation encompasses a range of processes beyond the direct plant uptake and metabolism, and it is best described as plant-mediated remediation (Van Aken 2008). Different phytoremediation processes can be summarized as follows: pollutants in soil and groundwater can be taken

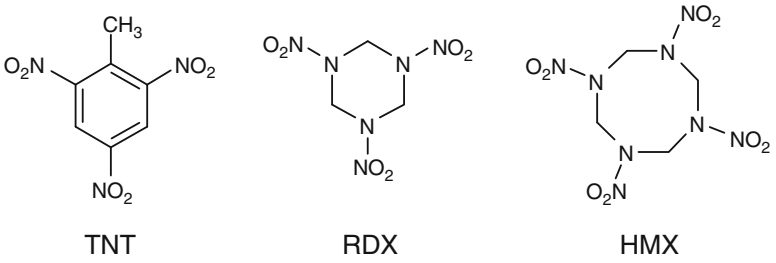

Fig. 1 Chemical structure of nitro-substituted explosives, TNT, RDX, and HMX (Reprinted with permission from Van Aken et al. 2004c. Copyright © 2004, American Society for Microbiology. All Rights Reserved)

up inside plant tissues (phytoextraction) or adsorbed to the roots (rhizofiltration); pollutants inside plant tissues can be transformed by plant enzymes (phytotransformation) or volatilize into the atmosphere (phytovolatilization); pollutants in soil can be degraded by microbes in the root zone (rhizosphere bioremediation) or incorporated to soil material (phytostabilization) (Dietz and Schnoor 2001; Pilon-Smits 2005; Van Aken et al. 2010).

However, unlike bacteria and mammals, plants are autotrophic organisms that lack the enzymatic machinery necessary for metabolizing efficiently organic compounds, often resulting in slow and incomplete biodegradation (Eapen et al. 2007). The discovery of endophytic bacteria living inside plant tissues has raised interest because of their potential involvement in the metabolism of xenobiotic pollutants. Bacteria exhibit a wide diversity of metabolic activities that allow them to take advantage of a range of substrates as source of carbon and/or energy (Van Aken and Doty 2009).

1.2 Explosive as Environmental Contaminants

Best known for their explosive properties, 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetra-zocine (HMX) are environmental pollutants contaminating numerous military sites in Europe and North America (Fig. 1). First synthesized in 1863, TNT became in the twentieth century the main conventional explosive used by military forces worldwide. However, because of a higher stability and detonation power, HMX and RDX are today the most widespread conventional explosives. Manufacture of nitro-substituted explosives, testing and firing on military ranges, and decommission of ammunition stocks have generated toxic wastes leading to large-scale contamination of soils and groundwater (Spain et al. 2000). TNT and RDX are listed as *priority pollutants* by the U.S. Environmental Protection Agency (USEPA) (Van Aken 2009). RDX was formerly used as a rat poison and it is considered as a *possible carcinogen* by the USEPA. HMX is listed by the USEPA as a *contaminant of concern* (Van Aken

2009). A *lifetime health advisory* of 2 μg l^{-1} of TNT in drinking water and a *water-quality limit* of 105 μg l^{-1} of RDX are recommended. Physicochemical properties, biodegradation, and toxicity of nitro-substituted explosives have been extensively reviewed in the literature (Hawari et al. 2000; Spain et al. 2000; Rosser et al. 2001; Van Aken and Agathos 2001). The toxicity of TNT has been reported since World War I among English ammunition workers. From laboratory studies TNT, RDX, and HMX have been found toxic for most classes of organisms, including bacteria, algae, plants, earthworms, aquatic invertebrates, and animals, including mammals and humans (Van Aken and Agathos 2001).

Traditional remediation of explosive-contaminated sites requires soil excavation prior to treatment by incineration or landfilling, which is costly, damaging for the environment, and, in many cases, practically infeasible due to the range of contamination. There is therefore a considerable interest in developing cost-effective biological alternatives based on microorganisms or plants. Because of its potential for the sustainable mitigation of environmental pollution, bioremediation has been recently listed among the *top ten technologies for improving human health* (Daar et al. 2002). Biodegradation of energetic pollutants, TNT, RDX, and HMX, have been reported by different classes of organisms, including bacteria, fungi, and plants (Thompson et al. 1998; Hawari et al. 2000; Spain et al. 2000; Van Aken and Agathos 2001). As a highly oxidized molecule, TNT is easily reduced to form toxic amino derivatives or hydride Meisenheimer complexes that undergo limited further transformation with the release of ammonium (NH_4^+) or nitrite (NO_2^-). No natural bacterium that is capable to achieve significant mineralization of TNT has been reported (Spain et al. 2000). In contrast to TNT, whose limiting degradation step is the aromatic ring fission, biotransformation of RDX and HMX involves either direct ring cleavage or reduction of the nitro groups, resulting in unstable nitroso and hydroxylamino derivatives (Hawari et al. 2000). The latter decompose into aliphatic nitramines that are eventually converted into nitrous oxide (N_2O) and carbon dioxide (CO_2). Due to different conformations, HMX (crown-type) is chemically more stable and therefore less amenable to biodegradation than RDX (chair-type) (Hawari et al. 2000).

1.3 The Genus Methylobacterium

The genus *Methylobacterium* involves strictly aerobic, facultative methylotrophic, Gram-negative, rod-shaped bacteria that are characterized by their capability to metabolize one-carbon substrates, such as methanol or methylamine (Green 1992; Nishio et al. 1997; Trotsenko et al. 2001). *Methylobacterium* bacteria belong to α-2 subclass of *Proteobacteria* and are distributed in a wide diversity of natural and human-made habitats, including soils, air, dust, fresh water, aquatic sediments, marine environments, water supplies, bathrooms, and masonry (Hiraishi et al. 1995). Some species are opportunistic human pathogens (Trotsenko et al. 2001).

Methylobacterium bacteria are frequently found in association with terrestrial and aquatic plants, colonizing the roots, leaf surfaces, and internal tissues (Austin and Goodfellow 1979; Yoshimura 1982; Corpe and Rheem 1989; Holland and Polacco 1994; Pirttilä et al. 2000; Trotsenko et al. 2001; Lidstrom and Chistoserdova 2002). *Methylobacterium* association with plants is based on a symbiotic relationship. Plants produce methanol (representing 50% of volatile atmospheric organic carbon), which is toxic for higher organisms, but can be used by *Methylobacterium* bacteria as sole source of carbon and energy (*methanol cycle*) (Trotsenko et al. 2001). In response, *Methylobacterium* bacteria produce phytohormones (cytokinins and auxins) that regulate and stimulate the plant growth (Ivanova et al. 2001; Koenig et al. 2002). *Methylobacterium* species have been shown to nodulate and fix atmospheric nitrogen in symbiosis with legumes (Sy et al. 2001). Finally, *Methylobacterium* symbiosis may help plants to fight pathogens (Holland and Polacco 1994). *Methylobacterium* bacteria are often pink to red due to the presence of carotenoid pigments and referred as *pink-pigmented facultative methylotrophs* (PPFM).

Beside their association with plants, *Methylobacterium* bacteria play important ecological functions. Methanotrophic species consume methane, which is an atmospheric pollutant and greenhouse gas (the radiative forcing of methane is 20-time higher than the one of carbon dioxide) (Hanson and Hanson 1996). Finally, because of their versatile metabolic capabilities members of *Methylobacterium* can degrade a wide diversity of organic pollutants, including methyl chloride (Tourova et al. 2001), methyl bromide (Goodwin et al. 1995), dichloromethane (Gisi et al. 1998), methyl *tert*-butyl ether (Mo et al. 1997), cyanate, and thiocyanate (Wood et al. 1998).

In the present chapter, we describe the discovery of a novel pink-pigmented bacterium, *Methylobacterium populi* BJ001^{T}, that was isolated from poplar tissues (*Populus deltoides* × *nigra* DN34) used to study the phytotransformation of toxic nitro-substituted explosives. Strain BJ001^{T} differed from other members of the genus *Methylobacterium* by its carbon source utilization pattern and its ecology as an endophyte of poplar trees. The bacterium was shown to efficiently transform the nitroaromatic explosive, TNT, and partially mineralize the heterocyclic nitramines, RDX and HMX. This discovery raises the question of the actual involvement of bacterial endophytes in the metabolism of xenobiotics inside plant tissues. Part of the results presented below was originally published in Van Aken et al. (2004a, c).

2 Isolation and Characterization of a Novel Species, *Methylobacterium Populi* BJ001^{T}, from Poplar Tissues

2.1 Bacterium Isolation

A pink-pigmented bacterium was isolated from poplar tissue cultures and plantlets (Imperial Carolina hybrid poplars, *P. deltoides* × *nigra* DN34) that were used to conduct hydroponic experiments on the phytoremediation of toxic explosives

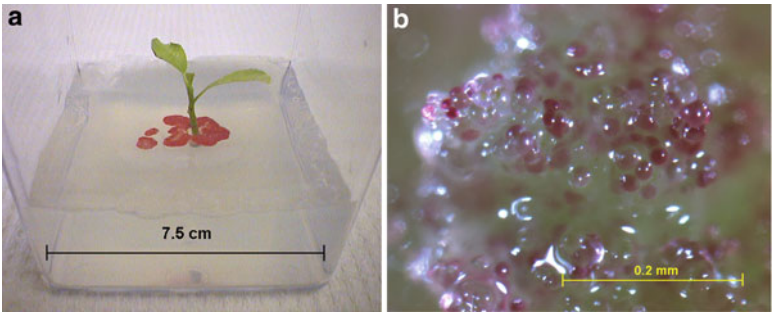

Fig. 2 (**a**) Poplar plantlet (*Populus deltoides* × *nigra* DN34) growing on semi-solid modified MS medium. Red colonies of strain BJ001^T are well visible. (**b**) Stereoscope microphotographs of poplar tissue cultures (*P. deltoides* × *nigra* DN34) contaminated with isolate BJ001^T. Plant cells containing BJ001^T appear as red inclusions on a green background (Van Aken et al. 2004a, c) (Reprinted with permission from Van Aken et al. 2004c. Copyright © 2004, American Society for Microbiology. All Rights Reserved)

(Van Aken et al. 2004b). Tissue cultures were prepared from surface-sterilized explants and cultivated under axenic conditions in modified Murashige and Skoog (MS) medium supplemented with sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D), and 6-furfurylaminopurine (kinetin) (Van Aken and Schnoor 2002). Poplar plantlets were regenerated from tissues cultures and grown on semi-solid modified MS medium. Tissue cultures in liquid medium and intact plantlets did not show microbial contamination. However, plant tissues plated on semi-solid modified MS medium frequently turned red and excised plantlets showed the development of bright-red colonies spreading from the plant material (Fig. 2), suggesting the presence of a bacterium associated with or within poplar tissues. Red or pink-colored colonies were collected manually from different plant materials and isolated by streaking on Luria-Bertani (LB) solid medium (2.5% agar). The isolated bacterium, referred as strain BJ001, was propagated routinely on LB solid medium at 28°C.

2.2 Bacterium Identification

Isolate BJ001 was identified by phylogeneic analysis based on ribosomal DNA (rDNA). Total DNA was extracted from pure cultures of BJ001 in exponential phase using DNeasy Tissue Kit® (Qiagen, Valencia, CA). 16S rDNA and 16S–23S intergenic spacer (IGS) were amplified by PCR (16S rDNA primers: 27f and 1513r, 16S–23S IGS primers: 926f and 115r/23S) and sequenced (University of Iowa DNA Core, Iowa City, IA) (Tan et al. 2001; Van Aken et al. 2004c). By comparison of the amplified rDNA with sequences in NCBI GenBank (U.S. National Library

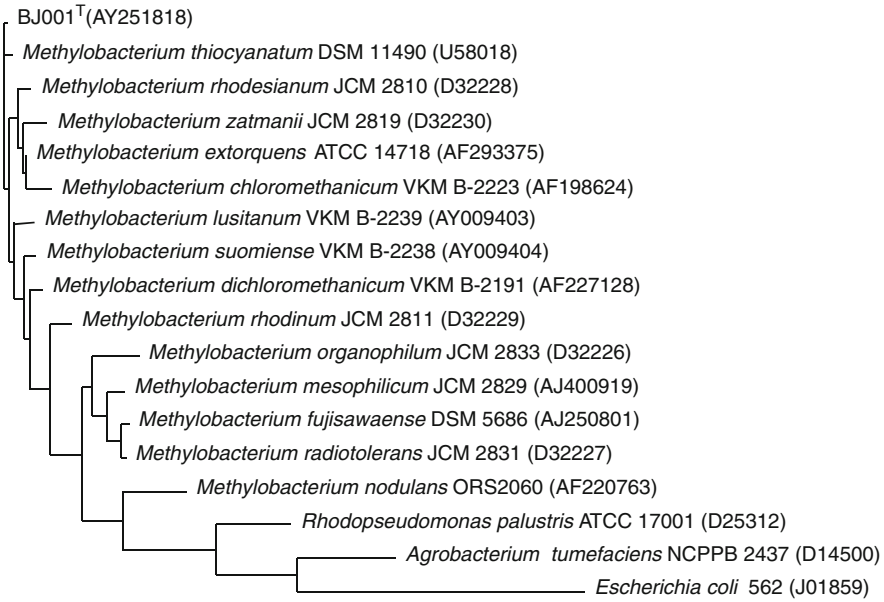

Fig. 3 Phylogenetic tree based on 16S rDNA sequences of members of the genus *Methylobacterium* and other representatives of *Proteoabacteria* showing the location of strain BJ001^T isolated from *Populus deltoides* × *nigra* DN34. Sequences were retrieved from NCBI GenBank and aligned by ClustalW Multiple Alignment (BioEdit, Raleigh, NC). The tree topology was inferred by the 'neighbor-joining' method using Mega2 software (Kumar et al. 2001). NCBI Genbank accession numbers are provided under parentheses (Van Aken et al. 2004a)

of Medicine, Bethesda, MD), isolate BJ001 was identified as a *Methylobacterium* sp. Based on the 16S rDNA sequence similarity matrix, the closest relatives to *Methylobacterium* sp. BJ001 were *M. thiocyanatum* (99.3%), *M. extorquens* (99.1%), *M. zatmanii* (98.6%), and *M. rhodesanium* (98.5%) (Fig. 3).

In order to show the close association between strain BJ001 and plant tissues, 16S rDNA fragments specific to *Methylobacterium* species were amplified from poplar DNA. Total plant DNA was extracted from various poplar explants and tissue cultures using DNeasy Plant Mini Kit® (Qiagen) and used perform the PCR amplification of *Methylobacterium* species-specific 16S rDNA fragments, Mb2, Mb3, and Mb4 (Nishio et al. 1997). Targeted fragments were amplified from both DNA extracts of pure cultures of *Methylobacterium* sp. BJ001 and leaves of *P. deltoides* × *nigra* DN34, but not from the control DNA extracted from tobacco leaves (*Nicotiana tabacum*) and from pure cultures of *Agrobacterium tumefaciens* C58 (data not presented). These results strongly suggest that *Methylobacterium* sp. BJ001 is an endophyte living in symbiotic association in the tissues of *P. deltoides* × *nigra* DN34.

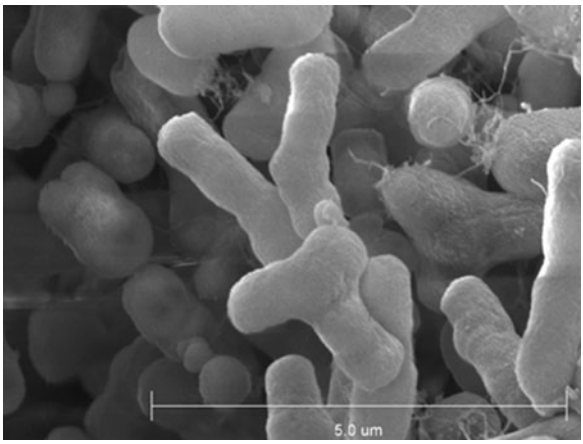

Fig. 4 Scanning electron microscope (SEM) photograph of strain BJ001^T isolated from *Populus deltoides* × *nigra* DN34. The fixed material (glutaraldehyde/osmium tetroxide) was critical point dried, sputter coated with gold/palladium, and visualized using a Hitachi S-4000 SEM (Tokyo, Japan) equipped with a field emission electron source (Van Aken et al. 2004a)

2.3 Bacterial Characterization

2.3.1 Microscopic Observations

Methylobacterium sp. BJ001 forms colonies that are pink to red, slow growing, and 1.0–2.0 mm in diameter after 4 days at 28°C on LB or nutrient agar (NA) plates. Standard staining procedures revealed a Gram-negative, non-sporulating bacterium. The isolate has characteristics of the *Methylobacterium* genus (Green 1992): cells are rod-shaped (0.8–1.0 × 1.0–10.0 μm) frequently branched, occurring singly or in rosettes (Fig. 4). They exhibit a polar growth and are motile by a single polar or lateral flagellum.

2.3.2 Carbon and Nitrogen Source Utilization

Methylobacterium sp. BJ001 was cultivated on minimal liquid medium supplemented with nitrogen (1.2 g l^{-1}, 30 mM-N NH_4NO_3) and various carbon sources (0.5% v/v for liquid substrates and 5.0 g l^{-1} for solid substrates, except formaldehyde: 0.05% v/v) (Green and Bousfield 1982). After 2 weeks of incubation, the bacterium was shown to utilize different one-carbon substrates, including methanol, methylamine, and formaldehyde, which is a particular attribute of the genus *Methylobacterium*. Other carbon sources sustaining growth of isolate BJ001 included fructose, glycerol, ethanol, and a wide range of organic acids. On the other hand, no growth was observed on glucose, saccharose, arabinose, galactose, *iso*-propanol, *n*-butanol, chloromethane, and dichloromethane. Based on the specific growth rate, fructose was the best carbon substrate that we tested for BJ001. Fructose is the first hexose synthesized from plant carbon fixation (photosynthesis), which is consistent with the association of *Methylobacterium* sp. BJ001 with poplar plants. Based on a dehydrated carbon-source utilization test including 49

compounds (API50CH, Biomerieux, Montalieu-Vercieu, France), isolate BJ001 was tested positive for growth on glycerol, D-fructose, and inulin.

Nitrogen-substrate utilization by *Methylobacterium* sp. BJ001 was tested using mineral liquid medium supplemented with fructose (5.0 g l^{-1}) and various nitrogen sources (30 mM-N). After 2 weeks of incubation, utilizable nitrogen-sources by BJ001 were determined as ammonium, nitrate, L-alanine, L-aspartate, L-glutamate, L-glutamine, glycine, L-tryptophane, and methylamine.

2.4 Discussion

Methylobacterium sp. BJ001 was isolated from poplar tissues and belongs to the α-2 subclass of *Proteobacteria*. It has been shown to be related to *M. extorquens*, a widely distributed methylotrophic bacterium, frequently associated with plant leaves and roots (Trotsenko et al. 2001). Even though association with poplar trees (*Populus* sp.) has not been previously described, members of the genus *Methylobacterium* are known to be common inhabitants of the rhizosphere and phyllosphere of various plants and described as chronic contaminants of plant tissue cultures (Holland and Polacco 1994; Lidstrom and Chistoserdova 2002).

In vitro poplar tissue cultures and plantlets have been routinely used in our laboratory for phytoremediation studies (Van Aken and Schnoor 2002; Van Aken et al. 2004b) and were frequently maintained for months without showing microbial contamination. A transient red coloration of plant tissues, as well as red colonies spreading from explants, indicated the presence of an endophytic bacterium. Indeed, surface sterilization of original explants and manipulations under sterile conditions should ensure microbe-free plant tissues, except in the case of endophyte. In addition to rhizosphere and phyllosphere, *Methylobacterium* bacteria are known to colonize internal plant tissues (i.e., endophytic bacteria) (Holland and Polacco 1994; Pirttilä et al. 2000). Also, the ability of *Methylobacterium* sp. BJ001 to metabolize fructose faster than any other carbon sources suggests an endophytic life style.

On the basis of its 16S rDNA sequence, carbon-source utilization pattern (including the use of methane), and endophytic association with poplar trees, the isolate was proposed and accepted as a novel *Methylobacterium* species, with the name *Methylobacterium populi* strain BJ001^T (Van Aken et al. 2004a).

2.5 Deposition of the Strain and Accession Numbers

The type strain, *M. populi* BJ001^T, has been deposited at the American Type Culture Collection as ATCC BAA-705^T and National Collection of Industrial, Food, and Marine Bacteria as NCIMB 13946^T. The 16S rDNA and 16S–23S IGS sequences from *M. populi* strain BJ001^T have been deposited to NCBI GenBank database under the accession number AY182525. The accession numbers for the sequences

used in the phylogenic analyses are *M. extorquens*, D32224; *M. mesophilicum*, AJ400919; *M. nodulans*, AF220763; *M. organophilum*, D32226; *M. radiotolerans*, D32227; *M. rhodesianum*, D32228; *M. rhodinum*, D32229; and *M. zatmanii*, L20804. The genome of *M. populi* BJ001^T has been recently sequenced (http://genome.jgi-psf.org/metpo/metpo.home.html).

3 Biodegradation of Explosives

3.1 Transformation of the Explosives, TNT, RDX, and HMX

Poplar plants are known to be capable to take up, translocate, and, to some extent, metabolize toxic explosives (Schnoor et al. 1995; Thompson et al. 1998; Van Aken et al. 2004b). The discovery of a bacterial endophyte inside poplar tissue cultures and plantlets raised the question of its involvement in the metabolism of explosives inside plant tissues. This hypothesis was tested by conducting biodegradation experiments using pure cultures of *M. populi* BJ001^T exposed to the nitro-substituted explosives, TNT, RDX, and HMX.

