Edited by
Karl Esser

THE MYCOTA

A Comprehensive Treatise on Fungi
as Experimental Systems for Basic and Applied Research

Environmental and Microbial Relationships

IV

Third Edition

Irina S. Druzhinina
Christian P. Kubicek
Volume Editors

Springer

The Mycota

Edited by
K. Esser

The Mycota

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Environmental and Microbial Relationships
3rd Edition

Volume Editors:
I.S. Druzhinina and C.P. Kubicek

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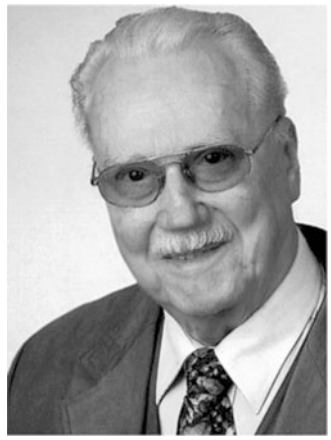

Karl Esser
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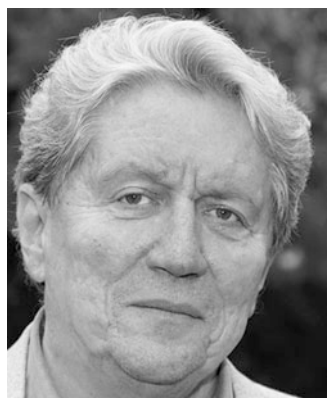

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Series Preface

Mycology, the study of fungi, originated as a sub discipline of botany and was a descriptive discipline, largely neglected as an experimental science until the early years of this century. A seminal paper by Blakeslee in 1904 provided evidence for self incompatibility, termed "heterothallism", and stimulated interest in studies related to the control of sexual reproduction in fungi by mating-type specificities. Soon to follow was the demonstration that sexually reproducing fungi exhibit Mendelian inheritance and that it was possible to conduct formal genetic analysis with fungi. The names Burgeff, Kniep and Lindegren are all associated with this early period of fungal genetics research.

These studies and the discovery of penicillin by Fleming, who shared a Nobel Prize in 1945, provided further impetus for experimental research with fungi. Thus began a period of interest in mutation induction and analysis of mutants for biochemical traits. Such fundamental research, conducted largely with *Neurospora crassa*, led to the one gene: one enzyme hypothesis and to a second Nobel Prize for fungal research awarded to Beadle and Tatum in 1958. Fundamental research in biochemical genetics was extended to other fungi, especially to *Saccharomyces cerevisiae*, and by the mid-1960s fungal systems were much favored for studies in eukaryotic molecular biology and were soon able to compete with bacterial systems in the molecular arena.

The experimental achievements in research on the genetics and molecular biology of fungi have benefited more generally studies in the related fields of fungal biochemistry, plant pathology, medical mycology, and systematics. Today, there is much interest in the genetic manipulation of fungi for applied research. This current interest in biotechnical genetics has been augmented by the development of DNA-mediated transformation systems in fungi and by an understanding of gene expression and regulation at the molecular level. Applied research initiatives involving fungi extend broadly to areas of interest not only to industry but to agricultural and environmental sciences as well.

It is this burgeoning interest in fungi as experimental systems for applied as well as basic research that has prompted publication of this series of books under the title *The Mycota*. This title knowingly relegates fungi into a separate realm, distinct from that of either plants, animals, or protozoa. For consistency throughout this Series of Volumes the names adopted for major groups of fungi (representative genera in parentheses) areas follows:

Pseudomycota

Division:	Oomycota (*Achlya, Phytophthora, Pythium*)
Division:	Hyphochytriomycota

Eumycota

Division:	Chytridiomycota (*Allomyces*)
Division:	Zygomycota (*Mucor, Phycomyces, Blakeslea*)
Division:	Dikaryomycota
Subdivision:	Ascomycotina
Class:	Saccharomycetes (*Saccharomyces, Schizosaccharomyces*)
Class:	Ascomycetes (*Neurospora, Podospora, Aspergillus*)
Subdivision:	Basidiomycotina
Class:	Heterobasidiomycetes (*Ustilago, Tremella*)
Class:	Homobasidiomycetes (*Schizophyllum, Coprinus*)

We have made the decision to exclude from *The Mycota* the slime molds which, although they have traditional and strong ties to mycology, truly represent nonfungal forms insofar as they ingest nutrients by phagocytosis, lack a cell wall during the assimilative phase, and clearly show affinities with certain protozoan taxa.

The Series throughout will address three basic questions: what are the fungi, what do they do, and what is their relevance to human affairs? Such a focused and comprehensive treatment of the fungi is long overdue in the opinion of the editors.

A volume devoted to systematics would ordinarily have been the first to appear in this Series. However, the scope of such a volume, coupled with the need to give serious and sustained consideration to any reclassification of major fungal groups, has delayed early publication. We wish, however, to provide a preamble on the nature of fungi, to acquaint readers who are unfamiliar with fungi with certain characteristics that are representative of these organisms and which make them attractive subjects for experimentation.

The fungi represent a heterogeneous assemblage of eukaryotic microorganisms. Fungal metabolism is characteristically heterotrophic or assimilative for organic carbon and some nonelemental source of nitrogen. Fungal cells characteristically imbibe or absorb, rather than ingest, nutrients and they have rigid cell walls. The vast majority of fungi are haploid organisms reproducing either sexually or asexually through spores. The spore forms and details on their method of production have been used to delineate most fungal taxa. Although there is a multitude of spore forms, fungal spores are basically only of two types: (i) asexual spores are formed following mitosis (mitospores) and culminate vegetative growth, and (ii) sexual spores are formed following meiosis (meiospores) and are borne in or upon specialized generative structures, the latter frequently clustered in a fruit body. The vegetative forms of fungi are either unicellular, yeasts are an example, or hyphal; the latter may be branched to form an extensive mycelium.

Regardless of these details, it is the accessibility of spores, especially the direct recovery of meiospores coupled with extended vegetative haploidy, that have made fungi especially attractive as objects for experimental research.

The ability of fungi, especially the saprobic fungi, to absorb and grow on rather simple and defined substrates and to convert these substances, not only into essential metabolites but into important secondary metabolites, is also noteworthy. The metabolic capacities of fungi have attracted much interest in natural products chemistry and in the production of antibiotics and other bioactive compounds. Fungi, especially yeasts, are important in fermentation processes. Other fungi are important in the production of enzymes, citric acid and other organic compounds as well as in the fermentation of foods.

Fungi have invaded every conceivable ecological niche. Saprobic forms abound, especially in the decay of organic debris. Pathogenic forms exist with both plant and animal hosts. Fungi even grow on other fungi. They are found in aquatic as well as soil environments, and their spores may pollute the air. Some are edible; others are poisonous. Many are variously associated with plants as copartners in the formation of lichens and mycorrhizae, as symbiotic endophytes or as overt pathogens. Association with animal systems varies; examples include the predaceous fungi that trap nematodes, the microfungi that grow in the anaerobic environment of the rumen, the many insect associated fungi and the medically important pathogens afflicting humans. Yes, fungi are ubiquitous and important. There are many fungi, conservative estimates are in the order of 100,000 species, and there are many ways to study them, from descriptive accounts of organisms found in nature to laboratory experimentation at the cellular and molecular level. All such studies expand our knowledge of fungi and of fungal processes and improve our ability to utilize and to control fungi for the benefit of humankind.

We have invited leading research specialists in the field of mycology to contribute to this Series. We are especially indebted and grateful for the initiative and leadership shown by the Volume Editors in selecting topics and assembling the experts. We have all been a bit ambitious in producing these Volumes on a timely basis and therein lies the possibility of mistakes and oversights in this first edition. We encourage the readership to draw our attention to any error, omission or inconsistency in this Series in order that improvements can be made in any subsequent edition.

Finally, we wish to acknowledge the willingness of Springer-Verlag to host this project, which is envisioned to require more than 5 years of effort and the publication of at least nine Volumes.

Bochum, Germany — Karl Esser
Auburn, AL, USA — Paul A. Lemke
April 1994 — *Series Editors*

Volume Preface

In their concept of *The Mycota*, Karl Esser and Paul Lemke (Series Editors) determined that there was a need for a volume that emphasizes research on fungal populations and communities and their biotic and abiotic interactions with environments. Two volumes written in this line have so far been published. We were invited to prepare a third edition of Volume IV that continues concentrating on fungal responses to physical environments, interactions with other fungi, but also bacteria, plants, and invertebrates, and their role in ecosystem processes.

The dramatic progresses in genetic manipulation of fungi, genome-wide analytical tools, and bioinformatics have also revolutionized research in the above-described area. Consequently, while several authors of the third edition were asked to rework and update their chapters for this volume, we also felt the need to also incorporate new chapters to emphasize important findings and trends in this area.

The first section highlights two important aspects of the fungal population: Sun and Heitman discuss the fundamental roles that recombination plays in fungal evolution by generating novel allele combinations, as well as by increasing the genetic diversity and facilitating natural selection. They thereby particularly focus on the diverse mating systems and mating-type determination mechanisms in fungi, which provide a rich resource to study the effects of recombination on mechanisms of sex determination and sexual development, as well as the evolution of sex determination systems and sex chromosomes in general. Leavitt and Lumbsch illuminate new insights into the geographical distributions of lichen-forming fungi and the factors that shape these distributions. Besides discussing general perspectives of biogeography of lichen-forming fungi, they focus on major themes directly related to ecological biogeography, such as dispersal and establishment of lichens, landscape genetics and gene flow, and the role of photobionts in determining distributional ranges.

The second section is dedicated to the determinants of fungal communities. Among these, sunlight is definitely one of the most important cues for all living organisms on earth. Casas-Flores and Herrera-Estrella describe how light regulates several physiological and developmental processes, including phototropism, synthesis of pigments, circadian rhythms, sexual and asexual development, and primary and secondary metabolism, among other processes. They also explain the fungal components involved in sensing light and how this signal is transduced downstream. Morris and coauthors deal with the impact of events that disrupts ecosystem, community, or population structure such as changes in resources, substrate availability, or the physical environment on fungal communities. They

show that such disturbances that alter the fungal community have major consequences for ecosystem dynamics by changing nutrient cycles and affect plant diversity. However, also the type of fungal community that reestablishes on a given site is affected by the reestablishing plant community structure and nutrient dynamics across the landscape.

Fungi are capable of the degradation, utilization, and/or transformation of a wide variety of organic and inorganic substances, including xenobiotics, metals, radionuclides, and minerals. Fungal populations are therefore intimately involved in element cycling at local and global scales and such processes have major implications for living organisms, notably plant productivity and human health. Geoffrey Gadd outlines in his chapter some important interactions of fungi with organic and inorganic pollutants and highlights the interdisciplinary approach that is necessary to further understand the important roles that fungi play in pollutant transformations, the chemical and biological mechanisms that are involved, and their environmental and applied significance.

Fungi are also the major organisms for the recycling of biomass on this planet and thus essentially contribute to the global carbon cycle. Ramoni and Seiboth illustrate how the plant cell wall that is composed of cellulose, different hemicelluloses, pectins, and the polymer lignin and represents material extremely recalcitrant to degradation and decomposition can be used as a nutrient by different fungi via different strategies and describe the enzymes involved in this process.

The largest section of this volume is dedicated to the field of fungal interactions which today is one of the hotspot of fungal research. Pawlowska starts this section by describing the accumulated data about newly discovered bacterial–fungal symbioses. Particularly, the heritable alliances formed by early divergent lineages of *Mucoromycotina* and *Glomeromycota* with beta-proteobacteria and Mollicutes yielded novel insights into the forms of evolutionary trajectories in mutualisms and into mechanisms of symbiont genome evolution. Tarrka and Deveau continue on this topic by discussing the interactions of fungi and bacteria, which are frequent because they coexist in various environments and often share niches. They consequently often undergo physical associations that have beneficial effects for both partners. They highlight recent contributions to the understanding of bacteria–fungi interactions and focus on the rapid methodological development in this area.

Fungal plant interactions have been known for a long while. "Mycorrhiza"—the beneficial and mutualistic associations between plant roots and fungi—thereby has a major impact on earth's plant growth. Marmeisse and Girlanda summarize the recent advances in the field of mycorrhizal ecology, particularly prompted by "omic" approaches, which have offered insights into the genome signatures of different fungal trophic strategies and the roles of mycorrhizal fungi in the functioning of terrestrial ecosystems. In addition, root endophytes—while not being mycorrhizal fungi—have only recently been recognized to have a significant impact on plant nutrition. Yuan and coauthors describe the ecological significances of fungal root endophytes, particularly those termed class 4 endophytes which represent the main root associates. They particularly introduce a new model system for studying this process—*Harpophora oryzae*—that is a root endophyte of wild rice. Interestingly, *H. oryzae* is a close relative of the most

devastating pathogen of rice, *Magnaporthe oryzae*, and the authors highlight the metabolomic and transcriptomic differences that have been found in the two binary fungal-root systems which reveal details about the key elements leading to either mutualistic or pathogenic interactions.

Moving on the further interactions performed by fungi, Chenthamara and Druzhinina focus on mycotrophic fungi and—on the basis of genome-wide investigations—reveal unique features in the intracellular mycoparasite *Cryptomycota* and outline similar and apparently convergent mechanisms employed by a diversity of fungicolous Asco- and Basidiomycota. Herrera-Estrella and coauthors deal with the ancient and diverse group of nematophagous fungi that use refined mycelial structures or their conidia to capture their preys. They review the current state of knowledge of their biology and molecular physiology and particularly highlight the recent genomic insights into the virulence factors of nematophagous fungi. Schigel illustrates the ecological complexity of fungus–beetle interactions from European boreal forests. Larvae or adults of the fungivorous species of the genus *Coleoptera* selectively feed on a primarily fungal diet, fruit bodies, mycelia, and spores. He describes how the evolutionary success and diversity of both fungi and the beetles result in complex patterns of co-occurrence and interactions, culminating in diverse species assemblage patterns and varying degrees of trophic specialization of beetles.

We hope that this volume will prove useful to both scientists who wish to update themselves in any of the research areas outlined above and students for a first overview before entering these areas in depth. We are grateful to all the authors who took the time and effort to collaborate with us on the updating of this volume and particularly that they all helped us getting this task finished within the expected time schedule.

Vienna, Austria
December 2015

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The Fungal Population

1 Running Hot and Cold: Recombination Around and Within Mating-Type Loci of Fungi and Other Eukaryotes

Sheng Sun[1], Joseph Heitman[2]

CONTENTS

I. Introduction

Genetic recombination is the process of generating offspring that have different genetic composition from their parents. In diploid cells, it can occur either during mitosis, which could result in loss of heterozygosity (LOH) in the progeny, or more commonly during meiosis, which reshuffles the genetic information of the two parents and produces haploid gametes with novel genetic combinations. Most of the genetic recombination events are induced by double-strand breaks (DSB) on chromosomes, and depending on how the DSBs are repaired, the outcomes could be either crossing-over or gene conversion (Andersen and Sekelsky 2010). Because recombination requires proper alignment of the homologous chromosomes, it is not surprising that for chromosomal regions that show high levels of nucleotide polymorphisms and/or chromosomal rearrangements, recombination is typically repressed. Additionally, recombination occurring within the rearrangements will typically result in products with abnormal chromosomal structure (e.g., acentric and dicentric) and content (e.g., duplications and deletions), which reduces the fitness of the individuals that harbor such abnormal chromosomes and thus further reduces the observed recombination frequencies in these rearranged chromosomal regions.

The most familiar examples of chromosomal regions with extensive rearrangements are the sex chromosomes. It has long been recognized that in species with heterogametic sexes, interchromosomal meiotic recombination between homologous sex chromosomes (e.g., between X and Y in mammals and between Z and W in birds) is repressed, except within the small defined pseudoautosomal regions (PAR) located at the tips of the sex chromosomes (Chen et al. 2014; Ciccodicola et al. 2000; Hughes and Rozen 2012; Hughes et al. 2005, 2010, 2012). However, it has also been shown that Y chromosomes undergo intrachromosomal gene conversion within palindromic regions, which likely have contributed to the stabilized evolution of the Y chromosomes after their initial fast degeneration (Hughes et al. 2012). Additionally, recent studies have shown that in the European tree frogs *Hyla arborea*, *Hyla intermedia*, and *Hyla*

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molleri, the X and Y chromosomes are homomorphic, and the Y chromosomes do not show extensive degeneration, which is likely due to occasional X-Y interchromosomal recombination (which may occur in sex-reversed females) that keeps homogenizing alleles from the same species, followed by selective sweeps by the newly arisen recombinant Y chromosomes that have reduced levels of deleterious mutations, thus maintaining Y chromosome integrity and preventing gradual degeneration (Stöck et al. 2011).

These studies illustrate that while interchromosomal crossovers are highly repressed between heteromophic highly rearranged sex chromosomes, recombination can still occur and is likely at higher frequencies than previously appreciated in the regions outside of the highly rearranged key sex-determining regions or even within these regions in the form of gene conversion. We discuss in the following sections how these general processes regarding recombination and sex determination regions also operate in many fungi and unicellular eukaryotes (protists). Additionally, recombination plays important roles in fungal sex determination, as well as in sexual development.

II. Mitotic Recombination in Mating-Type Determination

A. Mating-Type Switching in Fungi

Mating-type switching was first discovered in the model yeasts *Saccharomyces cerevisiae* and later also in *Schizosaccharomyces pombe*. This involves one active mating-type cassette that can be rewritten by gene conversion from either of two distant mating-type cassettes that are otherwise maintained in a silent fashion by specialized heterochromatin (Dalgaard and Klar 1999; Egel 2005; Haber 2012; Heitman et al. 2007; Klar 2007, 2010) (Fig. 1.1). Specifically, in the genome of *S. cerevisiae*, the active *MAT* locus defines a bipolar mating system with two alternative mating-type alleles, *MAT***a** and *MAT*α, and the two silent mating cassettes, *HMR* and *HML*, which carry epigenetically silenced copies of *MAT***a** and *MAT*α alleles, respectively. Mating-type switching occurs during the G1 phase of the cell cycle in haploid cells and starts with the excision and elimination of the allele at the active *MAT* locus by a specialized DNA endonuclease called *HO* and exonucleases. The gap is subsequently filled in through gene conversion with genetic information present at either the *HML* or the *HMR* locus. Typically, *MAT***a** cells repair the gap using the *MAT*α allele from the *HML* locus, and vice versa, thus resulting in mating-type switching. Mating-type switching in *S. pombe* shares similar features with *S. cerevisiae*, including the presence of one active *MAT* locus and two silent mating cassettes on the same chromosome, as well as efficient directional switching initiated by a DNA lesion during the mitotic cell cycle. However, the two systems evolved independently, and they differ in the genes encoded within the *MAT* locus, as well as the organization of the *MAT* locus and the silent cassettes. Additionally, the mechanisms by which the silent cassettes are maintained, and the means by which the DNA lesion that initiates switching is created, also differ between the two species. Instead of a double-strand break induced by the *HO* endonuclease, the switching in *S. pombe* is initiated by a stall at the DNA replication fork that creates a lesion at the active *MAT* locus, which is subsequently repaired using the sequence from a silent cassette, facilitated by various recombinational repair factors (Arcangioli and de Lahondes 2000; Dalgaard and Klar 1999; Kaykov and Arcangioli 2004; Vengrova and Dalgaard 2004, 2006). Thus, recombination, specifically gene conversion, plays a central role in mating-type switching in *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, cells typically switch their mating types every other generation and only mother cells that have divided twice switch. Thus, the frequency of mating-type switching is likely considerably higher compared to typical mitotic recombination frequencies in other chromosomal regions, which are typically 100- to 1000-fold less common than meiotic recombination (Roeder and Stewart 1988).

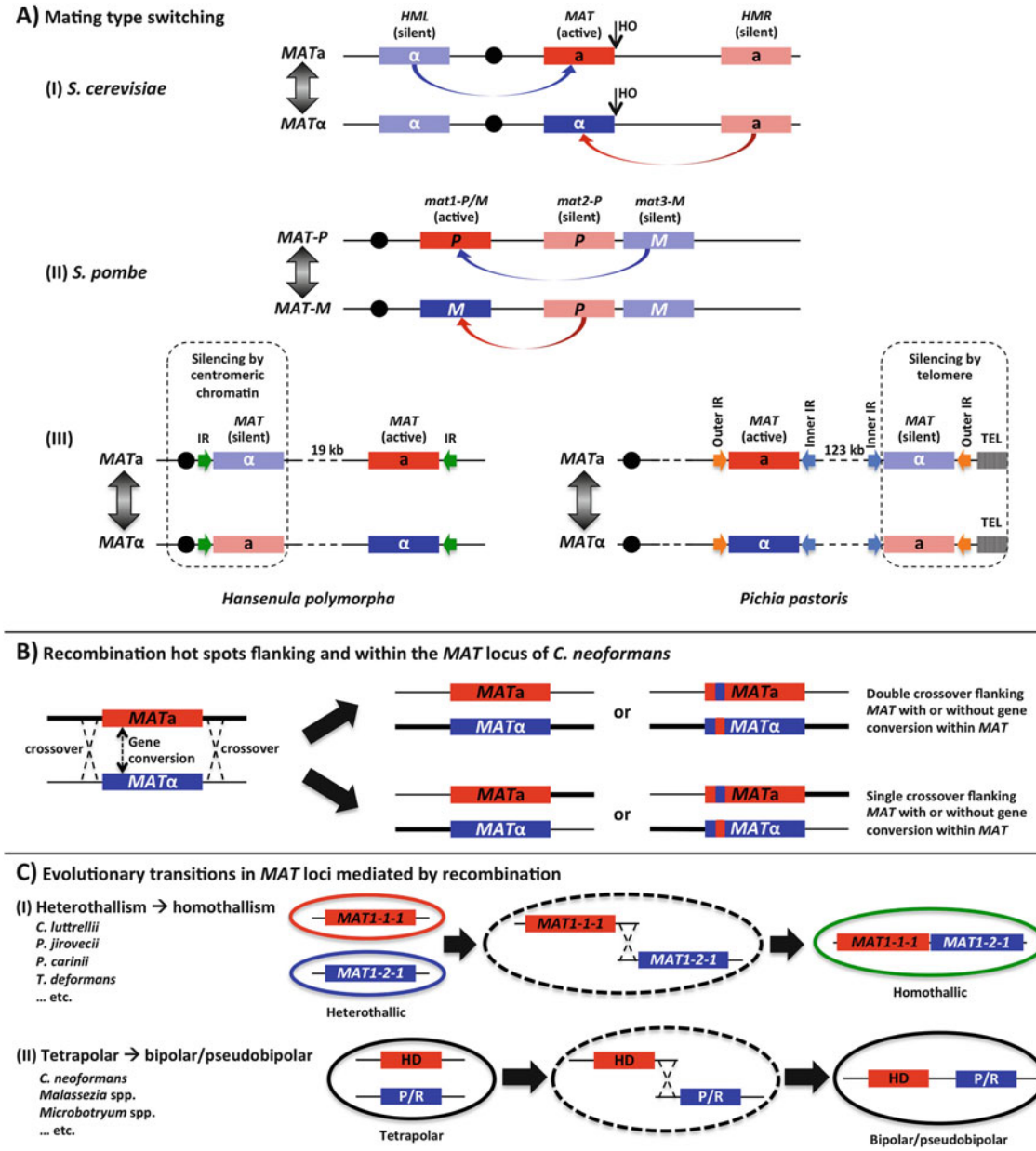

Fig. 1.1 Recombination associated with the *MAT* loci in different fungal species. (**a**) The three locus models of mating-type switching mediated by gene conversion in *S. cerevisiae* (I) and *S. pombe* (II) and the two locus models of mating-type switching mediated by inversion in *H. polymorpha* and *P. pastoris* (III) are illustrated. *IR* inverted repeat. (**b**) Recombination hot spots flanking the *MAT* locus *C. neoformans* break the linkage of *MAT* and its original genetic background during meiosis and increase the efficiency of natural selection. They may also function to repress crossover events which could have deleterious effects within the *MAT* locus. Gene conversion hot spot within the *MAT* locus of *C. neoformans* could be the result of DSB repair through non-crossover pathways. It could also function to maintain proper alignment of the two divergent *MAT* alleles during meiosis. (**c**) Evolutionary transitions of the *MAT* locus, including transitions between heterothallism and homothallism (I), as well as between tetrapolar mating system and bipolar mating systems (II), could be the result of ectopic recombination mediated by repetitive sequences or transposable elements

Remarkably, it has recently been shown that another budding yeast, *Kluyveromyces lactis*, also undergoes mating-type switching through a mechanism that is again distinct from that in *S. cerevisiae* (Barsoum et al. 2010; Rusche and Rine 2010). Additionally, it has been found that a specialized protein α3 is essential for switching in *K. lactis*. α3 is homologous to transposases, and the amino acids conserved among transposases are required for successful mating-type switching in *K. lactis*, suggesting that similar to the *HO* gene in *S. cerevisiae*, α3 originated through domestication of a transposase (Barsoum et al. 2010; Keeling and Roger 1995; Rajaei et al. 2014).

Recently, another novel mechanism for mating-type switching has been discovered in two closely related ascomycetous species, *Hansenula polymorpha* (=*Ogataea angusta*) and *Pichia pastoris* (=*Komagataella pastoris*) (Hanson et al. 2014; Maekawa and Kaneko 2014) (Fig. 1.1). In both species, there are just two mating-type loci, one active and one silenced, and the mating-type switching involves an inversion of the region that encompasses the two, essentially interchanging the chromosomal position of active and silent *MAT* locus to enable switching between the two opposite mating types, *MAT***a** and *MAT*α. Specifically, in *H. polymorpha*, the *MAT***a** and *MAT*α loci are located approximately 19 kb apart on the same chromosome, and the chromosomal region encompassing the two *MAT* loci lies beside the centromere. As a consequence, depending on the orientation of this chromosomal region, one of the two *MAT* loci will be silenced by the centromeric chromatin due to its proximity to the centromere, leaving the other *MAT* locus active and capable of defining the mating type of the cell (Hanson et al. 2014; Maekawa and Kaneko 2014). In *P. pastoris*, the two *MAT* loci are approximately 123 kb apart on the same chromosome, and depending on the orientation, one of the two *MAT* loci will be located close to the end of the chromosome and consequently silenced due to its close proximity to the telomere. The inversion that results in switching between the mating types occurs by recombination between inverted-repeat sequences flanking the *MAT* loci (Hanson et al. 2014) (Fig. 1.1). Additionally, while this simpler two-cassette inversion system differs from the more canonical three *MAT* cassette mating-type switching paradigms of both budding and fission yeasts, there are strong grounds to hypothesize that this was an ancestral state from which the more complex switching yeasts evolved. Models involving inversion of just two mating-type loci were in fact advanced many years ago by Jim Hicks and Ira Herskowitz (Hicks and Herskowitz 1977) and others as one possible model for mating-type switching. Given that there are other invertible genetic switches in biology that underlie flagellar phase variation in *Salmonella* (Andrewes 1922; Haykinson et al. 1996; Silverman et al. 1979) and plasmid copy number amplification of the two-micron circle in *S. cerevisiae* (Futcher 1986), it would not be surprising if other examples of similar inversion-mediated genetic switches remain to be discovered.

Additionally, mating-type switching has also been reported in one basidiomycetous species, *Agrocybe aegerita* (Labarère and Noël 1992), further demonstrating that this form of homothallism has evolved repeatedly and independently in different fungal lineages.

B. Recombination-Mediated Sex Determination in Other Small Eukaryotes

A recent study reports the exciting discovery of the complex and fascinating mating-type locus of the model ciliate *Tetrahymena thermophila*, solving an ~50-year-old mystery surrounding its unique mode of sex determination involving seven different sexes (Bloomfield 2014; Cervantes et al. 2013). *T. thermophila* was among the first microbial eukaryotes in which mating types were discovered in the 1950s following similar discoveries in the ciliate *Paramecium* and the yeast *Saccharomyces*. This opened a new era of invaluable microbial models for research in genetics and evolution for species with more than two sexes or mating types. While the yeast mating-type locus has become a textbook paradigm in genetics and molecular biology, the sex determination mechanisms in

the model ciliates languished in relative obscurity due to their complex biology and genetics.

Cervantes et al. took clues from the basic biology of *T. thermophila* and ingeniously applied RNA-seq to reveal the fascinating nature of the *MAT* locus in this species (Cervantes et al. 2013). They found that the *T. thermophila MAT* locus contains an array of up to seven tandem gene pairs, one pair corresponding to each mating type, in the germ line genome of the species. Only one of these gene pairs is assembled into a functional dual protein-coding unit through homologous recombination during differentiation of the somatic genome, and all of the other *MAT* modules are eliminated. Hence, different sibling progenies of the same parents may have different mating types depending upon which gene pair is assembled in their soma. This is astounding, especially because *Tetrahymena* biology ensures that all siblings are exactly genetically identical in their germ line genome, yet somatic differentiation generates different phenotypes (mating types). The discovery of the mating-type determination mechanism in this unicellular eukaryote thus provides a gripping account of how phenotypic plasticity can be generated through developmental genome rearrangements. In this context, the study salutes insights of the senior authors by supporting their molecular model predicted ~20 years ago based purely on classical genetic approaches.

Additionally, Cervantes et al. found that the two mating-type-specific proteins are large (161 and 194 kDa) and while they lack homology with any known proteins, they do contain a few conserved furin-like repeats similar to those observed in signaling receptors (Cervantes et al. 2013). This suggests either rapid evolution of ancestral proteins or an independent evolution of novel mating-type specificities, in line with the recent discovery of novel master sex determinants in fishes and novel sex determinants found in other ciliates.

Together with the recent discovery of the trisexual mating-type locus of the social amoeba *Dictyostelium discoideum* (Bloomfield et al. 2010), characterization of mating-type genes in *T. thermophila* has made the evolution of multiple mating types a genetically tractable research question. Discovery of the *MAT* locus, its genomic structure, and its differentiation in *T. thermophila* now provides biologists a foundation to obtain insights into the evolution of mating systems and further stresses the importance of studying the unique and unconventional biology and genetics of ciliates.

III. Meiotic Crossover and Gene Conversion Related to the *MAT* Locus

A. Recombination Hot Spots Flanking and Within the *MAT* Locus in *Cryptococcus neoformans*

Cryptococcus neoformans is a basidiomycetous human fungal pathogen. It has a bipolar mating system that is determined by the presence of one of two alternative alleles, **a** and α, at the unusually large *MAT* locus (>100 kb in size with >20 genes) (Fraser et al. 2004; Lengeler et al. 2002; Loftus et al. 2005). Mating typically occurs between isolates of opposite mating types (bisexual reproduction) (Kwon-Chung 1975, 1976a, b), although sexual reproduction can also occur between two MATα isolates (unisexual reproduction) (Lin et al. 2005, 2007, 2009). The *C. neoformans MAT* locus in many ways mirrors the sex chromosomes of other organisms. Specifically, extensive sequence divergence and chromosomal rearrangements, including translocations and inversions, have accumulated between the **a** and α *MAT* alleles, indicating meiotic recombination is likely repressed within the *MAT* locus. Additionally, studies have identified evolutionary strata within the *MAT* locus, suggesting it underwent gradual cessation of recombination between the two alleles during the evolution of the *MAT* locus (Fraser et al. 2004).