Experiments were conducted using 250-ml conical bioreactors containing 100 ml of liquid LB medium supplemented individually with TNT (25 mg l^{-1}), RDX (20 mg l^{-1}), or HMX (2.5 mg l^{-1}). Bioreactors were inoculated with 1.0% v/v of log-phase cell suspension (approx. 10^9 cells ml^{-1}) and incubated at room temperature under agitation (125 rpm). Nitro-substituted compounds (TNT, RDX, HMX, and their metabolites) were analyzed by reverse phase HPLC (Agilent 1100 Series, Palo Alto, CA) equipped with a UV-visible photodiode array (PDA) detector. The profiles of explosive concentration recorded over the 55 days of the experiment revealed that isolate BJ001^T was able to fully transform TNT, RDX, and HMX (Fig. 5). Bacterial biomasses (monitored by the OD_{600}) showed typical growth curves and were not significantly affected by the presence of TNT, RDX, or HMX (data not presented).

3.2 Mineralization and Metabolism of Explosives

In order to detect explosive metabolites and potential mineralization into CO_2, radioactive ^{14}C-labeled TNT, RDX, and HMX were obtained from DuPont NEN (Boston, MA) and Perkin Elmer Life Science (Boston, MA). For mineralization experiments, the bioreactors were closed by a rubber stopper and equipped with a CO_2 trap containing 1 ml of 1.0 N NaOH. The HPLC (Agilent 1100 Series LC/MSD) was equipped with a Radiomatic Flo-One β radio-chromatograph (Packard Bioscience, Meriden, CA) and a mass spectrometry detector with an electrospray ionization source (ESI) used in negative mode. ^{14}C-radioactivity in solution and CO_2 traps was

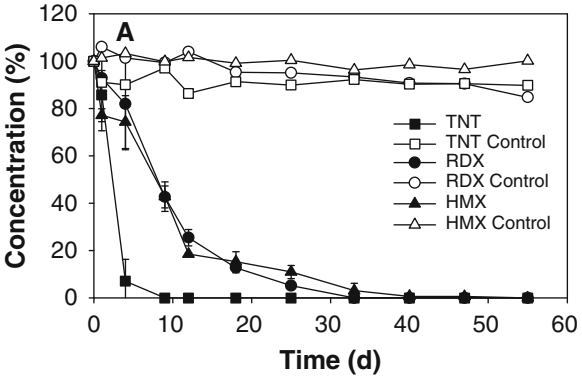

Fig. 5 Transformation of TNT (25 mg L^{-1}), RDX (20 mg l^{-1}), and HMX (2.5 mg l^{-1}) by pure cultures of *Methylobacterium populi* BJ001^T. TNT, RDX, and HMX concentrations remaining in solution were determined by HPLC (UV absorbance at 230 nm). Control experiments consisted of non-inoculated bioreactors. Concentrations are expressed as percentage of the initial level (Reprinted with permission from Van Aken et al. 2004c. Copyright © 2004, American Society for Microbiology. All Rights Reserved)

Table 1 Mass balance for ^{14}C-TNT (25 mg l^{-1}), ^{14}C-RDX (20 mg l^{-1}), and ^{14}C-HMX (2.5 mg l^{-1}) exposed to *Methylobacterium populi* BJ001^T growing in LB liquid medium after 55 days. Radioactivity is expressed as a percentage of the initial dose. Control experiments consisted in non-inoculated flasks (Van Aken et al. 2004c)

	^{14}C-TNT (25 mg l^{-1})		^{14}C-RDX (20 mg l^{-1})		^{14}C-HMX (2.5 mg l^{-1})	
Radioactivity (%)	Cells	Control	Cells	Control	Cells	Control
Initial solution	100.0 ± 0.0	100.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0
Final solution	93.3 ± 3.4	103.4	12.8 ± 1.5	104.4	12.5 ± 1.3	98.3
Bacterial cells	6.3 ± 1.3	0.2	1.0 ± 0.2	0.0	0.9 ± 0.2	0.2
Mineralization	0.7 ± 0.4	0.6	58.0 ± 3.0	1.2	62.0 ± 3.9	1.1
Mass balance	100.3 ± 4.7	104.2	71.8 ± 4.7	105.6	75.4 ± 5.4	99.6

analyzed with a liquid scintillation counter (LSC) LS 6000IC (Beckman Coulter, Fullerton, CA). Radioactivity in the cells was analyzed using a biological oxidizer OX600 (R. J. Harvey Instrument, Hillsdale, NJ). $^{14}CO_2$ contained into outgoing gases was trapped into 10 ml of Carbon-14 Cocktail® (R. J. Harvey Instrument) and the radioactivity was determined by LSC.

While TNT disappeared completely in less than 10 days, no significant mineralization (release of $^{14}CO_2$) was observed (Table 1). In contrast, RDX and HMX concentrations decreased more slowly, reaching non-detectable levels after 40 days, but were associated with significant release of $^{14}CO_2$, corresponding to 58.0% ± 3.0% and 62.0% ± 3.9% of the initial radioactivity, respectively (Fig. 6 and Table 1). The radioactivity remaining in solution decreased to 12.8% ± 1.5% and 12.5% ± 1.3% of the initial dose after 55 days.

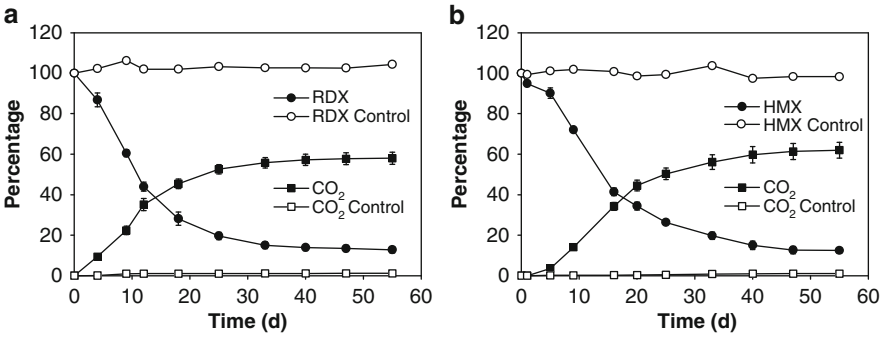

Fig. 6 Mineralization of (**a**) ^{14}C-RDX (20 mg l^{-1}) and (**b**) ^{14}C-HMX (2.5 mg l^{-1}) by pure cultures of *Methylobacterium populi* BJ001^{T}. Radioactivity remaining in solution and release of $^{14}CO_2$ are presented. Experiments were conducted with bacterial cell suspensions and controls consisting of non-inoculated bioreactors. Radioactivity in solution and release of $^{14}CO_2$ are expressed as percentage of the initial level (Reprinted with permission from Van Aken et al. 2004c. Copyright © 2004, American Society for Microbiology. All Rights Reserved)

The radio-chromatogram of the solution containing TNT at the beginning of the experiment showed a single peak (at 18.9 min) whose spectral analysis gave a mass of 226 [M-H] corresponding to TNT (Fig. 7). After 4 days, the radioactivity was distributed among three peaks (at 15.0, 5.1, and 2.7 min). Spectral analysis of the second and third peaks (at 15.0 and 5.1 min) gave masses of 196 [M-H] and 166 [M-H], suggesting the presence of amino-dinitrotoluenes (ADNTs) (MM = 197) and diamino-nitrotoluenes (DNATs) (MM = 167), respectively. At the end of the experiment, the radioactivity was concentrated in a single unidentified peak (at 2.7 min). The profile of metabolite formation showed the transient appearance of ADNTs and DNATs, reaching a maximum after 4 days, and the final accumulation of unknown compound(s) after 55 days.

The radio-chromatogram of the solution containing RDX at the beginning of the experiment showed a single peak (at 8.7 min) with a mass of 281 (M + 60-H) corresponding to RDX (data not presented). After 4 days, the radioactivity was distributed among three peaks (at 8.7, 7.8, and 2.7 min). The second peak (at 7.8 min) with a mass of 265 (M + 60-H) was identified as the mononitroso derivative of RDX (MNX). The third peak (at 2.7 min) with a mass of 135 (M-H) suggested the formation of methylenedinitramine ($O_2NNHCH_2NHNO_2$). The radioactivity after 55 days was concentrated in a single peak (at 2.7 min) corresponding to methylenedinitramine. The concentration of MNX reached a maximum after 4 days before decreasing slowly to undetectable level while unidentified metabolite(s) accumulated in the solution and accounted for 14.9% of the initial radioactivity after 55 days.

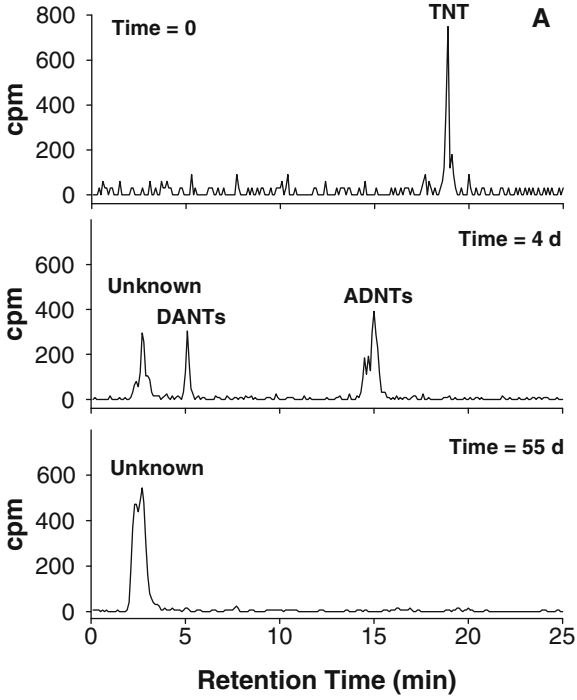

Fig. 7 Degradation of ^{14}C-TNT (25 mg l^{-1}) by pure cultures of *Methylobacterium populi* BJ001^{T}. Radio-chromatograms obtained from HPLC analyses of the liquid medium at time 0, after 4 days, and 55 days of incubation are presented (Reprinted with permission from Van Aken et al. 2004c. Copyright © 2004, American Society for Microbiology. All Rights Reserved)

3.3 Utilization of Explosives as Carbon and Nitrogen Sources

To determine whether the transformation/mineralization of nitro-substituted explosives was metabolic or co-metabolic, similar biodegradation experiments were conducted on minimal medium supplemented with TNT (25 mg l^{-1}), RDX (20 mg l^{-1}), or HMX (2.5 mg l^{-1}) in the presence and absence of carbon and/or nitrogen sources: fructose (5.0 g l^{-1}) and NH_4NO_3 (1.2 g l^{-1}). Significant bacterial growth, as recorded by the absorbance at 600 nm, was only observed in the presence of fructose, regardless the presence or absence of a nitrogen source (data not presented). These results suggest that *M. populi* BJ001^{T} can utilize nitro-substituted explosives as nitrogen source, but not as carbon source.

3.4 Biodegradation of Explosives by Other Members of Methylobacterium

Similar mineralization experiments were carried out with other members of the genus *Methylobacterium* grown on LB medium: *M. extorquens* (ATCC 14718), *M.*

Table 2 Mineralization of ^{14}C-RDX (20 mg l^{-1}), ^{14}C-HMX (2.5 mg l^{-1}), and ^{14}C-TNT (25 mg l^{-1}) by members of the genus *Methylobacterium* after 20 days of exposure. Cells were growing on LB medium supplemented with succinate (2.0 g l^{-1}). Control experiments were carried out with non-inoculated flasks (Van Aken et al. 2004c)

	$^{14}CO_2$ (%)		
Methylobacterium strain	^{14}C-RDX (20 mg l^{-1})	^{14}C-HMX (2.5 mg l^{-1})	^{14}C-TNT (25 mg l^{-1})
M. extorquens	15.2 ± 2.4	13.8 ± 1.9	0.2 ± 0.2
M. organophilum	8.4 ± 3.0	8.1 ± 0.7	0.4 ± 0.3
M. rhodesianum	13.7 ± 3.2	11.6 ± 2.0	0.3 ± 0.1
BJ001	18.5 ± 1.7	17.2 ± 2.3	0.5 ± 0.3
Control	0.1 ± 0.0	0.6 ± 0.2	0.2 ± 0.0
	Biomass (mg l^{-1})		
	^{14}C-RDX (20 mg l^{-1})	^{14}C-HMX (2.5 mg l^{-1})	^{14}C-TNT (25 mg l^{-1})
M. extorquens	1.01 ± 0.10	1.12 ± 0.06	1.07 ± 0.08
M. organophilum	1.23 ± 0.07	1.19 ± 0.10	1.13 ± 0.05
M. rhodesianum	0.95 ± 0.15	0.98 ± 0.08	1.00 ± 0.04
BJ001	0.98 ± 0.11	0.99 ± 0.06	0.97 ± 0.05
Control	0.02 ± 0.01	0.02 ± 0.02	0.03 ± 0.07

organophilum (ATCC 27886), and *M. rhodesianum* (ATCC 21611). As observed previously, all strains tested could partially mineralize ^{14}C-RDX and ^{14}C-HMX, while no significant mineralization of ^{14}C-TNT was observed (Table 2). Among the *Methylobacterium* species tested, *M. populi* BJ001^T was shown to achieve the highest mineralization rates.

3.5 Discussion

This study showed for the first time that a member of *Methylobacterium*, *M. populi* strain BJ001^T was capable to transform TNT and mineralize partially RDX and HMX into CO_2. The transient generation of reduction derivatives early in the degradation process (ADNTs and DANTs from TNT and MNX from RDX) indicates that the transformation of explosives by *M. populi* BJ001^T was started by a reduction step. Being a highly oxidized molecule, TNT undergoes easily stepwise reduction of the nitro groups, with generation of the reduction derivatives, ADNTs and DANTs (Spain et al. 2000). Similarly, bacterial transformation of the heterocyclic nitramines, RDX and HMX, frequently involves an initial reduction step and nitroso metabolites have previously been detected, both under aerobic and anaerobic conditions (Hawari et al. 2000).

Further biodegradation of the nitroaromatic explosive, TNT, and heterocyclic nitramines, RDX and HMX, by *M. populi* BJ001^T followed different pathways. Although no mineralization of ^{14}C-TNT was detected, extensive release of $^{14}CO_2$ was recorded from ^{14}C-RDX and ^{14}C-HMX. It is commonly accepted that the initial

reduction of RDX and HMX heterocycles destabilizes the molecule, leading to ring cleavage. Resulting aliphatic hydroxylamines and nitramines further decompose into methanol (CH_3OH), formaldehyde (CHO), and CO_2. In accordance with our biodegradation experiments on minimal medium, RDX and HMX have been reported to be used by nitramine-degrading bacteria as sole nitrogen source (Hawari et al. 2000). On the other hand, even though release of $^{14}CO_2$ was observed, our results suggest that *M. populi* BJ001 was unable to use RDX and HMX as carbon source and that explosive degradation occurred co-metabolically. The observed high mineralization rates of RDX and HMX may be related to the particular ability of *Methylobacterium* bacteria to metabolize one-carbon substrates, CH_3OH and CHO, generated from nitramine degradation. In contrast, biotransformation of TNT by *M. populi* BJ001^T did not result in significant release of $^{14}CO_2$, even though the molecule was very quickly reduced. Indeed, bacterial degradation of nitroaromatic compounds is known to lead to the formation of dead-end derivatives that are transformed no further (Spain et al. 2000).

The capacity to metabolize explosives is a common feature among bacteria (Hawari et al. 2000; Spain et al. 2000). However, this is the first time that a member of the genus *Methylobacterium* was shown to biodegrade TNT, RDX, and HMX. Although the transformation of TNT did not lead to mineralization, reduction metabolites are significantly less toxic than the parent TNT and may be bound to soil particles and humic acids or conjugated to organic molecules, resulting in a reduction of bioavailability and toxicity.

4 Conclusion

Nearly all plant species have been shown to harbor endophytic bacteria in their internal tissues establishing symbiotic or commensal relationships: endophytes may provide plants with growth-promoting compounds, fix atmospheric nitrogen, or mitigate the effect of plant pathogens (Doty 2008). In addition, many endophytes have been shown to metabolize toxic xenobiotic pollutants, potentially providing plants with enhanced detoxification capabilities. Even though plants have been shown to transform to some extent xenobiotic pollutants, they are autotrophic organisms that typically lack the enzymatic machinery necessary to fully metabolize organic compounds, often resulting in the accumulation of toxic metabolites (Eapen et al. 2007). The discovery of endophytic bacteria living inside plant tissues has raised interest because of their potential involvement in the phytoremediation of xenobiotic pollutants.

In this study, we isolated and characterized a novel bacterial species, *M. populi* BJ001^T that is an endophyte of poplar plants (*P. deltoides* × *nigra* DN34). Because poplar plants are known to be capable to efficiently take up and metabolize toxic nitro-substituted explosives (Schnoor et al. 1995; Thompson et al. 1998; Van Aken et al. 2004b), this discovery raised the question of the involvement of *M. populi* BJ001^T in the metabolism of explosives inside plant tissues. The observation that *M. populi* BJ001^T can transform TNT and mineralize RDX and

HMX strongly supports the hypothesis that the bacterium is indeed involved in the phytotransformation of explosives inside poplar tissues. However, even though bacterial degradation of energetic compounds occurred inside the plant, its actual contribution to the phytoremediation process may be limited: *Methylobacterium* bacteria are rather slow growers and their relative biomass in plants is likely to be small. A better understanding of plant endophytes and their symbiotic relationships with plants is needed to further evaluate the implication of endophytic bacteria in phytoremediation of organic pollutants.

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Part V
The Promise of Endophytes in Biocontrol of Trees

Foliar Endophytes of Spruce Species Found in the Acadian Forest: Basis and Potential for Improving the Tolerance of the Forest to Spruce Budworm

J. David Miller

Abstract This chapter describes the likely role of foliar fungal endophytes of the some ecologically- and commercially important spruce species widespread in the Acadian forest. Based on paleobotanical, historical, and photogrammetric data from 1945 to the present, the nature of the Acadian forest is reviewed in relation to cyclical epidemics of the needle herbivore *Choristoneura fumiferana*. The population ecologist Dr. Tom Royama proposed that there might be a factor that influenced the probability of *C. fumiferana* epidemics by affecting population structure. This formed the theoretical basis for studies of foliar endophytes that produced toxins that affected the growth of *C. fumiferana*, as is the case of the foliar endophytes of cool season fescues and their insect pests. This resulted in the discovery of reservoirs of toxin-producing endophytes in superior trees throughout the Acadian forest. There are various genotypes of several cryptic species that produce mixtures of compounds that individually or together reduce *C. fumiferana* growth. Many of these natural products were new to science and have been demonstrated to be produced in the tree and to persist the infection at least for 10 years in the field. Unlike the cool-season endophytes, which are transmitted vertically, these endophytes are transmitted horizontally. With reforestation or afforestation either by seedlings produced in a greenhouse or from natural regeneration after fire, agriculture or forestry, the diversity of the endophytes in the stands has been reduced.

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Abbreviations

Btk	*Bacillus thuringiensis* var. *kurstaki*
US EPA	US Environmental Protection Agency
PMRA	Health Canada's Pest Management Regulatory Agency

1 Introduction

Endophytes of some fescues, the seaweed *Ascophyllum nodosum* and of the needles of temperate and boreal species of conifers (Sumarah and Miller 2009) are all known to confer various benefits in exchange for protection and, in the case of the endophyte of *A. nodosum*, survival and dispersal (because it is obligate; Garbary 2009). Known benefits to the seaweed include increased drought tolerance, important in this intertidal species (Garbary and London 1995; Garbary 2009). On the basis of many comparative studies over several decades, *Balansia* endophytes of cool season fescues increase insect and drought tolerance and probably increased tolerance to fungal pathogens (Clay and Schardl 2002; Kuldau and Bacon 2008). The effects on productivity are large. In a review by Clay (1997), almost all (95%) of 17 studies comparing productivity of endophyte − and + fescues in field and greenhouse studies found an increased relative productivity of + fields. The average grass productivity increase was 30% (Clay 1997), but under conditions of greatest biotic and abiotic stress, can be much larger. Arising from a suggestion made by Carroll and Carroll (1978), recent extensive studies have demonstrated that foliar endophytes of various conifer species produce toxins that affect insect growth (Miller 1986; Sumarah and Miller 2009). Since 1984, their prevalence and ecology has been studied extensively in commercially important conifer species. This work has been done in the Acadian forests of Maine, New Brunswick (NB), Nova Scotia and extending somewhat into eastern Quebec.

Choristoneura fumiferana (eastern spruce budworm) causes cyclical large scale mortalities in the coniferous forests of eastern Canada and Maine. Large-scale chemical pesticide spraying was undertaken during the last major outbreak in this region which peaked in the mid-1970s. Since then, the chemical pesticides used have been deregistered (MacLean et al. 2002; Royama et al. 2005).

Carroll and Carroll (1978) proposed that foliar endophytes of conifers might be mutualistic symbionts. Carroll (1986) suggested that Douglas fir (*Pseudotsuga menziesii*) and the needle endophyte *Rhabdocline parkeri* provided some tolerance to a gall forming needle pest. Higher rates of mortality of the larvae were seen in galls in endophyte-infested needles (Sherwood-Pike et al. 1986). An extract of *R. parkeri* exhibited cytotoxicity and reduced growth of spruce budworm larvae in diets (Miller 1986).

In 1984, small collections were made of conifer endophytes near Fredericton, NB (Miller et al. 1985) followed by collections in 1987–1988 (Johnson and Whitney

1989, 1992). Some years later, two larger surveys were made. In 1992, branches were collected from superior conifers trees from the southern border of New Brunswick (NB) (beside Maine) to near Quebec City in a north-south transect representing 3,500 isolates (Sumarah and Miller 2009). A decade later, another large collection of 2,250 spruce needle endophytes (90% red spruce, 10% white spruce) was acquired. These endophytes were isolated from branches collected in forests in Maine, New Brunswick and Nova Scotia in an east-west direction. The coordinates were obtained of each tree sampled. This data demonstrated that in the northeast of maritime Canada and the USA, there are genetic populations of foliar endophytes of white and red spruce that produce mixtures of compounds affecting the growth of spruce budworm *in vitro* and *in vivo* (Sumarah and Miller 2009; Sumarah et al. 2008a, 2010). Subsequent work established that these were not present in seedlings produced in greenhouses, unlike the grass and seaweed endophytes, the infection transmission is horizontal as opposed to vertical in nature (Miller et al. 2009). This is conceptual similar to the effects of endophytes in cool season fescues described above.

2 Royama 1984

Aside from the starting point provided by Carroll (discussed in Miller 1986), another key paper influenced the decision to begin work in this area was the monograph "Population dynamics of the spruce budworm *Choristoneura fumerana*" (Royama 1984). Despite the fact that there had been in the past clear cyclical epidemics, Royama argued that the weight of evidence was against one epicenter spreading across the region. He then considered five potential drivers: parasitism, predation, food shortage, weather and the fifth agent. The proportion of survivors to the fifth and sixth instars (developmental stages in the budworm) was identified as potentially important factors early on in his analysis.

Parasitism: The data available in 1984 indicated that parasitism was a factor that was a major source of variation across the landscape. The percentage of the larval population that survived to reproduce (some survive but do not reproduce) was not inherently driven by a factor that was plausibly subject to region wide forces. The data acquired since then support that view. Although Cappuccino et al. (1998) suggested that parasitism was greater in patches of balsam fir in extensive stands of conifers, this does not seem to be generally applicable. Quayle et al. (2003) and Kemp and Simmons (1978, not cited by Royama 1984) showed that much of the variance could be explained by one or two parasitoids in conifers in mixed wood stands. A possible mechanism for this is that there are other insect species that are acceptable hosts for parasitoids that mainly affect spruce budworm. This general conclusion was largely supported by the subsequent analysis of Campbell et al. (2008). Possible variation in different years in this pattern appeared to be weather

driven a view supported by Quayle et al. (2003). MacKinnon and MacLean (2004) found only a modest or no detectable effect of surrounding forest type.

Predation: Based on data collected in 1952 analysis of the stomach contents of birds in Maine) and 1981 (experimental data on consumption of larvae by birds in NB), Royama found that bird predation could not be responsible for influencing the budworm cyclic epidemics. A subsequent study also from Maine found that songbirds consumed larvae as a function of their availability as a food supply. The authors suggested that silviculture could be employed to promote songbird populations and the birds possibly would reduce their severity (Crawford and Jennings 1989).

Food shortage: The analysis of available NB data did not provide evidence that food shortage affected survival of sixth instar larvae. On a mass basis, the bulk of defoliation occurs when the larvae are in later instars. This led Royama (1984) to conclude that the effect of food shortage was either not detected or not material. Independent theoretical analyses at the time were equivocal on the issue of food as a limiter or driver of epidemics (e.g. Fleming 1983).

Influence of weather: Royama (1984) concluded that there is little evidence that weather results in the cyclical epidemics of spruce budworm although weather does affect larval survival and moth dispersal. No data have appeared to change this determination (Royama et al. 2005).

Fifth agent: The last of the mortality factors discussed by Royama is what he believed was a complex of viral and protozoan diseases and "death from unknown causes." Neilson (1963) found microsporidioses and granulosis to be the most prevalent protozoan and viral diseases in his study area (Green River). Other viral diseases, such as nuclear and cytoplasmic polyhedrosis virus, and bacterial and fungal diseases were infrequent. Neilson collected weekly samples of budworm larvae from third instar until 80% pupation and reared each collection in the lab for 1 week, and examined dead larvae for diseases. In the majority of larvae that died there was no detectable disease and the pathogens identified are of low virulence. For various reasons, Royama offered a compelling argument that a fifth agent affected the population dynamics of spruce budworm (Royama 1984).

In summary, Royama felt that epidemics were driven by survival of older larvae by something that operated in the field but the reason was not obvious given the information at the time. He deduced that "... a combination of parasitism and the 'fifth agent' is the most likely cause of population oscillation" Royama (1984). Under nursery conditions, Miller et al. (2008) demonstrated that the presence of endophytes in white spruce resulted in effects on growth of spruce budworm and that among the expected effects would be immune suppression. The hypothesis that motivated this research was that the toxigenic endophytes were the fifth agent.

Writing two decades later, Royama et al. (2005) noted that although currently low, budworm populations in New Brunswick will rise again. Despite a great deal of study they noted that "whether the outbreak will be severe and extensive or light and

localized" cannot be predicted and that prediction of what will happen by correlating extent of infestation on the basis of "comparatively fixed or systematically changing environmental characteristics would be unproductive and even misleading".

Returning to the stand level (i.e. non theoretical data), Campbell et al. (2008) wrote that the weak relationship between spruce budworm impact and abundance occurs not just because of budworm densities but also "by the ability of the hosts to withstand the effects of a given level of these defoliators".

3 The Acadian Forest

The nature of the current Acadian forest deserves some consideration. This forest type is concentrated in Maine as well as New Brunswick, Nova Scotia and Prince Edward Island with pockets into eastern Quebec and New England. It is characterized by red, black and white spruce, balsam fir, yellow birch and sugar maple. Other tree species include red pine, eastern white pine, eastern hemlock and beech. These trees live an average of 150 years. Settled in by Europeans in the 1600s, much of the forest was harvested to support the new inhabitants. Pines were cut for ship masts and forests were cleared to make way for agriculture. Tree disease, fire and more importantly spruce budworm epidemics changed the structure of the forest over long time cycles (Loo and Ives 2003). Two recent re-analyses of historical data in northern Maine near New Brunswick have been done. The most recent (Lorimer 2008) indicates that reductions in pine attributed to nineteenth century harvesting, and of proportion of pine in the northern Maine forests were about half of previous values.

After the last ice age, the effects of spruce budworm on forest structure have apparently always been a major determinant of the precursor Acadian forest. Using macrofossil analysis, spruce budworm abundance was assessed in cores from an 8,600-year-old bog in eastern Quebec. From 8240 before present (BP), budworm feces were recovered in the peat profile indicating endemic and epidemic presence of the insect in the area. Two periods of insect activity were seen: 6815–6480 calendar year BP and during the twentieth century (Simard et al. 2006). A similar study was conducted on an 8,000 km^2 island in the Gulf of St. Lawrence. The earliest remains of spruce budworm and hemlock looper were found ca. 3220 and 2350 calendar year BP, respectively. Peaks of insect head capsules occurred from ca. 1640 to 625 year BP. Low balsam fir pollen concentrations during this period suggested a 1,000 year period of high insect activity resulting in extensive fir mortality. Insect activity and the relatively uncommon occurrence of fires affected the long-term dynamics of the forest (Lavoie et al. 2009). Schauffler and Jacobson (2002) reported a large scale stand scale pollen stratigraphy in several sites in Maine also comprising charcoal fragments. Two of the sites are near the Maine/New Brunswick border, one in the northern corner and another in the southern corner. They show that the large driver of tree species change in the last 5,000 years was the cooling of coastal areas (i.e. Gulf of Maine, Bay of Fundy, see their Fig. 5)

and consequent moisture/temperature regimes. They also note that there remained refugia of spruce species that rapidly expanded when climate allowed. The largest change in landscape tree species distribution occurred during the last 1,000 years was a rapid expansion of spruce and pine. Which spruce species that dominates the landscape is also highly affected by climate.

The best estimate of the pre-European aboriginal population in Maine and NB is ca. 20,000, soon reduced to 5,000 by disease. The earliest clear records (1672) suggest that aboriginal disturbance of the forest was modest (Loo and Ives 2003). However, forests were burned to encourage berry and nut production. It is known that a corn land race called Gaspé flint was grown in Eastern North America from ca. AD 2500. This was called "Micmac corn" and was seen in the sixteenth century then accessed in the seventeenth century from a farmer in that area as well as more recently (Kuleshov 1933; Labate et al. 2003). It is known to have been grown perhaps on some scale in the St. John River Valley in NB. This suggests there was deliberate burning for that purpose. It is also known that forest fires were less common because of the percentage that result from human as opposed to lightening in the last decades (Loo and Ives 2003). This is supported by the charcoal stratigraphy noted above (Schauffler and Jacobson 2002). Stand replacement was common. Pre-colonial fire rotations were 800 years to which 575 year rotations for moderate to heavy windthrow indicating a 35% stand turnover (Lorimer 2008). Early land surveys during the early Loyalist period required descriptions of anchor trees used as benchmarks (witness trees). Aside from the data in Loo and Ives (2003), they suggest that the largest changes have been that balsam fir and spruce have twofold increased area land occupied and modest to larger (0.5 fold) reductions in pine, depending on land type and loss of cedar, ash and larch, depending on soil type. These changes are consistent with the trends indicated by the pollen stratigraphy visible from 1,000 years ago, i.e. pre-European settlement (Schauffler and Jacobson 2002).

A detailed analysis of changes in a managed forest in northwestern New Brunswick from 1946 to 2008 is available based on high–quality data from J.D. Irving, Limited Black Brook district (Etheridge et al. 2005, 2006; Amos-Binks et al. 2010). These data are based on original copies of detailed expert cruises as well as aerial photographs taken in 1944–1945 and digitized to modern standards, as well as similar photographs from 1966, 1982, 2002 and 2006 and modern cruise data.

In this site, there have been three processes in play in the last 60–100 years. Perhaps the single most important of these is the spruce budworm which resulted in the deaths of large areas of balsam fir. The extent of this damage as well as stand senescence is of signal importance in understanding the changes that have occurred in modern times (see their Fig. 5, Amos-Binks et al. 2010, see also their Figure 2, Chang et al. 2009). The changes in mixed wood and soft wood areas in 1946–1966 were driven by the fact that these stands would be susceptible to mortality from wind, pathogens and spruce budworm, which arose from the budworm epidemic in the region in the 1870s (Etheridge et al. 2005; Amos-Binks et al. 2010). In response to the spruce budworm epidemic peaking in 1975 that was destroying the balsam fir,

forest managers planted white and red spruce. White spruce, red spruce and black spruce are ~70%, 40% and 30% as susceptible to budworm as balsam fir (Hennigar et al. 2008) (black spruce is slower growing). The difference between balsam fir and black spruce appears to be primarily related to the timing of budburst. Over the past 60 years, extensive forest management has been practiced, including the planting of 200 million softwood trees, but the percentage of softwood has not materially changed (Etheridge et al. 2005, 2006; Amos-Binks et al. 2010). As noted by Amos-Binks et al. (2010), caution is needed when monitoring changes in the abundance of mixed wood stands, because there are site-specific succession patterns.

4 Nutritional and Anti-nutritional Factors in Fir and Spruce Needles

There is a variety of chemicals in fir and spruce needles that change according to soil conditions type, needle age and location in the crown, including cross sectional area, as well as primary nutrients (N, presumably including protein), chlorophyll, tannin, and terpene concentrations. These might be anticipated to result from potential changes in needle chemistry due to variables including needle age and shade, and endophytic colonization. Shading (Lhotakova et al. 2007) and needle age, as well as soil conditions, are known to affect foliar chemical composition and this in turn is suggested to affect budworm growth (Carisey and Bauce 1997; Clancy et al. 2004; Nealis and Nault 2005).

There are data that suggest that tannins interact with the biopesticide Btk. Under laboratory conditions, tannins incorporated into diets affect consumption of diet and the growth of larval stage spruce budworm (Carisey et al. 2004). Also in the laboratory, tannins can negatively affect the lethality of Btk (Bauce et al. 2006). In synthetic diet, tannins and tannic acid apparently have either stimulatory or toxic effects, respectively (Cardinal-Aucoin et al. 2009). In needles, terpenes are toxic to spruce budworm, however the available data are conflicting (Carisey and Bauce 1997; Chen et al. 2002; Nealis and Nault 2005). None the less, the argument is presented that induced defense resulting from terpenoid synthesis has a material effect on budworm populations (Keeling and Bohlmann 2006). Needle nutrients have not been demonstrated to negatively affect budworm growth except perversely to result in increased susceptibility in some genotypes (Clancy et al. 2004). Collectively, the available data on the role of needle secondary compounds in relation to spruce budworm have not resulted in much clarity as to their impact on insect populations.

We have placed the most effort on the effects on the spruce budworm of the needle endophyte *Phialocephala scopiformis*. In growth-chamber studies, occurrence of the fungus and its toxins, assessed as its primary toxin rugulosin, reduced spruce budworm growth in needles. These latter experiments were done using detached needles from 4-month old seedlings (Miller et al. 2002). Based on field experiments

at the nursery (Sumarah et al. 2005) and field test level (Sumarah et al. 2008b; Miller et al. 2009), it is clear that once inoculated, the fungus persists and needle toxin concentrations reach 'effective' levels. This was critically examined by Miller et al. (2008). Spruce budworms were placed on needles of trees that were grown outdoors as described following. Two aspects of the effect of rugulosin in needles on *C. fumerana* growth were studied under outdoor nursery conditions. The first was a comparison of the impact of rugulosin on budworm growth on infected trees and uninfected trees. The second was to use a group of older trees such that needles from the infected tree served as a control. This strategy is typically used in toxicology to eliminate possible confounding variables arising from intra-individual variance. The intention was to look for evidence of a dose-response to rugulosin, which might be anticipated to result from potential changes in needle chemistry due to variables including needle age and shade (see above). The trees used in the study represented a diverse genetic population used for reforestation in eastern Canada and Maine.

The study on younger trees (2 years old) that received a carefully determined number of budworms per unit area branch in whole tree mesh bags resulted in two findings. The distribution of animal weights collected on infected needles at termination was different than the respective controls. There was also a statistically significant difference in budworm weights between the two groups. As in the growth chamber, the presence of the fungus and its toxin reduced spruce budworm growth (Miller et al. 2008). Experiments on infected and uninfected grass endophytes have resulted in similar findings, as well as in findings on effects on development (e.g. Hardy et al. 1986).

The second aspect of the present experiments was the use of older trees from which the effect of rugulosin on budworm growth on multiple branches from the same infected tree could be observed. Variation in rugulosin concentration was observed between individual branches within a single tree. As was found previously, rugulosin concentrations were typically above those that affect growth of spruce budworm in needles and *in vitro*. A comparison of those branches with concentrations above the threshold of rugulosin toxicity demonstrated that there was an inverse relationship between budworm weight and rugulosin concentration (-0.288, $P = 0.023$). This means that a dose-response was seen for rugulosin (Miller et al. 2008). From these experiments it was also seen that the minimum lowest observed effect level of >0.5 μg per needle is within the range bracketed by the growth chamber data published 6 years previous (Miller et al. 2002). Further discussion of this issue is in the article by Miller et al. (2008).

The primary effect of rugulosin *in vitro* and in the plant is to slow the growth rate of spruce budworm. Insects that are exposed to the toxin would therefore be exposed to environmental and biotic factors for longer durations. It is also possible that various groups would be put out of reproductive synchrony. Another but difficult to test possibility is that the toxins would result in immune modulation. This is a poorly understood topic but it is known that the moth immune system shares some features of the mammalian system along with more primitive features (Miller et al. 2008). As noted, the insects used in the study were disease-free and reared on trees grown under optimal conditions. Under field conditions, parasitoid infections

are common (see above). In addition, there is a prevalence of pathogenic *Bacillus* species on budworm collected on balsam fir in New Brunswick (Strongman et al. 1997). The facultative insect pathogen *Aspergillus fumigatus* is common in trees infested with spruce budworm and other insect defoliators (Miller et al. 1985; Sumarah et al. 2008b). Had the tests been done in the wild and thereby including other mortality factors, fitness on rugulosin-containing branches might have been lower. Ecologically, this population effect is called the top-down effect, i.e. where herbivore populations are limited primarily by natural enemies (Moreau et al. 2006). The other effect relates to the fact that herbivore populations are limited by the supply of food, i.e. the host (see discussion above regarding Royama 1984). In another herbivorous insect (the sawfly *Neodiprion abietis*) in the coniferous forest in Newfoundland, both effects operate but perhaps, unsurprisingly, in a partly compensatory and partly additive way (Moreau et al. 2006). Thus the overall impact on fecundity and fostering epidemics was not evaluated (i.e. following larvae through moth development and eggs laid). These experiments are in progress. Although the above text has focused on one endophyte, data have been collected on other species.

5 Conclusions

The sum of these data indicates that the hypothesis that the natural populations of toxigenic endophytes are the fifth agent proposed by Royama (1984) is correct. The use of foliar endophytes of conifers in the context of reforestation or aforestation and seedling stock appears now to be a useful approach. Over the past 25 years, our approach has been to determine how many populations of toxigenic endophyte genotypes are present in the region. Making use of representative cultures to inoculate seedlings used in reforestation is an attempt to restore all of the relevant genotypes at the stand level rather than to focus on one (Sumarah and Miller 2009). This is precisely the same approach that was taken leading to the mass planting of infected cool season fescues. These endophytes are not regulated by the US EPA or PMRA but rather promoted to reduce pesticide volumes on golf courses and lawns (Charbonneau 1997; Health Canada 2000) including for schools (Hamilton et al. 2008). Cultivars of grasses certified free of endophytes are used for pasture. For the grasses, as new cultivars are produced through plant breeding, those that pass agronomic trials are deliberately infected with the fescue endophyte and then sold for lawns and golf courses. These are not regulated because (1) there was a long history of published research that identified their effects and host response and (2) in nature, grass endophyte acquisition cannot be stopped because it is a natural process (Clay and Holah 1999; Saari et al. 2010). In the case of foliar endophytes the evidence is that the process is the same except because the ecology is different (horizontal transmission, Miller et al. 2009), the impact is potentially long-lasting and could change fungal population structure above the stand level on an anthropological time scale.

However, based on what is known now, endophyte seedling inoculation will provide several benefits. Aside from economic and ecological benefits associated with reduced forest damage, there are important carbon sequestration implications of this project. We used the Canadian Forest Service Carbon Budget Model (CFS3) and the CFS spruce budworm decision support model to assess potential benefits. Assuming 60% foliar protection (based on Miller et al. 2008) in a planting program of 10 million trees annually, the CO2 sequestered would be approximately 350,000 tons over a 50-year time horizon. These results assume a normal budworm epidemic at peak.

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Endophytes in Forest Management: Four Challenges

George Newcombe

Abstract In spite of exciting, new research, endophytes remain more of a potentiality than an actuality in forestry. Of many upcoming challenges to endophyte applications in forestry, four are discussed in this chapter: (1) the assay-based, selection problem, (2) the question of replacement dynamics within complex, endophytic communities, (3) the need to complement the objectives of tree improvement programs, and (4) the decisions that will need to be made on deliberate introductions of selected endophytes outside sites where they were initially discovered. Ideally, endophytes selected in assays would first be effective as inoculants in improving the survival, growth or defense of trees in the forest setting. Furthermore, inoculants would be resistant to replacement by combative, unselected endophytes, complementary to genetically improved traits of trees in plantations, and unlikely to switch hosts and ecological roles when moved from one part of the world to another. Progress towards applications will likely be made as foresters become more aware of the potential of endophytes to extend host adaptations to pathogens, herbivores, anthropogenic disturbance and climate change.

1 Introduction

Endophytes can extend the ecological adaptations of woody plants. A few are already being used for that purpose in forest management, and the rugulosin-producing endophytes, that are now inoculated into seedlings of *Picea glauca* in Canada (Sumarah et al. 2008), are a prominent example highlighted elsewhere in this book. Still, applications based on endophytes could become much more

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extensive and important to forestry than they are today. Holding back this expansion of applications are many looming challenges and this chapter will focus on four of them.

2 Challenges

2.1 The Assay-Based, Selection Challenge: Are Assays Worthwhile, and What Do They Tell Us About Functional Roles in Nature?

Assays can be dismissed as unrealistic, and no doubt their results sometimes are, but Fleming's observations of an interaction between *Penicillium* and a bacterium in a petri dish (Fleming 1929) eventually led to a revolution in medicine: the antibiotic era. Similarly, endophytes might eventually be employed in forests to manage insects, disease, and abiotic stress associated with climate change, even if, today, we are still at the preliminary stage of understanding the implications of assays. Assays do reveal the impressive range of functional roles that endophytes might assume in the forest (Rodriguez et al. 2004, 2009). Actually, if fully investigated, the functional range might expand further, given all of the diversity associated with endophyte communities in trees (Arnold et al. 2000; Ganley et al. 2004), and given how little of it has been sampled via functional assays.

Growing knowledge of the phylogenetic context of endophyte symbioses in relation to host families (Arnold 2007), and a new classification scheme of four functional groups or classes (Rodriguez et al. 2009) should help address the selection challenge. Still, the huge store of endophyte diversity can be overwhelming to the researcher interested in simply selecting a particular endophyte for a particular functional role, perhaps in response to a stakeholder's query. More questions occur to that researcher when he or she considers the following possibilities: (1) that a selected endophyte will be replaced when deployed, given the diversity of endophyte communities and the extent of our ignorance of endophyte-endophyte interactions; (2) that endophytes may appear to the tree improvement and forest genetics community as agents of small or even trivial effects if they are influencing highly heritable and artificially selectable traits in their hosts; (3) that the selection environment of endophytes may not match that of their deployment environment, reducing their efficacy or even leading to undesirable, non-target effects. These further questions will be discussed in turn below, as the second, third, and fourth challenges. But first, we will focus on the selection question.

This first challenge is premised on the assumption that unselected mixtures of the whole endophyte community are less advantageous than selections from the latter. This assumption seems reasonable because plants are in general subject to both positive and negative feedback from fungal symbionts (Klironomos 2002), and endophytes represent a continuum in plant symbiosis from mutualism to parasitism

(Saikkonen et al. 1998; Schulz and Boyle 2005; Sieber 2007). Native plants in particular may be subject to more negative feedback with pathogens than non-native plants (Klironomos 2002), but pathogenic endophytes can still be isolated from the latter along with mutualists (Newcombe et al. 2009). In native plants, endophytic mutualists may be relatively uncommon (Faeth and Fagan 2002), but they still can be selected via assays (Gure et al. 2005). Some selected mixtures have been shown to provide better defense against pathogens than other mixtures (Ganley et al. 2008). Thus, it appears that endophyte selection could be worthwhile.