It has been hypothesized that the extensive sequence divergence and chromosomal rearrangements between the **a** and α alleles pose physical barriers for crossing-over within the *MAT* locus during **a**-α bisexual reproduction in *C. neoformans*. Indeed, in a study in which

more than 150 meiotic progeny from **a**-α bisexual reproduction of serotype D *C. neoformans* were analyzed, no crossover was detected within the *MAT* locus. However, gene conversion does occur within the *MAT* locus around a GC-rich intergenic region between the *RPO41* and *BSP2* genes, which have relatively high sequence similarity between the two alleles (Sun et al. 2012). Interestingly, the *C. neoformans MAT* locus is also flanked by two GC-rich regions, and it has been shown that both GC-rich flanking regions contain recombination hot spots that elevate crossover frequencies in these regions during meiosis and crossover at one side of the *MAT* is typically associated with another crossover at the other side of the *MAT* (Hsueh et al. 2006) (Fig. 1.1). It is possible that during meiosis, DNA double-strand breaks are induced similarly at high frequency in the *MAT* flanking recombination hot spots and the intra-*MAT* gene conversion hot spot. While repair in the flanking regions can result in crossovers, breaks within the *MAT* gene conversion hot spot are more likely repaired through mechanisms that result in gene conversion (e.g., synthesis-dependent strand annealing), or the crossover products fail to survive, due to structural or genetic constraints exerted by chromosomal rearrangements between *MAT* alleles.

Recent studies also suggest that sequence divergence and structural variation between the two mating-type alleles are not the only mechanisms that act to repress meiotic recombination within *MAT*. In a study of meiotic recombination in α-α unisexual reproduction, during which two *MAT*α alleles align with each other in meiosis, and the physical constraints on recombination posed by sequence divergence and chromosomal rearrangements are no longer present, the authors found crossing-over is still highly repressed, albeit not completely eliminated (Sun et al. 2014). Specifically, among 156 unisexual meiotic progeny analyzed, only one crossover event occurred within the *MAT* locus, yielding a frequency of crossover within the *MAT* that is considerably lower than expected given the size of *MAT* and the average recombination frequencies in other chromosomal regions during both **a**-α bisexual and α-α unisexual reproduction, as well as the intervariety hybridization (Marra et al. 2004; Sun et al. 2014; Sun and Xu 2007). One possible explanation could be that the two recombination hot spots that flank the *MAT* locus repress recombination within *MAT* through crossover interference, as these flanking hot spots have been shown to be active also during α-α unisexual reproduction (Sun et al. 2014).

B. Pseudobipolar Mating System and Its Effect on Meiotic Recombination

Typically in fungi, the mating type is determined by restricted regions in the genome called *MAT* loci, which usually contain genes that encode pheromone/pheromone receptors and/or sexual development transcription factors (Heitman et al. 2013), although in some species, such as *Cryptococcus heveanensis*, *Cryptococcus amylolentus*, and *Cryptococcus neoformans*, the *MAT* loci apparently have undergone expansion by recruiting additional genes into *MAT* (Findley et al. 2012; Fraser et al. 2004; Lengeler et al. 2002; Metin et al. 2010). In basidiomycetes, the pheromone/pheromone receptors and sexual development transcription factors are typically located in two separate loci, called the PR locus and HD locus, respectively, that lie on different chromosomes, constituting a tetrapolar mating system. However, in some cases, such as *C. neoformans*, as well as *Ustilago hordei*, *Malassezia* species, and *Microbotryum* species, the two *MAT* loci have become fused, possibly through ectopic recombination or nonhomologous end joining, resulting in a bipolar mating system with linked PR and HD loci (Fig. 1.1). This structural transition can sometimes capture previously non-sexual autosomal genes between the two loci and subsequently represses recombination in these newly captured chromosome regions. In some cases, this repression of recombination can span the entire chromosome on which the PR and HD loci now reside, resulting in mating-type chromosomes that share features with the sex chromosomes of animals (Gioti et al. 2013a; Giraud et al. 2008; Hood et al. 2013; Lee et al. 1999; Petit et al. 2012; Votintseva and Filatov 2009; Whittle et al. 2015). In both *Malassezia*

and *Microbotryum* species, while the PR and HD loci are linked, more than two mating types have been identified in the natural population, which is different from canonical bipolar species where there are only two mating types, and thus, they have pseudobipolar mating systems (Gioti et al. 2013a). Additionally, given the large size and the syntenic nature between the two mating types in the region linking the PR and HD loci, crossover may still occur occasionally in this region, although this is hypothesized to be detrimental as it breaks up the proper allele associations of the PR and HD loci.

Several recent studies on the dimorphic mating-type chromosomes of an unusual group of closely related fungal species in the genus *Microbotryum* that have expansions in the *MAT* loci reveal unique features of their evolutionary trajectory. Both mating-type chromosomes (a1 and a2) were isolated and sequenced and also subjected to optical mapping to generate very high-quality assemblies and sequences for the reference genome. This scaffold then served to assemble data from 12 other related *Microbotryum* species. The mating-type chromosomes could be partitioned into two types of regions termed recombining regions (RR) and nonrecombining regions (NRR), analogous to sex-specific and pseudoautosomal regions for animal and plant sex chromosomes. By comparing these regions, recombination suppression in these chromosomes predates species divergence times and thus is ancient. Evolutionary strata were not clearly apparent, in contrast to studies on XY and ZW sex chromosomes and other studies of fungal mating-type loci, suggesting one major event may have driven this expansion to a fungal sex chromosome. Additionally, 11 examples of gene conversion events within the NRR regions were identified.

A key feature of XY and ZW sex chromosomes is the degeneration of genes resident on one of the two sex chromosomes, namely, those mutations that are sheltered on the Y or W chromosome that is only present in the heterogametic sex and not in a homogametic fashion that would allow repair from a homolog. In contrast to this pattern, an evolutionary signature of gene decay was observed for both the a1 and the a2 fungal sex chromosomes in *Microbotryum* species. The ratio of nonsynonymous to synonymous (dN/dS) substitutions in the NRR regions of the sex chromosomes compared to non-*MAT* chromosomes was significantly higher for 4 of 12 species examined based on a Codeml, PAML-based comparison, whereas the dN/dS ratio was significantly higher for 9 out of 12 species based on singleton analysis that focused only on more recent events. These observations provide evidence for a higher rate of deleterious mutations on both fungal sex chromosomes, consistent with the genetics of the a1/a2 genotype only being present in the zygote and heterokaryon stages of the life cycle, and the absence of an equivalent to the homogametic state enabling differential sex chromosome repair in species with XY- and ZW-specific sex-determining systems. Taken together, these studies reveal not only parallels in the evolutionary trajectory of sex chromosomes in fungi, plants, and animals but also lineage-specific features giving rise to distinct patterns of sex chromosome-linked gene mutation and decay in symmetric vs. asymmetric patterns.

IV. Consequences of Recombination in the Evolution of the *MAT* Locus

Recombination, or lack thereof, within and in the proximity of the mating-type locus can have broad effects on the evolutionary dynamics and trajectories of the *MAT* loci, as well as the mating-type determination systems.

First, recombination is required for efficient natural selection. Therefore, chromosomal regions in which recombination is repressed, such as the Y chromosome in animals, usually degenerate due to accumulation of deleterious mutations and transposable elements. Additionally, chromosomal rearrangements, such as translocations and inversions occurring within the recombination-repressed region, also cannot be corrected efficiently through recombination-mediated repair mechanisms. These will in turn further compromise proper

alignment of these regions during meiosis and consequently reinforce the repression of recombination within these regions. This is also true for fungal *MAT* loci that have undergone expansion. For example, in *C. neoformans*, the two mating-type alleles show elevated sequence divergence and extensive chromosomal rearrangements compared to other chromosomal regions. Additionally, repetitive sequences as well as transposable elements have been found within the *MAT* locus of *C. neoformans*, which could have played roles in mediating the chromosomal rearrangements within the *MAT* locus through ectopic recombination (Fraser et al. 2004; Lengeler et al. 2002). Additionally, natural *C. neoformans* populations have been found to be largely clonal, suggesting recombination may not occur at high frequency or be difficult to detect if recombination occurs in a clonal population. Thus, the recombination hot spots found flanking the *MAT* locus of *C. neoformans* could function to increase the effective population size of *MAT*, thus enhancing the selection efficiency on this functionally important locus.

Second, ectopic recombination likely mediated the emergence of homothallism from ancestral heterothallism in many fungal species. In heterothallic species, sexual reproduction only occurs between two individuals with different mating types. However, for some fungal species, a single spore/cell can initiate sexual reproduction without the presence of another individual with a different mating type, and we call these species homothallic. The aforementioned species that can undergo mating-type switching are examples of homothallism as the mother cell that underwent mating-type switching can mate with a daughter cell and the two mating partners are identical by descent (except at *MAT*). Homothallic sexual reproduction can also be achieved through special configurations at the *MAT* locus. For example, in species such as *Neurospora tetrasperma* (Merino et al. 1996), *Agrocybe semiorbicularis*, *Conocybe tenera* for. *bispora*, *Coprinellus ephemerus* (Raper 1966), and *Agaricus bisporus* (Li et al. 2004), the meiotic spores contain separate nuclei with compatible mating types and can germinate into heterokaryotic mycelia and proceed with sexual reproduction by themselves.

Homothallism can also be due to the presence of *MAT* loci of opposite mating types in one genome. The two loci can be unlinked, such as in *Aspergillus nidulans* (Galagan et al. 2005), or they can be linked, as found in *Neurospora pannonica* and *Neurospora tericola* (Gioti et al. 2012, 2013b; Pöggeler 1999), *Cochliobolus luttrellii* (Lu et al. 2011; Yun et al. 1999), as well as *Pneumocystis* species and *Taphrina deformans* (Almeida et al. 2015; Turgeon and Inderbitizen 2015). In these species, it is likely that the linkage between the two *MAT* loci originated from heterothallic ancestors through recombination at regions shared between alleles from opposite mating types (Fig. 1.1). For example, when the sequence from the *MAT* locus of the homothallic species *C. luttrellii* is aligned with the *MAT1-1* and *MAT1-2* alleles from the heterothallic sister species *Cochliobolus heterostrophus*, there is a stretch of eight nucleotides that is identical between the two species and lies at the fusion junctions between opposite mating-type alleles in *C. luttrellii*, which could have mediated the ectopic recombination in the heterothallic ancestor that resulted in the extant *MAT* configuration observed in *C. luttrellii* (Lu et al. 2011; Turgeon and Inderbitizen 2015; Yun et al. 1999).

Ectopic recombination mediated by repetitive sequences or transposable elements could have also played critical roles in the transition from an ancestral tetrapolar mating system to the extant pseudobipolar and bipolar mating systems observed in basidiomycetous species such as *U. hordei*, *C. neoformans*, as well as *Malassezia* and *Microbotryum* species (Findley et al. 2012; Fraser et al. 2004, 2007; Gioti et al. 2013a; Lee et al. 1999; Metin et al. 2010). Additionally, in species such as *C. neoformans*, which have regional centromeres that are enriched with common, shared transposable elements and their remnants, it is also possible that ectopic recombination that gave rise to the initial linkage of the P/R and HD loci was mediated by centromeres that brought the two *MAT* loci onto the same chromosome, and similar events of chromosomal arm exchanges have been observed in the evolution of the pathogenic *Cryptococcus* species complex (Janbon et al. 2014).

V. Conclusions

Recombination, both crossover and gene conversion, plays critical roles in sex determination in many fungal species. Additionally, the evolution of the *MAT* locus, including sequence divergence and chromosomal rearrangements between alleles of different mating types, as well as the accumulation of repetitive elements in some species, is likely strongly influenced by recombination (or lack thereof). On the other hand, the presence of a mating-type locus on a chromosome can also affect its recombinational landscape, such as in the cases of 1) pseudobipolar mating systems in *Malassezia* species and 2) *Microbotryum* species where recombination is repressed in a large portion or almost the entirety of the mating-type chromosome. Additionally, while in some cases the *MAT* locus is associated with repressed crossing-over within, there are also examples where recombination hot spots are present within or in the proximity of the *MAT* locus. Given the diverse mating systems and mating-type determination mechanisms in different species, fungi as well as other microbial eukaryotes provide rich resources to study the effects of recombination on mechanisms of sex determination and sexual development, as well as the evolution of sex determination systems and sex chromosomes in general.

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2 Ecological Biogeography of Lichen-Forming Fungi

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CONTENTS

I. Introduction

"In truth, any statements we make on lichen (bio)geography are bound to be modified, sometimes embarrassingly so, as new collections are made. . .."
(Hale 1990), p. 34

A long-standing interest in understanding factors that shape distribution patterns of biological communities and evolutionary lineages has resulted in diverse research programs attempting to elucidate meaningful biogeographic patterns and processes shaping those patterns (Lomolino et al. 2006). Biogeographic research generally fits within two major subdisciplines: one focuses on historic events and/or processes (e.g., plate tectonics, dispersal events, vicariance, etc.) that shape current distributions; and the other investigates the role of ecology in determining distributions. While these two perspectives are not necessarily mutually exclusive, they provide a useful framework for biogeographic research.

Contemporary distributions of species are the result of the dynamic interplay of multiple factors operating across multiple temporal and spatial scales (Hubbell 2001). While important advancements have been made in understanding both historical and ecological factors that determine species contemporary distributions, their respective relative importance and interplay remain largely unknown and difficult to tease apart. For example, Bonada et al. (2005) attempted to quantify the role of historical versus ecological biogeographic factors influencing distribution patterns of caddisflies in Mediterranean rivers. Their study revealed that ecological factors played a more important role in contemporary biogeography than historical factors (21 % vs. 3 %, respectively) and that only 0.3 % was explained by the interaction of history and ecology, with the rest unaccounted for. For most groups of organisms, including lichens, the relative importance and interplay between historical and ecological biogeographic factors in shaping species distributions remains unexplored.

In this chapter, we highlight the current state of knowledge of lichen biogeography, with a focus on ecological biogeography and potential avenues for future research. Lichens are mutualistic associations of a fungus (mycobiont) and a photosynthetic partner (photobiont), either a green alga and/or cyanobacterium, rarely also other groups of algae occur in lichen symbioses. In addition to these two main partners, there are

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also many other fungi present in lichen thalli, including endolichenic and lichenicolous fungi. Also bacteria are commonly found associated with lichens. Lichens are ubiquitous components of most terrestrial ecosystems, playing important ecological roles, for example, as pioneers or as stabilizers of soil.

Specifically, we briefly discuss general perspectives of biogeography of lichen-forming fungi, followed by a synthesis of four major themes directly related to ecological biogeography, including (1) dispersal and establishment of lichens, (2) landscape genetics and gene flow, (3) modeling lichen distributions, and (4) the role photobionts play in determining species distributions. We conclude by discussing the role of ecological biogeography in conservation and climate change research.

Reflection on the broad scope of both past and current views of lichen biogeography can improve our perspective into biogeographic patterns and the underlying processes shaping species distributions, in addition to directing future research. A comprehensive summary of the multifaceted and developing field of lichen biogeography is well beyond the scope of a single chapter. Rather, our emphasis is on summarizing our current understanding of contemporary factors that influence ecological and spatial distributions of species (Monge-Nájera 2008). Our hope is that this review will encourage a more comprehensive perspective of lichen biogeography by promoting the consideration of a wide variety of factors that potentially shape species distributions, in addition to historical processes (traditional phylogeographic perspective). This expanded focus of lichen biogeography should, in turn, promote creative biogeographic research incorporating the fields of ecology, landscape genetics, species distribution modeling, symbiont interactions in lichens, etc.

II. Perspectives of Biogeography of Lichen-Forming Fungi

There has been a tradition of studies attempting to understand distributions of lichen-forming fungi and the factors that shape these distributions (Galloway 1988, 2008; Galloway and Aptroot 1995; Culberson 1972; Werth 2011; Du Rietz 1940). However, with the increasing availability of molecular data, our understanding of lichen phylogeography has dramatically improved in recent years. This is due, in part, to the increased recognition of the importance of cryptic, or previously unrecognized, lineages in lichen-forming fungi (Lumbsch and Leavitt 2011; Crespo and Pérez-Ortega 2009; Crespo and Lumbsch 2010), large-scale collaborative projects (Lumbsch et al. 2011), and developments in analytical approaches (Yang and Rannala 2006; Rabosky et al. 2014; Ree and Smith 2008). A general perspective of lichen biogeography is presented by Galloway (2008), and Werth (2011) also provides a valuable review.

Lichens exhibit biogeography and/or ecological distribution patterns that in many cases differ from other co-occurring biota (e.g., vascular plants, animals, etc.; Galloway 2008; Culberson 1972). For example, a disproportionately high number of lichen-forming fungal species have bipolar distributions, meaning that they occur in polar regions of the Northern and Southern Hemisphere (Wirtz et al. 2008; Fernández-Mendoza and Printzen 2013; Myllys et al. 2003; Lindblom and Søchting 2008); other species occur in ecologically and geographically restricted regions (Lücking et al. 2014); and many species occur across incredible ecological and geographic distances (Leavitt et al. 2013b; Printzen et al. 2013). Traditionally, it has been thought that species with broad, intercontinental distributions have high dispersal abilities and general ecological preferences. Broad distributions have alternatively been explained as older species of lichen-forming fungi that have had more time to disperse and reach extensive distributions (reviewed in Werth 2011). However, increased interest in species delimitation research and estimating divergence times for groups of lichen-forming fungi have challenged these traditional perspectives (Amo de Paz et al. 2011; Divakar et al. 2012; Leavitt et al. 2015a; Otálora et al. 2010). For example, in the genus *Melanohalea*, many of the species-level lineages with more ancient diversification histories, including *M. multispora* s. lat. and *M. ushuaiensis* s. lat., have geographically restricted

distributions in western North America and southern South America, respectively. In contrast, species with more recent diversification histories, including *M. elegantula* and *M. exasperatula*, generally have much broader geographic distributions (Otte et al. 2005; Leavitt et al. 2013a).

Iconic examples of phylogenetic structure corresponding to major biogeographic regions or major tectonic events are relatively scarce in the groups of lichen-forming fungi that have been investigated to date, although other striking biogeographic patterns have been observed in a number of cases (Miadlikowska et al. 2011; Lücking et al. 2013; Del-Prado et al. 2013). Rather than biogeographic patterns driven by dispersal limitations or vicariance, it appears that major climatic shifts may have played the dominant role in the diversification and distributions of lichen-forming fungi in the family Parmeliaceae (Amo de Paz et al. 2011, 2012; Kraichak et al. 2015).

Ultimately, the interplay of climate-driven diversification, dispersal and establishment, and vicariance results in biogeographic patterns that may be difficult to generalize. The *Rhizoplaca melanophthalma* group (Lecanoraceae) provides an interesting example of the challenges inherent to elucidating biogeographic patterns in lichen-forming fungi. *Rhizoplaca melanophthalma* s. lat. occurs on all continents, except Australia, in a broad range of habitats, from extremely arid continental habitats to upper montane coniferous forests and the lower portions of the alpine tundra (Leavitt et al. 2013b). Although *R. melanophthalma* s. lat. was traditionally assumed to represent a single, cosmopolitan species, molecular sequence data support the conclusions that this nominal taxon is comprised of multiple species-level lineages, three of which occur across broad intercontinental distributions, and the remaining lineages are known exclusively from western North America (Leavitt et al. 2011, 2013b). All of the known species within the *R. melanophthalma* group can be found within a limited geographic region in the southwest USA; species have not been shown to have distinct ecological preferences nor do distributions of species, otherwise correspond to distinct biogeographic regions, with the exception of *R. shushanii* which is known exclusively from subalpine habitat on the Aquarius Plateau in southern Utah, USA (Fig. 2.1; Leavitt et al. 2011, 2013b).

Our limited understanding of morphological adaptations is also exemplified by the presence of cryptic species that are only distantly related but occur under similar ecological conditions. For example, *Parmelina quercina* was believed to occur in areas with Mediterranean-type climate throughout the world, but molecular studies have demonstrated that distinct species occur in each continent, with the Australian taxon now being classified in a separate, unrelated genus, *Austroparmelina* (Argüello et al. 2007; Crespo et al. 2010a, b). Another example is *Physcia aipolia*, which was believed to occur in temperate to subtropical regions of the Northern Hemisphere but also in Australia (Moberg 2001). However, the Australian samples were shown by molecular data to represent a group of three separate species, unrelated to the populations in the Northern Hemisphere (Elix et al. 2009).

Given our limited ability to make generalizable inferences and predictions on species distributions within a historical biogeographic framework, we emphasize that more effective incorporation of an ecological biogeographic perspectives into biogeographic research of lichen-forming fungi will provide an improved understanding of the range of factors shaping the geographical distributions of species.

III. Assessing Dispersal Capacity of Lichen-Forming Fungi

Accounting for differences in reproductive strategies and dispersal capacities of lichen-forming fungal species is central to understanding and interpreting biogeographic patterns. Lichen-forming fungi generally use two major reproductive strategies, sexual reproduction via meiotically produced fungal spores and vegetative reproduction using asexually produced diaspores. Sexual reproduction is restricted to characteristic fungal fruiting bodies (ascomata)

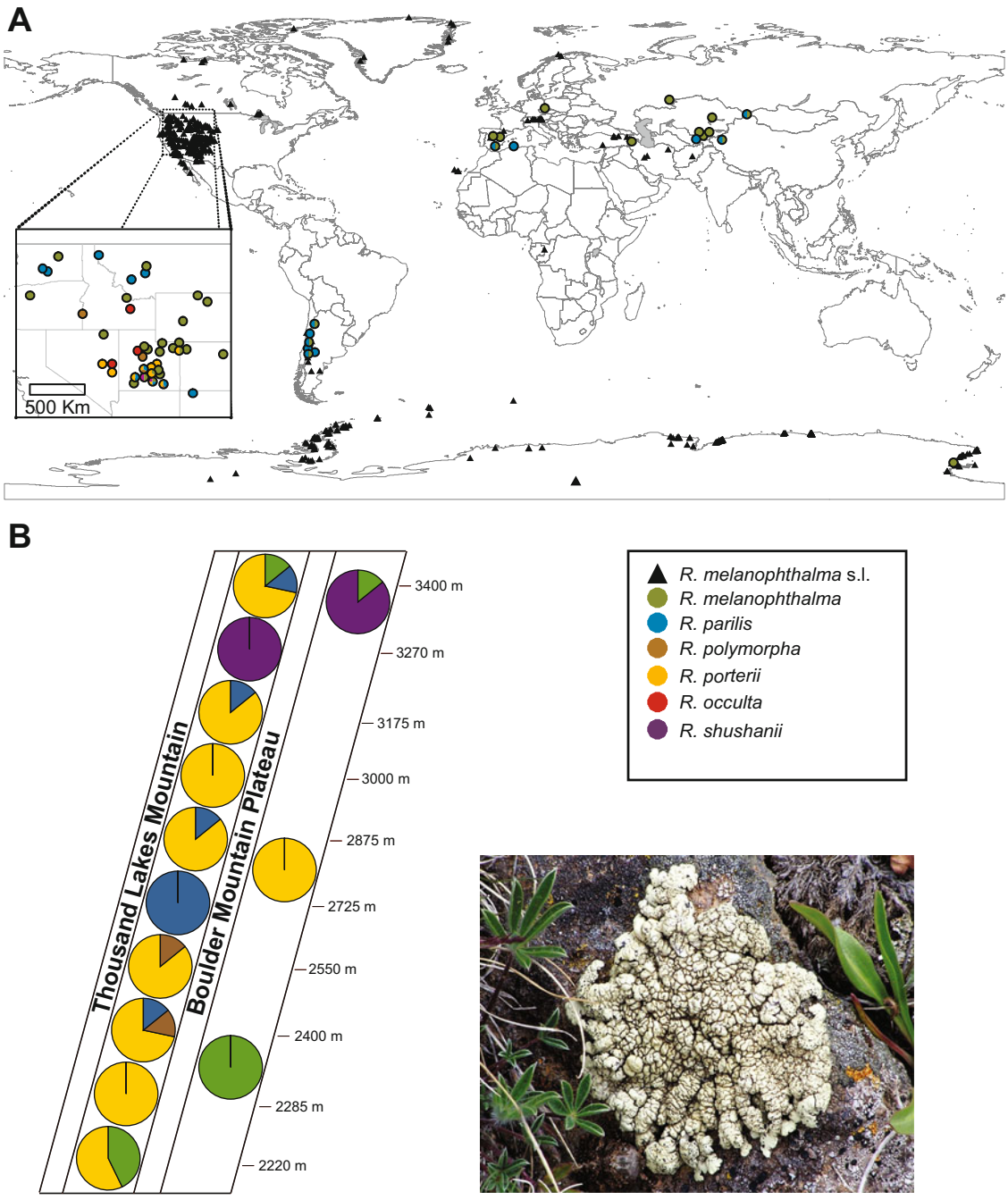

Fig. 2.1 (**a**) Worldwide distribution of the *Rhizoplaca melanophthalma* group; (**b**) Distribution of species along an altitudinal gradient on the Aquarius Plateau in southern Utah, USA; and habitat of *R. shushanii*, endemic to subalpine habitats in the southern Utah, USA. Modified from Leavitt et al. (2011) and Leavitt et al. (2013b)

that produce meiospores (ascospores). Ascospores are dispersed independently of the photosynthesizing partner (photobiont) and usually require acquisition of the appropriate photobiont partner in order to reestablish the lichenized condition. Ascospore ejection

appears to be temporally gauged toward maximum efficiency of dispersion and germination (Pyatt 1973; Favero-Longo et al. 2014; Trail and Seminara 2014). In contrast, other lichens commonly propagate asexually by means of vegetative diaspores produced in characteristic structures termed isidia or soredia. These specialized vegetative reproductive propagules contain both fungal and algal or cyanobacterial symbionts and potentially a core fraction of the lichen microbiome (Aschenbrenner et al. 2014), eliminating the need for independent acquisition of the appropriate symbiotic partners. Isidia and soredia are usually not actively dispersed but require physical separation from the parent lichen by wind, animals, or other physical disturbances. In some cases, it appears that hygroscopic movement also facilitates soredia dispersal (Jahns et al. 1976). Fertile lichens—those bearing ascomata which produce ascospores—only rarely produce vegetative diaspores (exceptions include *M. elegantula*, *Letharia vulpina* s. lat., *Lobaria pulmonaria*, and others); likewise, lichens that typically reproduce vegetatively rarely form apothecia (exceptions include *Hypogymnia physodes*, *Physcia tenella*, and others).

The general efficacy of dispersal for major reproductive strategies is now beginning to be better understood. Traditionally, lichen fungi reproducing via ascospores were considered to be more effective at long-distance dispersal due to the relatively smaller size of the meiospores when compared to vegetative diaspores, although Bailey (1966) suggested that soredia are more effective dispersal agents than ascospores. However, effective dispersal does not equate to successful establishment, and successful fungal meiospores establishment may be abysmally low due the small likelihood of encountering the appropriate symbiotic partners after dispersal. This has led to the widespread acceptance that sexually reproducing lichens commonly occur as juvenile parasites on other lichens from which they presumably obtain the photosynthetic partner (Poelt 1956, 1987, 1990; Poelt and Steiner 1971; Hawksworth 1982; Friedl 1987). In addition, the temporal viability of lichen-forming fungal spores remains largely unexplored.

While species-specific differences in morphological and physiological traits of the diaspores play a major role in successful establishment (Hilmo et al. 2011), Werth et al. (2014) demonstrated that size is not necessarily a good predictor of local or regional structure in three species of *Lobaria* that generally reproduce clonally. In fact, it has long been recognized that species using a vegetative reproductive strategy often have a larger distributional range than their fertile counterparts, at least in foliose and fruticose groups (Bowler and Rundel 1975). This conclusion is generally supported by molecular phylogenetic studies that commonly reveal broad intercontinental distributions and no phylogeographic substructure in many lichens reproducing via vegetative diaspores (Leavitt et al. 2012; Wirtz et al. 2008, 2012). In maritime Antarctic lichens, soredia were found to be the most abundant airborne propagules, more so than ascospores, further highlighting the importance of asexual reproduction in some lichen communities (Marshall 1996).

Although studies suggest that vegetative diaspores are able to effectively disperse and successfully establish across broad distributions, empirical studies generally reveal much shorter dispersal capacities. For example, the vegetative dispersal distance between host trees and *Lobaria pulmonaria* (Fig. 2.2) was found to be only 15–30 m (Jüriado et al. 2011). However, Schei et al. (2012) highlight the fact that the observed spatial distribution patterns in *Lobaria* species are the result of dynamic interactions of environmental factors with dispersal capacity and that single site patterns are not readily generalizable. It appears that lichen fungi that reproduce via passively dispersed small propagules tend to exhibit patchy populations with extensive distributions at the landscape scale (Gjerde et al. 2014). In the sorediate lichen *Hypogymnia physodes*, wind dispersal has been shown to deposit most soredia within 5 cm of the parent thallus, with a maximum measured dispersal distance of 80 cm in wind speeds of 9 m/s, empirically demonstrating that air currents directed over the surfaces of lichen thalli would be able to effectively disperse soredia vertically and horizontally within a tree canopy (Armstrong 1994).

Fig. 2.2 Habit of the lung lichen (mycobiont = *Lobaria pulmonaria*) in the Great Smokey Mountains, USA. This lichen has become a model species for ecological biogeographic research, including investigations into dispersal capability, landscape genetics, symbiont interactions, and conservation. Photo credit: Jason Hollinger, http://www.waysofenlichenment.net

Fig. 2.3 *Flavocetraria* sp. (Parmeliaceae) and *Thamnolia* sp. (Icmadophilaceae) used as nesting material in an American golden-plover (*Pluvialis dominica*) scrape near Coffee Dome, Seward Peninsula, Alaska, USA. Photo credit: Wikipedia CC BY-SA 3.0

Although wind is generally considered an important dispersal agent for lichen propagules, water-mediated dispersal is also a potential mechanism for propagule dispersal (Bailey 1966). Laboratory experiments have assessed dispersal by water droplets and suggest that falling water can effectively disperse lichen propagules on the scale of centimeters to meters (Armstrong 1987). Some lichen propagules may also be abundant in runoff water, although successful establishment may be limited by a shortage of suitable sites for the attachment of propagules (Armstrong 1981).