If endophytes are to be selected, the correlation between assay and field results becomes important. Addressed for many other aspects of woody plant biology (Cornelissen et al. 2003), discrepancies between field and assay results have been reported for endophytes (Krauss et al. 2007). The correlation can, of course, be approached in the traditional way by first obtaining assay-based results that are then followed with field trails, but endophyte researchers in forestry are likely to be tempted to simultaneously try both field experiments and assays.

Some may even start with field experiments without prior assay results. If selections were to be made in this circumstance they would have to be made on the basis of taxonomic identities and functional classification (Rodriguez et al. 2009). A typical field trial might be established with nursery-grown seedlings from local tree populations that would be inoculated with local endophytes prior to outplanting. The trial would then be monitored over time in the typical way for survival, growth, disease, and herbivory, to determine which selected endophytes improved host fitness with respect to these traits. We know that nursery-grown tree seedlings are typically depauperate in endophytes relative to natural regenerants (Miller et al. 2002; Ganley and Newcombe 2006) so the rationale for inoculation is to mimic a natural process in which seedlings become infected with endophytes drawn from their local forest. Inoculation ideally ensures that outplanted seedlings will not be without the endophytes that they need for optimal establishment and defense at a vulnerable phase in their life cycle. But what should the inoculum be?

Functional roles can be predicted on the basis of taxonomic identity in some cases. For example, when *Beauveria bassiana* was isolated as an endophyte in *Pinus monticola* (Ganley and Newcombe 2006) it was clear that it could be applied in a field experiment with the reasonable expectation that it would provide defense against insects. Isolates of *Trichoderma* might well be expected to induce host defense against pathogens (Bailey et al. 2006). An isolate of *Piriformospora indica* could promote growth (Varma et al. 1999), or provide stress tolerance or higher yield (Waller et al. 2005). But it is important to remember that many fungal taxa are characterized by functional diversity. This may be the case, for instance, even within species of arbuscular mycorrhizal fungi (Munkvold et al. 2004), and genera are obviously more functionally heterogeneous than species. Moreover, attempts to focus on function via taxonomy may be complicated by the extent of undescribed diversity among endophytes (Arnold et al. 2000; Arnold 2007).

Trichoderma endophytes can be mycoparasitic (Bailey et al. 2008), and that attribute might add to the potential of a defense mutualist. However, caution is needed in this regard. A mycoparasite could conceivably work against a defense mutualist,

as appears to be the case with *Hydropisphaera fungicola* (Rossman et al. 2008) that parasitizes an endophytic *Ulocladium* that itself reduces the severity of *Melampsora* leaf rust in *Populus* (Newcombe et al. 2010).

If selection were based on a classification scheme that emphasizes function, such as that described by Rodriguez et al. (2009), non-clavicipitaceous, Class 2 endophytes would seem like the logical choice. They are known to improve host fitness in a multitude of ways, and their abundance in both root and shoot systems of herbaceous plants would seem to provide a basis for their selection (i.e., selection of matching endophyte isolates from roots and shoots). Although surveys of endophyte communities in forest trees have largely been focused on shoot systems (Sieber 2007), roots have also been sampled in a number of tree genera (Stone et al. 2000). But, correlated studies of both root and shoot systems of individual trees appear to be absent. Endophytes from seeds of *Pinus monticola* included isolates of *Hormonema*, *Geomyces* and *Cladosporium* that were also isolated from healthy needles (Ganley and Newcombe 2006), so these fungi could be Class 2 endophytes that merit further assessment as candidate mutualists. It is sometimes argued that mutualists are favored in vertical transmission through seed, suggesting that selection of endophytes from seeds of forest trees might yield endophytes that enhance host fitness. But pathogenic isolates are also a possibility in tree seeds (Höfnagels and Linderman 1999), necessitating assays. With appropriate germination and inoculation assays, desirable, seed-associated fungi have been distinguished in *Podocarpus falcatus* from pathogenic endophytes (Gure et al. 2005).

2.2 *Endophyte Community Assembly Dynamics: Are Selected, Deployed Endophytes Replaced?*

Obviously, an individual forest tree is never interacting with one symbiont alone. Even discounting diverse endophyte and mycorrhizal communities, and even when one pathogenic symbiont has very strong effects on a host tree, as the Dutch elm disease pathogen has on susceptible elms, other symbionts are affecting both the pathogen and the host (Webber 1981). A bacterial endophyte promoting growth in lodgepole pine can be inhibited in an analogous manner by other rhizobacteria that roots might encounter in soil (Bent and Chanway 1998). Plant growth-promoting rhizobacteria may induce resistance to fusiform rust in loblolly pine seedlings (Enebak and Carey 2000), and thus indirectly affect other members of the biotic community that consume galled plant tissue (Peterson 1960).

Endophyte communities in forest trees are particularly diverse (Arnold 2007). Yet other examples will be discussed in this chapter and elsewhere in this book in which endophytes interact with one another, the host, mycorrhizal fungi, pathogens and arthropods. It goes without saying that community interactions must be much more complex than studied examples suggest, due to the number of players involved.

Whatever the level of complexity, net effects of the community on the host matter greatly inasmuch as this epigenetic variation is selectable (Gilbert et al. 2010).

Even recruitment into the community presents difficult questions. Attempts have been made to develop community assembly rules to predict which plants will become members of a community when larger numbers are available for recruitment (Weiher and Keddy 1999). Thus far, rules in plant community assembly have been "challenging to find" (Wilson 1999), in spite of a long tradition in this research stretching back to the nineteenth century. Succession, vegetation and habitat types, and plant community composition are all thought to be products of local environmental filters (Daubenmire 1968), even if these have been difficult to pin down.

Filters for endophyte communities have thus far been identified only in a very general way as the host and the environment (Ahlholm et al. 2002). Host genetic variation can significantly affect endophyte community composition, as can infection of the host with a systemic pathogen (Pan et al. 2008) that would itself be dependent on an environment conducive to infection at the appropriate time in development of a susceptible host. Endophyte-endophyte interactions *in planta* are undoubtedly significant but they have not been investigated yet to a meaningful extent.

The question of endophyte inoculants in particular exposes the relative absence of knowledge of assembly rules for, and interactions within, endophyte communities. It also automatically raises further questions about microbial community dynamics. In forestry circles, for example, the question brings to mind ectomycorrhizal inoculants that have commonly been replaced by other fungi of the site (Gagné et al. 2006). Concerns that endophyte inoculants might be similarly replaced cannot be adequately addressed at present. Nor can endophyte-endophyte interactions be studied in isolation; at least some endophytes can control mycorrhizal status with knock-on effects for competition between plants (Omacini et al. 2006). Even plant invasions may be facilitated by some *Neotyphodium* endophytes (Rudgers et al. 2005) that may themselves be affected by interactions with other endophytes. Assembly rules have been proposed that might eventually allow shifts between 'provider' mutualisms and the 'protector' variety to be predicted (Thrall et al. 2007). But, at present, questions about replacement of most endophyte inoculants cannot be addressed with any certainty.

On the other hand, this picture may change rapidly since microbial communities can be exploited as model systems. Their use has shown that the history of community assembly is an important factor in community membership (Fukami and Morin 2003). As first members of endophyte communities in trees, inoculants might be expected to influence subsequent assembly even if they were ultimately replaced. Moreover, as first members, protector inoculants, in particular, might last long enough as dominant elements in endophyte communities that they would usher their hosts through the youngest and most vulnerable phase in a tree's development: the seedling stage, that is highly vulnerable to negative feedback with soil pathogens (Mangan et al. 2010).

The importance of the first, or pioneering, symbionts of a host has also been demonstrated in systems that model human development (Xu and Gordon 2003). Mice raised under germ-free conditions possess abnormally primitive, submucosal networks of interconnected capillaries in their intestines. But, the pioneering bacterium *Bacteroides thetaiotaomicron* not only initiates an angiogenic program to complete the network; it also affects gene expression in its host to influence subsequent recruitment of other members of the distal gut microflora. It is easy to imagine analogous experiments with forest trees that would reveal the importance of the pioneering endophyte to subsequent community membership, even if the pioneering endophyte was eventually replaced. Candidates for the role of pioneering endophyte could presumably be selected from among the nonpathogenic isolates obtained from seed.

Related, pathogenic symbionts of a plant can competitively exclude one another from their host (Gold et al. 2009). Antagonism was actually found to increase with genetic distance among competitors. Although the exclusion of pathogens by endophytes tends to be considered as a separate concept (i.e., as a defense mutualism), it may be that endophyte-endophyte interactions are based primarily on competitive exclusion also. An overall pattern of competitive exclusion may emerge with more research that includes the whole continuum from mutualists to pathogens. Exclusionary interactions might be more likely if endophytes are functionally similar (e.g. a guild of defense mutualists), but less likely if the interactors are closely related enough to be vegetatively compatible (Gold et al. 2009). Replacement from within a guild would presumably not result in loss of function. It might also be true that interactions would be affected by responses of interactors to host defense, and plant organs might vary as venues. Foliar endophytes, for example, may be so limited in their colonization of leaf or needle tissue (Stone 1987) that interactions are relatively unlikely. In the long run, these and many other questions related to replacement interactions will need to be addressed.

There may be exceptional circumstances in which replacement dynamics do not matter. For example, when plant survival in geothermal soils is conditional upon symbiosis with a particular endophyte (Redman et al. 2002), endophytes that cannot confer thermotolerance on the symbiosis will not be replacements. Environments contaminated with phytotoxic pollutants may similarly act as strong filters and select for relatively few endophytes (Doty 2008). Plant community assembly rules have been easiest to formulate when recruitment was strongly limited by environmental filters (Booth and Larson 1999) and this may also prove true for endophyte communities.

2.3 Integration of Endophytes into Tree Improvement

Endophytes have not yet been integrated into tree improvement or breeding programs, and the reasons may span those covered here and then some others. Yet the greatest potential for endophyte applications in forestry probably is in

conjunction with tree improvement in that plantation forestry is rapidly growing in importance relative to other types of forestry (Sedjo 2005), and improved stock for planting is essential for plantations. Thus, this disconnect between endophyte research and plantation forestry is important and it may be heavily influenced by two conceptual problems: first, tree breeders know that the traits that endophytes influence are also strongly influenced by host genes, and secondly, they know that endophytes are themselves genetically dependent upon their hosts. To my knowledge, no researchers to date have distinguished and measured the relative contributions of endophytes and host genotypes to tree phenotypes. When trait heritabilities are high enough for breeders to pursue tree improvement objectives, what conceptual room is there for endophytes? Even the question that would arise in a heritability study of whether or not endophytes contribute to host genetic variation or to environmental variation, appears to be unanswered currently.

Secondly, the genotype of the host is a significant predictor of endophyte isolation frequency (Ahlholm et al. 2002; Todd 1988), at least in the few studied cases. So, trees could be selected for their ability to host particular endophytes. But, if selection criteria in a breeding program are already numerous, it may be hard to justify interest in yet another, especially when the additional criterion appears to be relatively difficult to evaluate. In *Populus* breeding for example, selection criteria are numerous ranging from yield, climatic adaptability, adventitious rooting, and disease resistance to wood quality and suitability as a bioenergy feedstock (Stanton et al. 2010). For the sake of illustration, let us consider the potential for endophytes to contribute to the selection of disease resistance in trees.

In general, disease or pathogen resistance in forest trees is heritable and tree breeders have been successful in improving populations in this regard (Namkoong 1991; Stanton et al. 2010). Disease resistance typically takes the form of either qualitative, major-gene resistance or quantitative resistance. There is no indication from the literature that endophytes can induce the hypersensitive responses to pathogens that characterize major genes for resistance. Quantitative resistance may be a different story. The absence of endophytes is typically assumed but not verified in such studies of quantitative resistance. Quantitative resistance can be highly (Lillemo et al. 2008) or more moderately heritable (Kayihan et al. 2005). The heritability of quantitative resistance to leaf rust of *Populus* can be as high as 0.87 (Lefevre et al. 1998). Uredinial size, a measure of quantitative resistance, can be highly heritable by itself (Dowkiw and Bastien 2004).

However, such high values are not necessarily proof that endophytes play little role in quantitative resistance. Endophytes could conceivably enhance expression of quantitative resistance within genotypes without changing the ratio of genetic to total phenotypic variation (i.e., heritability values). If this were the case for disease resistance, it could also be the case for some, or even all, of the selection criteria.

But, even so, and putting aside the other three challenges discussed here, tree breeders might still be reluctant to incorporate endophytes into their improvement programs. This is because microbes, including mutualistic endophytes, are commonly viewed as ubiquitous in the forest environment, such that there is no need for either selection or deployment via inoculation. In this view, nursery-grown seedlings

might be depauperate in endophytes, as mentioned previously, because the nursery is an environment set apart from nature. But, outplanted seedlings would not be depauperate as they would be immediately be colonized. The fastest correction to this erroneous view can likely be achieved by considering the phenomenon of enemy or pathogen release (Mitchell and Power 2003), whereby plants in their introduced ranges are beset by far fewer pathogens than they are in their native ranges. As mentioned already, ectomycorrhizal plants were also limited when introduced to new continents or regions by the lack of mutualist fungi (Richardson et al. 2000). However, if we accept that particular endophytes are not everywhere, we then have to confront the fourth challenge considered here.

2.4 Endophyte Host Range and Local Adaptation: Do We Move Selected Endophytes to New Environments, and If We Do, What Are the Likely Outcomes?

The temptation to move endophytes to other parts of the world will likely arise, as selections prove advantageous, and as related patents are filed. This seems especially likely for plants that are cultivated worldwide. For example, promising endophytes discovered in certain parts of banana's cultivated range (Akello et al. 2007; Vu et al. 2006) will probably appear in plantations elsewhere. If rugulosin-producing endophytes prove to be effective defense mutualists in Canada (Coyle et al. 2005), foresters elsewhere will be tempted to import them for suitable hosts. If *Piriformospora indica*, native to India (Verma et al. 1998), benefits model plants from *Arabidopsis* to *Populus*, why not employ it everywhere? The chief concern, of course, with deliberate endophyte introductions is that we currently have no basis for successfully predicting the extent or nature of the host-shifting that might occur. Evolutionarily naïve plants might be attacked by fungi that were mutualists in their native ranges, given that individual endophytes can shift from mutualistic to pathogenic roles, depending on their hosts (Rodriguez et al. 2009).

In general, humans have a poor track record in the area of deliberate introductions of non-native organisms (Olden et al. 2004). Even though most deliberately introduced organisms do not become invasive, some do, and membership in this latter group has been difficult to predict (Reichard and White 2001). Ectomycorrhizal fungi have been deliberately moved to allow dependent hosts, such as *Pinus*, to survive and grow in new environments, but at least some of these same mutualisms are now contributing to plant invasions (Richardson et al. 1994). Endophytic fungi have likely been inadvertently moved with plant materials, but the extent of such co-introductions is not known. Plant propagules with associated fungi have likely always accompanied migrating peoples, but the pace of introductions picked up notably around 1,500 with unknown implications for endophyte distributions. Thus, biotic homogenization during the last 500 years coupled with a general absence of baseline information on the native geographic ranges of endophytic

fungi (Newcombe and Dugan 2010) make it difficult to address the question of endophyte local adaptation. Moreover, the question of host range of endophytes remains inadequately studied.

Local adaptation is characterized by higher relative fitness in local habitats. For parasites this means greater virulence or aggressiveness versus local host populations, which would seem to place plant and parasite local adaptation at odds. Both parasite local adaptation (Springer 2007) and parasite local maladaptation (Kaltz et al. 1999) have been demonstrated in different host-parasite systems. Potential mutualist local adaptation has been observed with *Neotyphodium* in marginal habitats (Sullivan and Faeth 2008). In general, the overall, conceptual reconciliation of local adaptation of plants with diverse parasite and mutualist local adaptations and maladaptations has yet to be achieved. It is thus hard in general to argue in an evidence-based manner when it comes to the question of deliberate introductions of endophytes into new geographic ranges.

A final factor that may influence the geographic deployment of endophytes is climate change. Foresters are predicting widespread range shifts in tree species as the climate warms (Iverson and Prasad 1998), but endophytes can potentially extend the adaptations of their hosts (Rodriguez et al. 2004) to allow the latter to stand their ground. Some endophytes can regulate the thermotolerance of their hosts via effects on heat shock protein genes (McLellan et al. 2007), and these endophytes in particular might be pressed into service even if they were non-native to a particular site.

3 Conclusions

Endophytes could become much more important tools in forest management than they are at present because they affect fitness traits that matter to foresters: survival, growth, defense, and stress tolerance. In addition to the challenges to endophyte adoption discussed here, foresters may simply be unfamiliar with the endophyte research literature as it tends to be neglected or under-represented in the traditional forestry curriculum. Some forest pathology and ecology classes currently include some coverage of endophytes but the curricular upside is still considerable.

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Part VI
Endophytes as Sources of New Drug Compounds

Antimicrobial Compounds from Tree Endophytes

Anja Schueffler and Timm Anke

Abstract Endophytes are organisms that live at least parts of their life cycle asymptomatically within the plant tissue. Endophytic fungi include new species as well as latent pathogens and dormant saprophytes. The estimated high species diversity of endophytes and their adaptation to various plant habitats presumes a rich and almost untapped source of new secondary metabolites, some of which might become useful leads for pharmaceutical or agricultural applications. Forests are large reservoirs for fungal diversity, covering 30.3% of the land area in the world. This chapter focuses on bioactive natural compounds, which were isolated from tree endophytes described from 2007 on. Furthermore, an overview is given on research efforts of pharmaceutically significant plant compounds produced by endophytic fungi, namely: taxol, camptothecin, as well as podophyllotoxin and derivatives. In addition, recent literature on endophytes and the biological activity of their extracts is cited.

Abbreviations

ITS	internal transcribed spacer
MIC	minimal inhibitory concentration
IC_{50}	half maximal inhibitory concentration
HIV	human immunodeficiency virus
VOC	volatile organic compound

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1 Introduction

"Endophytes colonize symptomlessly the living, internal tissues of their host, even though the endophyte may, after an incubation or latency period, cause disease" (Petrini 1991). In literature the term "fungal endophytes" is normally used to describe fungal organisms, which in contrast to mycorrhizal fungi, reside entirely within the host tissues and emerge during host senescence (Rodriguez and Redman 2008). Fungal endophytes can be classified into two groups exhibiting differences in evolution, taxonomy, hosts, and ecology: the clavicipitaceous endophytes of some grasses and the nonclavicipitaceous endophytes of nonvascular plants, ferns, conifers and angiosperms (for review see Rodriguez et al. 2009). Most of the endophytes from woody plants belong to the ascomycetes of diverse phylogenetic origin. Endophytes also include latent pathogens and dormant saprophytes (Saikkonen et al. 1998; Osono 2006), which live at least parts of their life cycle asymptomatically within the plant tissue before conspicuous signs of infection (e.g. fruiting bodies) appear. The estimation of the total number of endophytic fungal species by Dreyfuss and Chapela (1994) is as high as 1.3 million which would represent by far the largest part of the 1.5 million fungal species postulated by Hawksworth (2001). More conservative estimations by Schmit and Mueller (2007) assumed that there were two microfungal species for every terrestrial plant species which would lead to at least 600,000 plant-associated microfungi. It has to be taken into account, however, that all these estimations strongly depend on the respective species concept.

During the last decades endophytes have come into the radar of research efforts for several reasons. Some studies showed that endophytes could promote the growth of the host plant, inhibit the growth of pathogens, and improve stress tolerance (e.g. Arnold et al. 2003; Herre et al. 2007; Rodriguez and Redman 2008), all of which could lead to an increased productivity. An interest in secondary metabolism of endophytes arose with the identification of toxins in endophyte-infected grasses causing toxicosis to grazing animals (Powell and Petroski 1992) and the discovery of a taxol-producing endophyte isolated from the Pacific yew, in 1993 (Stierle et al. 1993). Whereas only 75,000 fungal species were known in 2000 (Hawksworth 2001), their number should increase dramatically even if the estimations of endophytic species were exaggerated. In any case, the endophytes, especially the new species, offer new and unexploited sources of new secondary metabolites, some of which might become useful leads for pharmaceutical or agricultural applications.

Since ancient times, mankind has had to cope with various threats, such as famine caused by plant pathogens, and human diseases such as cancer, or microbial and viral infections. The approaches to new and useful biologically active compounds by the exploration of synthetic and natural products have both limits and drawbacks, e.g. complexity and chirality for the synthesis and the availability of producers and the supply of large amounts for the natural products. In the field of natural products, the most promising approach to finding new chemical entities has always been the exploitation of previously untapped biological sources, e.g. basidiomycetes

or teleomorphic ascomycetes. Now the endophytes, due to their adaption to very diverse plant habitats and their large number, offer great promise for the future.

There are several up-to-date reviews on endophyte metabolites with biological activity. A review by Pimentel et al. (2011) focuses on the role of endophytes in the production of bioactive compounds, and on an alternative method of microbial biotransformation to obtain compounds. Yu et al. (2010) review methods for the isolation and screening of endophytes, and categorized recently found endophytic compounds. Gunatilaka (2006) discusses the structures of over 230 metabolites isolated and characterized from over 70 plant-associated microbial strains during 2002–2006. Additional literature on endophytic metabolites and endophytes has been reviewed by Tan and Zou (2001), Strobel and Daisy (2003), Strobel et al. (2004), Zhang et al. (2006), Firáková et al. (2007), Guo et al. (2008), Weber (2009), and Aly et al. (2010).