Animal-mediated dispersal may be another important mechanism for dispersal, although reports of lichen propagule dissemination by animals are rather scarce. For lichens that disperse via thallus fragmentation, the thallus fragments may disperse effectively across short distances in open vegetation habitats but generally ineffective for long-distance dispersal; and wind and animals likely play a major role in the dispersal of thallus fragments across longer distances (Heinken 1999; Rosentreter 1993).

Birds are commonly implicated as agents of long-distance dispersal of lichens, particularly in cases where lichen distribution and not readily accounted for by atmospheric air current patterns (Bailey and James 1979). Birds frequently use lichens as nesting material (Fig. 2.3), and this presumably plays a role in lichen dispersal. In addition to lichen dispersal by birds for nesting, Bailey and James (1979) demonstrated a small-scale transport of lichen fragments in New Zealand by large seabirds, and this study indicates that, at least theoretically, these birds could be effective agents of long-distance dispersal of lichen propagules. Although bryophyte diaspores have recently been documented in the plumage of transequatorial migrant birds (Lewis et al. 2014), there is, however, little direct evidence at this time supporting long-distance dispersal of lichen propagules by birds.

Vertebrates, arthropods, and gastropods may also mediate dispersal of reproductive propagules. For example, in Central Maine, USA, a quarter of oribatid mites (*Humerobates arborea*) were found to have soredia externally adhered to their bodies (Stubbs 1995), although the estimated average distance for transferal via mite was estimated to be relatively short (<5 cm; also see Seyd and Seaward 1984). Insect-mediated soredia dispersal has also been reported for ants (Lorentsson and Mattsson 1999; Bailey 1970) and potentially lacewings larvae, which construct and carry lichen "packets" (Skorepa and Sharp 1971). Intuitively, one could assume that a wide range of insects groups that occur alongside lichens could serve as a potential means of propagule dispersal, including tardigrades, nematodes,

springtails, etc. (Pickup 1988; Stubbs 1989; Aptroot and Berg 2004).

Endozoochory, diaspores carried within an animal, plays an important role in seed plant dispersal, and it appears that gastropods grazing on lichen communities likely serve as important vectors for lichen dispersal (Boch et al. 2011; McCarthy and Healy 1978). Boch et al. (2011) found that two lichens, *Lobaria pulmonaria* (Fig. 2.2) and *Physcia adscendens*, were able to regenerate from fecal pellets after passing through their digestive tracts of common snail species occurring in temperate Europe. This gastropod–fungus–alga association represents another level of complexity in lichen symbioses, and endozoochory provides a previously overlooked mechanism for lichen dispersal. Viable lichen fungal spores and photobionts have also been in fecal pellets from slugs (McCarthy and Healy 1978), and lichenivorous mites have also been shown to distribute lichens with their feces including viable cells of both fungal and algal partners (Meier et al. 2002). Rotifers have also been shown to ingest ascospores of *Xanthoria parietina* and deposit viable spores in their feces (Pyatt 1968).

Given the range of distribution patterns in lichen-forming fungal taxa, increased interest in understanding mechanisms for fungal dispersal and variation in dispersal capacity will likely provide novel insight into biogeographic patterns in lichens. Recent advancements in sampling environmental DNA (eDNA) and "next-generation" sequencing technologies provide promising avenues for more accurately characterizing dispersal capacities of lichen-forming fungi (Shokralla et al. 2012). Rather than relying exclusively on visual observations of lichen propagules, eDNA collected from migratory birds and other animals can be used to determine if animals consistently carry evidence supporting animal-mediated lichen dispersal. Similarly, eDNA can be used to assess the presence of lichen-forming fungal DNA from air and water samples. In the foreseeable future it is reasonable to assume that source populations of eDNA samples could be accurately identified by using highly variable DNA markers, affording an exciting avenue for future research.

IV. Ecological Biogeography

In contrast to historical biogeography, the study of ecological biogeography attempts to elucidate contemporary factors that influence ecological and spatial distributions of species (Monge-Nájera 2008). The study of contemporary biogeographic relationships focuses on biotic interactions among organisms, environmental changes that potentially impact a species distribution, and how landscape and environmental features influence gene flow and population structure. While evolutionary patterns may not be explicitly of interest in ecological biogeography, there is no distinct boundary between historical and ecological biogeography; and ecological biogeography can extend back in time to reconstruct demographic histories, ecological interactions, environmental controls, and evolutionary relationships (e.g., Richardson and Meyer 2012; Chan et al. 2011; Lira-Noriega et al. 2015).

A. Ecological Biogeography and Lichens

The overall importance of ecology in determining the distributions of lichens is well known (Renhorn et al. 1996; Kantvilas and Minchin 1989; Culberson and Culberson 1967). The distributions of some lichen-forming fungal species and overall diversity are commonly determined by microclimatic differences (Palmqvist and Sundberg 2000; Renhorn et al. 1996; Hauck et al. 2007; Ranius et al. 2008), and a wide variety of contemporary factors potentially influence lichen distributions. For example, Nelson et al. (2015) recently demonstrated that different combinations of lichen functional traits, including choice of photobiont, dispersal capacity, microsite specificity, and water relations, peak along environmental and disturbance gradients.

The distribution and abundance of species can be explained by the combination of dispersal and environmental filtering. The occurrence of a particular species is the product of the probability of establishment (environmental filtering), and the number of propagules arriving at the site (dispersal) (Schei et al.

2012). Schei et al. (2012) underscore that the relative importance of local dispersal and environmental filtering varied widely among sites in deciduous forests in southwest Norway, particularly in terms of abundance patterns. However, environmental filtering by tree species was more important than local dispersal overall (Schei et al. 2012). Of course inferences from any study investigating the relative roles of dispersal and environmental filtering are scale dependent (Jackson and Fahrig 2014). Other studies of fine-scale epiphyte distribution patterns have revealed somewhat conflicting views of the overall importance of dispersal versus environmental filtering.

It has been found that traditional biogeographical variables explain little of the variance in lichen richness in the Antarctic Peninsula at local and regional scales (Casanovas et al. 2013). Interestingly, while the majority of variability in moss richness at a region scale in the Antarctic Peninsula was explained by summer mean sea surface temperature, lichen richness in the same region was not correlated with any of the variables investigated (Casanovas et al. 2013). These data suggest that site-specific habitat characteristics that were not investigated (substrate, water availability, etc.) likely play an important role in explaining variance in lichen richness.

In biological soil crusts in western North America, lichen community composition is strongly related to vascular plant species, soil texture and pH, and climate variables (Root and McCune 2012). While species-rich biotic crust lichen sites were scattered throughout the region, areas impacted by physical disturbances, including grazing, fire, etc., had the lowest overall lichen richness (Root and McCune 2012). Strikingly, a third of the nearly 100 lichen-forming fungal species encountered in this study were only observed a single time, highlighting the fact that a substantial proportion of lichen diversity may not be found ubiquitously even across relatively similar habitats. Interspecific competition among lichens and other biotic interactions have also been shown to be the major drivers of lichen community structure in soil crust communities in central Spain (Maestre et al. 2008).

In a saxicolous lichen community in coastal Norway, vegetation cover, rather than radiation, maritime influence, and microhabitat variables, was the predominant factor explaining variation in community composition (Bjelland 2003). However, in this study over 90 % of the total variation in lichen community composition remained unexplained. A combination of a variety of factors potentially contribute to our ability to more fully account for community composition and species' occurrences, including ignoring local historical factors affecting contemporary distributions, failing to account for influential environmental variables, stochasticity in species establishment, and/or lack of fit of data to response models.

Cladonia species occurring in the Wisconsin Pine Barren ground-layer lichen-moss community tend to occupy slightly different habitats (Lechowicz et al. 1974). Although there is some overlap in microdistributional patterns, differences in net photosynthesis temperature responses appear to underlie the distributions of *C. arbuscula* subsp. *mitis*, *C. carolinensis*, *C. rangiferina*, and *C. uncialis*, highlighting the putative role of temperature in structuring species distributions. While the eco-physiological responses observed for these *Cladonia* species provide some intuitive insight into their biogeography and microhabitat selection in the Wisconsin Pine Barrens, the nuanced interactions remain largely unaccounted for.

Assessing the combined biological effects of light, temperature, and humidity on photosynthetic activity of *Usnea sphacelata* in Antarctica, Bölter et al. (1989) demonstrated that although individual microhabitat conditions (e.g., light, humidity, and temperature) show long periods of favorable conditions for metabolic activity in *U. sphacelata*, the combined analysis of these variables considering threshold values for metabolism drastically shortens favorable periods for growth. Experimental manipulations revealed that the combination of light with desiccation has caused photoinhibitory damage in pendulous lichens that commonly occur in boreal forests and that the species-specific production of sunscreening fungal pigments plays a major role in the verti-

cal canopy gradient of epiphytes (Färber et al. 2014). Specifically, high-light-tolerant *Bryoria* species producing melanin are more commonly found in the upper canopy than light-susceptible species in the genera *Alectoria* and *Usnea* (sunscreening pigment = usnic acid) (Färber et al. 2014).

The interplay between the source of hydration and light availability plays a major role in structuring epiphytic lichen distributions (Gauslaa 2014). Lichens utilize a variety of sources for water, including rain, dew, and humid air, facilitating active photosynthesis. Sources of hydration vary on temporal and spatial scales (e.g., regional, landscape, stand, tree); and distinct atmospheric hydration sources influence and shape lichen diversity and distributions (Gauslaa 2014).

In short, the studies highlighted above demonstrate that a variety of environmental, ecological, and historical factors have the potential to significantly influence lichen distributions. However, identifying the specific factors and teasing apart their relative contributions remains challenging. Similarly, determining the most appropriate scales for sampling poses a significant challenge. The contribution of these factors in structuring lichen distributions may vary dramatically across different temporal and spatial scales, and extrapolations between studies can potentially be misleading.

1. Landscape Genetics and Gene Flow

From a landscape genetic perspective, researchers attempt to understand how geographic and environmental factors impact gene flow and genetic structure in populations and individuals. Two of the major objectives of modern landscape genetics are (1) to improve our understanding of how recent global change (e.g., climate change, land use, etc.) affects neutral and adaptive genetic variation and (2) understand if species are likely to adapt to ongoing global change on an ecological time scale (Manel and Holderegger 2013). While assessing gene flow is a central component in understanding landscape dynamics, it is worth noting that dispersal does not equal to gene flow. Some lichen-forming fungi may be able to effectively disperse, but without successful reestablishment and reproduction, the dispersal event has no impact. Only those dispersal events that occur in suitable habitats, with compatible symbiotic partners, and at the appropriate spatial and temporal scales will have the potential to contribute to gene flow and impact genetic structure.

Colonization rates of epiphytic lichens appear to be tied to a number of crucial factors, including connectivity to occupied patches and species traits (e.g., niche breadth and propagule size, local and long-distance dispersal, and patch dynamics) (Johansson et al. 2012). The majority of studies of landscape genetics in lichen-forming fungi have focused on the model epiphyte genus *Lobaria pulmonaria* (Fig. 2.2; Werth et al. 2006, 2007; Walser et al. 2005; Walser 2004; Widmer et al. 2012). Among the first studies of landscape dynamics in *L. pulmonaria*, Walser et al. (2005) used microsatellite loci to elucidate regional population differentiation and isolation by distance in populations in western North America (British Columbia, Canada) and central Europe (Switzerland). The use of highly variable microsatellite markers revealed striking genetic differentiation among populations of *L. pulmonaria*, with Swiss populations being distinct from those occurring in British Columbia, with additional differentiation between coastal and mainland populations in western North America (Walser et al. 2005). Subsequent studies of *L. pulmonaria* suggest that its occurrence may not be limited by dispersal capacities, but that ecological constraints at level of the sampled tree stands result in establishment limitations (Werth et al. 2006). However, dispersal characteristic of *L. pulmonaria* appears to be at least as important as landscape configuration in determining the spatial scale of population connectivity (Wagner et al. 2006). In fragmented relict stands in the boreal rainforest in central Norway, nearly all genetic variation found in *L. pulmonaria* could be attributed to variation within sites and spatial genetic structure was absent or appeared on very small scales (5–10 m; Hilmo et al. 2012). While this study high-

lights that relict stands may contain high levels of genetic diversity, disturbances (e.g., fire and intensive logging) may have long-lasting negative consequences on adaptive potential and reproduction in epiphytic lichens (Singh et al. 2014). Overall, studies of landscape genetics in *L. pulmonaria* support the conclusion underscored by Werth et al. (2007) that dispersal in *L. pulmonaria* is quite effective, but not spatially unrestricted.

Arctic and alpine ecosystems are facing significant impacts from global climate change, including ongoing increases in temperature and altered precipitation patterns (Ernakovich et al. 2014). Two species in *Flavocetraria* (Parmeliaceae), *F. cucullata* and *F. nivalis*, have widespread artic–alpine distributions in the Northern Hemisphere but also occur in a few disjunct populations in the Southern Hemisphere. Geml et al. (2010) found high genetic diversity and effective long-distance dispersal capacity among populations of both *F. cucullata* and *F. nivalis* occurring in the Northern Hemisphere. Long-distance gene flow appears to have prevented pronounced genetic differentiation among disjunct populations and suggests that these taxa are able to effectively track their potential niche during climatic shifts (Geml et al. 2010).

Long-distance, intercontinental dispersal and subsequent gene flow appear to be common in a number of other lichen-forming fungal species. For example, in the rock-dwelling lichen *Porpidia flavicunda* (Lecideaceae), a lack of fixed nucleotide polymorphisms and wide sharing of identical haplotypes between disjunct geographical regions indicates recurrent long-distance gene flow of propagules (Buschbom 2007). Similarly, lichens of the genus *Thamnolia* (Icmadophilaceae) occur at high altitudes/latitudes across incredibly broad geographic distances without any evidence of phylogeographic structure (Nelsen and Gargas 2009), although gene flow among disjunct populations has not been explicitly tested. The broad distribution and lack of phylogeographic structure is particularly striking given that this taxon is thought to reproduce almost exclusively via vegetative fragmentation and ascomata are unknown in this genus. While the charismatic epiphyte *Letharia vulpina* (Parmeliaceae) is known to occur in western North America, Europe, the Caucasus, and Morocco, European populations are genetically depauperate, resulting from an apparent genetic bottleneck caused by limited long-distance dispersal (Högberg et al. 2002; Arnerup et al. 2004).

Similar patterns of effective gene flow have also been observed at regional scales. In western North America, the epiphytic lace lichen (mycobiont = *Ramalina menziesii*) occurs in inland foothills along the Pacific Coast, ranging from Baja California northward to Alaska. Sork and Werth (2014) demonstrated that broad range of this epiphytic lichen has been shaped not only by long-distance dispersal across suitable habitats but also by lineage formation and persistence. While the majority of genetically distinct *R. menziesii* lineages tended to occur in distinct ecoregions, high migration out of populations in coastal and the Pacific Northwest into inland California populations was observed. In contrast, populations of *R. menziesii* in Baja California appear to be relatively isolated. Previously, a complete lack of local genetic structure was observed in *R. menziesii* populations growing on distinct oaks species in an oak-savanna ecosystem in southern California (Werth and Sork 2010). The low level of local genetic structure in some lineages within *R. menziesii* is consistent with high effective gene flow in other epiphytic lichen species. While population structure corresponding to different host trees was not observed in *R. menziesii*, population differentiation was observed in *Xanthoria parietina* populations occurring in different habitats (rock vs. bark) but not in populations occurring in the same habitat (Lindblom and Ekman 2006). Therefore, in some cases habitat isolation, rather than dispersal limitations, may play an important role affecting gene flow.

Population structure corresponding to geographic regions has been observed in some species of lichen-forming fungi with broad, intercontinental distributions. Fernández-Mendoza et al. (2011) demonstrated striking genetic structure in *Cetraria aculeata* (Parmeliaceae) corresponding to distinct geographic regions.

However, robust hypotheses of species boundaries in the *C. aculeata* group are lacking, and additional data will be required to accurately circumscribe species and adequately characterize gene flow among disjunct populations (Printzen et al. 2013).

Ongoing research into landscape genetics of model systems, such as *Lobaria pulmonaria*, will likely continue to provide novel insight into how geographic and environmental factors impact gene flow and genetic structure in lichens. Fortunately, many of the molecular techniques that have previously only been available for model systems can now be incorporated into studies of non-model groups using genomic data generated from high-throughput sequences (Ekblom and Galindo 2011). Incorporating non-model groups into landscape genetic studies of lichen-forming fungi will ultimately provide a much more nuanced perspective into how lichens respond to changing environments.

2. Modeling Distributions of Lichens

Modeling species distributions has the potential to play a pivotal role in biogeographic research. While species distribution models have become commonplace in biogeographic research, only a limited number of studies of lichen-forming fungi have incorporated modeling approaches to elucidate biogeographic patterns. Modeling distributions of species is most commonly based on pattern-recognition approaches, where associations between records of a species geographic occurrence and a suite of predictor variables are explored to allow an estimation of the species' ecological requirements. Accurate modeling depends, in part, on how the appropriate use of occurrence data and the adequacy of the predictors used for model building (Anderson et al. 2003).

Many lichens show significant relationships with macroclimatic variables (Giordani and Incerti 2008; Glavich et al. 2005), and overall it appears that there is general support for bioclimatic modeling in lichens (Braidwood and Ellis 2012). Species distribution models have been used in a number of ecological-, biogeographical-, and conservation-related studies. McCune et al. (2003) used habitat models to forecast the frequency of occurrence of epiphytic lichens under different forest management strategies in northwestern USA. These habitat models were able to successfully estimate the occurrence of lichens modeled, and forecast that forest management strategies reducing even-aged stands will increase the frequency of epiphytes associated with old-growth forests. Logistic regression models have been shown to accurately predict the occurrence of six lichen epiphytes in Switzerland, including two threatened or vulnerable lichens, by using the statistical relationships between response and explanatory variables to predict the distribution of lichens in previously unsampled geographic areas (Bollinger et al. 2007). Shrestha et al. (2012) used nonparametric multiplicative regression analysis to model the potential distribution of the sensitive indicator species *Usnea hirta* (Parmeliaceae) in western North America. Their predictive model suggested that average monthly minimum and maximum temperatures, precipitation, and solar radiation were the major macroclimatic factors influencing the distribution of *Usnea hirta*, providing a useful ecological niche-based approach to forecast the distribution of air pollution-sensitive lichens based on macroclimatic variables (Fig. 2.4). Similarly, ecological niche modeling has also played an important role in improving survey design of rare epiphytic macrolichens in the Pacific Northwest, USA (Edwards et al. 2005).

Recently, Martellos et al. (2014) utilized ecological niche modeling to characterize differences in the distribution of two varieties of the soil crust lichen *Squamarina cartilaginea* in Italy. Their study highlights not only the role the modeling plays in forecasting distributions but also the potential for using ecological niche modeling for species delimitation research. In fact, within an integrative species delimitation framework, distribution modeling serves as an important independent line of evidence to corroborate lineage separation (Leavitt et al. 2015a; Pelletier et al. 2014). The study by Martellos et al. (2014) provides the first implementation of ecological niche modeling for resolving taxonomic issues in lichen-forming fungi.

While distribution modeling can provide important insight into species distributions

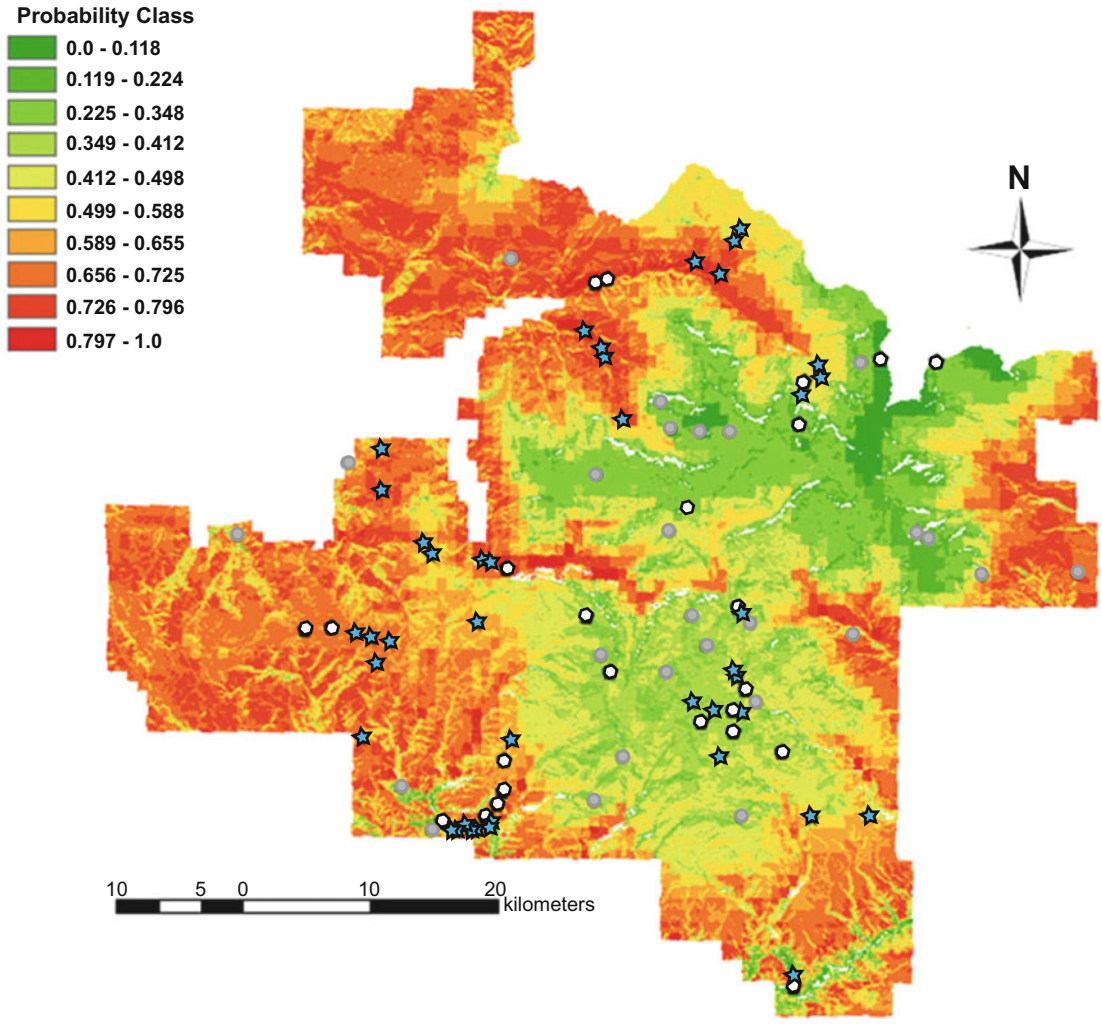

Fig. 2.4 Modeled distribution map for *Usnea hirta* in the White River National Forest in central Colorado using nonparametric multiplicative regression analysis. *Blue stars* represent sites where *U. hirta* was recorded, *white hexagons* represent sites with no *U. hirta*, and *grey circles* represent inaccessible sites. *White areas* represent no data value due to lower average neighborhood size. Modified from Shrestha et al. (2012)

and biogeographic patterns, effective distribution modeling faces a number of general challenges, including: (1) clarification of the niche concept; (2) weaknesses in sampling ability and design; (3) variation in parameterization within each technique, potentially providing different modeled distributions; (4) a need for improved model selection and predictor contribution; and (5) a need for more robust model evaluation strategies (Araújo and Guisan 2006).

Among the most practical issues of distribution modeling is effective sample survey design, the results of which will impact all downstream analyses (Edwards et al. 2006). Even when occurrence data is relatively well characterized, bioclimatic modeling may not provide results congruent with known species occurrences. Distribution models for the rare lichen species *Staurolemma omphalarioides*, known for its disjunct distribution in the Mediterranean region and central Norway, well outside of the main range of the species, indicate that either the species has not reached its potential distribution or the models fail to

accurately characterize the actual species distribution (Bendiksby et al. 2014). Furthermore, while models can incorporate macroclimatic variables, as indicated above, lichens are inherently sensitive to microhabitat variation, and measuring and incorporating this variation remains challenging. As an example, microhabitat conditions, including proximity to watercourses and bark pH, play important roles in determining the occurrence *Platismatia norvegica* (Parmeliaceae) in suboceanic habitats at the fringe of the species' distribution (Lidén and Hilmo 2005). Similarly, the distribution of some epiphytic lichens in different forest types is strongly influenced by vertical position within the forest canopy (Coxson and Coyle 2003; Antoine and McCune 2004). Land use intensity has been shown to influence the local variation of lichen diversity in Mediterranean ecosystems (Giordani et al. 2010). However, other lichen communities appear to be unaffected by human activity and are predominantly determined by macroclimatic factors (Werth et al. 2005).

Going forward, species distribution modeling holds important promise in ecological biogeographical research of lichens. Due to the fact that species distributions reflect the dynamic interplay of geographic and environmental processes with biotic factors, including species dispersal capabilities and interactions with other species, developing a stronger link between ecological factors and modeling will be beneficial for developing more approaches for distribution modeling. Advances in spatial data technologies, geographic information system (GIS) data, and modeling approaches will continue to facilitate ecological and evolutionary insight and predict distributions of lichen-forming fungi.

3. Role of Photobionts in Ecological Biogeography

Lichens represent iconic examples of symbioses, and therefore taking their symbiotic partners into account in biogeographic research is likely to provide valuable insight into elucidating biogeographic patterns. Generally, lichen photobionts are considered the subsidiary member of lichen associations; however, it now is clear that at least some photobionts exhibit differential preference for environmental factors (Peksa and Škaloud 2011). Algal preferences potentially limit the ecological niches available to lichens, further supported the idea of habitat-specific lichen guilds, where lichen communities growing in similar habitats share the same photobionts (Rikkinen et al. 2002). Factors such as photobiont availability (Werth et al. 2006; Rikkinen et al. 2002), fungal specificity in photobiont choice (Yahr et al. 2004), ecological constraints (Peksa and Škaloud 2011), and symbiont interactions (del Campo et al. 2013; Werth et al. 2013) have all been shown to have a major impact on the occurrence of lichens.

The spiny heath lichen (mycobiont = *Cetraria aculeata*; Fernández-Mendoza and Printzen 2013; Pérez-Ortega et al. 2012; Fernández-Mendoza et al. 2011; Printzen et al. 2013), lace lichen (mycobiont = *Ramalina menziesii*; Werth and Sork 2010, 2014), and lung lichen (mycobiont = *Lobaria pulmonaria*; Werth et al. 2006; Dal Grande et al. 2012; Singh et al. 2014) have become model groups for understanding the dynamic roles that photobionts play in determining lichen distributions. Based on broad geographic sampling of the spiny heath lichen (mycobiont = *Cetraria aculeata*), Fernández-Mendoza et al. (2011) provide evidence that photobiont switches played an important role in increasing the geographical range and ecological niche of lichen mycobionts by associating them with locally adapted photobionts in climatically distinct regions. In this specific case, the photobiont switch allows *C. aculeata* that is common in temperate and alpine habitats to extend into semiarid regions in the Mediterranean. In the lace lichen (mycobiont = *Ramalina menziesii*), ecological specialization of the photobiont (*Trebouxia decolorans*), geography, and climate shape the distribution of this lichen (Werth and Sork 2014). Algal specialization on local environmental conditions, including macroclimatic factors and substrate ecology, allows *R. menziesii* to associate with locally adapted photobiont strains. As discussed previously, dispersal limitations of the mycobiont

Lobaria pulmonaria is not the most important mechanism underlying differentiation among populations. Availability of the appropriate photobionts has been proposed as a potential mechanism generating population structure in *L. pulmonaria* (Werth et al. 2006, 2007).

While species richness of lichen-forming fungi is associated with both climate and forest structure variables, specific responses to these different variables were dependent on the type of photobiont. A study of patterns in lichen richness across Italy highlights the photobiont-dependent response of species richness to various environmental factors (Marini et al. 2011). Mycobiont species paired with chlorococcoid green algae was correlated with increasing forest cover. While species richness of cyanolichens—lichen-forming fungi associating with photosynthetic cyanobacteria—was related to area and precipitation, lichens with *Trentepohlia* algae were enhanced by rainy and warm climates (Marini et al. 2011). In some cases, the mycobiont's habitat preferences may be determined by factors that are independent of those of the photobiont at the landscape level (Nadyeina et al. 2014). The differential responses of lichens associating with different types of photobionts to ecological, climatic, and other environmental factors demonstrate the challenges in predicting lichens distributions.

These studies clearly indicate the potential importance of carefully taking the role of the photobiont into consideration when considering ecological biogeography. Currently, studies of species interactions in lichen symbioses are limited by uncertainty in the circumscription photobiont species (Kroken and Taylor 2000; Sadowska-Deś et al. 2014; Blaha et al. 2006; Dahlkild et al. 2001; Tibell 2001). For example, in spite of the fact that traditional morphology-based species circumscriptions have consistently been shown to be inadequate to characterize species-level diversity in *Trebouxia* (Kroken and Taylor 2000; Sadowska-Deś et al. 2014; Blaha et al. 2006; Dahlkild et al. 2001), only a limited number of studies have implemented objective methods for delimiting species-level lineages (Sadowska-Deś et al. 2014; Kroken and Taylor 2000). We argue that there is a pressing need to develop and adhere to a practical, species-level classification system for major groups of lichen photobionts in order to enhance communication about diversity, distributions, ecological patterns, and interactions in lichen symbioses (Leavitt et al. 2015b).

IV. The Role of Biogeography in Conservation and Climate Change Research

Lichens are not easy targets for conservation measures due to the fact that lichens represent the symbiotic phenotype of multiple interacting species (Scheidegger and Werth 2009). This challenge is further confounded by the fact that many lichens have specific habitat requirements that are not generally shared with other organisms. An ecological biogeographic perspective of lichen-forming fungi highlights the importance of seeing distributions of species as the result of dynamic interactions among symbionts and ecological and environmental factors. Scheidegger and Werth (2009) provide an invaluable perspective into lichen conservation biology, including potential strategies to effectively protect lichens and develop priorities for conservation approaches. Here we only highlight in brief a number of examples from lichen conservation biology literature, emphasizing the role of maintaining habitat quality, connectivity, and size.

Relative to more charismatic species, conservation of lichens generally receives very limited attention from governmental organizations and other institutions. Conservation status of most lichens is unknown, and therefore, conservation approaches may fail to protect vulnerable lichen and lichen communities. For example, the Natura 2000 Program was established in the European Union as the main instrument for nature conservation, focusing on protecting the most threatened habitats, ensuring the long-term survival of species, and reducing the loss of biodiversity caused by anthropogenic impact (http://www.natura.org/). While Martínez et al. (2006) demonstrate that the Natura 2000 network may include key habitat in conserved forests and mountain

ranges for a number of lichens in Spain, the effectiveness of the Natura 2000 network in protecting Mediterranean lichens is quite low (Rubio-Salcedo et al. 2013). Natura 2000's reserve network, based mainly on vascular plant data, may be ineffective for neglected taxonomic groups, like lichens, and highlights the need to include "noncharismatic" species to improve reserve design and other conservation strategies (Rubio-Salcedo et al. 2013). However, even when explicitly taking lichens into consideration when developing models to predict species richness, it appears lichen surveys are critical for assessing species abundance, dynamics, and viability (Waser et al. 2007).

Ultimately, successful lichen conservation is contingent on a wide variety of factors, including effective approaches for prioritizing the protection of vulnerable habitat and species (Bowker et al. 2008), promoting interdisciplinary collaborations (Campbell 2005), incorporating population/landscape genetics into policy decisions (Scheidegger and Werth 2009), and increasing the number of bio-inventory surveys which include lichens. Lättman et al. (2009) suggested that dispersal capability is likely to be commonly underestimated for lichens defined to habitat with long ecological continuity, and this perspective was corroborated for epiphytic lichens occurring in the boreal rainforest in central Norway (Hilmo et al. 2012). Therefore, successful conservation of some lichens may be possible even when a suitable habitat is highly fragmented.

Lichens are included among a suite of indicators of ecosystem health, which also includes bryophytes (Frego 2007; Pesch and Schroeder 2006), vascular plants (Coulston et al. 2003), some terrestrial and aquatic invertebrates (Hodkinson and Jackson 2005), and other sensitive species and/or communities (Leavitt and St. Clair 2015). Rather than abiotic metrics, bio-indicators are likened to canaries in a coalmine, serving as a direct surrogate for assessing disturbances on biological communities. Lichens are particularly useful as bio-indicators due to the fact that many live and grow continuously for decades, or even hundreds of years, showing cumulative responses to ecological changes, including climate change, land management practices, changes in atmospheric pollution levels, etc. Measurable responses of individual lichen thalli (e.g., differential accumulation of atmospheric pollutants) and lichen communities (e.g., changes in community composition and population density) can provide a means to quantitatively assess ecosystem health (McCune 2000).

Climate change is forecast to promote major ecological shifts worldwide (Parmesan and Yohe 2003; Araújo and Rahbek 2006). Species that are unable to tolerate or adapt in situ to altered environmental conditions or migrate to suitable habitats face potential extinctions. Alternatively, climate change may lead to major shifts in species distributions. Some components of cryptogamic communities, including lichens, have been shown to be particularly sensitive to climatic shifts (Cornelissen et al. 2001; Bjerke 2011). Although lichens are well-known indicators of air quality (Leavitt and St. Clair 2015), recent studies indicate that they may also be useful in assessing ecological shifts related to climate change (Bjerke 2011; Ellis et al. 2007a, b; Cornelissen et al. 2001). Some lichens, due to their sensitivity, may play an important role in monitoring the potential impacts of climate change.

In general, specific responses of most species, including lichen-forming fungi, to rapid climate changes in vulnerable habitats remain uncertain, and detailed, long-term monitoring will be essential to accurately assessing biologically meaningful shifts in community composition and species distributions (Eaton and Ellis 2012). On a global scale, macrolichens in climatically milder arctic ecosystems may decline if and where global changes cause vascular plants to increase in abundance (Cornelissen et al. 2001). In the UK, the southern elements of Britain' s lichen flora, and other lichen species adapted to warmer climates, are projected to expand northward, while the montane species appear to be disproportionally threatened by climate change (Ellis et al. 2007b). Other data suggest that a warmer, humid climate in Norway will likely be beneficial for the generalist species *H. physodes*, but detrimental to the subalpine birch specialist *Melanohalea olivacea* (Evju and Bruteig 2013).

These studies highlight the importance of continued research on specific responses of lichen species and communities to changing climate. Only by consistently coupling efficient quantitative methods with accurately characterized species- and/or community-specific responses to changing climatic conditions will we be able to effectively document climate change related impact on vulnerable lichen communities. However, biological communities are threatened not only by changing climate but a wide range of other environmental disturbances, including habitat degradation, invasive species, air pollution, etc. Dynamic interactions among multiple types of disturbances make characterizing risks to biological communities and their potential responses quite difficult (Ellis et al. 2014). Principles from ecological biogeography should play a central role in climate change research, facilitating more effective study design and appropriate interpretation of results.

V. Conclusions

Current research has dramatically increased our understanding of the geographical distribution of lichens and allowed new insights in the importance of the photosynthetic partners for shaping the spatial distribution of these symbiotic organisms. Novel methods, including the increased availability of data from environmental sampling, will further enhance and refine our hypotheses to explain distribution patterns. These are truly exciting times—with the help of next-generation sequencing techniques, increased number of species for which microsatellite markers have been identified, and improved analytical tools, biogeographical questions can be addressed that were beyond our reach only a decade ago. Improved understanding of species delimitations of the fungal partners has allowed us to better understand distribution patterns, and in tandem with enhanced knowledge of species diversity of photosynthetic partners, this provides an avenue to better understand patterns that explain distribution patterns at an ecological level. Currently, ecological biogeographical studies focus on a few species that provide great insights, but we look forward to seeing these exemplary studies extended to other lichens, including phylogenetically distant groups and also tropical species, which are currently severely understudied. The extension of research to include a wider amplitude of species will also strengthen predictions of the impact of global climatic change to lichen distribution.

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Determinants of Fungal Communities

3 The Bright and Dark Sides of Fungal Life

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CONTENTS

I. Introduction

Life on earth depends on the energy of the sun, which is harvested from the radiations that reach our planet by photosynthetic organisms (plants, algae, and some bacteria) and is used to synthesize energy-rich molecules (sugars) from carbon dioxide and water. Besides being the source of energy that maintains life on earth, electromagnetic radiations emitted by the sun have had a profound impact in the evolution of all forms of life. This effect derives from the properties of sunlight (intensity, duration, polarization, and spectral composition) that provide patterns, which in turn have important consequences for living organisms due to their potential use as source of information. In their natural habitat, all forms of life are continuously obtaining and decoding information from their environment (including that contained in light), which they use for their benefit. In addition, the energy contained in light can initiate photochemical reactions that lead to physiological responses in photosensitive organisms.

The use of light either as energy or as information source depends on the interaction of light with the molecular system of the organisms. Sunlight ranging from ultraviolet (UV) to infrared (IR) regulates several biological processes including circadian rhythms, photomorphogenesis, phototropism, and synthesis of pigments, among others. Shorter wavelength radiation (of higher energy), corresponding to the UV, can also initiate damaging photochemical reactions.

DNA is perhaps the most important molecule that can be affected by UV, since a photochemical reaction can result in a modification that is transmitted to the next generation as a mutation, if it is not repaired before DNA replication. Additionally, visible light can indirectly give raise to reactive oxygen species (ROS) as a product of photosensitive reactions through energy transfer from a molecule that can be activated by light such as flavin or porphyrin. It is in this way that blue light is potentially harmful to living beings (Lledias and Hansberg 2000; Aguirre et al. 2005).

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Fungi, like other organisms, require information about which way is up or down, how to orient themselves, and clues to whether the fungus is in the soil, inside a host, or exposed to air and other types of stress. In contrast with photosynthetic organisms, fungi use sunlight not as a source of energy but to obtain information from their environment. The ability of a fungus to respond to light is likely to contribute to its survival, its fitness, and its ability to compete. Visible light, while not necessarily damaging in itself, can provide early warning of impending UV damage (Gressel and Rau 1983; Berrocal-Tito et al. 1999).

Although fungi typically grow in damp, dark habitats, numerous studies have revealed that exposure to light is accompanied by major changes in their physiology. A response to light might be very rapid, simply changing the direction of growth, or might provide the initial cue to alter transcriptional profiles. These, in turn, provide the enzymes and metabolites needed for protection from damage as well as programming the development of new structures. Developmental decisions triggered by light, depending on the intensity, require from nanoseconds to minutes of exposure and amounts as low as 10^{-10} molm^{-2} (Betina and Zajacova 1978; Horwitz et al. 1990; Corrochano 2007). Fungi display very rapid responses to light (e.g., phototropism), some of which were discovered more than 150 years ago (Payen 1843). In fact, many alterations in fungal morphology provoked by light have been described in hundreds of fungi (Marsh et al. 1959). However, it was Max Delbrück who in his pioneering work established the basis for the development of fungal photobiology in the 1960's (Delbrück and Shropshire 1960; Delbrück and Varju 1961).

Rhythmic changes such as day and night, to which most organisms are well adapted, require anticipation of dusk and dawn and proper reaction to the changing environment represent a major evolutionary adaptation. Fungi are no exception and possess biological clocks, including a ca 24 h clock (the circadian clock), which uses light as one of the main cues to keep time. The machinery involved in the regulation of the circadian clock helps fungi distinguish day from night and alters their physiology accordingly.

Notably, the yeast *Saccharomyces cerevisiae* is absent from the long list of photoresponsive fungi. Although exposure of this yeast to increasing illumination results in a reduction in growth rate, the effect of light is weak, and it appears to occur only at low temperatures (Edmunds et al. 1978). Additionally, cell division and amino acid transport in *S. cerevisiae* are synchronized to light/dark (LD) cycles (Edmunds et al. 1979). The lack of robust light responses could mean that photoresponsiveness was selected out, at least in laboratory strains.

To respond to light fungi require photoreceptors (proteins or protein complexes) able to perceive light that generate a signal, which is propagated into the cell to initiate a cellular response. Their light-absorbing cofactors, known as pigments or chromophores, are typically small molecules. Among these chromophores, riboflavin (vitamin B12), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and porphyrins such as a heme group absorb light in the visible range. Consequently, most—if not all—biological responses to visible light must be initiated by one of these pigments (Casas-Flores and Herrera-Estrella 2013).

II. The Visual System of Fungi

A. Flavin-Binding Photoreceptors

Light regulates growth and development in fungi, including sexual and asexual development, phototropism, biological rhythms, metabolism, and gene transcription, among others (Herrera-Estrella and Horwitz 2007; Idnurm and Heitman 2005; Casas-Flores and Herrera-Estrella 2013; Fuller et al. 2014). As mentioned above, to respond to this environmental cue, fungi require photoreceptors that enable them to respond to different light qualities and quantities.

Phycomyces was probably the first fungus in which light perception was analyzed. Max Del-

brück concentrated in studying the capacity of this fungus to "see." He described in great detail the properties of the response to light of *Phycomyces* and the genetic zapping of the signal transduction pathways which was initiated in his laboratory but faced serious difficulties in the molecular characterization of the first step of these pathways, photoperception (Delbrück and Shropshire 1960; Bergman et al. 1973). Another fungus for which the search for elements involved in light perception was initiated at about the same time was *Trichoderma* (Gressel and Hartmann 1968; Kumagai and Oda 1969). In both cases it was concluded that the most likely receptor for UV-blue light was a flavoprotein. Accordingly, the operation of cryptochromes was suggested—a hypothesis corroborated many years later by the presence of genes encoding potential flavoproteins that could act as photoreceptors in several fungi (Ballario et al. 1996; Casas-Flores et al. 2004; Idnurm et al. 2006).

Flavin-binding photoreceptors contain a specialized PAS (Per-Arnt-Sim) domain called LOV (light-oxygen-voltage), which is a highly conserved domain among proteins that sense UV-blue light in plants and fungi (Fig. 3.1a). LOV domains are about 110–120 aa (amino acids) in length and present a highly conserved structure from bacteria to plants, which adopt a conserved α/β-fold that binds FAD or FMN (Figs. 3.1b and 3.2). Upon exposure to blue light, a covalent adduct between the flavin molecule and a conserved cysteine residue in the LOV domain is formed. Once in the darkness, this adduct is dissociated and the photocycle is completed (Salomon et al. 2000).

The founding member of blue-light fungal photoreceptors is the White Collar-1 (WC-1) protein from *Neurospora crassa*; however, the existence of this kind of light receptors was first suggested in *Trichoderma* spp. during the first half of the nineteenth century that was further supported by the cloning of the blue-light receptor 1 (Blr1), the WC-1 orthologue (Casas-Flores et al. 2004). Fungal photoreceptors belonging to the WC-1 family contain in their structure three PAS domains, where the first one is a LOV domain, specialized in sensing blue light (He et al. 2002). The other two PAS domains are involved in protein-protein interaction (Taylor and Zhulin 1999). These photoreceptors, in general, also contain GATA zinc finger DNA-binding domains, nuclear localization signals (NLS), and activation domains in several cases (Corrochano 2011), but there are exceptions to the presence of the latter (Fig. 3.2), which implies the participation of additional factors for the activation of transcription (Casas-Flores et al. 2004). The *N. crassa* WC-1 protein dimerizes with its transcriptional partner, the WC-2 protein, which contains a PAS, a GATA zinc finger domain, and an activation domain to form the White Collar Complex (WCC) (Fig. 3.2). Mutants in either *wc-1* or *wc-2* are affected in all known blue-light responses. In a similar way, mutants in other fungal models such as *Trichoderma* spp. and *A. nidulans* are also affected in blue-light perception (Casas-Flores et al. 2004; Castellanos et al. 2010; Purschwitz et al. 2008).

In *Mucor circinelloides*, three *white collar-1* genes (*mcwc-1a*, *mcwc-1b*, and *mcwc-1c*) orthologous to the *N. crassa* WC-1 have been identified, and all three contain a LOV domain. *mcwc-1a* regulates phototropism, whereas *mcwc-1c* regulates photocarotenogenesis (Silva et al. 2006). The *mcwc-1b* product is also involved in carotenoid synthesis in some mutant background strains (Silva et al. 2008).

Some fungi contain in their genomes a secondary photoreceptor (Vivid/Envoy), which is entirely conformed by a LOV domain (Fig. 3.2) and binds either FAD or FMN as chromophore (Zoltowski and Crane 2008; Lokhandwala et al. 2015). These photoreceptors are important for photoadaptation under constant light and to respond to changes in light intensity. Vivid fine-tunes these light responses by interacting with the WCC through the WC-1 LOV domain, to quench these responses (Vaidya et al. 2011).

Cryptochromes (Cry) are flavoprotein photoreceptors with a structure closely related to that of DNA photolyases (Fig. 3.2), which directly bind DNA or RNA, are sensitive to blue and UV light, but display strongly reduced or have lost DNA-repair activity (Daiyasu et al. 2004; Lin and Todo 2005). Crys regulate growth and development in plants and circadian rhythms in plants and animals (Daiyasu et al.

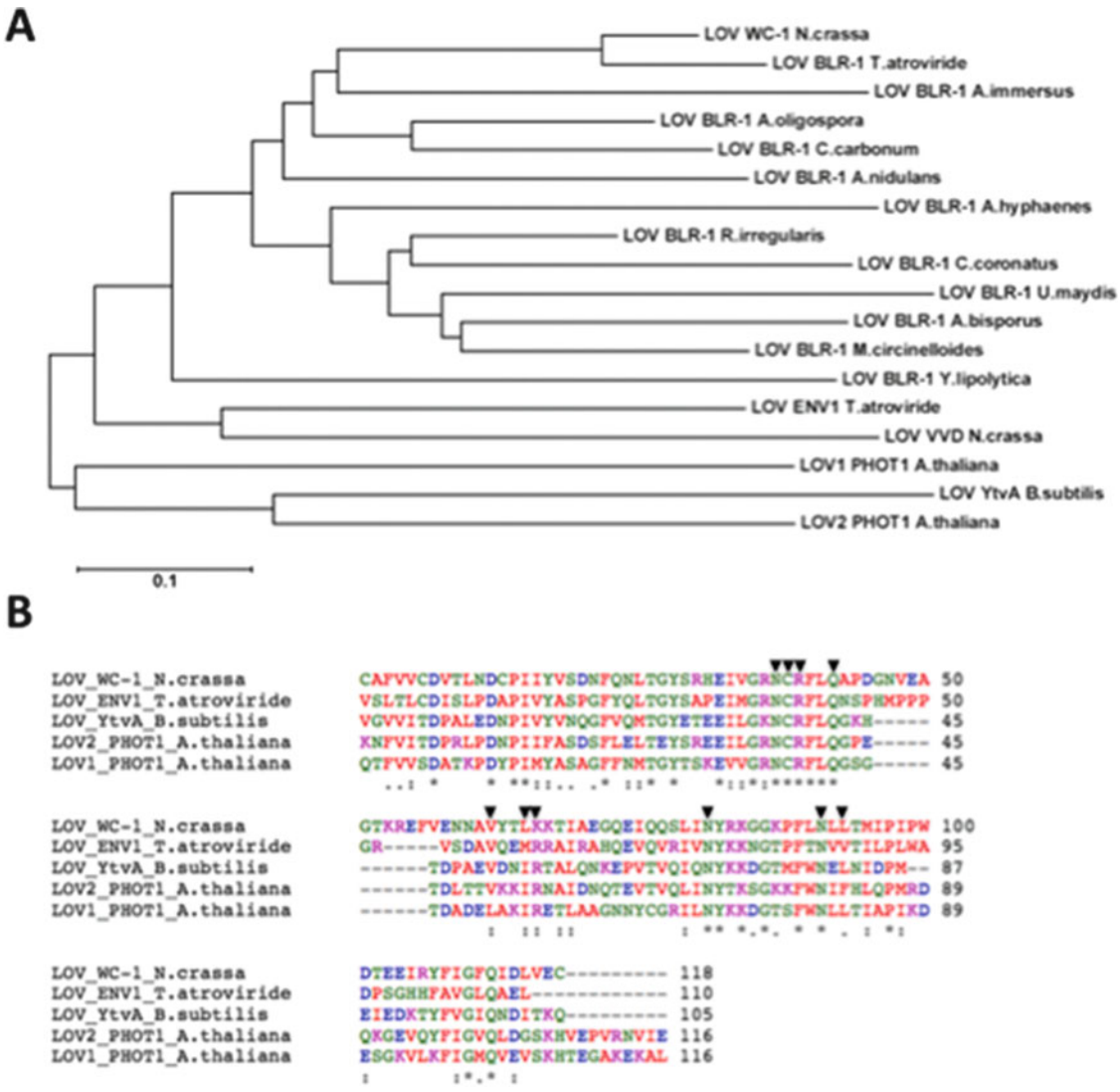

Fig. 3.1 (**a**) Evolutionary relationships of fungal LOV domains taxa. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 5.27804499 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 18 LOV domain amino acid sequences which are listed in (**b**). All positions containing gaps and missing data were eliminated. There were a total of 93 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). (**b**) LOV-domain amino acid sequences alignment, which depicts the flavin-interacting residues, and the forming adduct cysteine with the chromophore. The sequence analysis includes the LOV1 and LOV2 domains from *Arabidopsis thaliana* phototropin (GenBank accession no. AEE78073.1), *Neurospora crassa* WC-1 (GenBank accession no. Q01371), and *T. atroviride* ENV1 (GenBank accession no. AAT40588.1), the blue-light photoreceptor from *Bacillus subtilis* (GenBank accession no. CEI58264.1). LOV domains representative of the different classes of fungi were also included—*Agaricostilbum hyphaenes* (ID: 234740; Pucciniomycotina), *Ustilago maydis* (GenBank accession no. XP_011389633.1; Ustilaginomycotina), *Agaricus bisporus* var. *bisporus* (ID: 186808; Agaricomycotina), *Ascobolus immersus* (ID: 242339; Pezizomycetes), *Arthrobotrys oligospora* (ID: 7017; Orbiliomycetes), *Aspergillus nidulans* (ID: 8655; Eurotiomycetes), *Cochliobolus carbonum* (ID: 21495; Dothideomycetes), *Yarrowia lipolytica* (ID: 65092; Saccharomycotina), *Rhizophagus irregularis* (ID: 88544; Glomeromycota), *Mucor circinelloides* (ID: 117241; Mucoromycotina), *Conidiobolus coronatus*

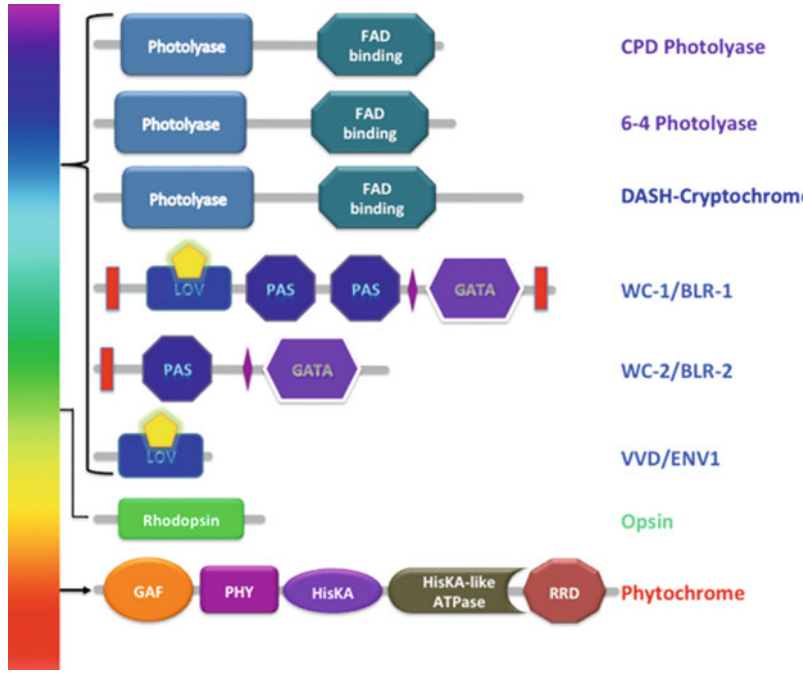

Fig. 3.2 Domain organization of the different classes of known and putative fungal photoreceptors and photolyases. The flavin-binding photoreceptors contain a specialized LOV domain, which is known to bind FAD or FMN as pigment to harvest light (WC-1, VVD/ENV1, and CRY). The WC-1 orthologous also contains two PAS domains, a GATA zinc finger DNA-binding domain, a nuclear localization signal, and activation domains. The WC-2 orthologous, the transcriptional partners of WC-1, contain one PAS domain, a GATA zinc finger domain, and activation domain. The DNA 6-4 and CPD photolyases and cryptochrome are related flavoproteins, containing a DNA photolyase domain, which uses folate as harvesting cofactor. They also contain a FAD-binding domain of DNA photolyase. Opsins resemble the microbial opsin-related proteins, which bind rhodopsin to perceive *green light*. The phytochrome binds biliverdin as chromophore, and its structure includes a GAF small ligand-binding domain, a phytochrome region (PHY), a histidine kinase A (phosphoacceptor) domain, one dimerization, one phosphoacceptor domain (HisKA), a HisKA-like ATPase domain, an RRD, and a signal receiver domain that receives the signal from the sensor partner in a two-component system. It contains a phosphoacceptor site that is phosphorylated by HisKA

2004; Lin and Todo 2005). Based on phylogenetic analysis, cryptochromes have been classified in plant, animal, and DASH (from *Drosophila*, *Arabidopsis*, *Synechocystis*, human) cryptochromes (Partch and Sancar 2005). At the structural level, cryptochromes have an amino-terminal photolyase-related region (PHR) and a carboxy-terminal domain of variable length. However, DASH cryptochromes lack the latter domain (Fig. 3.2). *A. nidulans* possesses only one cryptochrome/photolyase-like photoreceptor, named CryA, which includes a PHR domain containing both DNA photolyase and FAD-binding domains. This protein shows sensory and regulatory roles during *A. nidulans* development and DNA-repair activity when expressed in *Escherichia coli* (Bayram et al. 2008a).

Fig. 3.1 (continued) (ID: 58233; Entomophthoromycotina), *Ramicandelaber brevisporus* (ID: 90268; Kickxellomycotina), and *Catenaria anguillulae* (ID: 114754; Blastocladiomycota)—but are not shown by space limitation. LOV residues that interact with FMN are marked with *arrowheads*. *Colored residues* in LOV domains are according to their physicochemical properties

In the filamentous fungus *N. crassa*, the CRY-DASH-type cryptochrome binds FAD and MTHF (methenyltetrahydrofolate). The robust accumulation of *cry* and CRY upon a light pulse is *wc-1* dependent. Purified CRY protein binds to both single- and double-stranded molecules of DNA or RNA in vitro (Froehlich et al. 2010). As described for *N. crassa*, the CRY-DASH cryptochrome transcript (*cryD*) from *Fusarium fujikuroi* is highly induced by light and is regulated by the WC-1 orthologue. Recently, a member of the cryptochrome/photolyase family, Cry1, was characterized in *T. reesei*. The *cry1* transcript is positively regulated and modulated by the Blr and Env1 proteins. Heterologously expressed Cry1 binds to undamaged and 6-4PP damaged DNA and photorepairs it but does not repair CPD and Dewar DNA lesions (Guzmán-Moreno et al. 2014).

B. Opsins or Rhodopsins

Opsins/rhodopsins comprise seven transmembrane domain proteins that bind retinal via a conserved lysine residue to form green-light-responsive ion pumps to transport ions (H^+ or Cl^-) across membranes or sensory receptors in microorganisms (reviewed by Spudich et al. 2000; Spudich 2006). In fungi, the funding member of this protein family is NOP-1 from *N. crassa*, which was isolated from an expressed sequence tag from a mycelial library (Bieszke et al. 1999).

The *nop-1* transcript is prominently expressed in conidia and sexual structures under light conditions. However, the *nop-1* transcript level was not affected in the WC-1-defficient mutant during conidiation (Bieszke et al. 2007). NOP-1 binds retinal in vitro and undergoes a very slow photocycle, whereas proton pump activity was not detected, which indicated a putative role in light sensing in *N. crassa* (Bieszke et al. 1999). In *Leptosphaeria maculans*, the opsin LR (*Leptosphaeria* rhodopsin) shows a fast bacteriorhodopsin-like photocycle and pumps protons light dependently. In this case the LR transcript is not induced by light, which contrasts with *nop-1* from *N. crassa*. Even though when NOP-1 and opsin (OPS) show high sequence similarity, their biochemical characteristics suggest different roles in light sensing and proton pumping, respectively (Furutani et al. 2006). The differences between the photochemical behavior of LR and NOP-1 are due to the replacement of the cytoplasmic proton donor Asp with Glu (Furutani et al. 2006).

The filamentous fungus *Fusarium fujikuroi* possesses two genes, which encode putative retinal-binding opsins called CarO and OpsA, and a gene for an opsin-related protein (*hspO*). OpsA was classified as NR-like rhodopsin by sequence similarity to that from *N. crassa*, which suggests also a very slow photocycle and lack of proton pump activity. On the contrary, CarO was classified as an auxiliary ORP-like rhodopsin that contains all conserved amino acids required to function as proton pump and, together with LR-like rhodopsins, shows fast photocycles. The *carO* and *opsA* genes are light regulated, and their induction is WC-1 (*wcoA*) dependent. Interestingly, *carO* and *opsA* transcripts are not accumulated in carotenoid-overproducing mutants (Estrada and Avalos 2009). Indeed, CarO is a green-light-driven proton pump as demosntrated by patch-clamp electrophysiological experiments with heterologously expressed CarO. Fusion of CarO with GFP led to determine that CarO is strongly accumulated in conidia, upon exposure of mycelia to light (García-Martínez et al. 2015).

In the brown leaf spot fungus *Bipolaris oryzae* two opsin-like genes, *ops1* and *ops2*, were identified. The *ops1* transcript is highly expressed in mycelia under near-UV irradiation, but the *ops2* transcript is constitutively expressed in mycelia under dark conditions and weakly induced after near-UV application. The expression of *ops1* and *ops2* is Blr1 (the orthologue of WC-1 in *B. oryzae*) dependent (Kihara et al. 2009).

In *Blastocladiella emersonii* light perception is accomplished by the function a novel type I (microbial) rhodopsin domain and guanylyl cyclase catalytic domain encoding gene (BeGC1), which is highly expressed in late sporulation cells, during zoospore biogenesis.

Photobleaching of rhodopsin inhibits the accumulation of cGMP and phototaxis of zoospores when exposed to green light, whereas depletion of guanylyl cyclase activity negatively impacts phototaxis. The BeGC1 protein was located in the external surface of the zoospore eyespot positioned close to the base of the swimming flagellum. Therefore, Blastocladiomycota fungi have a cGMP signaling pathway involved in phototaxis similar to the vertebrate vision-signaling cascade integrated in a novel gene fusion encoding a protein composed of the different protein domains and of distant evolutionary ancestry to type II rhodopsins of animals (Avelar et al. 2014).

C. Phytochromes

Phytochromes are a widespread family of proteins that covalently attach to bilin-type (or linear tetrapyrrole) chromophore and absorb in the red/far-red region of the spectrum. The bilin-type chromophores enable photoconversion between red-absorbing (P_r) and far-red-absorbing (P_{fr}) forms of phytochromes (Rockwell and Lagarias 2010). The most common structural architecture of bacterial, plant, and fungal phytochrome is composed of an N-terminal photosensory core with three conserved PAS-GAF-PHY domains (Per/Arndt/Sim-cGMP phosphodiesterase/adenylyl cyclase/FhlA-phytochrome specific) and a C-terminal regulatory histidine kinase-related domain (HKRD; Fig. 3.2) (Rockwell and Lagarias 2010). Phytochrome means "plant pigment" and was first discovered in higher plants in the mid-twentieth century, based on the ability of red and far-red light to control several processes of plant growth and development (Quail 2002; Smith 2000). *A. nidulans* possesses a phytochrome (FphA) that is more closely related to bacterial than to plant phytochromes. FphA binds biliverdin as chromophore and is located in the cytoplasm, which indicates that red-light perception occurs in the cytoplasm (Blumenstein et al. 2005). Furthermore, FphA has kinase activity and is likely to act as red-light sensor. Orthologues of FphA have also been found in the genomes of *A. fumigatus*, *Gibberella moniliformis* (ascomycetes), and *Ustilago maydis* and *Cryptococcus neoformans* (basidiomycetes) but not in the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, or *Ashbya gossypii* (Blumenstein et al. 2005). Intriguingly, FphA forms a complex with LreA and LreB, the orthologues of WC-1 and WC-2, the central components of blue-light perception in *N. crassa*.

III. Growth and Primary Metabolism

Filamentous growth of a variety of fungi is affected in diverse ways by light. Branching in *N. crassa* hyphae increases in cultures grown in the light, as compared to cultures grown in the dark, resulting in more compact colonies (Lauter et al. 1998). This effect of light requires the WC proteins and has also been observed in the truffle *Tuber borchii* (Lauter et al. 1998; Ambra et al. 2004). The inhibition of mycelial growth by light may promote the growth of *Tuber* underground (Ambra et al. 2004). A similar effect of light has been observed in another ascomycete, *Trichoderma atroviride*, whose hyphal growth is inhibited by light but in a Blr (WC) independent fashion (Casas-Flores et al. 2004). Promotion of hyphal branching has also been documented for the plant pathogen *Colletotrichum trifolii* (Chen and Dickman 2002).