2 Isolation of Endophytes

Endophytes colonize the living, internal tissues of their host. Therefore the isolation methods usually consist of steps for a surface-sterilization of plant parts and outgrowth of fungi from the plant specimen on suitable media. The strains thus obtained are usually assumed to be endophytes and not strains e.g. resistant to surface sterilization or latent pathogens. The proof that a fungus thus isolated is indeed an endophyte is tedious and time consuming and could for example consist of a comparison of genetic markers of fungal tissue in the plant or successful reinfection and reisolation. This, however, is very rarely done. Another complication is that sometimes, especially in reports on new natural compounds, there is no indication on the region where the specimen were collected, the plant species, the plant tissue, or the details on the isolation procedures. There are several publications available that describe appropriate isolation methods (e.g. Stone et al. 2004; Gallo et al. 2008; Filip et al. 2003). The subsequent cultivation, fermentation and testing for biological activities usually follow the well established methods for asco- and basidiomycetes.

3 Compounds First Isolated from Trees, Later Found to be Produced by Endophytic Fungi

3.1 Taxol

Taxol was first isolated from the stem bark of the conifer *Taxus brevifolia* (western yew, Taxaceae). The pronounced cytotoxicity of taxol made it an interesting candidate for further development (Wani et al. 1971). This, however, was greatly

hampered by the limited supply of the compound as the removal of the bark kills the tree, causing a drawback of its clinical development (Jennewein and Croteau 2001). Researchers worldwide tried to solve the problem with different approaches: Potier (Guenard et al. 1993) using the leafs of the European Yew instead of the bark resulting in the semisynthetic TAXOTÈRE®, chemical synthesis (e.g. Nicolaou et al. 1994; Danishefsky et al. 1996), semisynthetic efforts (e.g. Ojima et al. 1992), plant tissue cell culture (e.g. Zhong 2002; Tabata 2004), and microbial fermentation (e.g. Jennewein and Croteau 2001; Frense 2007). Such hope exists that the isolation of taxol-producing fungal endophytes could lead to another option for a reliable production of the large quantities needed for cancer treatment. To date, there are several fungal species isolated from trees reported to produce taxol, e.g.

- *Taxomyces andreanae*, isolated from the phloem of the Pacific yew, *Taxus brevifolia* (Taxaceae; Stierle et al. 1993)
- *Pestalotiopsis guepinii*, an endophyte of the Wollemi pine (*Wollemia nobilis,* Araucariaceae; Strobel et al. 1997)
- *Periconia* sp. from the evergreen tree *Torreya grandifolia* (Taxaceae; Li et al. 1998)
- *Seimatoantlerium nepalense*, a coelomycete from Himalayan yew (*Taxus wallachiana*; Bashyal et al. 1999)
- *Tubercularia* sp. strain TF5 from *Taxus mairei* (Wang et al. 2000)
- *Bartalinia robillardoides* isolated from the medicinal tree Bael (*Aegle marmelos*, Rutaceae; Gangadevi and Muthumary 2008)
- *Phyllosticta spinarum* from *Cupressus* sp. (Cupressaceae; Kumaran et al. 2008)
- *Xylaria* sp., *Sordaria* sp., *Metarhizium anisopliae*, and *Coniothyrium diplodiella*, isolated from *Taxus chinensis* (Liu et al. 2009)
- *Pestalotiopsis* species isolated from *Taxus cuspidate* (Kumaran et al. 2010)

However, currently the levels of taxol produced by fungal isolates are far too low for industrial production (Aly et al. 2010). Several questions arise from the fact that endophytic fungi can form the same metabolites as their host plants taking into account that the possibility of the independent evolution and development seems unlikely. Since genes coding for taxol biosynthesis (10-deacetylbaccatin-III-10-*O*-acetyl transferase, C-13 phenylpropanoid side chain-CoA acyltransferase) showing high sequence similarity with the plant counterparts were confirmed in three out of 90 endophytic fungi from *Taxus* species (Zhang et al. 2008) and in *Taxomyces andreanae* (Staniek et al. 2009), the horizontal gene transfer hypothesis should be investigated further.

Young et al. (1992) showed that taxol exhibits antifungal properties especially towards oomycetes, which could be one of the ecological functions of this compound. In strong support of this theory is the fact that the production of taxol could be enhanced through addition of fungal elicitors (Wang et al. 2001). In recent years, several reviews were published on taxol-producing endophytes, most recently Flores-Bustamante et al. (2010) and Zhou et al. (2010).

3.2 Camptothecin

The topoisomerase I inhibitor camptothecin was isolated from stem wood of the tree *Camptotheca acuminata* (Cornaceae; Wall et al. 1966). Later on, the compound was isolated from the small tree *Nothapodytes foetida* (Icacinaceae) found in India (formerly known as *Mappia foetida*; Govindachari and Viswanathan 1972). The semisynthetic camptothecin-derivatives irinotecan and topotecan are currently in clinical use. Although total synthesis has been accomplished by several groups (Li et al. 2006), the main source of camptothecin is still plant material. Recently several publications reported camptothecin-producing endophytes. Puri et al. (2005) isolated a fungus from *Nothapodytes foetida* showing 99.8% similarity to *Entrophospora infrequens* and belonging to the phylum Glomeromycota. From the same plant species Rehman et al. (2008) isolated a *Neurospora* species producing camptothecin. From *C. acuminata*, the endophytic *Xylaria* sp. M20 was isolated by Liu et al. (2010b) and *Fusarium solani* by Kusari et al. (2009b), which both produced camptothecin or analogs. Another camptothecin-producing *Fusarium solani* was described by Shweta et al. (2010) as an endophyte of *Apodytes dimidiata* (Icacinaceae), a medium-sized tree from the Western Ghats. This was the first communication that *Apodytes dimidiata* itself contains detectable amounts of camptothecin. As for taxol, one ecological role of camptothecin could be its antifungal activity. Li et al. (2005) studied the antifungal activity of several *C. acuminata* metabolites and showed that camptothecin is active against pathogens which are known to infect this tree (*Alternaria alternata*, *Epicoccum nigrum*, *Pestalotia guepinii*, *Drechslera* sp., *Fusarium avenaceum*).

3.3 Podophyllotoxin and Desoxypodophyllotoxin

Podophyllotoxin and desoxypodophyllotoxin were first described from *Podophyllum* species (Berberidaceae), perennial herbs used in traditional medicine (Liu et al. 2007 and references cited therein). In recent years podophyllotoxin and derivatives were found in other plant species, such as trees belonging to Juniperaceae and Cupressaceae. The aryltetralin lignan podophyllotoxin is highly toxic but semisynthetic derivatives such as etoposide and teniposide are used in cancer therapy. The semisynthetic compounds interact with the DNA/topoisomerase II complex leading to double-strand breaks and cell cycle arrest, whereas podophyllotoxin itself interacts with tubulin and blocks mitosis. The amounts of podophyllotoxin needed for therapy are extracted from *Podophyllum emodi* and *Podophyllum peltatum.* Chemical synthesis is not yet economically feasible (for review see Petersen and Alfermann 2001; Gordaliza et al. 2004; Canel et al. 2000). Two fungi, *Phialocephala fortinii* (Eyberger et al. 2006) and *Trametes hirsuta* (Puri et al. 2006) isolated from *Podophyllum* itself were described to produce podophyllotoxin. The basidiomycete *Trametes hirsuta* causes white rot of wood and is usually not

considered an endophyte. The first report of podophyllotoxin isolated from a tree endophyte was issued by Kour et al. (2008). The fungal strain JRE1, which showed 98% homology to gene sequences of *Fusarium oxysporum,* was isolated from a fully matured *Juniperus recurva* (Cupressaceae). Recently Kusari et al. (2009a) isolated a deoxypodophyllotoxin-producing *Aspergillus fumigatus* from twigs of common juniper. Additionally they proved by LC-MS quantification that different *Juniperus* species contain measurable quantities of deoxypodophyllotoxin. Kusari et al. (2009a) suggest that the isolated fungus could serve as source for aryl tetralin lignans for scientific and commercial uses.

4 Tree Endophytes as Source for Bioactive Natural Products

Every plant harbours at least one endophytic fungus. Bills and Polishook (1991) investigated twigs of *Carpinus caroliniana* (American hornbeam, Betulaceae) and Sieber-Canavesi and Sieber (1987, 1993) needles of *Abies alba* (European silver fir, Pinaeceae) and both found more than 120 species. The diversity is high even in a small tissue sample like one needle of Douglas fir (Pinaceae) where Carroll (1995) could isolate up to four fungal species. Forests cover 30.3% of the land area in the world (FAO 2006) forming a large reservoir of fungal diversity.

In the following we will focus on tree endophytes and on bioactive metabolites described between 2007 and 2010. The family of the plant species is provided in brackets and phylogenetic data were taken from the NCBI taxonomy database (Sayers et al. 2009). Table 1 summarizes the endophytes, their host trees and the produced metabolites.

4.1 Gymnosperm Endophytes Producing Bioactive Metabolites

4.1.1 Conifers

Conifers are gymnosperms with nearly 550 species in 67 genera and worldwide distribution. Widespread genera are *Pinus* (pines), *Abies* (firs), *Picea* (spruces), and *Juniperus* (juniper) and representatives found mainly in the boreal forests (Eckenwalder 2009).

4.1.1a *Microdiplodia* sp. KS 75-1 from *Pinus* sp. (Pinaceae)

Microdiplodia sp. strain KS 75-1 was isolated from the stem of a *Pinus* sp. (Japan) by Hatakeyama et al. (2010). The authors isolated two new eremophilane sesquiterpenes, 8α-acetoxyphomadecalin C and phomadecalin E, together with the known compounds phomadecalins C and D (Fig. 1). 8α-Acetoxyphomadecalin

Table 1 Metabolites isolated from tree fungal endophytes

Plant	Endophyte	Isolated metabolite	Reference
Pinus sp	*Microdiplodia* sp. strain KS 75-1	**8α-Acetoxyphomadecalin C**	Hatakeyama et al. 2010
		Phomadecalin E	
		Phomadecalins C, D	
Picea glauca	Strain CBS 120379 (unidentified)	**2,8-Dihydroxy-3-methyl-9-oxoxanthene-1-carboxylic acid methylester**	Sumarah et al. 2008b
		(3R)-Mellein	
	Strain CBS 120380 (unidentified)	**2-Acetyl-4,6-dimethoxybenzoic acid**	
		5,7-Dimethoxy-3-methylpthalide	
		3,4-Dihydro-8-hydroxy-6-methoxy-(3R)-propyl-isocoumarin	
	Strain CBS 120381 (unidentified)	(3R)-5-Carbomethoxymellein	
		(3R)-5-Formylmellein	
		(3R)-5-Methylmellein	
		3-Butyl-4-methyl-5H-furan-2-one	
		tyrosol	
Picea rubens	Strain CBS 121942 (unidentified)	**(9E,12Z)11-Hydroxy-6-methyl-7,15-dioxabicyclo [12.1.0]penta-deca-9,12-diene-2,8-dione**	Sumarah et al. 2010
		4-Oxo-5-phenyl-pentanoic acid	
		5-Benzyl-dihydro-furan-2-one	
	Strain CBS 121944 (unidentified)	Isopetasol	
		15-Hydroxy-3-epiisopetasol	
	DAOM 239833 (related to *Dwayaangam colodena*)	Cordyanhydrides A, B	
		7-(4-But-1-enyl-2,5-dioxo-2,5-dihydro-furan-3-ylmethyl)-4,5-dicarboxy-non-5-enoic acid	

(continued)

Table 1 (continued)

Plant	Endophyte	Isolated metabolite	Reference
Torreya jackii	*Xylaria* sp. NCY2	**1-(Xylarenone A)xylariate A**	Hu et al. 2010
		Xylarioic acid B	
		Xylariolides A-C	
		Methyl xylariate C	
		Xylariolide D	
		Taiwapyrone	
		Xylarenones A-B	Hu et al. 2008
		Xylarenic acid	
Ginkgo biloba	*Chaetomium globosum*	Chaetomugilin A	Qin et al. 2009
		Chaetomugilin D	
		Chaetoglobosins A, C	
	Xylaria sp.	**7-Amino-4-methylcoumarin**	Liu et al. 2008a
Kandelia candel	*Talaromyces* sp. ZH-154	**7-Epiaustdiol**	Liu et al. 2010a
		8-*O*-methylepiaustdiol	
		Stemphyperylenol	
		Skyrin	
		Secalonic acid A	
		Emodin	
		Norlichexanthone	
Angiosperm mangrove	*Xylaria* sp. (#2508)	**Xylopyridine A**	Xu et al. 2009b

Azadirachta indica	*Chloridium* sp.	Javanicin	Kharwar et al. 2009
	Phomopsis sp. YM 311483	**8α-Acetoxy-5α-hydroxy-7-oxodecan-10-olide**	Wu et al. 2008
		7α,8α-Dihydroxy-3,5-decadien-10-olide	
		7α-Acetoxymultiplolide A	
		8α-Acetoxymultiplolide A	
		Multiplolide A	
Laurus azorica	*Phomopsis* sp.	**Cycloepoxylactone**	Hussain et al. 2009
		Cycloepoxytriols A, B	
		Phomolactones A-C	
Piptadenia adiantoides	*Cochliobolus* sp. UFMGCB-555	Cochlioquinone A	Campos et al. 2008
		Isocochlioquinone A	
	Fusarium sp. UFMGCB-551	T2-Toxin	Campos et al. 2010
		8-n-Butyrylneosolaniol	
		8-Isobutyrylsolaniol	
Sandoricum koetjape	*Xylaria* sp.	**2-Chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione**	Tansuwan et al. 2007
		Xylariaquinone A	
		2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione	
		4-Hydroxymellein	

(continued)

Table 1 (continued)

Plant	Endophyte	Isolated metabolite	Reference
Vatica mangachapoi	*Endothia gyrosa* IFB-E023	Cytochalasins H, J Epoxycytochalasin H **Cytochalasins Z10, Z11**	Xu et al. 2009a
Garcinia dulcis	*Xylaria* sp. PSU-D14	**Xylarosides A, B** Sordaricin 2,3-Dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one	Pongcharoen et al. 2008
Piper aduncum	*Xylaria* sp.	**9,15-Dihydroxy-presilphiperfolan-4-oic acid** **15-Acetoxy-9-hydroxy presilphiperfolan-4-oic acid** Phomenone Phaseolinone	Silva et al. 2010
Ligustrum lucidum	*Xylaria hypoxylon* AT-028	**Xylariols A, B**	Gu and Ding 2008
Quercus variabilis	*Penicillium sp.* IFB-E022 f	**Penicidones A-C**	Ge et al. 2008
Unidentified tree	*Pestalotiopsis theae*	**Pestalotheols A-D**	Li et al. 2008
Unidentified tree	*Pestalotiopsis fici*	**Pestaloficiols A-E**	Liu et al. 2008b

Bold: Newly described compounds

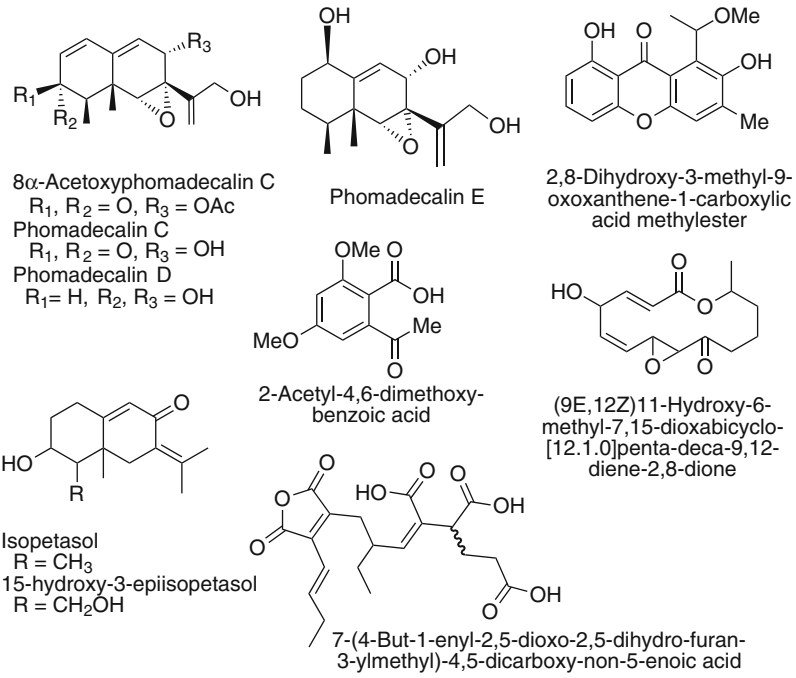

Fig. 1 Natural products from endophytes isolated from the genera *Pinus* and *Picea*

C and phomadecalin E were evaluated by agar diffusion assay against Gram-positive and Gram-negative bacteria, yeasts, and fungi. No activity was observed against *Staphylococcus aureus*, *Candida albicans*, *Fusarium* sp. and *Aspergillus clavatus* but against *Pseudomonas aeruginosa* 8α-acetoxyphomadecalin C and phomadecalin E showed moderate activities with inhibition zones of 12 and 11 mm in diameter (200 µg/disk). The phomadecalins C and D originally described in 2002 by Che et al. showed moderate antibacterial activity and were isolated from a *Phoma* species found on *Hypoxylon stomata* (Che et al. 2002).

4.1.1b Endophytes of *Picea* Species (Pinaceae)

Sumarah et al. (2008b) tested 250 foliar endophytes isolated from needles of *Picea glauca* (white spruce, North America) for their inhibitory activity against *Choristoneura fumiferana* (spruce budworm) and *Saccharomyces cerevisiae*. Three of these strains were selected for further study based on their ability to be cultured and to produce secondary metabolites under laboratory conditions. The strain CBS 120381 (ITS data indicated *Xylariaceae* related to *Nemania serpens*) yielded known compounds; three mellein derivatives [(3R)-5-Carbomethoxymellein, (3R)-5-Formylmellein, (3R)-5-Methylmellein], as well as 3-butyl-4-methyl-5H-furan-2-

one, and tyrosol. The new xanthone-derived compound 2,8-dihydroxy-3-methyl-9-oxoxanthene-1-carboxylic acid methylester (Fig. 1) and (3R)-mellein were isolated from strain CBS 120379 (unidentified). The third strain CBS 120380 (unidentified) yielded the new compound 2-acetyl-4,6-dimethoxybenzoic acid (Fig. 1), as well as the known 5,7-dimethoxy-3-methylpthalide and 3,4-dihydro-8-hydroxy-6-methoxy-(3R)-propyl-isocoumarin.

Recently, a similar study was conducted on red spruce (*Picea rubens*) where 150 foliar endophytes were assayed (Sumarah et al. 2010). Again, three fungi were selected for further study. A new compound (9E,12Z)11-hydroxy-6-methyl-7,15-dioxabicyclo[12.1.0]penta-deca-9,12-diene-2,8-dione (Fig. 1) together with 4-oxo-5-phenyl-pentanoic acid and 5-benzyl-dihydro-furan-2-one, not isolated as natural products before, were derived from the strain CBS 121942 (unidentified). The known sesquiterpene isopetasol (Fig. 1) was isolated together with the new derivative 15-hydroxy-3-epiisopetasol (Fig. 1) from CBS 121944 (unidentified). DAOM 239833, conspecific with *Dwayaangam colodena* (determined by ITS sequence) yielded four maleic anhydrides, the known cordyanhydrides A and B, as well as new stereoisomeric 7-(4-But-1-enyl-2,5-dioxo-2,5-dihydro-furan-3-ylmethyl)-4,5-dicarboxy-non-5-enoic acid (Fig. 1). In both studies, the biological activity of the pure compounds against spruce budworm was not specified although the extracts showed high activity (Sumarah et al. 2008b, 2010).

In addition, field studies were performed by Sumarah et al. (2008a). They inoculated *Picea glauca* with the rugulosin-producing endophyte 5WS22E1 (*Phialocephala* sp. based on DNA sequencing) that was initially reported to produce rugulosin by Calhoun et al. (1992). Four years after inoculation, they were able to show by an ELISA assay that the endophyte and rugulosin were present in the plants. Rugulosin, a known toxin produced by several fungi was first reported by Breen et al. (1955) from *Penicillium rugulosum*. Calhoun et al. (1992) showed that this compound is toxic to *Choristoneura fumiferana* larvae. Besides *C. fumiferana*, Sumarah et al. (2008a) tested rugulosin against the hemlock looper (*Lambdina fiscellaria*) and the spruce budmoth (*Zeiraphera canadensis*). Reduction in bodyweight was shown for them in response to 25 and 150 mM rugulosin. Subsequently Miller et al. (2008) studied the effects of endophyte-infected trees and uninfected trees on the spruce budworm growth. They demonstrated that the budworms recollected from the infected trees which contained rugulosin were smaller than those from untreated trees and that the observed growth reduction was concentration-dependent. The authors concluded that rugulosin produced by the endophyte is the reason for the insect's growth inhibition.

4.1.1c *Xylaria* sp. NCY2 from *Torreya jackii* (Taxaceae)

The nutmeg yews (*Torreya*) are evergreen trees and shrubs. *Torreya jackii*, a species of conifer in the Cephalotaxaceae, is endemic to southeastern China and belongs to the rare and endangered plants (Eckenwalder 2009). The endophyte *Xylaria* sp. NCY2 isolated from *Torreya jackii* was described by Hu et al. (2010) to produce

seven novel polyketides, namely, 1-(xylarenone A) xylariate A, xylarioic acid B, xylariolide A, xylariolide B, xylariolide C, methyl xylariate C, and xylariolide D and additionally the known taiwapyrone (Fig. 2), described initially as a metabolite of the crop pathogen *Cercospora taiwanensis* (Camarda et al. 1976). In an assay for cytotoxicity, the compounds exhibited weak inhibitory activities against the human cell lines HepG2 and HeLa (10 µg/ml). In an antibacterial assay the metabolites inhibited the growth of *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* with MIC values above 10 µg/ml and had no effect on the growth of yeasts (*Saccharomyces cerevisiae* and *Candida albicans*, 10 µg/ml).