In the case of *Neurospora*, the Ser/Thr protein kinase Cot-1 is involved in the regulation of branching and colonial growth, and expression of the corresponding gene is regulated by light through an as yet not fully understood mechanism (Yarden et al. 1992; Lauter et al. 1998; Gorovits et al. 1999). The *C. trifolii* homologue of the *Neurospora cot-1* gene (*tb-3*), like *cot-1*, is regulated by light, but TB3 may act as a transcriptional regulator for hyphal branching, as suggested by its nuclear localization and the presence of putative transcriptional activation domains (Chen and Dickman 2002).

As we have seen, light influences growth in the fungi, which is very likely linked to the major changes in metabolism that have been detected when a fungus grows exposed to light or darkness.

In an extensive early study by Cantino and Horenstein (1956) on the effect of light on the growth of the water mold *B. emersonii*, they observed that illuminated cultures had higher growth rates than those grown in the dark, and stimulation of carbon dioxide fixation by light was demonstrated. These authors showed that the increase in carbon dioxide fixation could be the result of the stimulation of the activity of a succinate-α-ketoglutarate-isocitrate cycle (Cantino and Horenstein 1956, 1959), which results in the provision of additional succinate and glyoxylate for biosynthetic purposes, consequently leading to an increased supply of thymidine, altered nucleic acid metabolism (28 % higher accumulation), and increased growth. Interestingly, exposure of mycelium to light also stimulates polysaccharide synthesis and reduces glucose-6-phosphate dehydrogenase activity (Goldstein and Cantino 1962). Based on their biochemical data, the authors postulated that it would be reasonable to assume that these events were related and that the increased polysaccharide synthesis could result from a diminished glucose oxidation, derived from the lessened activity of the enzyme.

It appears that intracellular synthesis and/or degradation of carbohydrates is altered in response to illumination. In this regard, stimulation of polysaccharide biosynthesis by light has been observed in *Penicillium isariiforme* (Graafmans 1977).

In addition, light provokes higher excretion of citric acid in this fungus likely due to a lower supply of pyruvate in light. Under illumination, accumulation of intermediates such as glucose-6-phosphate, glucose-1-phosphate, and fructose-6-phosphate was observed (Graafmans 1977). Thus, in this fungus light appears to stimulate the pentose phosphate pathway and block the supply of pyruvate for citric acid synthesis. Given that sucrose uptake is not altered in light in *P. isariiforme* (Graafmans 1974), it would seem that less endogenous substrate is channeled for citric acid biosynthesis when the fungus is grown in light conditions (Graafmans 1977).

The influence of light on carbohydrate metabolism has also been observed in *Phycomyces blakesleeanus*, where the metabolites glucose-6-phosphate, 2-phosphoglycerate, phosphoenolpyruvate, and pyruvate accumulated to higher levels in cultures grown in the dark, whereas fructose-6-phosphate, glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, and lactate reached higher concentrations in the light than in the dark. In this case, the levels of acetyl coenzyme A and citrate were higher in the light than in the dark (Rua et al. 1987).

Interestingly, it has been reported that phosphoglycerate kinase (*pgk*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) are downregulated at the late stages of conidiation induced by a light pulse in *T. atroviride* (Goldman et al. 1992; Puyesky et al. 1997).

Not only utilization but also nutrient uptake is regulated by light (Hill 1976), which consequently impacts metabolism. Thus, stimulation of growth could be the result of efficient nutrient uptake, whereas growth inhibition could be due to limited uptake of essential nutrients. In the case of *Aspergillus giganteus*, growth is positively affected by light and glucose.

The dry weight of *A. giganteus* grown in the presence of light and a glucose concentration of 20 % is about 60 % higher than when cultivated in the dark. The increase in biomass accumulation occurs only on medium amended with yeast extract, which was at most reestablished by high concentrations of nitrogen and phosphorus in the medium (Zurzycka 1991). In this case, the amount of glucans or glycogen content depends in part on these two factors (Fiema et al. 1991; Zurzycka 1991). Similarly, a decrease in glycogen content upon cultivation in light was observed in *T. reesei* (Farkas et al. 1990). However, glucose uptake decreases significantly when *Aspergillus ornatus* is grown under illumination, and it has been suggested that this effect is due to the biosynthesis of a small molecule that inhibits glucose uptake (Hill 1976).

Although the molecular mechanisms operating on the regulation of glucose uptake are not fully understood, light-dependent differences in regulation of biosynthesis and activity of enzymes could set the scenario to begin understanding what appears to be a complex regulation scheme.

Light-grown cultures of *N. crassa* showed no significant changes in growth or in the pH of the medium; however, significant changes were determined in the activities of several enzymes of carbohydrate metabolism. For instance, the light-grown cultures presented an enhanced activity of proteases and cytosolic malate dehydrogenase, whereas the mitochondrial malate dehydrogenase, isocitrate dehydrogenase, and cytosolic glucose-6P-dehydrogenase, isocitrate dehydrogenase, and isocitrate lyase showed decrease activity. In this sense, it has been demonstrated that light mediates changes in enzyme activities mainly in the carotenoids biosynthetic pathway. Furthermore, it was also found that light increases lipid concentration.

In this regard, the factors influencing lipid and carotenoid synthesis are (1) the provision of reducing power, which can be met mainly with glucose-6P-dehydrogenase, and (2) the availability of acetyl CoA, which can be provided by the cleavage of an excess of extramitochondrial citrate in the cytosol. Therefore, the cytosolic malate dehydrogenase, which showed higher activity in the light-grown culture, could provide reducing power for the synthesis of lipids and carotenes. Furthermore, the low isocitrate dehydrogenase activity of mitochondria detected in light-grown mycelia could play a significant role in increasing lipids and carotenoids in light-grown cultures. The fact that light-grown cultures showed low isocitrate lyase activity led to suggest that the glyoxylate bypass could be driving at higher rate in the dark as compared to light. Therefore, acetyl CoA may be driven to the glyoxylate bypass and consequently may be less available for lipid and carotenogenesis in the darkness (Ram et al. 1984). Interestingly, a link between light perception and carbon metabolism (redox state) was suggested by Casas-Flores and coworkers, since *blr* mutants were unable to conidiate in response to carbon deprivation. These data led to the hypothesis that LOV and PAS domains could be involved in sensing the energy status of the cell and reactive oxygen species (Casas-Flores et al. 2004). In this regard, it has been shown that the LOV domain of Envoy (Env1) is involved in oxidative response in a carbon source-dependent manner in vivo (Lokhandwala et al. 2015).

Tisch and Schmoll interpreted the data available on the intermediary metabolites of glycolysis and proposed that conversion of glucose to glyceraldehyde-3-phosphate may be enhanced, while downstream in the cascade glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, which activity decreases in light, appear to inhibit the metabolic flux resulting in lower levels of downstream metabolites (Tisch and Schmoll 2010).

Based on the data outlined above, they also proposed that if intracellular processes involved in utilization of carbon sources were working at different levels in light, uptake of a carbon source could be adjusted accordingly. They further proposed that regulation of these processes could in turn lead to adjustment of expression of extracellular enzymes. However, they explained that control of extracellular enzymes would only apply if the organisms were forced to degrade insoluble compounds such as cellulose or lignin (Tisch and Schmoll 2010). Consequently, the extracellular enzymes responsible for the degradation of nutrients outside the fungal cell should be regulated by light (Fig. 3.3).

Indeed, the *N. crassa* secretome in response to light revealed an increase in the expression of several polypeptides in constant light, whereas other polypeptides were markedly reduced in this condition, and vice versa.

Exposure of *Neurospora wc-1* and *wc-2* mutants to constant light showed the same pattern as when grown in constant darkness that was similar to the wild-type pattern in the dark (Kallies et al. 1992). Furthermore, Env1, an orthologue of the *N. crassa* photoreceptor Vivid, was identified in a screening for genes involved in cellulase signaling in *T. reesei* (Schmoll et al. 2004). Later, it was shown that cellulase gene expression is modulated by light and that such regulation involves the Blr photoreceptor complex and Env1 (Castellanos et al. 2010; Schmoll et al. 2005). Another interesting link to light regulation of cellulase expression is that Šesták and Farkaš (1993) found that the

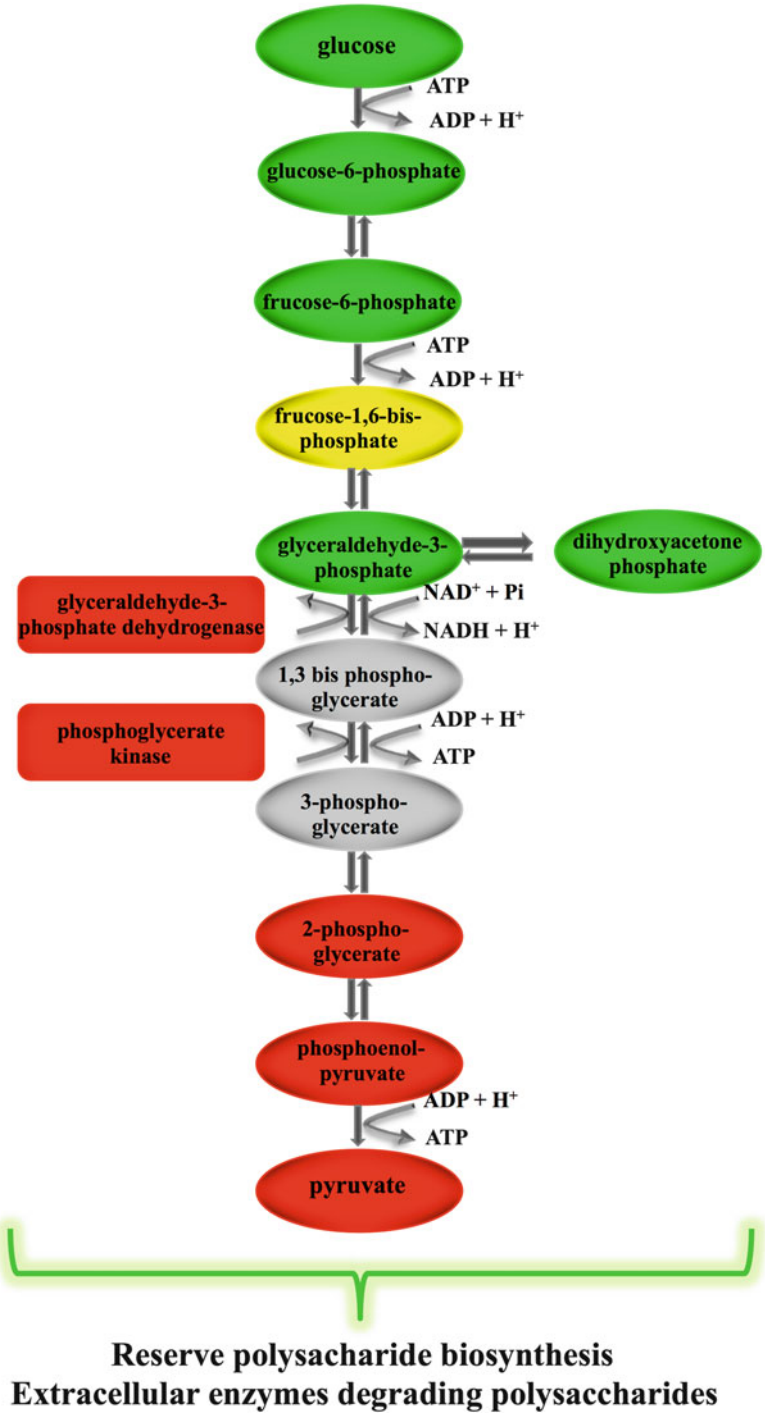

Fig. 3.3 Schematic representation of the impact of light on the glycolytic pathway and the consequent effect on polysaccharide biosynthesis and utilization. *Green ovals* indicate increased levels of the metabolite when a fungus is cultivated in light, and *red ovals* represent decreased levels. For fructose-1,6-bisphosphate (*yellow oval*), no alteration was found, and in case of *gray ovals*, no data on the levels of these metabolites in light are available (as interpreted by Tisch and Schmoll 2010). *Red rectangles* indicate lower transcript and enzymatic activities of the indicated protein

addition of dibutyryl cyclic AMP (dBcAMP), an analog of cAMP, to *T. reesei* cultures in the presence of a potent cellulase inducer repressed or activated the synthesis of endoglucanase depending on the dBcAMP concentration. Consistently, the addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) provoked an increase in intracellular cAMP levels and promoted the synthesis of endoglucanase. In this regard, it has been established that light induces changes in intracellular cAMP levels (Gresik et al. 1988) and that IBMX stimulates photoconidiation in *Trichoderma* (Berrocal-Tito et al. 2000; Sulová and Farkás 1991).

Another example of the regulation by light of extracellular enzymes is found in *Aspergillus niger*, which when exposed to blue or red light showed enhanced glucoamylase activity (Zhu and Wang 2005).

Regarding nitrogen metabolism, until recently there were only some indications to its relation with light. However, there is increasing evidence of the effect of light on the abundance of amino acids.

In *A. ornatus*, the uptake of many amino acids clearly decreases upon cultivation in light (Hill 1976), and in *P. blakesleeanus*, illumination of dark-adapted cultures decreased ornithine decarboxylase activity (Lapointe and Cohen 1983). In the basidiomycete *Polyporus hispidus*, phenylpropanoid metabolism and hispidin biosynthesis are regulated by light (Nambudiri et al. 1973). Further, in *T. viride* glutamic acid decarboxylase, catalyzing alpha-decarboxylation of L-glutamic acid to produce gamma-aminobutyric acid (GABA) is also stimulated by light (Pokorny et al. 2005; Strigacova et al. 2001), which is considered a pathway required for conidial germination in *N. crassa* (Schmit and Brody 1975). In addition, in *N. crassa* several genes involved in nitrogen metabolism are controlled by the circadian clock (Correa et al. 2003), and nitrate reductase activity decreases (Klemm and Ninnemann 1979). Finally, a recent study on the role of the light-induced gene *blu7*, encoding a putative transcription factor, revealed that it is involved in the expression of several amino acid transporters, and deletion of the gene results in clearly reduced growth in nitrogen-limited media but not in nitrogen-rich media (Cetz-Chel et al. 2016).

IV. Development

Fungal development is often modified or triggered by the incidence of light. The main developmental transitions in the fungal life cycle are spore germination, hyphal growth and branching, and the formation of resting or reproductive structures. Fungi take advantage of the presence of ambient light, and changes in its quality and quantity, to modulate several steps of their development. The regulation by light of fungal development is generally referred to as fungal photomorphogenesis, and can be measured precisely, allowing us to determine useful parameters such as action spectra and thresholds, and has attracted the attention of scientists worldwide. Within the spectrum of visible light, blue is in general most effective in fungal photomorphogenesis but other wavelengths are also important.

The fact that light induces initiation and development of sclerotia—multicellular resting structures, resistant to adverse conditions—in various filamentous fungi has been known for a long time (Chet and Henis 1975; Carlile 1956; Heath and Eggins 1965; Rai et al. 1967; Rudolph 1962; Tarurenko 1954). Back in the 1960s, Kaiser (1964) found that in *Verticillium alboatrum*, red light promoted microsclerotia production while blue light inhibited it, and ultraviolet radiation completely abolished microsclerotial formation in one isolate (Brandt 1964). Similarly, cultures of *Sclerotium rolfsii* produce considerably greater numbers of sclerotia when exposed to light (Gruelach and Mohr 1947), and Trevethick and Cooke (1973) found that in *Sclerotium rolfsii*, *Sclerotium delphinii*, and *Sclerotinia sclerotiorum*, both number and size of sclerotia could be determined by photoperiod length.

Until very recently, the mechanism by which light influenced sclerotial morphogenesis was not understood at all. However, a putative histidine kinase (HK) gene Bcphy3 from *B. cinerea*, containing a conserved GAF and a PHY

domain (putative phytochrome chromophore-binding sites), in which expression is induced by light, was recently cloned. Mutants in which Bcphy3 was disrupted were found to produce a substantially reduced amount of sclerotia compared to the wild-type strain (Hu et al. 2014). These results indicated that Bcphy3 might encode a highly divergent phytochrome-like HK. The exact reason why Bcphy3-deleted mutants lost the ability to produce sclerotia is still unknown. Nevertheless, as in the case of *V. albo-atrum*, sclerotia production in *B. cinerea* appears to be promoted by red and far-red light (Tan and Epton 1973). Thus, it is possible that the functional loss of the phytochrome-like HK gene Bcphy3 may affect the sclerotia production process.

As in the case of sclerotia formation, copious literature on the effects of light on asexual reproduction in ascomycetes and the related Fungi Imperfecti exists since the 1930s. Already in 1933 it was reported that light is required for the production of macroconidia in *Sclerotinia fructigena* (Hall 1933), whereas *Sclerotinia fructicola* requires darkness.

Conidiation may be triggered by several factors related to stress such as light, nutrient availability, oxidant state, and mycelial injury (Aguirre et al. 2005; Casas-Flores et al. 2004; Hernández-Oñate et al. 2012; Horwitz et al. 1985).

The fact that continues darkness results in a delay or failure of asexual sporulation of *Trichoderma* was reported in the 1950s, as well as the considerable differences between isolates in their capacity to respond to illumination (Gutter 1957). In contrast, abolishment of the conidiation process in the phytopathogenic fungi *B. cinerea* and *A. solani* by blue light was also described in the middle of the past century; nevertheless, this process was reverted by exposure to red light (Lukens 1963; Tan 1973).

A good example of light-induced conidiation is *T. atroviride*, which grows as mycelium if nutrients are not limiting but upon exposure to a brief pulse of blue light produces conidia in a nearly synchronous manner (Gressel and Galun 1967). Early photobiology studies carried out using *T. atroviride* as a model indicated that blue-light-activated conidiation was regulated by a single photoreceptor (Horwitz et al. 1990). Accordingly, the main blue-light receptor involved in this process is the Blr complex (Blr1/Blr2), which is also essential for the control of gene expression (Casas-Flores et al. 2004; Rosales-Saavedra et al. 2006). Similarly, mutants in the *Trichoderma reesei* homologues of the *blr* genes do not conidiate in response to light (Castellanos et al. 2010).

Interestingly, overexpression of *blr2* resulted in enhanced sensitivity to light in *T. atroviride* (Esquivel-Naranjo and Herrera-Estrella 2007). Nevertheless, recent biochemical and molecular data suggest the participation of at least two light perception systems that regulate photoconidiation (Berrocal-Tito et al. 2000; Rocha-Ramirez et al. 2002; Rosales-Saavedra et al. 2006; Casas-Flores et al. 2006). In this sense, increases in cAMP-dependent protein kinase (PKA) activity after a pulse of blue light have been observed (Casas-Flores et al. 2006). However, such activation occurred even in Δ*blr1* and Δ*blr2* mutant strains, confirming the existence of an alternative system for light perception linked to cAMP. The same authors showed that transformants expressing an antisense version of *pkr-1*, a gene encoding the regulatory subunit of PKA, with higher levels of PKA activity did not produce conidia after exposure to a pulse of blue light. Unfortunately, the light receptor responsible for the activation of the cAMP pathway has not been identified.

Conidiation in *Aspergillus* involves a complex regulatory network of gene expression and cell differentiation (reviewed by Adams et al. 1998) and is induced by light, but other aspects of *Aspergillus* development are also influenced by light. *A. nidulans* produces hyphae and sexual structures in the dark and mainly conidiophores and conidia in the light. Thus, the ratio of sexual to asexual development is altered by light. In this case apparently only red light stimulates conidiation, and the stimulatory effect may be reversed by far-red light, which suggested the participation of a phytochrome-like photoreceptor in *Aspergillus* (Mooney and Yager 1990). In this regard, the discovery of *fph*A in the *A. nidulans* genome allowed Blumenstein et al. (2005) to demonstrate that FphA acts as a red-light sensor and represses sexual development under red-light conditions.

Surprisingly, FphA is part of a protein complex containing LreA (WC-1) and LreB (WC-2), two central components of the blue-light-sensing system. FphA interacts with LreB and with VeA, another regulator involved in light sensing (Purschwitz et al. 2008). A mutation in *veA* allows *Aspergillus* to conidiate in the dark (Mooney and Yager 1990). Additionally, LreB interacts with LreA. The FphA-VeA interaction depends on the presence of the linear tetrapyrrole in FphA, whereas the interaction between FphA and LreB is chromophore independent (Purschwitz et al. 2008).

These observations suggest that photomorphogenesis in *A. nidulans* is mediated by a network consisting of FphA, LreA, LreB, and VeA acting in a large protein complex, sensing red and blue light, even though FphA represses sexual development and LreA and LreB stimulate it.

Amazingly, white light induces conidiation, but neither blue nor red light induces it, when applied separately. However, when applied at the same time, conidia formation is induced to the same level as upon exposure to white light (Purschwitz et al. 2008). In this sense, Δ*lreA* and Δ*lreB* strains produce slightly more conidia than the wild type, independently of the presence or absence of light, which suggests a role for *lreA* and *lreB* products as repressors in conidiation. Moreover, double mutation of *lre*A or *lre*B with *fph*A or the triple mutation leads to a drastic decrease in the number of conidia. Therefore, these results confirm at the molecular level the synergistic effect between blue- and red-light photoreceptors as it was observed at the phenotypic level.

VeA is largely localized in nuclei when grown in the dark, but VeA presence in the nucleus is decreased when grown in the light (Stinnett et al. 2007). VeA levels in the nucleus are partly dependent on FphA, and nuclear concentration of VeA is prevented by blue light, reflecting that both red- and blue-light-sensing systems are required for its effective nuclear localization (Purschwitz et al. 2008; Stinnett et al. 2007).

Blue light plays a dual role in conidial production of *Paecilomyces fumosoroseus*, an entomopathogenic fungus widely used as biocontrol agent. Conidia are the means for dispersal and transmission of the entomopathogen, which upon contact with the insect cuticle germinate and penetrate the host. In the dark, the fungus grows only vegetatively, lacking reproductive structures. However, exposure of *P. fumosoroseus* to a 5 min pulse of blue light is sufficient to induce conidial development (Sánchez-Murillo et al. 2004). Nevertheless, blue light has a stimulatory and inhibitory effect, depending on the fluence rate used. In the laboratory, the fungus shows a period of competence for this response, since light is effective only when applied between 72 and 96 h of growth. The minimal fluence required for the photomorphogenetic response is 180 μmol m^{-2}, and maximum conidial yield is obtained with 540 μmol m^{-2}. Higher light intensities greatly decrease conidiation, suggesting the existence of a complex photosensory system (Sánchez-Murillo et al. 2004).

Light is a crucial cue for fruiting-body formation in many fungi. The first studies reported of light effects on fruiting-body formation described that apothecium formation is completely light dependent and that dark-grown mycelia were sterile in *Pyronema confluens* and *Pyronema domesticum* (Claussen 1912; Moore-Landecker 1979). There are multiple examples of both the stimulatory and inhibitory effect of light on sexual reproduction of fungi. Almost a century ago, it was reported that light represses perithecium production but stimulates conidium formation in *Aspergillus glaucus* (Chona 1932). In contrast, light is essential for the initiation of apothecia formation in *P. confluens* (Carlile and Friend 1956) and *Ascobolus magnificus* (Yu 1954) and for perithecium initiation in *Pleurage setosa* (Callaghan 1962).

Light is frequently needed for one or more phases in fruiting-body development in the Basidiomycetes. In various species excessive elongation of the stipe occurs in darkness, an effect that appears to be associated with phototropism (see below). Pileus expansion often requires light, as shown in the polypore *Polyporus brumalis* (Plunkett 1956) and the agaric *Collybia velutipes* (Aschan-Aberg 1960). Actual initiation of fruiting-body formation usually occurs in darkness, although light is needed

for initiation in *Polyporus arcularius* (Gibson and Trapnell 1957) and speeds up the process in *Coprinus lagopus* (Madelin 1956), while in *Poria ambigua* light is required for hymenium development (Robbins and Hervey 1960).

Light is required throughout fruiting in *Sphaerobolus stellatus*. Interestingly, in this case high intensities are needed early in development, but very low intensities suffice at a later stage (Alasoadura 1963). These early observations suggested that light may play important roles at different stages of sexual development, but no much information was provided in the study on the qualitative and quantitative parameters or on the mechanisms involved. Later on, more in-depth reports on the effect of light in sexual development started to appear in the literature, as in the case of the photoinduction of fruiting-bodies by light of defined wavelengths in *Schizophyllum commune*. In this case, several properties of the induction were established. The exposure-response relationship for induced fruiting was determined for light of 448 nm. The Bunsen-Roscoe law of reciprocity—which establishes that a given quantity of photons delivered in pulses of different duration should have the same final response—was found to hold for the photoinduction of fruiting-bodies for the interval from half a minute to half an hour. Although the photoreceptor involved in this phenomenon is still unknown, it is thought to be a flavoprotein because fruiting-body formation is induced by UV- and blue-light. Neither red light nor far-red light induced fruiting bodies or affected the sensitivity of the fungus to blue light (Perkins and Gordon 1969).

In contrast with the effect of light on *S. commune*, sexual development of *P. blakesleanus* is inhibited by light. Effective wavelengths for this inhibition are shorter than 490 nm, but the shape of the action spectra and the most effective wavelength depend on the stage of sexual development. Longer wavelengths are more effective for the inhibition of the final stages of sexual development. Furthermore, biphasic fluence-response curves were observed using some wavelengths, providing additional support of the presence of a complex photosensory system for photoinhibition of sexual development (Yamazaki et al. 1996). Interestingly, the threshold for this photoresponse is unusually high for *Phycomyces*. The unique shape of the action spectrum with maximum efficiency at 350–410 nm indicates that the photosystems involved in the inhibition of sexual development have special features not shared by other photoreceptor systems in *Phycomyces*.

Studies in *N. crassa* reported that fruiting-bodies are produced in the dark and located on the surface of the growing medium even without illumination. However, the perithecial necks are ever oriented to the light source, whereas in the dark they point to any direction (Harding and Melles 1983). Additionally, the position of the neck on the perithecium is also light dependent (Oda and Hasunuma 1997). Moreover, the number of protoperithecia is greatly increased upon a blue-light pulse (Innocenti et al. 1983). These processes completely depend on the *wc-1* and *wc-2* products, since such responses were completely abolished in Δ*wc-1* and Δ*wc-2* strains (Harding and Melles 1983; Degli-Innocenti and Russo 1984; Oda and Hasunuma 1997).

A rather interesting example of the influence of light in the control of fungal reproduction is that of *Coprinus cinereus*. The basidiomycete *C. cinereus* forms dikaryons after fusion of two homokaryon mycelia of compatible mating type and develops a fruiting body where meiosis takes place to produce basidiospores (reviewed in Kües 2000; Kamada 2002). Additionally, *C. cinereus* develops different types of reproductive and specialized cells: haploid unicellular spores (oidia) develop on oidiophores in the aerial mycelium, and large chlamydospores appear in submerged, old mycelium. Hyphal knots can give rise to sclerotia in old cultures or serve as primordia of fruiting-bodies. As in most fungi, different environmental conditions determine which developmental pathway will be followed, light conditions being the major signal (reviewed in Kües 2000; Fischer and Kues 2003).

Sclerotia are produced in the dark, and initiation of the fruiting body occurs only in the light. Fruiting-body development depends on dark/light cycles; hyphal knot formation is inhibited by light; the formation of fruiting-body initials, maturation of primordia, and

karyogamy are induced by light; and meiosis completion is inhibited by light (reviewed in Kües 2000). The effect of light/dark cycles in meiosis, and possibly of the whole fruiting-body development, seems to allow the maturation of fruiting-bodies for spore dispersal shortly after daybreak, regardless of night duration (Lu 2000). Blind mutants, as well as other mutants altered in different steps of fruiting-body development, have been isolated (Muraguchi and Kamada 2000). The blind mutants are blocked in fruiting-body development, forming only "dark stipes." A gene affected in one of these mutants was cloned, and its sequence was found to be similar to that of the *N. crassa wc-1*, suggesting that this gene plays a similar role in *C. cinereus* photomorphogenesis (Terashima et al. 2005).

Cryptococcus neoformans is a heterothallic yeast and a human pathogen (Hull and Heitman 2002), in which blue light inhibits mating and haploid fruiting. As proposed for *C. cinereus*, mutations in any of the *Cryptococcus wc* genes resulted in a blind-mating phenotype (Idnurm and Heitman 2005; Lu et al. 2005), and their overexpression resulted in a stronger light-dependent inhibition of mating (Lu et al. 2005).

A. nidulans develops fruiting-bodies in the dark, whereas upon exposure to far-red light, their production is repressed (Mooney and Yager 1990). As mentioned before, light control of development in *A. nidulans* is regulated by a number of photoreceptors, including the phytochrome FphA, which senses red light, and it is responsible for repression of fruiting-body formation, while it positively regulates asexual spore development (Blumenstein et al. 2005). Interestingly, integration of blue- and red-light signaling affecting the developmental programs of this fungus was recently demonstrated. Mutants in *lreA* or *lreB* do not produce cleistothecia under light conditions, which is largely suppressed by deletion of *fphA*. Intriguingly, double and triple mutants of *lreA*, *lreB*, and *fphA* exposed to light produced similar quantity of cleistothecia when compared with dark conditions.

Sexual development of *T. reesei* requires light to take place, which has been shown to occur not only with laboratory strains but also with natural isolates (Seibel et al. 2009; Chen et al. 2012). Mating of Δ*env1*, Δ*blr1*, and Δ*blr2* (Seibel et al. 2009) with the wild-type strain was fruitful, and discharge of ascospores was not abated. These data indicated that deletion of the photoreceptors does not affect male fertility (Seibel et al. 2012). Sexual reproduction in fully male and female wild-type background, bearing mutation in *blr1* and *blr2*, showed altered fruiting-body formation compared to the wild type with fewer, but larger, fruiting-bodies. The *blr1* and *blr2* products repress the formation of stromata under constant light and are necessary to speed up the formation of stromata under 12 h light/12 h dark (LD) regime (Chen et al. 2012). In contrast, crosses of Mat1-1 and Mat1-2 bearing a deletion in *env1* provoked a defect in sexual development characterized by the lack of fruiting bodies, and Env1 was shown to be essential for female fertility. Recently, the participation in sexual development of the pheromone precursor gene *hpp1* and that of the orthologous gene to the pheromone transporter in yeast *ste6* is regulated by the phosducin-like protein (PhLP1), and the G-protein β (Gnb1) and γ (Gng1) subunits were demonstrated. It has been proposed that phosducin-like proteins mediate light transduction through a G-protein signal transduction pathway (Tisch et al. 2011). The participation of the protein kinase A (PkaC1) and the adenylate cyclase (Acy1) in sexual development in *T. reesei* was also recently elucidated. Deletion of *pkac1* and *acy1* provokes delayed fruiting-body formation but does not alter their development. Similar results were reported for Δ*cr-1* (adenylate cyclase) in *N. crassa* (Ivey et al. 2002). Based on these results, it can be concluded that the cAMP pathway is a positive input in the regulation of sexual development; however, it is not essential for this process in this fungus (Schuster et al. 2012). As mentioned before, light impacts in cAMP synthesis and protein phosphorylation (Gresik et al. 1988); however, the effect of light on mating using *pkac1* and *acy1* mutant backgrounds was not investigated.

Recently, the role of Velvet (Vel1) in light response, development, and secondary metabolism in *T. reesei* was described (Bazafkan et al.

2015). Lack of *vel1* leads to defects in growth, conidiation, and mating in the dark, whereas in light the *vel1* product was not necessary for female fertility. However, this protein was essential for female fertility in both mating types. Additionally, the absence of *vel1* negatively affected the transcription of *hpr1, hpr2, hpp1*, and *ppg1*, genes that are part of pheromone system, in a mating-type-dependent mode and depending on the mating partner of a given strain. These mating defects only occurred for the pheromone precursor *hpp1* and *hpr2* and receptor genes associated with the Mat1-2 mating type and for the mating-type gene mat1-2-1. It has also been demonstrated that a Δ*vel1* strain secretes different metabolites compared to the wild type; when the wild-type strain was confronted with a compatible partner, it produced a set of secondary metabolites, but the set changed if a Δ*vel1* strain was the partner. Therefore, this fungus uses both pheromones and secondary metabolites to interact with mating partners, which is conducted in part by Vel1 (Bazafkan et al. 2015).

V. Phototropism

As mentioned above, light may serve as a cue to change the directionality of growth of fungi, which helps them "decide" on the positive or negative consequences of approaching this signal (positive or negative phototropism). The best-studied phototropic response is that observed in the sporangiophore of *P. blakesleeanus*. The *Phycomyces* sporangiophore is a single coenocytic cell of 2 mm length and 100 mm diameter that elongates at an astonishing rate of 2–3 mm h^{-1}. Light perception, growth modulation, and phototropism all occur in the small growing zone extending 2–3 mm below the sporangium (Bergman et al. 1969). When sporangiophores are exposed for 6–8 h to unilateral light, they bend in response to near-UV and blue light of fluence rates extending 10 orders of magnitude (10^{-9} and 10^{2} Wm^{-2}) and transiently accelerate their growth rate (Galland et al. 1985; Bergman et al. 1969; Galland and Lipson 1987). This remarkable sensory dexterity resembles that of the human eye and is achieved through the action of two photosystems that operate at different light intensities (Galland and Lipson 1987). Trifluoperazine affects both sporangiophore growth and phototropism, suggesting that microtubules and calmodulin are involved in the response of the fungus to light (Valenzuela and Ruiz-Herrera 1989). Additionally, the role of calcium in dark adaptation of phototropism of *Phycomyces* suggests that the corresponding signaling pathway may play a relevant role in phototropism (Sineshchekov and Lipson 1992).

A genetic screen for phototropic mutants, conducted in the Delbrück's lab, led to the isolation and physiological characterization of *mad* mutants and the first draft of the signal transduction pathway for *Phycomyces* (Bergman et al. 1973). The discovery of additional *mad* mutants and detailed genetic characterization allowed the identification of ten unlinked *mad* genes (madA to madJ) (Alvarez et al. 1992; Orejas et al. 1987; Campuzano et al. 1995). The blue-light photoreceptor eliciting phototropism in *Phycomyces* is a molecular complex, similar to the *N. crassa* WCC constituted by the proteins MadA and MadB (Idnurm et al. 2006; Sanz et al. 2009). The MadA-MadB complex acts as a light-regulated transcription factor eliciting phototropism and other responses pertaining to photodifferentiation. Further, madC is understood as an early phototropism mutant with a 10^{6} higher threshold, and the corresponding gene encodes a Ras GTPase-activating protein (Polaino-Orts et al. 2013).

Sporangiophores of another zygomycete, *Mucor circinelloides,* also respond to light, exhibiting positive phototropism. Analysis of the phototropic response of knockout mutants in the three *wc-1* homologues present in the *M. circinelloides* genome revealed that while the wild-type strain is responsive to white, blue, and green light, the *mcwc-1a* mutant was unable to respond to light of any of these wavelengths. In contrast, photocarotenogenesis is induced only by blue light and unaffected in the *mcwc-1a* mutant (Silva et al. 2006). Therefore, multiple photosystems are involved in triggering photoresponses in this organism.

Although not studied in as much detail as in Zygomycetes, many other fungi display pho-

totropic responses. Such is the case of *A. giganteus*, in which conidiophores show positive phototropism toward white light, restricted to the apical 240 μm. During the response, the rate of extension of the proximal wall (relative to the direction of the light source) decreases by about 5 %, while that of the distal wall increases by about 5 %; thus, there is no net change in the rate of wall growth during phototropism. The sign of the phototropic response is reversed when conidiophores are unilaterally stimulated with UV-light (280 nm) (Trinci and Banbury 1968). Young sporangiophores of *Pilobolus kleinii* also respond to unilateral illumination by bending or growing toward near-UV and blue light and exhibit a negative phototropic response to UV light. The use of small beams of light and the behavior of sporangiophores submerged in mineral oil suggested that the photosensitive region is located in the tip of the young sporangiophore (Page and Curry 1966).

Positive phototropism was also shown for *N. crassa* perithecial beaks as beak bending in maternal structures. This effect is induced by blue light and abolished by deletion of *wc-1* and causes sexual spores to be ejected toward the direction of light (Harding and Melles 1983; Froehlich et al. 2010). In *Sordaria fimicola*, phototropism of the perithecial necks has been observed, with wavelengths below 550 nm being more effective. These observations are in agreement with early findings on the sensitivity of light-stimulated spore discharge in this fungus (Ingold and Hadland 1959).

The germ tubes of the phytopathogen *B. cinerea* present negative phototropism to near-UV and blue (300–520 nm) light followed by far red (700–810 nm), whereas red light (600–700 nm) induces positive phototropism significantly. Near-UV and blue light, triggering negative phototropism, promoted infection-hyphae formation on both onion scale and broad bean (*Vicia faba*) leaf epidermal strips. Conversely, red light that induces positive phototropism suppressed the formation of infection-hyphae (Islam et al. 1998).

Dermatophytes, such as *Trichophyton rubrum*, *Trichophyton mentagrophytes*, and *Microsporum canis*, also show phototropic responses. In these cases they have been shown to present negative phototropism upon repetitive exposure to UV-light, likely aimed at evading its harmful effects (Brasch and Menz 1995).

VI. Circadian Rhythms

The biological answer to the changing conditions brought about by the rotation of earth are circadian rhythms. A requirement for photoperiodism—the assessment of the light or the dark period—entails a timer, which in plants and animals is generally linked to the circadian system. As described above, the pattern of spore release in many fungi, which discharge their spores, is influenced by light. This is clearly shown when, with periods of light and dark alternating, spore discharge may show a marked periodicity. Studies of circadian periodic discharge related to light were reported back in the 1950s and 1960s for *Pilobolus*, *Daldinia* and *Sordaria*, *Sphaerobolus*, *Sporobolomyces*, and an extended range of Pyrenomycetes (Callaghan 1969).

Perhaps the first indication of endogenous rhythms in the fungi derives from the dung-loving fungus *Pilobolus*, which shoots spores as far as 2 m. Klein (1948) reported that when *Pilobolus* was submitted to various symmetrical non-24-h LD cycles, the spore-shooting rhythm was absent. His experiments probably represent the first systematic investigation of the influence of photoperiod on the sporulation rhythm. Short (LD 4:20) and long (LD 20:4) photoperiods appear to suppress rhythmicity, while using LD 9:15 and 15:9, rhythmicity persists with a low amplitude and with high amplitude in LD 12:12. The first report providing strong evidence for the endogenous nature of this rhythmicity included experiments both in constant light (LL) and darkness (DD) (Schmidle 1951). When *Pilobolus* is maintained in a LD 12:12 cycle and released to constant light, the rhythm rapidly dampens and continues when released to DD. Rhythmicity in DD also persists after release from several days in LL.

Later, Uebelmesser (1954) reported species-specific phase angles in LD cycles (*P.*

sphaerophorus peaks at ZT 03, *P. crystallinus* at ZT 08). In contrast with Klein's report, she observed the rhythm in all photoperiods (except LL) with a systematically different phase angle. Uebelmesser also investigated the ranges of entrainment and found that synchronization persisted in 29 h cycles. She submitted *Pilobolus* to symmetrical LD cycles, ranging from 18:18 down to 2:2. While in *N. crassa* accumulation of conidia appears to be driven with a constant phase angle in reference to lights off (Merrow et al. 1999), the phase angle of the spore-shooting rhythm in *Pilobolus* varied with varying cycle lengths, possibly reflecting circadian entrainment. However, a more in-depth investigation revealed that the *Pilobolus* sporulation rhythm is also driven by the LD cycle but by lights on.

Sordaria fimicola and *Daldinia* also show circadian rhythmicity in sexual spore shooting in the dark, which are entrainable in light-dark illumination cycles (Austin 1968; Ingold and Cox 1955). In *Daldinia*, circadian rhythmicity persists under continues light, damping after several days, while in *Sordaria*, exposure to continuous light appears to suppress rhythmic spore release (Austin 1968). Interestingly, *S. fimicola* shares a functional *frequency* (*frq*) orthologue with *N. crassa* (Merrow and Dunlap 1994), suggesting that the mechanics in the light input and the circadian programs of these species are similar. Like *Daldinia*, spore release of the basidiomycete *Pellicularia filamentosa* is circadian in either continues light exposure or complete darkness and entrainable by light-dark cycles (Carpenter 1949). There are also species that show some kind of rhythmicity in light-dark cycles, but this rhythmicity is lost in continues light or in the dark (e.g., *Pyricularia*; see Barksdale and Asai 1961), indicating that sporulation, although rhythmic, is not always controlled by a circadian clock.

Fungal circadian clocks can be exquisitely light sensitive, in terms of fluence and duration. Amazingly, the *Pilobolus* clock requires less than half a millisecond of light to be completely reset (Bruce et al. 1960), and conidial banding in *Neurospora* is driven by light fluences as low as moonlight levels (Merrow et al. 1999). In *N. crassa*, conidial banding occurs about once per 22 h in the dark. Under continues light exposure, the banding pattern stops, a phenomenon observed in many fungi. Light blocks the circadian system completely, which is supported by nonrhythmic, elevated clock gene (cg) expression in constant light (Crosthwaite et al. 1995), in addition to a set phase relationship of the free-running circadian rhythm upon release to darkness.

Undoubtedly, the reproduction of fungi is just as seasonal as in other organisms, especially if they live far enough away from the equator. Like in other organisms whose internal temperature varies considerably, fungal seasonality is controlled both by photoperiod and temperature. Any collector or gourmet of mushrooms knows that fungal fruiting-bodies appear only in certain times of the year. The amount of spores they produce also differs drastically over the course of a year. Studies carried out in the Antarctic showed that all airborne fungal spore types were most abundant in the summer months, except for chlamydospores, which were most numerous during the winter (Marshal 1997). These annual rhythms have been directly correlated to environmental conditions such as nutrient availability, humidity, wind speed, day or night length, temperature, and other climatic parameters, such as humidity and rainfall, or even triggered by other organisms in the form of food and, in the case of pathogens, host availability (Ingold 1971; Roenneberg and Merrow 2001).

Similarly to many photoperiodic plants, some diurnal sporulators require light induction followed by a dark period to initiate their seasonal reproduction (Durand 1982; Leach 1967). Like with short-day plants, their maturation process is highly light sensitive and is inhibited when exposed to a short (seconds) interrupting light pulse (Durand 1982). Timing of the light pulse and duration of the dark period are crucial for the inhibitory effect.

Despite the unquestionable seasonality in fungi, investigations into photoperiodic mechanisms and/or photoperiodic memory are scarce. Due to their economic importance, many of the investigations into the influence of photoperiod on fungal development and reproduction have focused on host-infecting pathogens and more recently litter decompo-

sers. When the entomopathogenic fungus *Metarhizium anisopliae* is grown in different temperatures (25, 28, and 30 °C) and photoperiods (24, 16, 12, and 8 h), its colony size and the number of conidia produced are maximal at 28 °C and a day length of 16 h (Alves et al. 1984). Mycelial growth of the phytopathogen *Colletotrichum manihotis* is greater in complete darkness than under continues light (Makambila 1984). When grown under light-dark cycles, the inhibitory effect of light depends on photoperiod length, as it is less in LD 12:12 cycles, compared with longer and shorter photoperiods. The amplitude of the response increases with light fluence and temperature and is not observed at low temperatures (20 °C). Chlamydospore formation of the corn pathogen *Exserohilum turcicum* is also significantly affected by photoperiod and temperature (Levy 1995). Furthermore, day or night length can also modify the development of sexual spores, when seasonal changes in fungal abundance, activity, and community composition were investigated in the litter and organic and upper mineral soils of a temperate *Quercus petraea*. The litter community was found to exhibit more profound seasonal changes than the community in the deeper horizons. In the litter, saprotrophic genera reached their seasonal maxima in autumn, but during summer the highest abundance of ectomycorrhizal taxa was detected. Although the composition of the litter community changed over the course of the year, the mineral soil showed changes in biomass (Voříšková et al. 2014).

In fungi, the mechanisms by which circadian rhythmicity is achieved are best studied at the molecular level in *N. crassa* (Brunner and Kaldi 2008; Heintzen and Liu 2007). As in other eukaryotes, the circadian clock consists of positive and negative transcriptional/translational feedback loops, resulting in cycles of gene expression covering various functions (Brunner and Kaldi 2008). Expression of most clock-controlled genes (ccgs) peaks just before dawn, reflecting the preparation of the cell to deal with exposure to DNA-damaging radiations, desiccation, and other stresses provoked by the imminent exposure to sunlight (Vitalini et al. 2006).

A key component of the central oscillator in *N. crassa* is frequency (FRQ); modifications of this protein result in altered periodicity or oscillation abolishment (Cha et al. 2011; Diegmann et al. 2010). Nevertheless, clock-controlled genes which oscillate independently of FRQ have been found (Correa et al. 2003), indicating the existence of an additional oscillator, the frequency-less oscillator (FLO) (Heintzen and Liu 2007; Lakin-Thomas and Brody 2004). In 2010 Brody and coworkers (Brody et al. 2010) suggested that components regulating the conidiation process or part of the RAS-cAMP-protein kinase pathway could contribute to the FLO. Recently, using genetic approaches, an oscillator mechanism that drives rhythmic spore development in the absence of the FRQ/WCC oscillator and in constant light was uncovered in *N. crassa*. While this novel oscillator does not require the FRQ/WCC oscillator for its activity, it requires the cryptochrome (CRY). The CRY-dependent oscillator has a period of ~1 day in constant light and is temperature compensated (Nsa et al. 2015). Another component involved in the regulation of the circadian clock is epigenetics, for which more and more evidence is being accumulated (Ripperger and Merrow 2011). Nevertheless, in fungi it is not clear if there is epigenetic control of the circadian clock, perhaps due to our poor understanding of epigenetic control in general.

Research with *Trichoderma* spp., in contrast, did not yet provide any hints as to the presence of circadian rhythms or their regulation by homologues of WC-1, WC-2, and FRQ in these fungi. Nevertheless, given the ubiquity of circadian regulation in countless organisms, it would be surprising if this mechanism would not be operative in *Trichoderma* spp. Thus, it seems that robust circadian rhythms in conidiation cannot be observed under the culture conditions currently used in the laboratory.

Since most *Trichoderma* species require light for conidiation, colonies growing in the dark were induced by LD cycles, determining that conidiation occurred, corresponding to the interval of illumination. When *Trichoderma* is returned to the dark after a pulse of light, conidiation is observed only at what was the

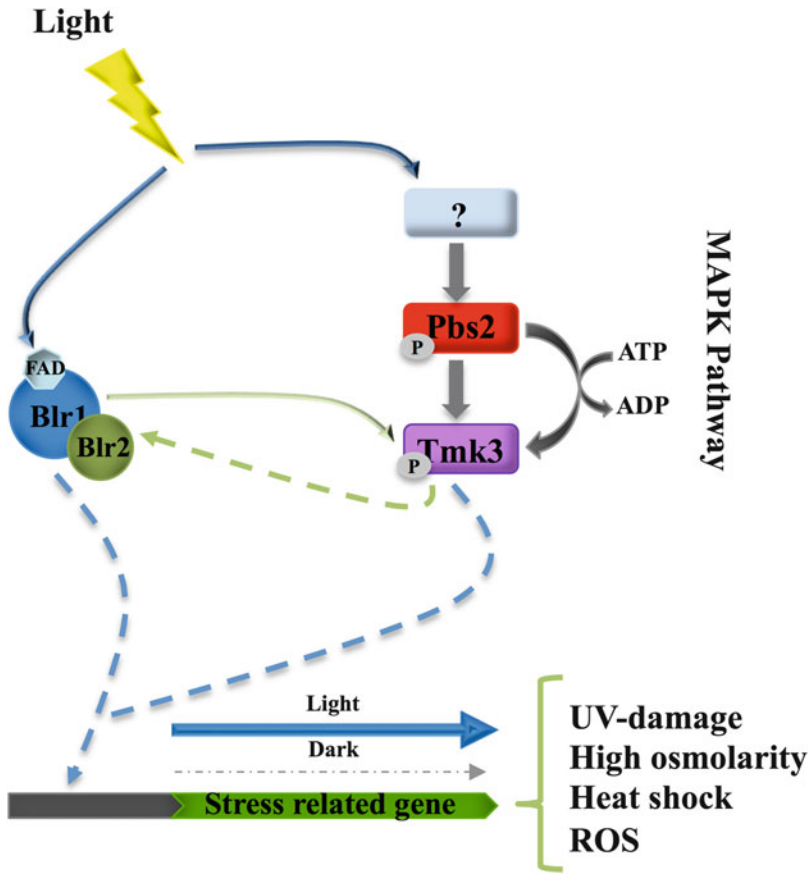

Fig. 3.4 Model for the light-dependent regulation of stress responses in *T. atroviride*, integrating components (Pbs2 and Tmk3) of the SAPK pathway and the *blue-light* receptor complex (Blr1/Blr2). *Solid lines* indicate interactions fully supported by experimental evidence and *dotted lines* indicate interactions suggested by these evidences

colony perimeter at the time of exposure to light. Such result allowed to conclude that conidiation in *T. viride* is not rhythmic but can be synchronized by a light pulse (Betina and Zajacová 1978). Similarly, under continues light, conidiation is not rhythmic; however, banding patterns are formed under light/dark cycles. Interestingly, a *Trichoderma* mutant (B119) that conidiates rhythmically in the dark has been described, and composition of the growth medium influences the period length of conidiation. Medium containing sodium deoxycholate, an ionic detergent that delays the growth of *Trichoderma*, increased the interval between dark bands from 12 to 24 h. As in the wild-type strains, the application of light induced a ring of conidia, but it also delayed the reappearance of the dark banding pattern (Deitzer et al. 1988). Recently, Steayert and coworkers described that dark-grown cultures of *T. pluroticola* form rings of green conidia at interval ca. 24 h. Light, however, did not induce rhythmicity in conidiation (Steayert et al. 2010). Nevertheless, similarly to what has been described in *N. crassa*, the *frq* gene of *T. atroviride* is regulated by light, and such regulation depends on functional *blr1* and *blr2* (García-Esquivel et al. 2015; Liu and Bell-Pedersen 2006). Orthologous genes to those described as involved in the regulation of circadian rhythms (*wc*-1, *wc*-2, and *frq*) in several fungal species have been also found in all *Trichoderma*

species, whose genomes have been sequenced. However, the role of their products in regulation of circadian clocks has not been established (Casas-Flores and Herrera-Estrella 2013). Further research on the molecular aspects of circadian rhythms in *Trichoderma* should lead us to understanding this phenomenon in the genus.

VII. Light as Warning Signal for Abiotic Stress

As mentioned above, the sunlight that impinges on our planet comprises harmful wavelengths that can generate mutations and/or damaging reactive oxygen species. Therefore, fungi must display a rapid reaction to exposure to UV light. Accordingly, blue light appears to be a cue for fungi to start a rapid response to illumination.

Photolyases play a major role in protecting the cell from UV damage by repairing DNA damage, through a process called photoreactivation. The cryptochrome/photolyase family consists of 55–70 kDa proteins that bind FAD, noncovalently, and an antenna chromophore of pterin (MTHF) or flavine type (8-HDF, FMN, or FAD) in the photolyase homology region (PHR) (Sancar 2003; Chaves et al. 2011; Fig. 3.2). CPD photolyases repair cyclobutane pyrimidine dimers (CPDs) and 6-4 photolyases repair the pyrimidine (6-4) pyrimidone photoproducts (Sancar 2008; Weber 2005). In fungi genes encoding proteins similar to cryptochrome/photolyases have been found, and most, if not all of them, are transcriptionally activated upon exposure to blue light (Herrera-Estrella and Horwitz 2007; Corrochano 2007). Further, DNA-damaging agents, including UV and ROS, can reset the clock (Gamsby et al. 2009).

Members of the cryptochrome/photolyase family with functions, both as regulator cryptochrome-photolyase type and photorepair enzyme, have been reported. Phr1, a protein with CPD photolyase activity that regulates its own expression, and modulates the expression of other light-responsive genes in *T. atroviride*, and its closest orthologue from *A. nidulans*, CryA, shown to control sexual development in the fungus, are examples of the dual functions that these types of proteins might play (Berrocal-Tito et al. 1999; Berrocal-Tito et al. 2007; Bayram et al. 2008a). Other examples are Phl1 from *Cercospora zeae-maydis*, which as a member of the cryptochrome/6-4 photolyase subfamily is involved in the repair of DNA damage by UV, but is also involved in the regulation of the CPD photolyase and participates in the development of the fungus and secondary metabolism (Bluhm and Dunkle 2008); *cry1* from *Sclerotinia sclerotiorum* is a DASH-type cryptochrome encoding gene expressed in response to UV-A light, which deletion results in diminished sclerotial biomass (Veluchamy and Rollins 2008). Yet another example is the *T. reesei* cryptochrome/6-4photolyase (Cry1), whose transcript levels are induced by light in a *blr*1-dependent manner and are repressed by Env1 (Guzmán-Moreno et al. 2014). Similarly, the second-most highly light-induced gene in *A. umigatus* is *phr*1, a gene encoding a predicted CPD photolyase (Fuller et al. 2013). Surprisingly, and although induction of *phr*1 is lost in a Δ*lreA* mutant, mutants in the photoreceptors *lreA*, *fphA*, and a double *lreA-fphA* mutant are as resistant to UV treatment as the WT in *A. fumigatus*, suggesting that additional photoreceptors may be functioning to promote UV resistance in response to light (Fuller et al. 2013).

The molecular chaperones known as heat-shock proteins (HSPs), together with their partners, are critical components for the normal function of stress signaling pathways, particularly high temperature (Nollen and Morimoto 2002). In mammalian cells, it is known that HSP expression can be induced by UV light and is suggested to mediate protection from UV-induced cell death (Trautinger et al. 1996). HSPs interact with the MAPK pathway and show mutual regulation in response to heat shock (Dorion and Landry 2002). In *N. crassa*, a protein related to HSP30, belonging to the group of LLRGs, was found to be responsive to light (Chen et al. 2009). Similarly, the *P. blakesleeanus* HSP encoding gene *hspA* is induced by heat shock and light (Rodriguez-Romero and Corrochano 2004, 2006).

High-throughput RNAseq analyses of the response to light in *T. atroviride* showed that at least 331 genes are differentially expressed early after illumination (García-Esquivel et al. 2016). In accordance with the above-described findings, at least 39 light-regulated genes are related to different stress responses. It is of major relevance that among these genes, 17 are related to oxidative stress responses, and 8 more have been related to different types of stress. This set of genes includes key elements such as components of the MAPK cascade *p38/hog1/sty1* and the corresponding MAPKK. In addition, elements involved in the nucleotide excision repair and mismatch repair systems are induced. This is in agreement with the light induction of the UV endonuclease encoded by *uvdE-1* in *N. crassa*, *A. fumigatus*, and *A. nidulans* (Fuller et al. 2013). Other effects of light on transcriptional activation are directed to genes encoding proteins that participate in protein folding (including two *hsp20*) and ubiquitination, as well as vesicle and protein transport. The induction of genes involved in protein folding could also be interpreted as a stress response, with the cell verifying that its proteins are working properly (García-Esquivel et al. 2016). Similarly, in a recent proteome analysis of the response to light of short wavelength (450 nm) in *Penicillium verrucosum*, most induced protein accumulation corresponded to proteins involved in response to stress (e.g., antioxidative proteins, heat-shock proteins) and general metabolic processes (Stoll et al. 2014).

In *A. fumigatus* exposure to light results in enhanced resistance to acute ultraviolet and oxidative stresses and an increased susceptibility to cell wall perturbation (Fuller et al. 2013). In this sense, cells possess stress-activated protein kinase (SAPKs) signaling pathways, which are activated practically in response to any cellular insult, regulating responses for survival and adaptation to harmful environment changes. In accordance, in *T. atroviride*, it was recently found that mutants lacking the MAPKK Pbs2 and the MAPK Tmk3 are highly sensitive to different cellular insults such as osmotic and oxidative stress, cell wall damage, high temperature, and UV irradiation. Interestingly, the function of this pathway was more evident in Δ*pbs2* and Δ*tmk3* mutant strains when combining oxidative stress or cell wall damage with exposure to light. Light was also found to stimulate tolerance to osmotic stress through Tmk3, suggesting the interaction of Tmk3 with the Blr complex (Esquivel-Naranjo et al. 2016; Fig. 3.4). Similar results have been reported for *A. fumigatus* upon exposure to the cell wall perturbing agent Congo red, where Δ*fphA* and Δ*lreA*Δ*fphA* mutants were more susceptible than the WT when grown under illumination, despite the finding that red light did not impact the susceptibility of the organism (Fuller et al. 2013).

In *Beauveria bassiana*, it was recently found that the putative phytochrome (*Bbphy)* acted not only as a photosensor essential for asexual development but also as a regulator of the fungal responses to nutritional, osmotic, oxidative, thermal, and UV-B stresses. The decreases observed in stress tolerance in a Δ*Bbphy* mutant were accompanied with downregulation of phosphorylation of the MAPK Hog1. Bbphy controls conidiation by responding to daylight length and red/far-red light and regulates multistress responses perhaps because of an involvement in the Hog1 pathway (Qiu et al. 2014).

VIII. Secondary Metabolism

Perhaps one of the best-studied cases of secondary metabolism (SM) is that of mycotoxin production, given its relevance to man as either a source of antibiotics or because of their potential harmful effects on animals, including humans. It was recently found that light of varying wavelength influences ochratoxin A biosynthesis in several species of *Aspergillus* (*A. carbonarius*, *A. niger*, *A. steynii*) and *Penicillium* (*P. nordicum* and *P. verrucosum*). In general the *Aspergilli* seem to be more resistant to light treatment than the *Penicillia*. Interestingly, both red and blue light have strong inhibitory effects on growth and ochratoxin A biosynthesis, although blue light generally had

a stronger effect. Depending on the intensity, complete cessation of growth and/or inhibition of ochratoxin A biosynthesis was observed. Interestingly, exposure to light has the opposite effect on ochratoxin A and citrinin production, two mycotoxins, which can be produced simultaneously in *P. verrucosum*. Citrinin was produced essentially under conditions that inhibited ochratoxin A biosynthesis. Similarly, a derivative of ochratoxin β in *A. carbonarius* was produced in higher amounts under blue light, conditions in which the production of ochratoxin A ceased (Fanelli et al. 2012a; Schmidt-Heydt et al. 2011).

In *Fusarium verticillioides*, wavelengths across the visible spectrum, from red to blue, stimulate growth and fumonisin production, by up to 150 % as compared to the levels observed in the dark. However, high blue-light intensities reduced fumonisin biosynthesis. Pulses of white light had no effect on growth but reduced fumonisin production to half of that observed in the dark. Analysis of gene expression showed a good correlation between the mRNA levels of *fum1*, *fum21*, and *Fvve1*, which encode proteins involved in fumonisin biosynthesis (Fanelli et al. 2012b).

As described earlier in this chapter, fungal development is strongly controlled by light, and an important correlation has been found between the developmental stage of a fungus and its capacity to produce secondary metabolites. Such is the case of *Alternaria alternata*, a phytopathogen that produces more than 60 different secondary metabolites and causes considerable loss of economically important crops for feed and food worldwide. Among the most important mycotoxins produced by this fungus are alternariol (AOH) and altertoxin (ATX). In this case, mycotoxin production and spore formation are regulated by light in opposite ways. Whereas spore formation is greatly inhibited by blue light, the production of AOH is stimulated two- to threefold, and ATX production is strictly dependent on it. Deletion of the orthologue of the *N. crassa* white collar 1 (*wc-1*) gene (*lreA*) results in derepression of spore formation and ATX formation, regardless of the light conditions, suggesting a repressing function of LreA, which appears to be released in the wild type after blue-light exposure. Additionally, light induction of AOH formation was partially dependent on LreA, which suggests also an activating function (Pruß et al. 2014). Similarly, in deletion mutants in the white collar orthologues (Fgwc-1 and Fgwc-2) of *Fusarium graminearum*, conidiation was derepressed in constant light and impaired early onset of carotenogenesis, photoreactivation, and the maturation of perithecia (Kim et al. 2014). However, in contrast with *A. alternata*, it was not derepressed in darkness, and individual mutants produced more aurofusarin and trichothecenes than the wild-type strain in both constant light and darkness (Kim et al. 2014).

A significant breakthrough in fungal biology was the discovery of the heterotrimeric VelB/VeA/LaeA transcriptional complex (known as the Velvet complex) which connects secondary metabolism (SM) with light signaling and development (Bayram et al. 2008a). The founding member of this complex is velvet (VeA), discovered in a *veA* mutant, which was described more than 40 years ago. VeA and the other subunits of the complex are highly conserved in several fungi (Myung et al. 2012). To coordinate SM biosynthesis with fungal development, different members of the velvet protein family partner with each other (reviewed in Bayram and Braus 2012). However, the way in which Velvet controls SM production is still not fully understood.