In 2008, Hu et al. described the isolation of three new sesquiterpenoids, xylarenones A and B, as well as xylarenic acid (Fig. 2) from the same *Xylaria* sp. NCY2. The isolated compounds showed modest cytotoxic activity against HeLa and HepG2 cells (10 µg/ml). Bacterial growth was very weakly inhibited at 50 µg/ml and no activity was observed against yeasts.

4.1.2 *Chaetomium globosum* and *Xylaria* sp. from Ginkgo (Ginkgoaceae)

The gymnosperm *Ginkgo biloba* is a tall dioecious tree representing a living fossil, not closely related to any other known living plant and is therefore classified as a separate taxon – Ginkgoaceae. Preparations of this plant (leaves, seeds) are used to treat hypertension, insomnia, anxiety neurosis, and to ameliorate memory loss. In general, this plant is used to treat diseases appearing at an advanced age (Daniel 2006). *Ginkgo biloba* is native to China and occurred probably in mixed-mesophytic forests (Tredici 2000).

Qin et al. (2009) studied the endophyte *Chaetomium globosum* which was isolated from the sterilized leaves of *Ginkgo biloba* (China). The extract showed activity against brine shrimp (*Artemia salina*) and activity-directed fractionation led to the isolation of the two azaphilones chaetomugilins D and A (Fig. 2), and two cytochalasan alkaloids chaetoglobosins A and C. Chaetomugilin D, a chlorine-containing azaphilone, is a new metabolite. The compounds showed significant toxicity against brine shrimps after 24 h at a concentration of 10 μg/ml with mortality rates of more than 75%. The chaetoglobosins A and C also exhibited marked inhibitory effects on *Mucor miehei* in an agar diffusion assay (10 μg/disk). These two compounds, earlier isolated from *Chaetomium globosum,* showed cytotoxic activity against HeLa cells at 10 μg/ml (Sekita et al. 1973; Umeda et al. 1975). The cytotoxic chaetomugilin A was described in 2008 by Yamada et al. from *Chaetomium globosum* isolated from the marine fish *Mugil cephalus* (Yamada et al. 2008).

An endophytic *Xylaria* sp. isolated from *Ginkgo biloba* exhibited broad antimicrobial activity. From culture extracts of this fungus Liu et al. (2008a) isolated 7-amino-4-methylcoumarin (Fig. 2) by bioactivity-guided fractionation. This compound showed antibiotic activity against several bacteria with MIC's ranging from 4 to 25 μg/ml and against fungi from 15 to 40 µg/ml.

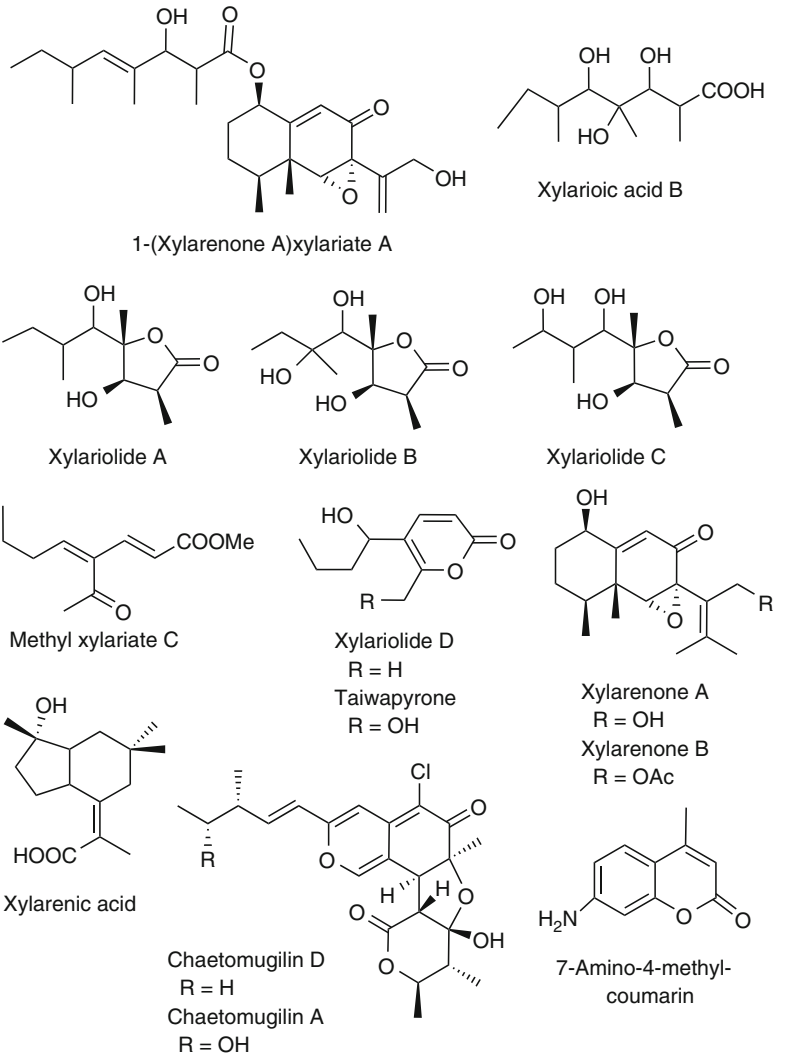

Fig. 2 Natural products from endophytes isolated from *Torreya jackii* and *Ginkgo biloba*

4.2 Angiosperm Endophytes Producing Bioactive Metabolites

4.2.1 Mangroves

Living between the sea and the land and being affected by the tides, the mangrove forests form a unique ecosystem. The plants growing in this transition zone are exposed to permanent changes in moisture, salinity, and oxygen content in the soil, conditions to which they have had to adapt. Mainly located in Asia, Africa, the

Americas, and Australia, mangrove estuaries are estimated to cover 15.2 million hectares (FAO 2008). These special ecosystems are expected to harbor an adapted fungal community which might give rise to unusual metabolites. Endophytes of mangroves have been examined extensively (for reviews see e.g. Kumaresan and Suryanarayanan 2001; Ananda and Sridhar 2002; Cheng et al. 2009). Recently Zhu et al. (2009) summarized the metabolites of the mangrove *Avicennia marina* (Acanthaceae) and the endophytic compounds together with their biological activities. This mangrove is cosmopolitan and widely distributed along tropical and subtropical coastlines.

4.2.1a *Phomopsis* sp. ZSU-H76 from *Excoecaria agallocha* (Euphorbiaceae)

Phomopsis sp. ZSU-H76 was isolated from the stem of the mangrove tree *Excoecaria agallocha* (China). Huang et al. (2008) isolated five octaketides: three new ones named phomopsin A, B, and C (Fig. 3) and the two known metabolites cytosporone B and C. Phomopsin A, B, and C had no significant antibiotic activity against three bacterial and two fungal strains. Cytosporone B and C inhibited *Candida albicans* and *Fusarium oxysporum* with MIC's ranging from 32 to 64 μg/ml. None of the metabolites had significant antibacterial activity. The known octaketides cytosporone B and C were initially described from endophytic fungi *Cytospora* sp. and *Diaporthe* sp. isolated from *Conocarpus erecta* (Button Mangrove, Combretaceae) and *Forsteronia spicata* (Apocynaceae; Brady et al. 2000).

4.2.1b *Talaromyces* sp. ZH-154 from *Kandelia candel* (Rhizophoraceae)

Talaromyces sp. ZH-154 was isolated from the bark of the mangrove tree *Kandelia candel* (China; Liu et al. 2010a). Two new metabolites were described and named 7-epiaustdiol, a new stereoisomer of austdiol and 8-*O*-methylepiaustdiol (Fig. 3). Additionally some known compounds were described (stemphyperylenol, skyrin, secalonic acid A, emodin, and norlichexanthone). 7-Epiaustdiol had inhibitory activity towards *Pseudomonas aeruginosa* (MIC 6.25 μg/ml) and *Staphylococcus aureus* (MIC 12.5 μg/ml). The methylated derivative 8-*O*-methylepiaustdiol showed less activity (MIC 25 and 50 μg/ml, respectively). The known compounds exhibited antibacterial and antifungal activity with MIC values ranging from 3.12 to 50 μg/ml. With the exception of secalonic acid ($IC_{50} \leq 1.05$ μg/ml), all isolated secondary metabolites displayed moderate cytotoxic activity.

4.2.1c *Xylaria* sp. from an Angiosperm Mangrove Tree

Xu et al. (2009b) isolated xylopyridine A (Fig. 3), a compound with a novel symmetrical pyridine dimer skeleton. The producing endophyte *Xylaria* sp. (#2508)

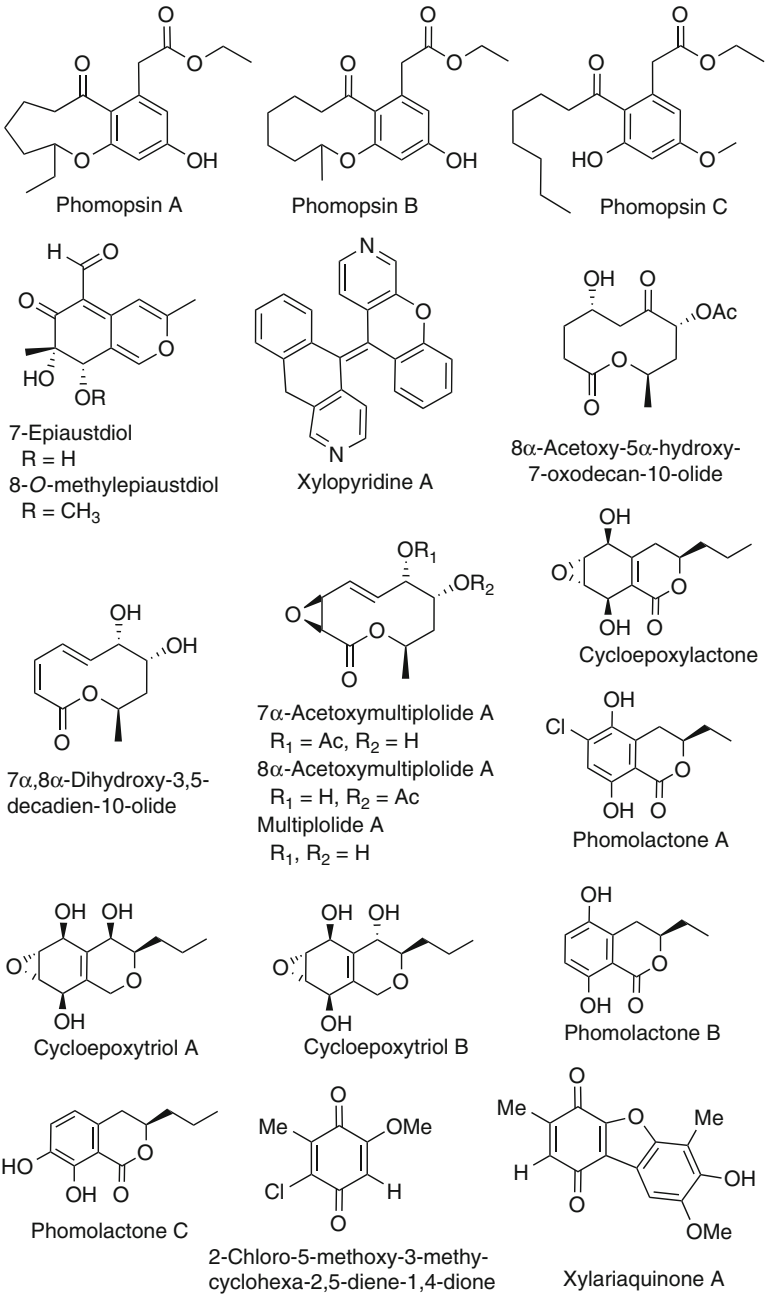

Fig. 3 Metabolites from endophytes isolated from different angiosperms I: Mangrove species, *Azadirachta indica*, *Laurus azorica*, and *Sandoricum koetjape*

was isolated from the seeds of an angiosperm mangrove tree (China). Xylopyridine A showed strong DNA-binding affinity towards calf thymus DNA.

4.2.2 *Chloridium* sp. and *Phomopsis* sp. YM 311483 from *Azadirachta indica* (Meliaceae)

The neem or niemtree (*Azadirachta indica*) is known for its medicinal properties, insecticidal, herbicidal, pesticidal activity as well as a soil stabilizer. Leaves, bark and seeds of this evergreen tree native to India and the Indo-Pakistan subcontinent have been used since ancient times. *Azadirachta indica* grows in tropical dry deciduous and evergreen forests (Arora et al. 2008; Barceloux 2008; Swaminathan and Raguraman 2008).

A *Chloridium* sp. was isolated from the roots of the niemtree and the antibacterial naphtoquinone javanicin, a known metabolite of *Fusarium javanicum* (Arnstein and Cook 1947), was found by Kharwar et al. (2009). Javanicin showed antifungal (*Cercospora arachidicola*; MIC 5 μg/ml) and antibacterial activity against *Pseudomonas aeruginosa* and *P. fluorescens* (MIC 2 μg/ml).

Phomopsis sp. YM 311483 was isolated from surface-sterilized fresh stems of a healthy *Azadirachta indica* specimen (China). Wu et al. (2008) isolated four new ten-membered lactones named 8α-Acetoxy-5α-hydroxy-7-oxodecan-10-olide, 7α,8α-Dihydroxy-3,5-decadien-10-olide, 7α-Acetoxymulti-plolide A, and 8α-Acetoxymultiplolide A, as well as the known multiplolide A (Fig. 3). These compounds showed weak antifungal activity against seven plant pathogens (*Aspergillus niger*, *Botrytis cinerea*, *Fusarium avenaceum, F. moniliforme*, *Helmintho-sporium maydis*, *Penicillium islandicum*, *Ophiostoma minus*). 8α- Acetoxymultiplolide A was the most potent one with MIC values ranging from 31.25 to 500 μg/ml. Interestingly, 8α-acetoxymultiplolide A was more active than 7α-acetoxymultiplolide A. Their structures differ only in the position of the acetoxy substituent. The known multiplolide A was first described by Boonphong et al. (2001) from the wood decaying fungus *Xylaria multiplex* BCC 1111. In agreement with the data of Wu et al. (2008) the compound showed antifungal activity against *Candida albicans* with an IC_{50} value of 7 μg/ml, exhibited no cytotoxicity, and was inactive towards *Plasmodium falciparum*.

4.2.3 *Phomopsis* sp. from *Laurus azorica* (Lauraceae)

Laurus azorica is a tree species common in Laurel forests which are specialized evergreen rain forests common to the eastern side of southern China, Australia, Japan, South America, and south-east Africa (Thomas and Packham 2007). From leaves of *Laurus azorica*, Hussain et al. (2009) isolated *Phomopsis* sp. which produced three new metabolites, cycloepoxylactone, cycloepoxytriol A, and cycloepoxytriol B (Fig. 3). In order to obtain minor components, three additional new isocoumarins, named phomolactones A–C (Fig. 3) were found. Cycloepoxylactone

showed good antibacterial, antifungal, and algicidal activities against *Bacillus megaterium*, *Microbotryum violaceum*, and *Chlorella fusca*, respectively, whereas cycloepoxytriol B had good algicidal activity. Cycloepoxytriol A was inactive in these tests. No data was available for the isocoumarins.

4.2.4 *Cochliobolus* sp. and *Fusarium* sp. from *Piptadenia adiantoides* (Fabaceae)

The genus *Piptadenia* includes shrubs or treelets distributed in South America and Australia (Brenan 1955). Campos et al. reported in 2008 that the crude extract of the endophytic fungus *Cochliobolus* sp. strain UFMGCB-555 isolated from *Piptadenia adiantoides* (Fabaceae) killed the amastigote-like forms of *Leishmania amazonensis*, the causative organism of leishmaniasis. Via bioassay-guided fractionation the known cochlioquinone A and isocochlioquinone A were isolated. Both compounds were active against *L. amazonensis* with EC_{50} values of 1.7 and 4.1 μM and showed no cytotoxicity against three human cancer cell lines (MCF-7, TK-10, and UACC-62) indicating an interesting selectivity. Cochlioquinone A was initially isolated from *Cochliobolus myarbeanus* (Carruthers et al. 1971) and later from *Helminthosporium sativum* by Schaeffer et al. (1990). The compound exhibited nematocidal activity and competed to the ivermectine binding site. Furthermore an inhibition of diacylglycerol kinase (Ogawara et al. 1994) and NADH oxidase (Lim et al. 1996) was reported. In addition, cochlioquinones and epi-cochlioquinones were described as antagonists of the human chemokine receptor CCR5 displacing the macrophage inflammatory protein-1α (Yoganathan et al. 2004).

Recently Campos et al. (2010) described that extracts of the endophytic fungus *Fusarium* sp. strain UFMGCB-551 isolated from *Piptadenia adiantoides* exhibited antifungal activity against the human pathogen *Paracoccidioides brasiliensis*. Bioactivity guided isolation lead to three known trichothecenes (T2-toxin, 8-n-butyrylneosolaniol and 8-isobutyrylsolaniol) with MIC's ranging between 75 and 640 nmol/l. These highly cytotoxic mycotoxins are common metabolites of *Fusarium* species.

4.2.5 *Xylaria* sp. from *Sandoricum koetjape* (Meliaceae)

Tansuwan et al. (2007) described two new benzoquinone metabolites, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and xylariaquinone A (Fig. 3), which were isolated along with two known compounds (2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and 4-hydroxymellein) from *Xylaria* sp. derived from healthy leaves of *Sandoricum koetjape* (Meliaceae, Thailand). This plant, known as Santol, is a fast-growing deciduous tree with edible fruits, native to south-east Asia. It is esteemed as a landscaping and ornamental shade plant (Janick and Pauli 2008). 2-Chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and xylariaquinone A showed *in vitro* activity against *Plasmodium*

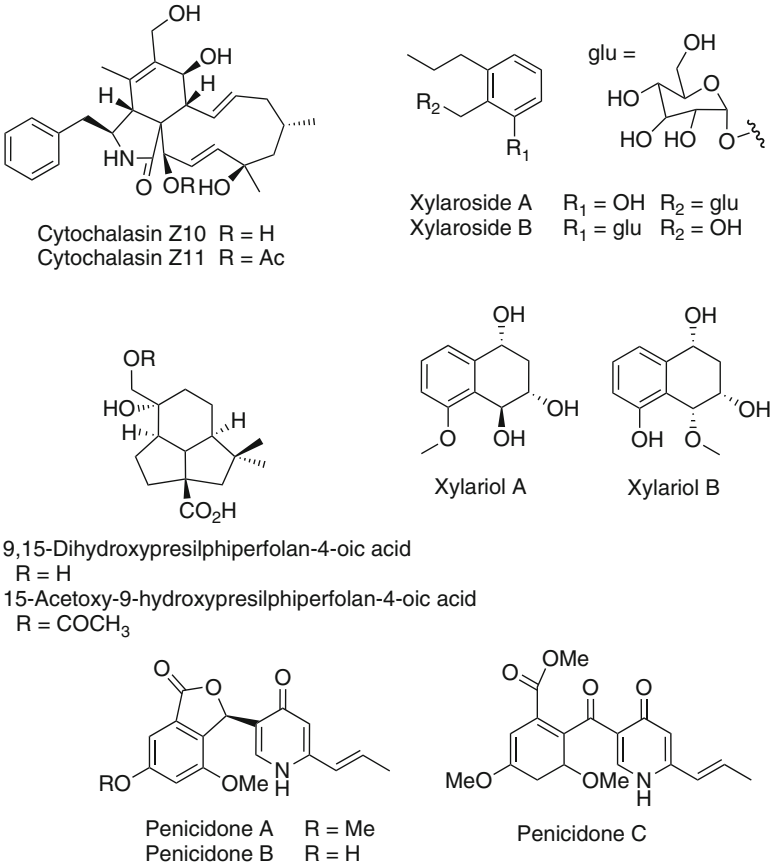

Fig. 4 Natural products from endophytes isolated from different angiosperms II: *Vatica mangachapoi, Garcinia dulcis, Piper aduncum, Ligustrum lucidum, and Quercus variabilis*

falciparum, with IC_{50} values of 1.84 and 6.68 μM and cytotoxicity against African green monkey kidney fibroblasts (VERO cells) with IC_{50} values of 1.35 and >184 μM, respectively. 2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and 4-hydroxymellein showed no activity against *P. falciparum*.

4.2.6 *Endothia gyrosa* IFB-E023 from *Vatica mangachapoi* (Dipterocarpaceae)

Xu et al. (2009a) examined the fungus *Endothia gyrosa* IFB-E023 isolated from the tree *Vatica mangachapoi* (Dipterocarpaceae, China). Three compounds were identified as cytochalasin H, cytochalasin J, and epoxycytochalasin H. Two new cytochalasins were named Z10 and Z11 (Fig. 4). These exhibited cytotoxic activities

against the human leukemia K562 cell line with an IC_{50} of 24.4 and 28.3 μM respectively. To date over 100 cytochalasans are known from ascomycete and basidiomycete species. For a recent review on their chemistry and biology see Scherlach et al. (2010).