Because many genes for the synthesis of SMs are found in clusters in fungal genomes (Keller and Hohn 1997), regulation of SM genes can be in part explained by transcriptional control through hierarchical levels of transcriptional regulatory elements including cluster-specific regulatory elements, global regulators, and transcriptional complexes like Velvet (reviewed in Yin and Keller 2011). In *A. nidulans*, the methyltransferase-domain protein LaeA apparently links light regulation to chromatin modification. In the dark, VeA forms a heterotrimeric complex with VelB and the global regulator of secondary metabolism LaeA (Bayram et al. 2008a; Bok and Keller 2004). A third velvet protein (VosA) also interacts in the dark with VelB, and it has been

proposed that this heterodimer can repress conidia formation as well as control spore maturation and trehalose biogenesis (Ni and Yu 2007; Bayram et al. 2010). Shuffling of VelB between the VelB-VosA and VeA-LaeA complexes is determined by the LaeA protein, which plays a fundamental role in the regulation of secondary metabolism and development in *A. nidulans* (Bayram et al. 2010). The function of the fourth member of this family (VelC) is much less understood (Bayram and Braus 2012). The trimeric velvet complex might directly perceive the light signal to coordinate secondary metabolism and development. In this sense, it has been proven that VeA can physically interact in the nucleus with the red-light phytochrome receptor FphA, which is also associated with the blue-light receptors LreA and LreB (Purschwitz et al. 2008; Bayram et al. 2010).

The *A. nidulans laeA* mutant does not express AflR, the transcriptional activator that controls the secondary metabolite cluster for the production of the aflatoxin precursor sterigmatocystin. LaeA is not only required for sterigmatocystin and penicillin biosynthesis but also for lovastatin, an important compound used to lower cholesterol to prevent cardiovascular diseases (Bok and Keller 2004). Similarly, *laeA* homologues of other fungi are involved in the control of secondary metabolism.

A velvet complex was recently detected in the filamentous fungus *Penicillium chrysogenum*, the main industrial producer of penicillin. The core components of this complex are PcVelA and PcLaeA, which in addition to secondary metabolite production regulate hyphal morphology, conidiation, and pellet formation. As in *A. nidulans*, other subunits of the velvet complex have been found, such as PcVelB, PcVelC, and PcVosA. Functional analyses using single- and double-knockout strains served to determine that the velvet subunits have opposing roles in the regulation of penicillin biosynthesis. PcVelC, together with PcVelA and PcLaeA, activates penicillin biosynthesis, while PcVelB represses it (Kopke et al. 2013). The phytopathogen *F. fujikuroi* also requires LaeA for gibberellin production (Hoff et al. 2010; Wiemann et al. 2010).

In several fungi, a regulatory connection between sporulation and the production of secondary metabolites has been reported (Calvo et al. 2002). The fact that in *Trichoderma* Δ*blr*1 and Δ*blr*2 strains do not conidiate in response to light allowed hypothesizing that they are altered in secondary metabolism and synthesis of peptaibols. Peptaibols are a family of short peptides ($\leq$20 residues), synthesized by non-ribosomal peptide synthetases (NRPS). Naturally occurring peptaibols are isolated from soil fungi and often exhibit antibacterial and antifungal activities. There are over 300 annotated sequences of non-ribosomal peptides (Whitmore and Wallace 2004), and the list is continuously increasing since a single NRPS can produce up to three distinct peptaibols. Neuhof et al. (2007) analyzed 28 phylogenetically related *Trichoderma* strains, identifying 48 different classes of peptaibols.

Recently, it was determined by mass spectrometry that *T. atroviride* did not produce peptaibols during vegetative growth; however, the production of peptaibols was evident when the fungus initiated conidiation. Therefore, different stimuli that induce conidiation were tested to determine if in all cases conidiation and production of peptaibols were intimately correlated. Interestingly, conidiation induced by light promoted high production of peptaibols, whereas in the dark it was observed in minimum quantities. Light-associated synthesis of peptaibols was dependent on *blr*1 and *blr*2 products. As expected, no conidiation was observed in the *blr*1 and *blr*2 mutants. On the other hand, carbon deprivation also induces the production of both peptaibols and conidia, independent of *blr*1 and 2, hence light independent. These data are apparently contradictory, since it was reported that the carbon deprivation signal is *blr*1 and 2 dependent; however, such dependence is only observed when cultures are subject to sudden carbon deprivation (Casas-Flores et al. 2006). Mechanical injury of mycelia also triggered peptaibol production; however, it depended completely on light stimulation, and no peptaibol production was observed in the absence of light, in spite of sporulation. These discoveries were also

observed in *blr* mutants. Together these results indicate the existence of a Blr-independent pathway for peptaibol production stimulated by light (Komon-Zelazowska et al. 2007).

Recently, it was shown that mycelia of *T. virens* wild-type strain (Gv29.8) exposed to light exhibited a slight increase in the expression of *veA* transcript (Mukherjee and Kenerley 2010). Mutants in *T. virens veA* are defective in induction of genes that encode secondary metabolism enzymes and showed a null phenotype in the synthesis of gliotoxin. Lack of gliotoxin correlates with low expression levels of *gliP*, the NRPS encoding gene responsible for gliotoxin production in *A. nidulans*. In *A. nidulans* VeA interacts with the phytochrome-white collar light regulator complex (FphA-LreA-LreB) and bridges VelB and LaeA to form the velvet complex (VelB/VeA/LaeA). The latter complex coordinates light signal with fungal development and secondary metabolism (Bayram et al. 2008b; Bayram et al. 2010). Furthermore, mutants in the *wcoA* gene, the *blr*1 orthologue in *Fusarium fujikuroi*, sustained carotenoid synthesis in response to light. On the contrary, production of fusarin showed a drastic reduction in the light and less synthesis of gibberellins and more bikaverins when mycelia were growing under nitrogen-limiting conditions. These results indicate that *blr*1 orthologous genes play pivotal roles in secondary metabolism in *F. fujikuroi*. It is noteworthy that *F. fujikuroi* does not sporulate when exposed to light; on the contrary, this stimulus seems to have a repressing role on conidiation in this fungus. However, Δ*wcoA* showed reduced levels of conidiation on minimal agar when compared with the WT strain (Estrada and Avalos 2008). Together, these results indicate an important role of WcoA in nitrogen-regulated processes, as well as for gibberellin and bikaverin synthesis in a light-independent pathway.

The available data indicate notable differences in the use of VeA and Blr1 and its partner in secondary metabolism. Further studies on single, double, and triple mutants as well as the demonstration of the interactions between the different components of VelB/VeA/LaeA and phytochrome-white collar complexes in different fungi will allow us to understand the complexity in regulation by light in secondary metabolism.

IX. Impact on Pathogenicity

Although the effects of light on pathogenicity of some fungi have been known for quite some time, the molecular basis of this effect is still only poorly understood. Light may directly or indirectly influence disease development through modulation of sporulation, motility, adhesion, toxin biosynthesis or activation, or host defense responses (Idnurm and Crosson 2009). An example of this is how the photoperiod—comparable with the length of a day—influences the infectivity of the insect pathogen *P. fumosoroseus* (Avery et al. 2004), where increasing the duration of the light phase results in an increase in colonization of whitefly nymphal hosts by blastospores of *P. fumosoroseus*. Furthermore, the well-known effects of light on secondary metabolism in *Aspergillus* spp. and many other fungi (Fox and Howlett 2008; Yu and Keller 2005) clearly indicate the importance of light in pathogenicity.

In *F. oxysporum*, knockout mutants lacking the putative photoreceptor Wc1 used in infection experiments with tomato plants and immunodepressed mice revealed that Wc1 is dispensable for pathogenicity on plants but required for full virulence on mammals (Ruiz-Roldan et al. 2008). The injection of microconidia of the wild-type or the Δ*wc1*+*wc1* strain into immunodepressed mice resulted in 65 % and 80 % mortality, respectively. By contrast, infection with the Δ*wc1* mutant produced only 20 % mortality rate, although during the infection of mice, the fungus remains in the dark. Similar results had previously been reported for mutants in the orthologous gene (Bwc1) of the human pathogen *C. neoformans*, which, although not essential for virulence, significantly contributes to disease severity (Idnurm and Heitman 2005).

As mentioned above, the germ tubes of *B. cinerea* show negative phototropism to near-UV and blue light followed by far red,

whereas red light induces positive phototropism. Exposure to near-UV and blue light also promotes the formation of infection-hyphae on onion and broad bean, whereas red light suppresses it, resulting in a high proportion of germ tubes without infection hyphae. In broad bean leaf infection, the number of infection points and area of necrosis are higher under near-UV-blue light than that of a dark control. Conversely, lower numbers of infection points and very small necrotic lesions develop under red light (Islam et al. 1998). In this sense, the role of the *B. cinerea* phytochrome like (Bcphy3) in pathogenesis was recently addressed on wounded capsicum, tomato, grape berry, cucumber, carrot, and intact lettuce. The wild-type strain caused obvious radial lesions 2–6 days postinoculation. In contrast, the invasion symptoms produced by the corresponding mutant were drastically weakened on different host plants, evidently suggesting the involvement of Bcphy3 in pathogenicity (Hu et al. 2014). Nevertheless, the authors of the mentioned report suggest that the disruption of the gene may affect the growth rate and general chitin synthesis of the cell wall, both of which in turn increased the susceptibility of the mutant to plant defense responses.

Recently, by a random mutagenesis approach, a novel virulence-related gene encoding a GATA transcription factor (BcLTF1 for light-responsive TF1), which expression is strongly induced by light, was identified (Schumacher et al. 2014). This transcription factor has homologues previously characterized in *A. nidulans* (NsdD) and *N. crassa* (SUB-1). By deletion and overexpression of *bcltf1*, it was confirmed that the transcription factor plays a role in virulence. The results obtained by the authors indicate that BcLTF1 is required to cope with oxidative stress that is caused either by exposure to light or arising during host infection. BcLTF1 is an important modulator of the transcriptional responses to light influencing the expression of most light-responsive genes in *B. cinerea*. While deletion of *bcltf1* mainly affects advanced stages of infection, its overexpression impairs penetration by germ tubes (Schumacher et al. 2014).

In addition to the possible direct involvement of photoreceptors in pathogenicity and the light control of secondary metabolism by members of the velvet family, genes belonging to the latter have been found to be important for conversion of *Histoplasma capsulatum* from the filamentous form typically found in the soil to the pathogenic yeast found inside macrophages, although it is not known whether the function of Velvet in this fungus involves a response to light (Webster and Sil 2008).

Exposure of pepper plants to *Colletotrichum acutatum*, the causative agent of anthracnose, under white, blue, red, and green light resulted in larger lesion size than when incubated in the dark (Yu et al. 2013). On the contrary, inoculation of rice plants with *Magnaporthe oryzae* under light conditions suppressed the infectious process, and such suppression was clearly dependent on the WC-1 orthologs (Kim et al. 2011). Canessa et al. (2013) demonstrated that BcWCL1, the putative blue-light photoreceptor of *B. cinerea*, plays an important role during plant infection in the presence of light. *Arabidopsis thaliana* and *Phaseolus vulgaris* plants inoculated with a Δ*bcwcl1* mutant showed reduced lesion sizes, with a more dramatic effect under constant light than in photocycles. Interestingly, it was found that excessive light impairs the growth of the mutant due to the generation of ROS, indicating that the mutant is affected in its capacity to cope with ROS produced by the plant within the extent of an oxidative burst, hampering the ability of the fungus to colonize the plant tissue (Canessa et al. 2013).

X. Relevance in Biotechnological Applications

Cellulose is the main component of plant biomass and it is associated mainly with hemicelluloses, lignin, and other components such as proteins, fats, waxes, terpenes, phenols, alcohols and alkanes, which together form the complex and rigid structure of the cell walls. The filamentous fungus *T. reesei* has been considered one of the most prolific cellulase produ-

cers in industry, since it could be used for conversion of cellulosic waste material into glucose (Buchert et al. 1998; Galante et al. 1998). It was thought that the cellulase enzyme complex of *T. reesei* consisted of at least three types of enzymes to convert crystalline cellulose to glucose (Lynd et al. 2002). However, the genome of this fungus contains a lower number of cellulolytic enzyme-encoding genes than any other sequenced fungus able to hydrolyze plant cell wall polysaccharides, including cellulases, hemicellulases, and pectinases (Martinez et al. 2008). The *T. reesei* genome encodes two cellobiohydrolases, four endo-β-1,4-glucanases, and several β-glucosidases, hemicellulases, and accessory enzymes (Häkkinen et al. 2012). As briefly mentioned in the section on metabolism, cellulase gene expression in *T. reesei* is potentiated by light, and Env1 is involved in the light-dependent transcriptional regulation of the cellobiohydrolase I-encoding gene (*cbh1*). Intriguingly, light does not lead to cellulase gene expression in the absence of an inducer (Schmoll et al. 2005).

A positive role of the Blr1 and Blr2 proteins (the *Trichoderma* spp. putative photoreceptor complex) on cellulase gene transcription was established, since mutants in *blr* genes showed noninducible cellulase gene transcription in response to light (Castellanos et al. 2010). In *T. reesei* the induction of endoglucanase activity by sophorose was stimulated by dBcAMP, as well as by IBMX. On the other hand, the addition of exogenous dBcAMP or IBMX did not induce endoglucanase activity (Šesták and Farkaš 1993). The exposure of *T. viride* to a blue-light pulse led to a transient increase in the intracellular level of cAMP (Gresik et al. 1988). Together, these results point to the participation of Pka through phosphorylation of transcription factors in the synthesis of cellulases. In this regard, in *T. reesei*, cellulase gene transcription is not only exerted by cellulase but also by lactose. Contrasting with cellulase gene transcription of *T. reesei* growing in cellulose, a considerable increase in transcript abundance of cellulase genes in darkness was determined compared to light upon growth on lactose. This effect was considerably enhanced in a mutant lacking the cAMP-dependent protein kinase A (PkaC1) or the adenylate cyclase (Acy1) encoding genes. Acy1 exerts a positive effect on cellulase gene expression in light and darkness compared to the wild type. PkaC1 stimulates the accumulation of mRNA of cellulase encoding genes in light but exerts a negative regulation in darkness. Transcriptional analysis of cellulase regulator genes showed that the regulatory output of the cAMP pathway might be settled by the regulation of Xyr1 abundance, which is an essential transcription factor for cellulase gene expression. These data point to a role of adenylate cyclase and protein kinase A in light-modulated cellulase gene expression in this fungus (Schuster et al. 2012). Additionally, *T. reesei* strains lacking the *env1* gene present reduced intracellular levels of cAMP (Tisch et al. 2011), which exert a considerable light-dependent influence on transcription of the major cellulase gene cbh1/cel7a. It was also demonstrated that Gna3 strongly regulates cAMP levels, whereas Gna1 has only moderate influence on them (Schmoll et al. 2009; Seibel et al. 2009). Therefore, Env1 links the light response to carbon source signaling, probably through heterotrimeric G-protein cascades by modulating the levels of *gna1* and *gna3* messengers provoking effects on the cAMP levels.

In *T. reesei* the expression of cellulases and hemicellulases is dependent on the function of the methyltransferase Lae1. Furthermore, it was demonstrated that this regulation is dependent on *xyr1* and that *xyr1* overexpression does not rescue the *lae1* deletion phenotype (Seiboth et al. 2012). On the other hand, a genome-wide analysis of H3K4 and H3K9 methylation patterns in *lae1* mutants did not show any methylation changes at the cellulase loci (Karimi-Aghcheh et al. 2013), which contravene with the hypothesis that LaeA plays a role removing the repressive chromatin (Reyes-Dominguez et al. 2010). In *T. reesei* *vel1* mRNA expression is regulated by both, light and darkness, and Vel1 is necessary for cellulase gene expression. This is in agreement with the hypothesis that the regulation of cellu-

lase gene expression by Lae1 takes place through the Velvet complex (Karimi-Aghcheh et al. 2014).

Analysis of the cellulolytic activity of *N. crassa* wild-type Δ*wc-1*, Δ*wc-2*, and Δ*vvd* mutants points to a role of WC-1 and WC-2 in promoting cellulose utilization, given that the Δ*wc-1* and Δ*wc-2* strains showed the lowest activity among all tested strains. In contrast, VVD appears to antagonize cellulose utilization, since a Δ*vvd* mutant presented higher endoglucanase activity than the wild type. Transcription analysis of the *N. crassa cel7a* and *cel6a* homologues to *T. reesei cbh1* and *cbh2*, respectively, presented significantly lower expression levels in the Δ*wc-1* and Δ*wc-2* mutants, whereas their expression levels in Δ*vvd* were comparable to those of the wild-type strain. Transcriptomic analysis of *wc* mutants showed significant downregulation of the major predicted cellulase and hemicellulase genes in this fungus. On the other hand, the Δ*vvd* strain showed an enrichment in carbohydrate metabolism genes, which explains the enhanced cellulose degradation by the Δ*vvd* strain. The transcriptional analysis on predicted cellulolytic showed that in addition to *cel7a* and *cel6a*, six other cellulolytic genes were downregulated in the Δ*wc-2* mutant, whereas the Δ*vvd* strain had increased and extended expression levels of genes regulated by the WCC, including those encoding cellulolytic enzymes. Furthermore, all three photoreceptors showed a negative effect on transcription of the carbon metabolism regulator encoding gene *cre*-1, which point to their regulatory contribution to carbon catabolite repression (CCR) and not only subject to photoadaptation. However, optimal expression of genes encoding plant cell wall-degrading enzymes and cellulolytic activity and release from CCR strictly require the presence of inducing molecules. These data point to a role of the WCC in regulating genes involved in plant cell wall degradation, probably through the activation of a WCC-dependent transcription factor. Together, the data derived from the analysis on *T. reesei* and *N. crassa* indicate that they use common mechanisms for the control of enzymes involved in plant cell wall and cellulose degradation, which is most probably also conserved in other ascomycete species (Schmoll et al. 2012).

XI. Concluding Remarks

Fungi are central to the global carbon cycle, constitute the major group of plant pathogens in managed and natural ecosystems, and serve as symbionts with heterotrophic and autotrophic organisms alike. The ecological success and importance of fungi to life can certainly be attributed to the large diversity of enzymes and metabolites that they produce, which allows them to access a large diversity of carbon sources and ecological niches. On the other hand, the influence of light on their growth, reproduction, and metabolism has been known for a long time. Thus, it is only logic to expect that such strong influence of light be reflected in their ecological behavior, consequently impacting life on earth.

It is clear now that any defect in their ability to perceive light represents an ecological disadvantage regardless of their habitat, as light is a cue that alerts them of the conditions of an ever-changing environment.

During the last 20 years, but particularly in the last decade, significant advances have been made toward understanding light perception and the molecular events triggered by this environmental cue. Nevertheless, we are still far from elucidating the unexpectedly complex network involved in all light responses observed. Most efforts have been focused on understanding blue-light responses through what is perhaps the main blue-light perception system.

The advent of omics technologies (genomics, transcriptomics, proteomics, metabolomics, etc.) has allowed us to discover new players in the physiology of fungi and to construct metabolic and regulatory pathways when interacting with sunlight. Until today, over 100 fungal genomes have been sequenced, including human and plant pathogens (https://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/fungal-genomics). In

addition, the transcriptomes, proteomes, and metabolomes of several fungi under different conditions have been obtained. These data together will lead us to a better understanding of why and how sunlight provokes detrimental effects in some fungi, whereas in other cases it has beneficial effects. Importantly, in addition to the classical models such as *N. crassa* and *A. nidulans*, other fungi of industrial, economical, medical, and agronomical importance are now being studied, thanks to these new technologies. Further, the discovery of new players in the physiology of fungi in response to light will allows us to exploit more efficiently the capacity of these organisms, to produce antibiotics, and to improve the biosynthesis of pigments, flavors, and aromas, among other important products.

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4 Disturbance in Natural Ecosystems: Scaling from Fungal Diversity to Ecosystem Functioning

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CONTENTS

I. Introduction

Disturbance is an ecosystem-level phenomenon that determines biotic and abiotic characteristics of a system. As defined by Pickett and White (1985), "a *disturbance* is any relatively discrete event in time that disrupts ecosystem, community or population structure and changes resources, substrate availability, or the physical environment." The breadth of this definition highlights the importance of scale and process, which are the components that make each disturbance unique and are therefore required to understand the response of organisms to disturbance.

Fungi, by their physiology, structure, and life history, are different from many other organisms on the planet. These characteristics can make fungi both sensitive and resilient in the face of disturbance. Even within the group of organisms currently identified as fungi, there is great diversity in terms of substrate need, habitat use, and functional roles within the community. At the global scale, patterns of fungal diversity appear to be strongly determined by climatic factors, followed by edaphic and spatial patterning (Tedersoo et al. 2014). Only mycorrhizal species composition was strongly associated with plant species composition. Disturbances alter fungal community composition through changes in vegetation, substrate, environment, and a number of other components characteristic of the specific ecosystem in which the fungal community is located. However, the responses of fungal species to disturbances will be different across functional groups.

Fungi play a number of important functional roles in ecosystem dynamics. Focusing specifically on soils, fungi are involved in soil food web dynamics as predators, prey, and decomposers (Coleman et al. 2004).

Fungi are directly involved in and alter competitive relationships. As mediators of nutrient availability, mutualistic fungi impact competitive relationships for the organisms with which they interact through more

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efficient nutrient capture and by expanding the resources available to plants (Hart et al. 2003). As pathogens, fungi alter vigor and reproduction of other organisms which are very important for maintaining biodiversity and mediating competition (Winder and Shamoun 2006; Hodge and Fitter 2013). As saprophytes, fungi are the primary decomposers of substrates and are essential for recycling nutrients to plants (van der Wal et al. 2013). Disturbance at any scale has the potential to alter the biomass or reproductive activities of fungi, and can affect the diversity of fungi in ecosystems. Population or community dynamics of the fungi involved in these activities will be altered to differing degrees based on physical location of organisms in the ecosystem and type of disturbance (Snyder et al. 2002). With the differences in characteristics of these functional groups in mind, we will attempt to discuss fungi in general with examples from each of these groups. Unfortunately, there is greater information available about how disturbance alters some of these groups than others. This constrains our conclusions here, but more importantly, limits our general understanding of the impacts of disturbance on fungi. It certainly brings to the forefront the necessity to understand the response of these groups of organisms to disturbance and to understand their impacts on ecosystems.

Fungi have unique physiology, morphology, and reproductive biology. This makes attempts to describe their dynamics difficult in the rather conventional terms used for higher plants and animals. Further, Taylor et al. (2014) provide data that suggest our current estimates of 0.6–1.5 million species of fungi may significantly underestimate the true number. Their estimate is closer to six million species of fungi. This would suggest that our current understanding of fungi in soil is woefully inadequate. The major limitations studying fungi are their microscopic size and a marginal understanding of their life history strategies. Recent uses of molecular techniques for evaluating fungal communities have allowed us to move a long way toward improving understanding of the needs and existence of fungi in ecosystems, especially compared to evaluation through direct observation using culture techniques and microscopes. However, we have not yet been able to determine aspects of biology important for studying these organisms, such as cues involved in sporulation, germination characteristics of spores, sensitivity of hyphae vs. spores to physical or chemical change, etc. These limitations in methodology pose large challenges for those characterizing fungal structure and function in response to any environmental change. This is especially problematic because differences in life history strategies among fungal species may mask their response to environmental change, altering the interpretation of presence-absence data depending on the techniques employed (Brundrett and Ashwath 2013).

Fungi, because of the size of individual hyphae or yeast cells, and because they are predominantly studied in the laboratory under a microscope, are viewed as microorganisms that primarily respond to minute quantities of substrates in their environment. In mass and spatial extent, fungi can be macroorganisms, often extending across large patches. These patches may be rather static in space, existing over long time periods, such as the matforming ectomycorrhizal (ECM) fungi or the *Armillaria* mycelial networks (Allen et al. 1995). However, because these large organisms are still made up of microscopic hyphae with the potential to function independently, detailing their life-history characteristics means understanding both the macroscopic and microscopic aspects of their existence. As a result of this dichotomy in the structure of fungal "individuals," any disturbance, from an individual gopher mound to a volcano, has important ramifications to the types of organisms and roles they play in ecosystem dynamics. For example, a large disturbance such as a volcanic eruption can destroy the entire fungal community, requiring subsequent reinvasion and establishment subject to classical models of succession (Allen et al. 1992, 2005b). In contrast, disturbances such as mound building by gophers, or ants can disrupt mycelial networks within multispecies patches and within "individual" fungal types. These scenarios allow for some recolonization from existing species should the mycelial network rebuild from the remnant hyphal fragments. Each of these disturbance extremes directly and indirectly regulate the composition of the plant community and, in turn, the animal community. It is this range in the activity that we will describe. Rather than just providing examples, we will develop a set of conceptual models that will allow us to begin to link our understanding of fungal biodiversity and ecosystem functioning.

II. Ecosystem Disturbance: A Conceptual Framework

It has long been a goal of ecologists and mycologists to understand the factors influencing the diversity, distribution, and abundance of

organisms in a community. Disturbance can fundamentally alter these characteristics of fungal communities; however, the mechanism by which disturbance impacts any organism is a consequence of the intensity and frequency of the disturbance. The effect of a disturbance on a community depends on how far the disturbance shifts the community from equilibrium or how often it recurs compared to the life span of the organisms. For example, disturbance has been proposed to influence community diversity and structure through a variety of mechanisms. Small-scale disturbances such as burrowing animals or insects can alter resource type or availability. A single disturbance type, e.g., gopher mounds, can enhance (Allen et al. 1984, 1992) or retard (Koide and Mooney 1987) the rate of succession by altering soil resources or species diversity. At even larger scales, such as that of plowing, soil disturbance was found to alter mycorrhizal community dynamics more than host plant identity (Schnoor et al. 2011). Disturbances may also affect communities by creating new sites for colonization (Platt 1975; Collins 1987) or creating new substrates (Cooke and Rayner 1984; Gams 1992). Disturbances that decrease resources for one functional group of fungi may result in an increase of resources for other functional groups of fungi (Lindahl et al. 2010; Xiong et al. 2014). For example, disturbances that decrease photosynthate available for mycorrhizae will provide increased substrate for saprophytes as hyphal networks become available for decomposition. Ultimately, the degree to which any disturbance type modifies the quantity or quality of a substrate on which a fungal community exists will determine the extent to which the community will change. These modifications can include alterations to substrate chemistry (e.g., differing litter types) or to the environmental factors under which those substrates can exist, such as changes in soil moisture content with removal of the litter layer. If disturbances differentially affect substrate and environment, then disturbances caused by a variety of agents will alter fungal species diversity within a community in different ways.

We have developed a conceptual model of natural disturbance that focuses on processes that scale from patch to ecosystem-level processes (Fig. 4.1). Our overview includes elements of these disturbance scales and the factors that affect the various states of the disturbance cycle. The conceptual model integrates processes operating at the level of the patch disturbance, such as soil enrichment, with those at the ecosystem level, such as spatial variation in natural disturbance density (e.g., fire, animal digging), soils, and microbial and plant community structure (Fig. 4.1). We believe that emphasis on the feedback between patch dynamics and local ecosystem processes is an important key to increasing our understanding of the role of natural disturbance in fungal community structure and ecosystem function.

III. Fungal Community Dynamics: A Natural Disturbance Model for Fungi

Communities are characterized by all of the interacting species that occur within a given area. The area is determined by the extent of those interactions. Fungal communities can involve organisms that span many functional roles. To adequately describe the impacts of disturbance on fungal community dynamics, a disturbance model would need to integrate understandings of structural and functional impacts to fungi across spatial and temporal scales.

A. Species Diversity

A natural disturbance model that evaluates fungi must examine species diversity in the context of the concomitant impacts on ecosystem dynamics. Diversity within a given system is characterized by richness and evenness. However, it is virtually impossible to delineate evenness due to the microscopic size of the individual organisms.