4.2.7 *Xylaria* sp. PSU-D14 from *Garcinia dulcis* (Clusiaceae)

Xylaria sp. PSU-D14 (Pongcharoen et al. 2008) was isolated from the leaves of "Mundo" (*Garcinia dulcis*). Parts of this plant are used in traditional medicine. For example, fruits are used for diarrhea, and the gum is used for the external treatment of wounds, cancerous sores, and indolent ulcers (Li 2008). Pongcharoen et al. (2008) described two new glucoside derivatives, xylarosides A and B (Fig. 4) together with two known compounds, sordaricin and 2,3-dihydro-5-hydroxy-2-methyl-4H-1-benzopyran-4-one. Sordaricin, the aglycone of sordarin, exhibited the highest antifungal activity against *Candida albicans* with an MIC of 32 μg/ml. The remaining compounds showed only weak antifungal activities (MIC > 128 μg/ml). Previously, the highly antifungal sordaricin derivative xylarin had been described from a wood-inhabiting *Xylaria* species by Schneider et al. (1995). Hypoxysordarin and sordarin were described from cultures of *Hypoxylon croceum* found on driftwood in a mangrove estuary (Daferner et al. 1999). Sordarin was originally isolated as an antifungal compound from the terrestrial ascomycete *Sordaria araneosa* (Hauser and Sigg 1971). Because of its unique mode of action (Gomez-Lorenzo and García-Bustos 1998; Justice et al. 1998) it has long been considered as a promising candidate for further development.

4.2.8 *Xylaria* sp. from *Piper aduncum* (Piperaceae)

Silva et al. (2010) isolated two new presilphiperfolane sesquiterpenes, 9,15-dihydroxy-presilphiperfolan-4-oic acid and 15-acetoxy-9-hydroxy presilphiperfolan-4-oic acid (Fig. 4) along with two known eremophilane sesquiterpenes, phaseolinone and phomenone from *Xylaria* sp. This fungus was obtained from leaves of *Piper aduncum*, an evergreen small tree used in traditional medicine to treat headache, diarrhea, insect bites, and fungal infections (Siges et al. 2005). The two new compounds did not show any antifungal or cytotoxic activity. Phaseolinone displayed moderate cytotoxic (CHO cells: IC_{50} 200 μM) and phomenone antifungal activity (against *Cladosporium cladosporioides* and *C. sphaerospermum* at 10 μM). Earlier, phaseolinone and phomenone were isolated from another *Xylaria* sp. BCC 1067 and were shown to exhibit antiplasmodial and cytotoxic activities (Isaka et al. 2000). Both compounds are known phytotoxins that inhibit plant growth, isolated earlier from e.g. *Phoma exigua* (Riche et al. 1974) and *Macrophomina phaseolina* (Dhar et al. 1982).

4.2.9 *Xylaria hypoxylon* AT-028 from *Ligustrum lucidum* (Oleaceae)

Xylaria hypoxylon strain AT-028 was isolated from stems of *Ligustrum lucidum* (glossy privet, China; Gu and Ding 2008). Fruits of this plant were used in traditional Chinese medicine to treat leukopenia, chronic bronchitis, and acute dystery (Huang 1999). Gu and Ding (2008) describe the isolation and characterization of two new tetralone derivatives named xylariol A and B (Fig. 4). Both compounds showed moderate cytotoxicity against HepG2 cells with IC_{50} values of 22.3 and 21.2 μg/ml, respectively.

4.2.10 *Penicillium* sp. IFB-E022 from *Quercusvariabilis* (Fagaceae)

Ge et al. (2008) described three new alkaloids, the penicidones A-C (Fig. 4), from *Penicillium* sp. IFB-E022 isolated from the oriental oak (*Quercus variabilis*). The cytotoxicity was moderate with IC_{50} values between 21.1 and 90.8 μM against four human cancer cell lines (SW1116, K562, KB, HeLa). Penicidones A-C possess a γ-pyridone nucleus that had not earlier been encountered in natural products.

4.3 Two Pestalotiopsis Species from Unidentified Trees

Pestalotiopsis theae was isolated from an unidentified tree (China; Li et al. 2008). Bioassay-guided purification with an HIV-1 replication assay in C8166 cells afforded the discovery of four new chromenone-type structures, the pestalotheols A-D (Fig. 5). Pestalotheol C showed inhibitory effects with EC_{50} and CC_{50} values of 16.1 and 163 μM. Antimicrobial activity against a panel of organisms was not detected.

Liu et al. (2008b) isolated *Pestalotiopsis fici* from an unidentified tree (China). Five new metabolites named pestaloficiols A-E (Fig. 5) were discovered. Pestaloficiols A, B, and D inhibited HIV-1 replication in C8166 cells with EC_{50} values of 26.0, 98.1, and 64.1 μM and showed CC_{50} values greater than 200 μM. Pestaloficiols C and E were not tested due to sample limitations.

4.4 Volatile Organic Compounds from Muscodor Species (Xylariales)

In recent years notable work was done on volatile organic compounds (VOCs) produced by *Muscodor* species, belonging to the order Xylariales. Studies by Strobel et al. (2001) on the endophyte *M. albus* showed antimicrobial activity of the volatiles, and *M. vitigenus* isolated by Daisy et al. (2002) showed insecticidal

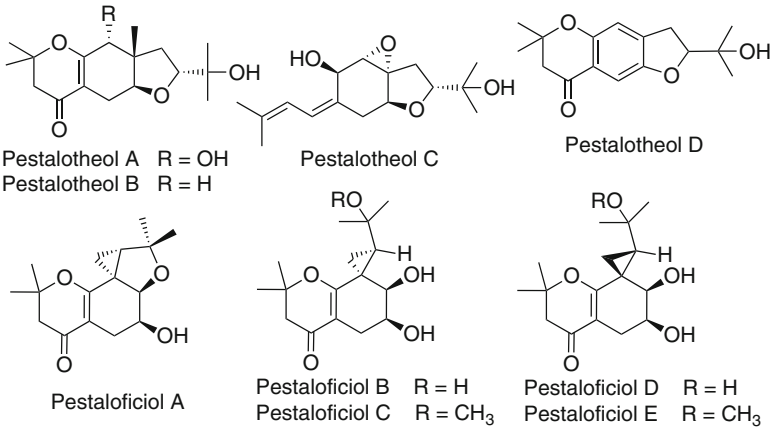

Fig. 5 Natural products from endophytes of the genus *Pestalotiopsis* isolated from unidentified trees

activity due to the production of naphthalene. Recent studies on VOCs of *M. albus* on pupal mortality and adult emergence of western cherry fruit fly (Yee et al. 2009) and codling moth (Lacey et al. 2009) proved insecticidal effects.

Macías-Rubalcava et al. (2010) isolated the new species *M. yucatanensis* (Gonzalez et al. 2009) from leaves of *Bursera simaruba* (Burseraceae, Mexico). The VOCs of this species were lethal to endophytic and phytopathogenic fungi (*Guignardia mangifera*, *Colletotrichum* sp., *Phomopsis* sp., *Alternaria solani*, *Rhizoctonia* sp., *Phytophthora capsici*, *P. parasitica*) but had no effect on *Fusarium oxysporum*, *Xylaria* sp. or the producer itself. Additionally, root elongation of the plants amaranth, tomato, and barnyard grass was inhibited by these compounds. Some of the VOCs, such as octane, 2-methyl butyl acetate, 2-pentyl furan, caryophyllene, and aromadendrene, had not earlier been reported from other *Muscodor* species.

Banerjee et al. (2010) isolated *M. albus* from *Ginkgo biloba* (USA), and Zhang et al. (2010) isolated the new species *M. fengyangensis* from leaves of *Actinidia chinensis*, *Pseudotaxus chienii* and an unidentified broad-leaf tree (China) producing antimicrobial VOCs.

Because of the mentioned bioactivities, the *Muscodor* species could provide an alternative to broad spectrum chemical fumigants (Strobel 2010).

5 Perspective and Conclusion

The vast existing work describing the isolation of endophytes and examining them for diverse biological activities reflects the general interest in these fungi to various areas of research:

- Hazalin et al. (2009) isolated 300 endophytes from various rain forest plants in Malaysia and 3.3% of the extracts showed potent cytotoxic and 8% antibacterial activity.
- Crude extracts of 68 endophytes of plants from the dipterocarpous forest of Thailand were investigated by Sutjaritvorakul et al. (2010) and 50 extracts were active against *Bacillus subtilis*, 26 against *Escherichia coli*, and 13 inhibited the growth of *Candida albicans*.
- Rosa et al. (2010) studied 121 endophytic fungi from leaves of Brazilian plant species. The crude extracts were tested in different bioassays using *Leishmania amazonensis*, *Trypanosoma cruzi*, and human cancer cell lines and 33 extracts exhibited at least one activity.
- Janeš et al. (2007) studied the antibacterial activity of higher fungi (89 species) and endophytes (12 species) from Slovenia. Especially the endophytes *Epicoccum purpurascens* and *Truncatella hartigii* were found to have significant antibacterial effects.
- Phongpaichit et al. (2007) tested 65 crude extracts from 51 selected endophytic fungi isolated from *Garcinia* species (Clusiaceae; Thailand) and 80% of the extracts displayed at least one activity: antimycobacterial (76.9%), antimalarial (14.1%), antiviral (16.7%), antioxidant (22.2%), antiproliferative (11.1% against NCI-H187 and 12.7% against KB cells), and cytotoxic (40.0% against VERO cells).
- Fernandes et al. (2009) isolated 22 endophytes from *Coffea arabica* (Rubiaceae) and the crude extracts were tested in agar diffusion assays against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with 18, 13, and 8 extracts showing activity.
- Mahapatra and Banerjee (2010) presented a detailed survey on endophytic fungi from the milky pine (*Alstonia scholaris*, Apocynaceae). In total, 1,152 endophytes from 1,002 different plant segments from seven locations in India were investigated and eight culture filtrates showed antimicrobial activity.

However the compounds responsible for the biological activities described in these recent examples remain to be identified.

In general, many of the genera and species identified as endophytes in these studies had previously been isolated from soil or from fruiting bodies on decaying plant material. In some cases, this reflects the similarity of the metabolites produced by fungi isolated from different environments, or an overlap in the fungal community across habitats. On the other hand, there are examples of unexpected and new compounds, which make extended investigation of endophytes and their metabolites worthwhile and promising. How endophytic fungi circumvent the defense of the host plant, and how they modulate their own metabolism and that of the plant, remain intriguing questions.

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Potential of Tree Endophytes as Sources for New Drug Compounds

Mysore V. Tejesvi and Anna Maria Pirttilä

Abstract The novel or designer metabolites produced by fungal endophytes are increasingly recognized by natural chemists due to their diverse structures and as candidates for drug discovery and development. Many of the metabolites belong to different classes i.e. alkaloids, benzopyranones, coumarins, chromones, cytochalasines, enniatines, isocoumarin derivatives, quinones, peptides, phenols, phenolic acids, semiquinones, steroids, terpenoids, xanthones and lactones. One of the most widely studied endophytic genera is *Pestalotiopsis,* from which more than 140 metabolites are reported with antimicrobial, antioxidant and antitumor activities. Besides reviewing the advances made in identifying bioactive metabolites with drug development potential from endophytic fungi, this chapter discusses possibilities and bottlenecks involved in employment of endophytic fungi and their products by the pharmaceutical industry. Furthermore, issues involved in anti-infective discovery and timeline of drug development are discussed in the view of developing new drug compounds from endophytic products.

Abbreviations

ACE	angiotensin I-converting enzyme
AIDS	acquired immune deficiency syndrome
DGGE	denaturing gradient gel electrophoresis
EMEA	European agency for the evaluation of medicinal products
FDA	food and drug administration
HI	human immunodeficiency
IC_{50}	the half maximal inhibitory concentration

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MIC	minimum inhibitory concentration
NDM-1	New Delhi metallo-beta-lactamase
RFLP	restriction fragment length polymorphism
SARS	severe acute respiratory syndrome
TB	tuberculosis

1 Introduction

The drastic rise in the number of publications on compounds from fungal endophytes within the past two decades is due to the creative ability of these fungi to produce secondary metabolites. There is also a rise in the need for new antibiotics, anti-malarial drugs, chemotherapeutic or pharmaceutical agents that are highly effective, possess low toxicity and have a minor environmental impact. The development of resistance in infectious microorganisms like *Staphylococcus*, *Mycobacterium* and *Streptococcus* to existing drugs and the presence of naturally resistant organisms are causing threat to mankind (Mwangi et al. 2007; Hugonnet et al. 2009; Richter et al. 2009). Emerging diseases such as AIDS, SARS and NDM-1 necessitate the discovery and development of new drugs (Kumarasamy et al. 2010). The weakened immune system due to AIDS not only requires specific drugs for treatment but also needs new therapies to combat the secondary problems arisen from it, and, furthermore, the HI virus is developing resistance towards the existing drugs (Richman et al. 2004). Opportunistic pathogens such as *Aspergillus*, *Cryptococcus* and *Candida* are also virulent in immunocompromised patients, and in patients, who need an organ transplant. In addition, parasitic protozoan and nematodal infections such as malaria, leishmaniasis, trypanomiasis and filariasis are causing major problems in many countries and effective drugs against them are needed. Malaria is claiming more lives each year than diseases caused by any other infectious agent, with the exception of AIDS and TB (NIAID Global Health Research Plan for HIV/AIDS 2001), and enteric infections claim more lives of children each year than any other disease (Strobel et al. 2004).

For all these reasons, there is a continuous search for novel natural products. The compounds produced by microorganisms have a history of offering opportunities for innovation in drug discovery and development, and therefore many scientists and researchers have turned their looks back to the microbial world. Exciting possibilities exist for those who are willing to take a risk and venture into the unexplored territories of the world to experience the excitement and thrill of engaging in the discovery of endophytes, their biology and potential usefulness (Strobel 2003). In the past decade, endophytic microbes have attracted considerable attention as completely new sources of novel pharmaceuticals (Strobel 2002).

A number of microbial metabolites have been available in quantities of up to hundreds of kilograms by fermentation technology (Grabley and Thiericke 1999). From the screening of a huge number of microbial extracts, an unexpected diversity of natural compounds with a broad variety of biological activities has

been found (Grabley and Sattler 2003). Therefore, microorganisms associated with plants, rather than the plants themselves, can be raw material with promising therapeutic potential (Strobel 2002). Until now, endophytic microbial metabolites have been studied for a wide range of activities like antioomycete, antibacterial, antifungal, anticancerous and immunosuppressive activities. Endophytes were given considerable credibility as sources of novel compounds through the discovery of Taxol® biosynthesis, and a variety of other antibacterial, antifungal and anticancer metabolites from the endophytic fungi *Taxomyces andreae* and *Pestalotiopsis* spp. (Stierle et al. 1993; Strobel 2003). Endophytes can be used as sources of novel metabolites for medicine, agriculture and industrial uses. The best strategy for finding new bioactive compounds is to survey endophytes from plants restricted to special areas, as a means to isolate fungi that likely were never studied in earlier screening programs (Pelaez et al. 1998).

2 Current Status of Endophyte Research with Respect to Drug Discovery

The research on endophytes is growing enormously, as >650 research articles covering both bacteria and fungi were published during the period between 1991 and 2010 (www.sciencedirect.com) (Fig. 1). When the bibliographic search was restricted to endophyte and metabolite there were 253 published research articles, which shows that roughly 40% of the endophyte researchers were looking for sec-

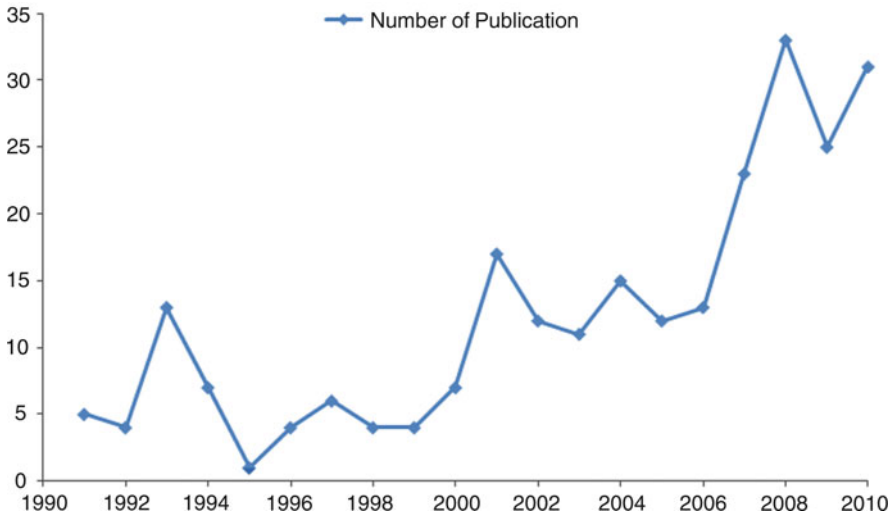


Fig. 1 Number of publication on endophytes from 1990 to 2010 (data used from science direct with a keyword Endophyte + Metabolite). Two hundred and fifty three published articles were found from two decades of research on endophytic metabolites

ondary metabolites (Fig. 1). The potential of endophytic fungi as a source of novel drugs can be seen in terms of number of patents filed and granted on endophytes. When searched with the keyword endophyte (http://www.freepatentsonline.com) >650 patents were filed and granted for using an endophyte as a source for new processes or industrial applications on bioactive metabolites.

3 Medicinal Plants

The plant kingdom is a rich source of structural biodiversity offering a variety of natural products. Plants have been utilized to produce various types of medicines for thousands of years (Samuelsson 2004). These medicines were initially used in the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations (Balick and Cox 1997; Samuelsson 2004). More than 50,000 medicinal plants (Schippmann et al. 2002) out of the total of 4, 22,000 flowering plants reported worldwide have been used for various medicinal purposes (Govaerts 2001). The information on the plants usable for these purposes, and the methods of applying them for a particular ailment, were passed down orally through successive generations. Eventually the information on medicinal plants was recorded in herbals. More recently, the use of plants as medicines has focused on the isolation of active compounds, for example the isolation of morphine from opium poppy in the early nineteenth century (Kinghorn 2001; Samuelsson 2004). According to the World Health Organization (WHO 1991), 80% of the world's population is dependent on health-care provided by medicinal plants.

A wide range of medicinal plant parts is used as extracts that can be considered raw drugs that possess specific medicinal properties. The different plant products used to cure various infectious diseases include root, stem, flower, fruit, root, twigs, exudates, and modified plant organs. Whereas some of these raw drugs are collected in small quantities for local use by the native communities and folk healers, many other raw drugs are collected in large quantities and traded in the market as the raw material for herbal industries (Uniyal et al. 2006). The same medicinal plants provide a good source for isolation of endophytic fungi and screening for bioactive metabolites. In this way, the need to sacrifice plants that in some cases are rare or endangered can be avoided.

4 Endophytic Fungi

Endophytic fungi are found practically in every plant species including terrestrial plants such as grasses (Bacon and White 1994; Groppe et al. 1999; Saikkonen et al. 2000), palms (Taylor et al. 1999; Frohlich et al. 2000), banana (Brown et al. 1998; Photita et al. 2004), mangroves (Suryanarayanan et al. 1998; Kumaresan and Suryanarayanan 2002; Ananda and Sridhar 2002) and halophytes (Suryanarayanan

and Kumaresan 2000), and in every subclass including mosses, liverworts, pteridophytes, gymnosperms and angiosperms (Provorov et al. 2002). Endophytes are found in all plant tissues including seeds and ovules (Siegel et al. 1987), fruits (Baayen et al. 2002), stems (Gutierrez-Zamora and Martinez-Romero 2001), roots (Germida et al. 1998), leaves (Cannon and Simmons 2002), inner bark of trees (Tejesvi et al. 2005, 2006), tubers (Sturz et al. 1998), buds (Pirttilä et al. 2000, 2003; Ragazzi et al. 2001), xylem (Hoff et al. 2004) and rachis (Rodrigues and Samuels 1999). Numerous publications are available on their biology (Jennings and Lysek 1996; Clay 1998; Brem and Leuchtmann 2001; Arnold et al. 2003), evolution (Carroll 1998; Saikkonen et al. 2004), occurrence (Kumar et al. 2004; Tejesvi et al. 2006), taxonomy (Petrini 1986; Guo et al. 2000, 2003) and biotechnological applications (Tomita 2003; Strobel 2007).

The impact of endophytic fungi on host plants is largely unknown compared with that of fungal pathogens or mycorrhizal symbionts. Endophytic fungi may influence other fungi present in the same host, existing between the tropical niches of pathogen and mutualist (Hoff et al. 2004). This influence can be expressed directly by inhibition or stimulation of fungal growth, or indirectly via effects on host physiology and morphology. Thus, the genetic variability and unpredictability of pathogen interactions with the host plants can be attributed to endophytes (Saikkonen et al. 1998; Hoff et al. 2004).

Schulz et al. (1993, 1995, 1998) obtained >6,500 endophytic isolates from different organs of more than 500 plants of diverse temperate habitats. The majority of the isolates belonged to ubiquitous genera (e.g. *Acremonium*, *Alternaria*, *Cladosporium*, *Coniothyrium*, *Epicoccum*, *Fusarium*, *Geniculosporium*, *Pestalotiopsis*, *Phoma*, *Pleospora*), concurring with previous results, reviewed by Petrini (1986), that many endophytes are from ubiquitous taxa. The assemblages of endophytes vary with habitat, as different ubiquitous genera are isolated from tropical than from temperate climates (e.g. see the chapters by M. Unterseher and T.S. Suryanarayanan, this volume). Some genera like *Fusarium, Phomopsis* and *Phoma* are common in both tropical and temperate climates, whereas members of Xylariaceae, *Colletotrichum*, *Guignardia*, *Phyllosticta* and *Pestalotiopsis* predominate in the tropics (Frohlich and Hyde 1999; Cannon and Simmons 2002; Suryanarayanan et al. 2003; Arnold 2008). An interesting aspect to investigate is how the occupation of an inter- or intracellular niche within a plant by one fungal group affects the subsequent establishment and evolution of other fungal partnerships (Schulz and Boyle 2005).

5 Antimicrobials from Endophytic *Pestalotiopsis* Species

Pestalotiopsis species have gained much attention in recent years as they produce many important secondary metabolites (Strobel 2002; Tejesvi et al. 2007; Xu et al. 2010). At present, more than 30 *Pestalotiopsis* species have been reported as endophytes, there are 235 species listed in *Index Fungorum* (http://www.indexfungorum.org/Names/Names.asp) and they are usually found in tropical and subtropical plants

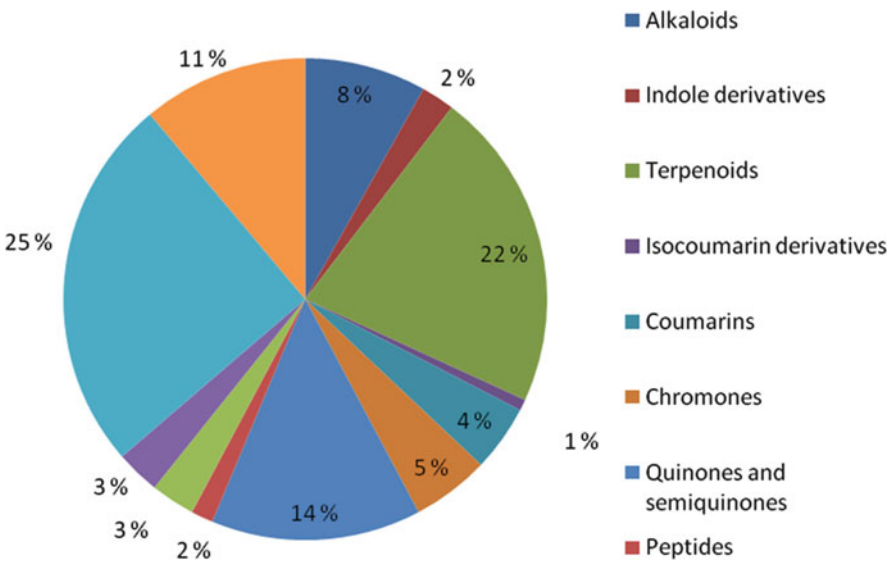
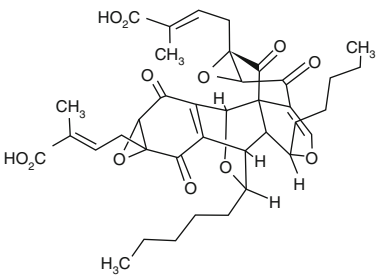


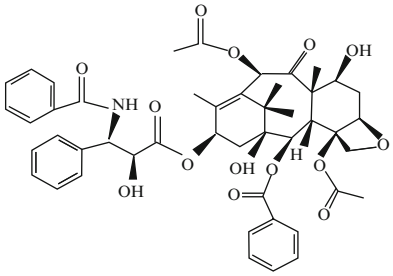
Fig. 2 Different class of secondary metabolite isolated from the genus *Pestalotiopsis*

throughout the world (Tejesvi et al. 2006, 2007; Ding et al. 2009; Liu et al. 2009a). A group of *Pestalotiopsis* species produces secondary metabolites, which have great potential as antioxidants, antimicrobials and anti-tumor compounds (Tan and Zou 2001; Zhang et al. 2006; Xu et al. 2010). However, many endophytic *Pestalotiopsis* species have been unidentified due to the limitation and difficulty in applying classification based on existing morphological characters (Okane et al. 1998; Suryanarayanan and Kumaresan 2000; Suryanarayanan et al. 1998; Toofanee and Dulymamode 2002; Tejesvi et al. 2009). There are >140 metabolites that have been identified and characterized from *Pestalotiopsis* spp., belonging to different classes of compounds such as alkaloids, terpenoids, isocoumarin derivatives, coumarins, chromones, quinones, semiquinones, peptides, xanthones, xanthone derivatives, phenols, phenolic acids, and lactones (Fig. 2), of which some examples with antimicrobial activity are given in the following paragraph.

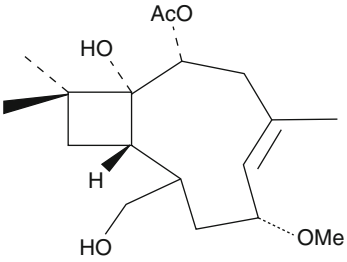
The crude extract of the endophytic *Pestalotiopsis* sp. from the lichen *Clavarioides* sp. yielded six ambuic acid derivatives and a torreyanic acid (Fig. 3), which showed antibacterial activity against *Staphylococcus aureus* with IC_{50} values of 43.9 and 27.8 μM, respectively (Ding et al. 2009). Ambuic acid has also been identified from different *P. microspora* strains isolated from *Taxus baccata*, *Torreya taxifolia*, *Taxodium disticum*, *Wollemia nobelis* and *Dendrobium speciosum* showing potential antifungal activity against plant pathogens (Li et al. 2001). Three new caryophyllene-type sesquiterpene alcohols, 6-hydroxypunctaporonin E, 6-hydroxypunctaporonin B and 6-hydroxypunctaporonin A were isolated from culture filtrate of *P. disseminata*. The compounds 6-hydroxypunctaporonin E and



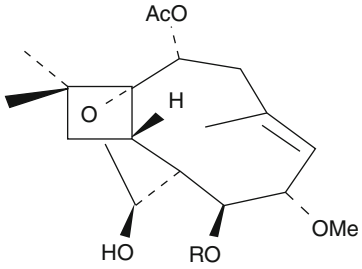
Torreyanic acid



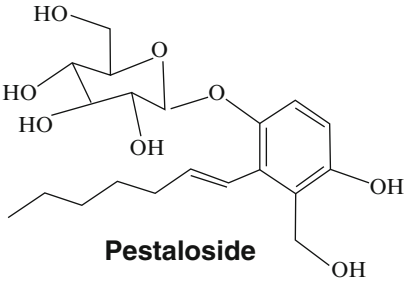
Taxol



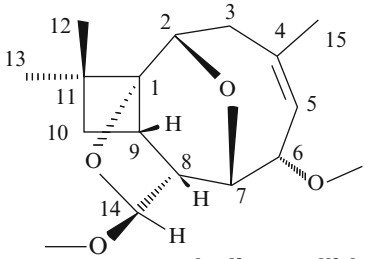
Pestalotiopsins B



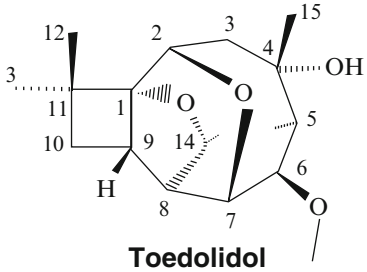
R = H: Pestalotiopsin A
R = CH3: Pestalotiopsin C



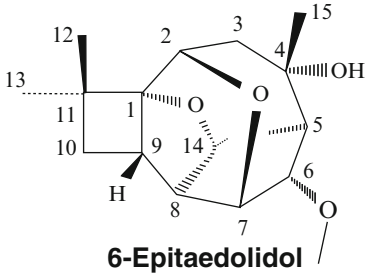
Pestaloside



pestalodiopsodlide A



Toedolidol



6-Epitaedolidol

Fig. 3 Structures of bioactive metabolites produced by endophytic *Pestalotiopsis* spp.

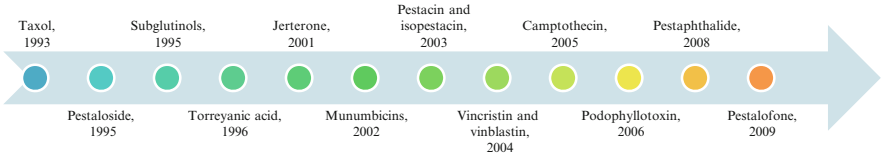


Fig. 4 Timeline of discovery of important secondary metabolites produced by endophytic fungi

6-hydroxypunctaporonin B exhibited antibacterial activities in agar diffusion plate assays at 100 μg/disk against *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 29213) (Deyrup et al. 2006). Pestalachloride A, an alkaloid isolated from an endophytic *Pestalotiopsis adusta*, displayed potent antifungal activity against *Fusarium culmorum* with an IC_{50} value of 0.89 μM (Li et al. 2008). *Pestalotiopsis foedan*, isolated from the branches of an unidentified tree, yielded a novel spiroazaphilone derivative, pestafolide A, which exhibited antifungal activity against *Aspergillus fumigatus* (ATCC10894) (Ding et al. 2008).

Jesterone and hydroxy-jesterone are novel cyclohexenone epoxides isolated from a newly described endophytic fungal species *P. jesteri*, which was isolated from the bark of *Fragraea bodenii* (oak tree, family Loganiaceae) (Li and Strobel 2001). Jesterone displayed selective antimycotic activity against the oomycetous fungi such as *Pythium ultimum*, *Aphanomyces* sp., *Phytophthora citrophthora*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, and *Pyricularia oryzae* with MIC values of 94.7, 24.6, 94.7, 24.6, 94.7 and 94.7 μM, respectively (Li and Strobel 2001). Bioassay-guided separation of the culture of *P. fici* yielded five new compounds, pestalofones A–E. Pestalofones C and D exhibited inhibitory effects against *Aspergillus fumigatus* with IC_{50}/MIC values of 1.10/35.3, 0.90/31.2 μM, respectively (Liu et al. 2009b).

Two novel phenols, pestacin and isopestacin (Fig. 4) were isolated from *P. microspora* associated with the combretaceaous plant *Terminalia morobensis* (Strobel et al. 2002; Harper et al. 2003). Pestacin showed a moderate antifungal activity against *Pythium ultimum*, and isopestacin displayed moderate antimycotic activities against plant pathogenic oomycete *Pythium ultimum*, ascomycete *Sclerotinia sclerotiorum* and basidiomycete *Rhizoctonia solani* (Strobel et al. 2002; Harper et al. 2003). Pestaphthalides A and B having moderate antifungal activity were isolated from *P. foedan* of an unidentified tree near Dongzai, Hainan Province, China (Ding et al. 2008). Pestalachlorides B and C are two chlorinated benzophenone derivatives isolated from endophytic *P. adusta*. Pestalachloride B exhibited antifungal activity against the fungal plant pathogen *Gibberella zeae* with an IC_{50} value of 1.1 μM (Li et al. 2008). *Pestalotiopsis microspora* was isolated from *Torreya taxifolia* and it produced pestalopyrone, hydroxypestalopyrone and pestaloside phytotoxins in axenic cultures. Pestaloside exhibited broad-spectrum antifungal activity against the fungi *Cladosporium* sp., *Rhizoctonia solani*, *Geotrichum candidum* and *Agricus campestris* (Lee et al. 1995).

Pestalotiopsis microspora isolated from the inner bark of a small limb of Himalayan yew, *Taxus wallachiana* produces Taxol® in mycelial culture. Taxol® was identified by spectroscopic and chromatographic comparisons similar to authentic Taxol® (Fig. 3). Optimal Taxol® production occurred after 2–3 weeks in still culture at 23°C. ^{14}C Acetate and ^{14}C phenylalanine served as precursors for the fungal ^{14}C Taxol® (Strobel et al. 1996a). A number of unrelated fungal endophytes including *Pestalotia*, *Pestalotiopsis*, *Fusarium*, *Alternaria*, *Pithomyces*, *Monochaetia* also produce taxol in vitro (Strobel et al. 1996b).

6 Methods for Gaining Industry-Level Production Rates of Endophytic Metabolites

Industrial production of bioactive substances (e.g. pharmaceuticals, drugs) requires reproducible, dependable productivity. Microbial fermentation as a means of producing bioactive substances has several advantages: (1) If a microbe is the source organism, in an optimal case it can be grown in tank fermentors, producing an inexhaustible supply of material, (2) microorganisms typically respond favorably to routine culture techniques, whereas tissue culture or growing of plants requires either specialized techniques, or months of growth before harvesting is profitable, (3) product escalation is relatively easy in microorganisms. Various biosynthetic pathways can be optimized by changing the culture conditions for effective development and discovery of the lead compounds. For example, aplasmomycins were produced by *Streptomyces griseus* in the medium only after addition of NaCl (Nakamura et al. 1977; Stierle and Stierle 2005; Imada et al. 2007).

Developing a productive microbial source for anti-infectives and immunosuppressants not only would lower the production cost of the compound but would also make it widely available. The sources of new drugs during the period from 1981 to 2006 indicate that over 60% of the drugs are natural products, and close to 70% of anti-infectives and 63% of anticancerous drugs are derived from natural products (Cragg and Newman 2009). However, even though endophytic fungi show great potential as sources for industry-scale production of Taxol®, so far they have not been applied to industrial use (Ji et al. 2006). Problems such as low fungal biomass produced during fermentation, low yield of Taxol® in culture and lack of knowledge of regulation of the biosynthesis pathway limit the industrial use of these fungi (Ji et al. 2006). A yield of 1 mg/l for Taxol® production would be profitable at industrial scale, but the highest yields reported so far are 15 to 20 times lower (Deng et al. 2009). Taxol® has become a successful natural compound that is widely used as anti-tumor agent, with a higher demand than production rates. Therefore, knowledge of biotechnological tools for large-scale production of Taxol® are needed to engineer endophytic strains, or to produce Taxol® in heterologous hosts.

An endophytic organism can produce secondary metabolites in relatively high yield in culture, particularly when subjected to strain improvement programs

(Penalva et al. 1998). It is feasible to produce and isolate mutants that could readily be cultivated or could generate either additional products or modified products with a higher therapeutic index (Piepersberg 1994). Moreover, the metabolites they produce are largely generated by enzymatic pathways that have the potential to biosynthetically link existing structures to chemical adjuncts in a reproducible manner, at yields that are acceptable for industrial scale (Verdine 1996). To improve Taxol® production, one strain of *Nodulisporium sylviform*, has been subjected to mutagenesis and production of 314–393 μg/L was obtained (Zhao et al. 2005; Zhou et al. 2005). Another way to modify the fungal secondary metabolome is to alter their epigenetic status (Williams et al. 2008). By treating bioactive fungi with enzymes such as DNA methyltransferases or histone deacetylase inhibitors, enhanced chemical diversity can be obtained (Williams et al. 2008). In this sense, natural products produced from microbes exhibit a number of properties that make them excellent candidates for industrial processes.

A typical problem encountered with endophytes is that they produce the bioactive metabolite only for a short while *in vitro* and then die during subculturing, or become impaired in production of the secondary metabolite, or do not grow at all *in vitro*. Similar problems encountered earlier with other microbes lead to the development of metagenomics and metatranscriptomics tools, to access the vast microbial wealth without restrictions of culturability or growth (Handelsman 2004; Green and Keller 2006; Bailly et al. 2007). The culture independent analysis techniques such as terminal restriction fragment length polymorphism (T-RFLP) (Nikolcheva and Bärlocher 2005), denaturing gradient gel electrophoresis (DGGE) (Duong et al. 2006), or direct sequencing of ribosomal sequences (Seena et al. 2008; Tejesvi et al. 2010) are generally used for analyzing the diversity of unculturable fungal communities in plants. However, these methods are not suitable for functional studies of unculturable endophytes, and metagenomic and metatranscriptomic tools are still waiting to be applied to endophytes.

Another reasonably new and highly innovative approach is the biosynthesis of bioactive compounds in heterologous hosts. Heterologous expression of biosynthesis pathway of a compound through the transfer of the pathway genes from the producer organism to another, foreign host, can enable the production of the compound in higher quantities (Wenzel and Muller 2005). *Escherichia coli* is a widely used host for expression of even complex metabolic pathways, such as that of erythromycin (Pfeifer et al. 2001), echinomycin and triostin A (Watanabe 2008). Other hosts used recently are e.g. *Myxococcus xanthus* and *Pseudomonas putida* for the production of epothilone or myxochromide S (Fu et al. 2008), the thermopilic isolate *Corallococcus macrosporus* GT-2 for the production of myxochromide (Perlova et al. 2009) and *Streptomyces lividans* for the production of meridamycin (Liu et al. 2009c). An engineered *Streptomyces avermitilis* mutant was developed very recently and used for the heterologous expression of three different biosynthesis pathways, streptomycin, cephamycin C and pladienolide. Another *Streptomyces* mutant was optimized for terpenoid production by introduction of a synthetic gene optimized for *Streptomyces* codon usage, and this mutant was capable

of producing the plant terpenoid intermediate, amorpha-4,11-diene (Komatsua et al. 2010). With respect to Taxol, already in 2001 Huang et al. accomplished in vivo production of the intermediate taxadiene in *E. coli*, and taxadiene and taxadien-5a-ol were produced in yeast by Engels et al. in 2008. As for now, the production of Taxol itself in a heterologous host remains to be fulfilled. Yeast might be a promising host for such attempts, as the plant-derived artemisin has been successfully produced in yeast (Ro et al. 2006). However, there is an on-going debate on the host selection between *E. coli* and yeast for the heterologous production of plant- and fungi-derived compounds. Although yeast might appear more suitable, there is a conflict of the yeast metabolism interacting with, and/or contaminating the expression of the heterologously introduced pathway (Zhang et al. 2011). Another host that could become useful for the heterologous expression of endophytic products is *Aspergillus nidulans*. This host has been tested and used, e.g. for the heterologous production of Monacolin J (a lovastatin intermediate) (Zirkle et al. 2004). As these methodologies develop further, and access to the genome data of endophytic fungi by pyrosequencing becomes available, further and most promising developments within this arena can be expected.

7 Drug Development Life Cycle

The development of anti-infectives is known to take longer than that of the agro-chemicals or industrial enzymes, because these compounds have to undergo three to four clinical studies. The drug development cycle from laboratory to market is long-lasting with the different phases involved, starting from drug discovery (for example, 10,000 compounds), preclinical trials (about 1–2% of the molecules), human clinical trials (2–5% of the molecules), Food and Drug Administration (FDA) or European Agency for the Evaluation of Medicinal Products (EMEA) review, FDA or EMEA approval and post-market clinical trials (Fig. 5). At the moment, the success rate for anti-infective molecules is about 16% for those approved between 1993 and 2004 (DiMasi et al. 2010) and the time to reach the markets varies between 10 and 12 years, depending on the drug. Because antimicrobials have a short-lived nature as drugs, as they normally are taken only for short periods of time and easily develop resistance, the resulting low profit expectancy and, subsequently, a low interest by pharmaceutical companies complicate the efforts of developing completely new drug compounds (Bradley et al. 2007). The pharmaceutical industry is therefore preferably investing in anti-inflammatory, ACE inhibitors, diabetes and anti-cancerous drugs, which are known to generate long-term revenue. Even though pharmaceutical and biotechnology companies are testing increasing numbers of compounds against various targets by high-throughput screening technologies, the question regarding drug development is whether the products can reach the markets in time for procurement of the disease. Regardless, or due to these problems, the efforts to look for safe, novel compounds from the nature should be persistent and continuous.

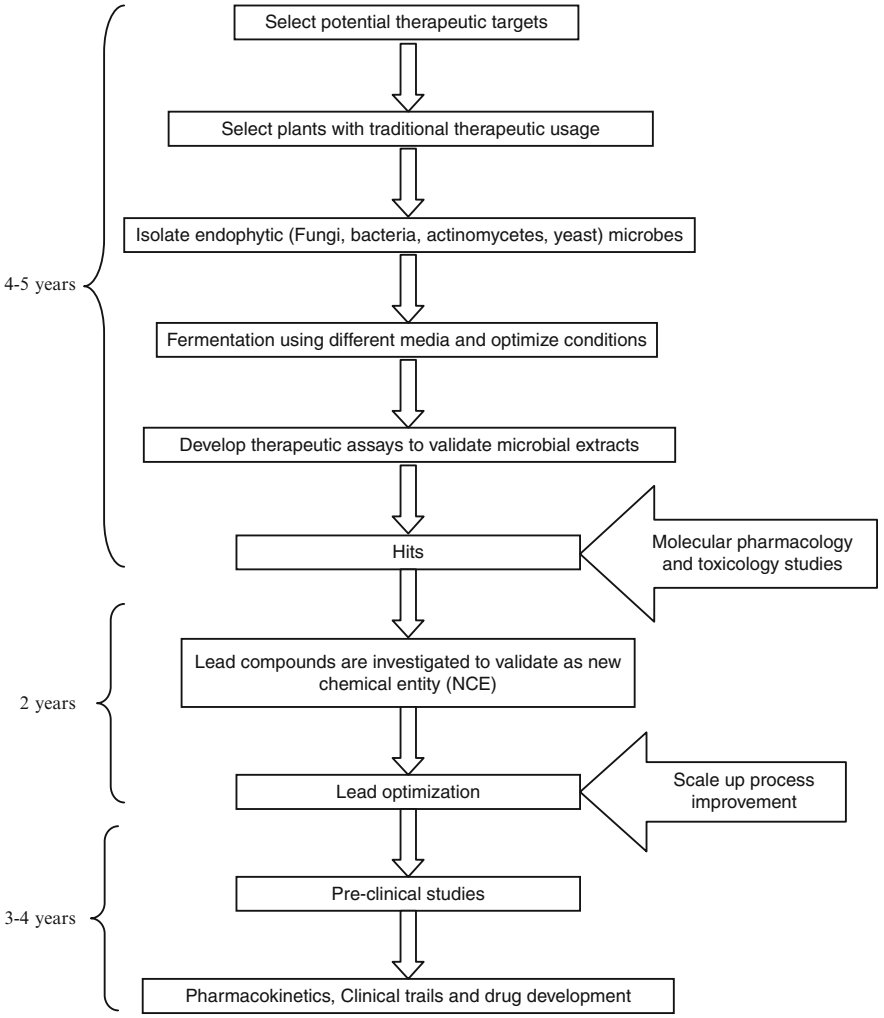


Fig. 5 The endophytes from ethnophamaceutically used plants as a source for new therapeutic leads

8 Conclusion

Endophytic fungi are prolific producers of secondary metabolites and *Pestalotiopsis* species, in particular, are of considerable interest to researchers and pharmacists due to their ability to synthesize a wide range of economically important bioactive molecules. The fermentation and high-throughput screening of a wide array of secondary metabolites with bioassay-guided fraction can yield various new metabolites for various therapeutic targets in the future. The new biotechnological tools have

great promise in enabling the industrial use of endophytic fungi and their products. There is a continuous need for international co-operation to identify and develop antimicrobial drugs to combat various infectious diseases.

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