Regardless of the disturbance size, fungal biomass, and therefore evenness and diversity will be altered. More problematic is richness. The size of the organism matters here. If a fungus occupies tens to hundreds of m^2,

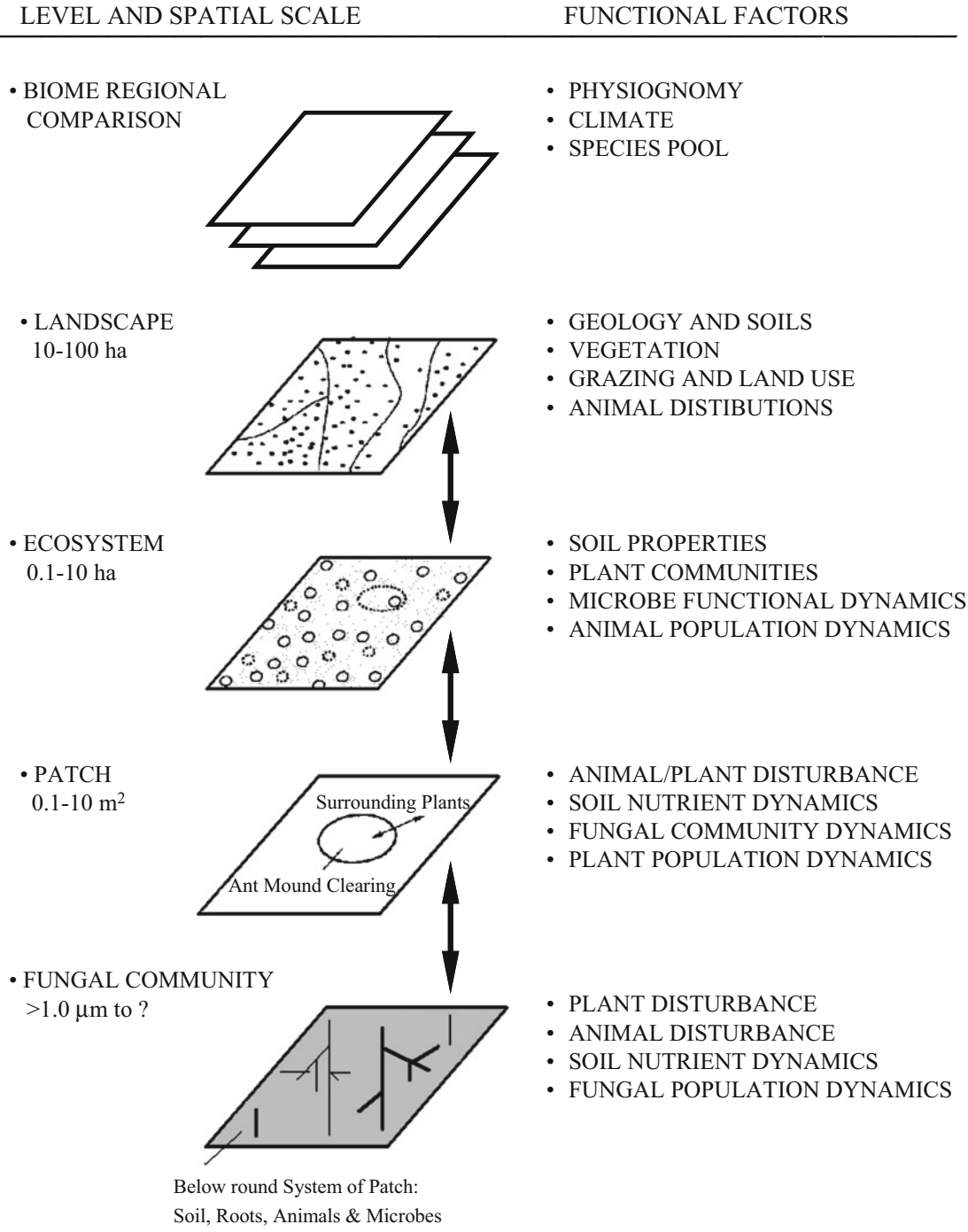

Fig. 4.1 Conceptual framework illustrating how ecological concepts of hierarchy and scale can be used to integrate and model the relationship of small-scale phenomenon, such as the impact of disturbance on microbial functional groups, to larger-scale spatial patterns and processes (e.g., ecosystem dynamics) (Friese et al. 1997)

then richness might not be affected by a small disturbance. However, individual yeast and microfungi could well be eliminated. How these diversity elements alter specific ecosystem characteristics is dependent on the disturbance event itself, the substrates that exist following the disturbance, the subsequent microclimate and its effects on substrate, and the surrounding organisms. A disturbance event directly alters the fungal community by destroying hyphae and propagules of existing species, although impacts of any disturbance will be modified by the intensity of the disturbance and the seasonality of the event (Gochenaur 1981). Understanding how these modifications regulate outcomes is important for predicting the impacts of disturbance on communities. Many recent studies have identified legacy effects wherein the long-term effect of the disturbance is moderated by the specific circumstances of the disturbances such that there is a unique response from the community of interest. Legacy effects, defined by Wurst and Ohgushi (2015) as "effects that persist after the biotic interaction that caused the effect ceases", occur when the disturbance differentially removes or impacts the pre-disturbance biotic community. Such was the case following the volcanic eruption at Mt. St. Helens wherein the existence of snowpack protected some of the plant community from initial ash fall but not others areas causing significant differences in disturbance severity and differences in recovery trajectories for plant and soil communities. (Crisafulli et al. 2005)

An additional dilemma in determining the effects of disturbance at the patch or ecosystem scale is determining the extent of a fungal community (Cooke and Rayner 1984). These effects can be studied if the interpretation that communities are made of individuals with definable sets of species interactions (MacMahon et al. 1978) is used to define our concept of community. Fungal communities are comprised of organisms with different functional roles (mutualist, saprophyte, pathogen), and these functional groups or guilds may have different responses to the same disturbance (Lindahl et al. 2010; Šťursová et al. 2014; Xiong et al. 2014). Large-scale disturbances can alter an entire landscape (Turner and Dale 1998), while small-scale disturbances may affect only a patch within the community. For this reason, the model developed here (Fig. 4.1) predicts the effects of disturbance within the patch, yet in some circumstances, the patch may encompass an entire community. Following disturbance, the changes within the patch may affect the community of which it was a part or may become a community of its own.

B. Disturbance Types and Characteristics

There are many types of disturbance that will affect a fungal community. In the fungal literature, disturbances have been broken into two main groups, enrichment and destructive disturbances (Cooke and Rayner 1984). For understanding fungal community dynamics, these classifications are often too broad because disturbances at the scale that relates to the fungal community can never be entirely enriching or entirely destructive. For example, Lindahl et al. (2010) found that the disruption of carbon to root systems from girdling in trees decreased the ECM in the humus layers; however, it increased opportunistic saprophytic fungi. Šťursová et al. (2014) found similar results following a bark beetle-induced tree die back. What was a destructive disturbance for the mycorrhizal species was an enriching disturbance for the saprophytic fungi.

Disturbances that alter plant and animal community dynamics will resonate through the fungal communities. Wind storms strip leaves and blow down canopy trees in a forest. These impacts are usually greater near the edge which decreases habitat quality especially for small stands. These wind events provide substrate for saprophytic fungi; however, they decrease host density for mycorrhizal fungi. In complicated food webs, such as those that involve truffle-eating flying squirrels, windthrow events can decrease food resources and canopy connectivity, which can decrease squirrel populations (Carey 2000; Ransome et al. 2004) ultimately decreasing squirrel dispersal of the mycorrhizal fungal spores contained in the truffles. As fungi participate in many different relationships in ecosystems, a single disturbance, regardless of size or enrichment/destructive capacity, has potential to impact fungi directly or indirectly through the organisms upon which fungi depend. Ultimately, this will feed back to alter fungal community dynamics.

C. Biotic and Abiotic Characteristics of the Disturbance Model

The greatest effect of disturbance on the fungal community will be through influences on key

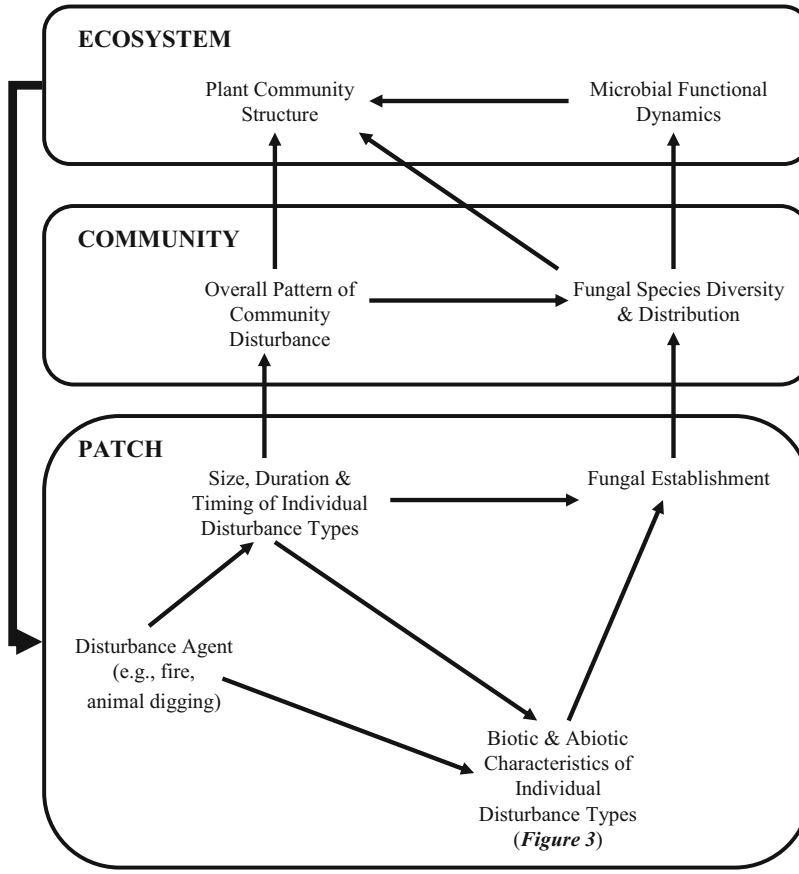

Fig. 4.2 Theoretical conceptual model depicting how patch-level disturbance processes can affect microbial functional dynamics, which, in turn, can shape the larger-scale patterns and processes of community- and ecosystem-level dynamics (Friese et al. 1997)

factors that impact the growth of fungal structures and germination of spores. These factors, denoted as biotic and abiotic characteristics in Figs. 4.2 and 4.3, include physical characteristics, resources, soil flora and fauna, and fungal propagules or hyphal fragments (Boddy 1984; Gams 1992). The physical components of the fungal community affected by disturbance include light, temperature, moisture, and pH (Gentry and Stiritz 1972; Rogers and Lavigne 1974; Mandel and Sorenson 1982). The resources include water, oxygen, organic matter, and a variety of mineral nutrients for growth and sporulation, for which some may be required in higher quantities than for vegetative growth (Moore-Landecker 1990). Another key resource included here is the physical environment within which organisms grow. For fungi in most terrestrial ecosystems, the complex system of decomposing wood and litter on the forest floor or simply litter in grassland systems is an essential part of their ecosystem. The litter and wood debris layer can provide substrate directly, or it can modify the soil quality below it.

Bässler et al. (2012) and Abrego and Salcedo (2013) found fungal diversity linked to the diversity of decaying wood types or wood quality and structural heterogeneity rather than biomass of materials available. McGuire et al. (2010) investigating the response of fungal taxa to specific substrates, identified patterns of response to substrate availability such community response to substrate availability could be predicted. However, they also found difference in substrate use

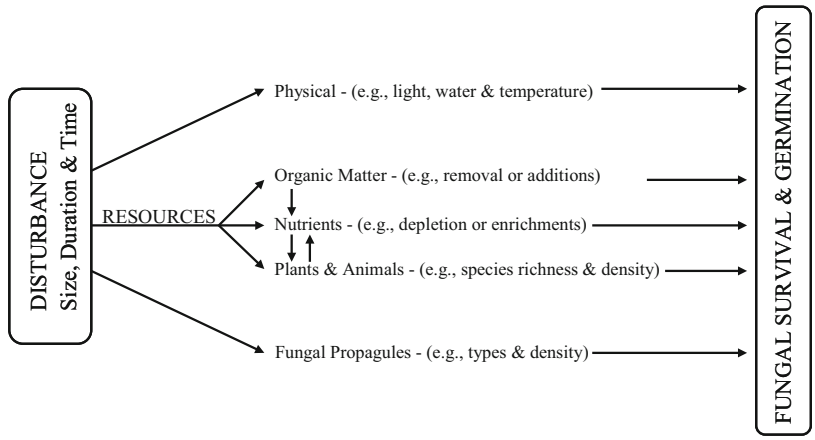

Fig. 4.3 Detailed model of the biotic and abiotic characteristics of individual disturbance types. This model is a subset of the complete conceptual model depicted in Fig. 4.2. The disturbance agent determines such aspects as the size, duration, and timing of an individual disturbance event. All of these variables create a wide diversity of disturbance patches with unique biotic and abiotic characteristics. If each disturbance type creates a unique set of biotic and abiotic characteristics, then it is hypothesized that the fungal community will also be differentially affected within each of these disturbance types (Friese et al. 1997)

profiles among individual species suggesting that loss of rare species could alter nutrient turnover. As such the forest floor composition and characteristics, and impact of disturbances on the litter layer can have severe consequences for the fungal biomass as well as diversity in terrestrial systems.

Plants and animals also regulate the composition of fungi in soils but often in a nonlinear manner. Plants can provide both energy and carbon for fungal growth, but they also provide many inhibitory substances. Many studies recently have documented impacts of plant species on belowground communities through addition or removal of tissue (Bouasria et al. 2012; Grove et al. 2012) or photosynthate and exudate release (Lindahl et al. 2010). Animals can remove (graze), disperse, provide housing, or provide substrate (defecate or die) for different members of the fungal community depending on fungal requirements. Additionally, changes in more than one of the parameters can act synergistically or antagonistically, to further change the emergent fungal community (Snyder et al. 2002).

In one case, the addition of CO_2 increased plant production, and C inputs to soil fungi. However that input was matched by the increased grazing by soil animals (Allen et al. 2005c). So, although the standing crop of fungal biomass remained the same under elevated CO_2, the total through-put actually increased. Fire can also alter fungal-animal relationships. For example, the loss of the litter layer, a physical factor and/or substrate, by environmental factors such as fire, results not only in substrate loss, but also in increases in soil temperatures and decreases water-holding capacity. As this happens, soil organisms such as mites and collembolans will migrate more deeply into the soil to escape drought, reducing grazing on fungi but also reducing fungal dispersal near the surface (Klironomos and Kendrick 1995). Thus, changes in each of the above characteristics will be dependent on the type and intensity of the disturbance and the interaction among the response variables.

The spatial structure of systems, as a consequence of environmental patterning or small-scale disturbance, has the potential to impact our ability to determine the effects of disturbance on organisms. For example, the existence of an ant mound has potential to alter the impact of a disturbance such as fire on an ecosystem. Alterations may be a consequence of different physical structures, for example, the movement of wind across the raised surface or decreased burn heat as a consequence of increased mound moisture. The difficulty is separating the impacts of fire on the system from the impacts of prefire small-scale disturbance. Failure to detect the specific impacts of fire may be the

result of sampling a mound without incorporating the specific characteristics of a mound. Boerner et al. (2000) found that enzyme dynamics related to bacterial and fungal activity in single-tree influence circles (see Zinke 1962) were altered by fire. Detecting changes in fungal dynamics following disturbance requires understanding of pre-disturbance spatial dynamics and scale-appropriate post-disturbance sampling efforts. Failure to understand the impacts of patch-level disturbances and other agents capable of creating spatial structure in fungal communities will decrease likelihood of detecting post-disturbance impacts.

IV. Scaling from Patch to Catastrophic Disturbance

Disturbances impact fungi at a number of levels. As discussed earlier, the smallest level reasonably available for description of disturbance impacts is the patch, and the largest is the ecosystem. While in theory large-scale disturbances can influence entire landscapes and the last ice age certainly disturbed entire biomes, our discussion focuses on individuals, communities, and impacts at the ecosystem level as these are the most common levels currently studied. Landscapes can be viewed as large mosaics of divergent ecosystems across a large integrated spatial distance, or they can be discussed as groupings of similar ecosystems at different successional stages recovering from similar disturbance regimes (Forman 1995). This point is important as adjacent ecosystems house communities that provide propagules for the reestablishment of similar communities. While not addressing this directly, the availability of propagules similar to pre-disturbance communities is an essential component of reestablishment which, over evolutionary time, may have produced the mechanisms and community dynamics discussed below.

A. Individuals

Our understanding of impacts of disturbance on fungal individuals or species has been limited by lack of good techniques for identifying either. While culture techniques allowed us to examine some species, the availability of molecular techniques has greatly increased our knowledge of fungi as they exist in their natural environment. However, molecular identification does not always help with understanding the role of fungi in ecosystems, as within genera, closely related species can occupy widely divergent niches (Taylor et al. 2014). Further, several studies examining arbuscular mycorrhizal (AM) fungi have determined that spore numbers, often used to predict existence or survival of fungal species after disturbance, are not always related to presence as mutualists within roots, in the same way that seeds and plant mass are not necessarily related. The relationship between hyphal presence and spore production and cues for spore production may be species specific, which would alter our interpretation of the presence or importance of specific taxa (Vargas et al. 2010; Brundrett and Ashwath 2013). As many studies carried out before the use of molecular techniques were used to develop our understanding of patch dynamics and the role of individual, we are now gaining new knowledge of the complexity of these relationships. Many of the questions generated regarding individuals and impacts of disturbance are just beginning to be addressed in greater detail; our discussion of individuals of necessity depends on data derived from a number of different approaches.

Patch level disturbances often have greatest impact at the individual level. Plants link with multiple fungi, and individual fungi infect multiple individual plants. Through interconnections, disturbances alter fungal mycelial networks, disrupt the hyphal networks of individual fungi, and alter environmental conditions for production and germination of fungal spores. Individuals will therefore change in response to patch level disturbances yet this may or may not impact fungal communities. The loss of the species from the ecosystem will only occur if the individual impacted is rare, has poor sporulation capacity or mechanisms, poor dispersal ability, is unable to germinate under new conditions or is not represented in another reasonably close resource patch. Differences in these characteristics among fungi and consequences for continued existence following disturbance have been explored. Often patch dynamics are linked to fragmentation and connectivity among patches. Nordén et al. (2013) found that connectivity amongst patches was, not surpris-

ingly, more important for highly specialized species, compared to the generalist species. Given that the species impacted by the lack of connectivity were more likely to be red listed species, it is important to understand impacts of connectivity at the species level for predicting the impact of patch level dynamics on the survival of rare species.

Further research on the impacts of disturbance, such as tillage, on agricultural soils suggests that soil disturbance decreases fungal biomass and alters soil structure (Denef et al. 2001; Rillig and Mummey 2006). In natural systems, tillage was also found to alter mycorrhizal community composition, with the disturbance increasing variability in the community structure among disturbed plots (Schnoor et al. 2011). While impacts from these types of disturbances often occur at the patch level in natural systems, the degree to which they impact individuals and species has been little studied. It is likely that the level of patch disturbance by digging animals and insects alters species composition at the patch while maintaining fungal community composition at the ecosystem. Studies that examine soil disturbances such as ant mounds have detected some of these patterns in arid systems.

Friese and Allen (1993) found biotic enrichment of microorganisms in ant nests at the study sites in Colorado and Wyoming. In this case disturbance by harvester ant digging increased total number of AM fungal propagules in the ant nests compared to adjacent soil from blue grama grass (*Bouteloua gracilis*) at a site in Colorado and under shrubs (*Artemisia tridentata*) in a site in Wyoming. The assemblages and dominant species of fungi also differed in mound vs. non mound sites, with more fungi (characteristic of mesic sites) occurring in ant nests (Friese et al. 1997). Fungal species richness was higher from mound associated material than from soil adjacent to mounds, or soil collected under shrubs at the Wyoming site, but not from soil adjacent to the mounds at the Central Plains Experimental Range location. It appeared that for both locations, microenvironments selected a distinct assemblage of dominant fungi with *Fusarium spp.* dominating the root material, and *Aspergillus* and *Penicillium* species predominating in seed cache soil. However, *Aspergillus fumigatus* had high densities in off-mound soil from the Colorado site. Mucoraceous taxa (i.e., *Cunninghamella*, *Rhizopus*, and *Syncephalastrum*) were isolated primarily from mound material, suggesting that the ant mounds may represent refugia for these more mesic adapted fungi. These results suggest the greatest impacts of these types of patch disturbances were on individual species through changes in biomass and organic matter and overall fungal community diversity across the site was impacted to a much lesser degree.

Most recently, studies have begun to identify individual fungal species that are sensitive or insensitive to a given disturbance allowing for predictions based on understanding the characteristics of survivor species. Duan et al. (2011) found, within the AM community, fungi that were insensitive to disturbance and could compensate for those that were sensitive to disturbances, supporting the importance of diversity within communities for resilience following disturbance. Similarly, Gehring et al. (2014) found that multiple stressors on a community of ECM decreased fungal diversity and moved communities to more generalist species; however, there was some suggestion that those fungi may have been better mutualistic partners for hosts under the prevailing disturbance regime. In contrast, Lekberg et al. (2012) failed to find impacts on the AM fungal community following a disturbance involving plant removal suggesting that resilience may be a community characteristic that is highly influenced by patch dynamics and disturbance scale.

B. Community-Level Effects

Change in the species composition within a patch may cause changes within the larger ecosystem-level fungal community. The establishment of fungi within the patch can affect the diversity and distribution of fungi within the community or it can cause the establishment of a new community that exists only within the patch. The exact outcome will be determined by the characteristics of the disturbance, especially the size of the disturbance, but also by the heterogeneity of the landscape prior to disturbance. A landscape matrix is established by a series of disturbances of increasing scale. The patches are nested within a habitat that can be relatively homogenous or heterogeneous depending on the scale and intensity of other disturbances that occur across the landscape. From an area of no disturbance, the smaller-

scale disturbances are overlaid by the increasing intensity of the larger-scale disturbance. The impact of the smaller-scale disturbances may alleviate the effects of the larger-scale disturbances by acting as islands of inoculum or dispersal agents. This was the case for the arbuscular mycorrhizal (AM) fungi at Mt. St. Helens. Disturbances by gopher digging and elk droppings returned the AM fungal inoculum to the site that was completely decimated by the volcano blast (Allen et al. 1984, 1992, 2005b). Initially, ECM fungi were predominantly dispersed back onto the site by wind (Allen 1987). Thus, across the pyroclastic flow zone, small AM fungal patches reformed along animal pathways, whereas the ECM plants initially established at random locations. It was the reestablishment of these individuals or the small eclectic group of fungi that existed in the deposited inoculum that led to recolonization following the most severe disturbance type. Similarly Jumpponen (2003) found the fungal community in the youngest soils adjacent to a receding glacier to have dormant mycorrhizal fungi even before the arrival of the plant community. As these fungi are biotrophs dependent on plant hosts, airborne spore deposition preceded plant arrival providing symbionts for arriving seeds.

The composition of the post-disturbance community is also dependent on the competitive interactions among residuals and immigrants. A good deal of work has been undertaken studying the interactions of competitive (C), stress-tolerant (S), or ruderal (R) strategies in saprophytes following disturbance (Cooke and Rayner 1984). Immediately following a disturbance, the R-strategist is likely to predominate although, depending on the disturbance, many S-strategists may remain as residuals. Presumably the C- and more S-strategists will predominate later. Fungi such as *Mucor* and *Rhizopus* are presumed to be ruderals because they exploit simple carbohydrates rapidly (R-strategy). Alternatively, *Phanerochyte* grows slowly but can degrade almost any type of substrate (S-strategy). *Cephalosporium* is an outstanding competitor because it expends a large amount of resources to produce antibiotics that restrict access to its own resource base. However, these separations are highly artificial, and organisms exist along gradients of these extremes. For example, a common fungus of burned pine forests is *Morchella*. Is that because it tolerates the fires and harsh conditions following fires (S), competes well with other residuals and immigrants by growing hyphae fast and utilizing resources in the early spring before other saprophytes are getting started (C), produces a massive sporulating fungus that disperses spores by wind and animals when released from competition (R), or (most probably) has some effective combination of all strategies?

Taylor and Bruns (1999) examined the ECM community structure in a mature pine forest. They detected minimal overlap in the active mycorrhizal community and the community present as resistant propagules. This suggested that differences in colonization strategies such as the C, S, and R described above and resource preferences, combined with resistant fungal structures, allow diversity to be maintained in forest communities so that organisms can respond to environmental cues and disturbances that have been historically part of the ecosystems. Peay et al. (2007) found that ECM communities were very strongly structured at the tree level, with each tree acting as an island of diversity rather than a homogenous mixture across a landscape. Given their findings they suggest that the trade-off between competition and dispersion strongly determines composition of the island patches and the overall landscape community. The "island" structure of the community would protect diversity in patches that would be available for reestablishment following disturbance.

Other studies on mycorrhizal communities suggest that community development is dependent on the type (enrichment vs. destructive), intensity, and frequency of disturbance. Lilleskov and Bruns (2003) found the timing of root colonization by two ECM fungi was altered by soil nutrient status. *Rhizopogon occidentalis*, an early successional species, colonized roots early and then was replaced as a dominant species by *Tomentella sublilacina* as the forest matured. Under high-nutrient conditions, however, this replacement was delayed suggesting that interspecific interactions between the two species were mediated by soil nutrient content.

Fungal pathogens have unique roles in disturbances. Pathogen density and diversity can

be impacted by disturbances. The role of pathogens in natural ecosystems, especially in relation to historical disturbance regimes, has not been addressed well in the literature. Increases in the loss of plants, most often forest trees through pathogen attack, have increased focus on fungi as pathogens. This may be a consequence of recent attention in the face of economic loss or may be as a consequence of increased importance of pathogens as regulators of community dynamics. Allen et al. (2005a) suggested that altered weather patterns in the tropical dry forest they examined may have resulted in increased incidence of an indigenous fungal pathogen. Hence, altered historical disturbance regimes may change relative abundance and impact of pathogens on hosts. Parker et al. (2006) found that fire suppression resulted in increased levels of fungal pathogen activity in North American forests. This activity is now being altered by large-scale wildfires and the reintroduction of more historically representative fire regimes using prescribed burns. However, these new disturbances are also causing increases in pathogen activities. Reintroduction of natural disturbance regimes is needed to reduce pathogen activities, yet the way to achieve these reductions may require greater understanding of the impacts of fire on the physiology and structure of these pathogens in their natural habitat. Changes in historical disturbance patterns and physical characteristics of the environment such as altered temperature and moisture may be resulting in increases in pathogen activity.

Fungal pathogens also make interesting contributions as disturbances to plant communities. Fungi such as root rot fungi increase the likelihood of canopy gaps in forests (Bendel et al. 2006) directly through impacts on trees. These fungi increase substrate for saprophytes and decrease habitat for mycorrhizal species. Other fungal pathogens have been found to increase the severity of natural disturbances. Papaik et al. (2005) found that beech bark disease does more damage to trees, by decreasing the resistance of beech to disturbance events such as windthrow, than it does by directly impacting the plant it infects.

C. Ecosystem Characteristics and Feedback Loops

Changes in the fungal community structure will have the greatest impact on the ecosystem if functioning changes with composition or if the fungi affect plant diversity (e.g., Renker et al. 2004). Changes in primary production, quality of material produced, decomposition rates, or nutrient pool conversion can affect the stability, productivity, and, ultimately, the functioning of ecosystems (Chapin et al. 2012). Consequently, disturbances that alter soil processes or the vegetation will alter corresponding ecosystem dynamics. Ecosystem-level feedback loops (Figs. 4.1 and 4.2) can influence patch structure through effects on disturbance types, characteristics, and the biotic and abiotic characteristics of the patch. Ecosystem dynamics will affect small-scale disturbance by influencing such things as animal types and densities, and large-scale disturbances by fuel loads, and litter layer thickness. All scales of ecosystem disturbance ranging from landslides to fire, to introduced species, to animal burrowing, to hyphal grazing by microarthropods can disrupt critical points in the hyphal network that exist in the soil. Altering this hyphal network changes nutrient availability and transfer to plant hosts, which in turn alters ecosystem productivity.

The most significant impacts of disturbance to fungal communities will be through changes in structure. It is predicted that a disturbed site tends to return to a community structure that does not entirely resemble the pre-disturbance state (Gochenaur 1981). The existence of a new assemblage of species, even of the same species of different age or density, may restrict the ability of the community to return to a pre-disturbance state. This was observed in the simple experiment on cultured *Penicillium* and *Aspergillus* by Armstrong (1976), which demonstrated that although *Aspergillus* would exclude *Penicillium* in plated cultures of the same age, if *Aspergillus* spores were plated with *Penicillium* spores of a younger culture, then both would be maintained. While terres-

trial ecosystems have a great deal more complexity than these two species model, it is likely that changes induced by disturbance have differential effects on the species present which will ultimately affect the composition of the post-disturbance community.

More complex ecosystem impacts and feedback loops in understanding fungal community dynamics are being identified in studies examining invasive species. Invasive species can be understood as a disturbance to an ecosystem. Further, many studies have identified that invasive species more easily enter an ecosystem following a disturbance or a change to a disturbance regime (Moles et al. 2008) often resulting in a secondary or interactive impact on belowground communities from the invasive interacting with the disturbance impact. Studies on the invasive species *Alliaria petiolata* (garlic mustard), for example, have shown decreases in AM fungi (Rodgers et al. 2008) and ECM fungi (Wolfe et al. 2008) following invasion. Garlic mustard may, in large part, be an effective North American invader because in altering the mycorrhizal inoculum, it can shift the competitive advantage from the native species, a feat it does not accomplish in its native range (Callaway et al. 2008). However, Barto et al. (2010) were able to demonstrate that native plants associated with AM fungi were able to withstand the invader suggesting that the impact of the invasive species was temporally mediated such that the allelochemicals released by garlic mustard may only be effective against the establishment of symbioses, rather than established mutualisms.

Mycorrhizal fungi may provide the links necessary to evaluate the impact of community structure and changes in community structure on ecosystem function (Read and Perez-Moreno 2003). The effects of individual fungal species on plant communities are expressed through their impacts on aboveground productivity and diversity. Fungal species can increase productivity through increased resource acquisition. They can alter plant diversity directly by presence or absence through mechanisms of specificity and by altering the outcome of aboveground competition. They can cause changes to litter and tissue quality through differences in nutrient acquisition. The associations that tie belowground community structure to aboveground dynamics are established, maintained, and disrupted by disturbances that alter fungal community dynamics.

Understanding the impacts of changes in communities of saprophytic fungi on ecosystem function is also essential for understanding the overall impacts of a given disturbance type. Modeling has been used recently to examine linkages between communities and ecosystem dynamics. While these models have been important for understanding global change scenarios, they have also allowed independent evaluation of the key components of belowground communities on ecosystem dynamics.

Hunt and Wall (2002) modeled effects of species loss on biodiversity. They found that deletion of saprophytic fungi and bacteria caused changes to net primary productivity. As they were modeling a large number of belowground groups and detected little change with deletions of other groups, these results emphasize the importance of saprophytes as determinants of ecosystem characteristics. It also emphasizes that changes in this group as a result of disturbance has potential to alter larger scale functioning. Fisk et al. (2011) demonstrated that alterations to substrate through disturbance had significant impacts on the community of fungi involved in decay. There is currently a great deal of interest in tying community structure to function at the ecosystem level. The response of fungal communities to disturbance events might provide good systems to evaluate these linkages.

D. Disturbance and Species Diversity

Disturbances influence the composition and species richness of communities through a variety of mechanisms. Some disturbances result in decreases in the fungal biodiversity, while others are hypothesized to release ecosystems from unproductive states of "retrogression" (Wardle 2006).

Within a short time scale, some disturbances affect the entire community simultaneously, such as volcanic eruptions (Andersen and MacMahon 1985; Allen et al. 1984, 2005b; Allen 1988) and catastrophic winds (e.g., Dunn et al. 1983), while other disturbances such as animal diggings (Koide and Mooney 1987; Allen et al.

1992; Friese and Allen 1993), defoliation (Williamson and Wardle 2007) or invasive species (Eviner and Chapin 2003; Bohlen 2006; Wolfe et al. 2008) influence only a relatively small portion of the community at a time. Others initially start small and expand over longer time scales such as invasive species (van Mantgem et al. 2004), N deposition and fertilization (Egerton-Warburton and Allen 2000; Miao-Yan et al. 2009), glacial expansions and retreats (Helm et al. 1999; Jumpponen et al. 2002). The scale and intensity of disturbances can, in turn, significantly affect the response of organisms and resulting successional patterns (e.g., Bazzaz 1983; Mooney and Godron 1983; Sousa 1984; Pickett and White 1985; Pickett et al. 1989; McClendon and Redente 1990; Egerton-Warburton and Allen 2000). Further, the frequency of the disturbance may alter community composition by encouraging the growth of rapid growing more generalist species, where in less frequent disturbance regimes may allow the establishment and continued growth of species that grow more slowly. (Koide et al. 2011; Gehring et al. 2014)

Small-scale disturbance can affect the diversity of fungal communities in a variety of ways. These disturbances are often poorly characterized because they create a mosaic of heterogeneous patches within the landscape that are often functionally and structurally different from the landscape that surrounds them. Patches are defined ecologically as discrete spatial patterns with easily identifiable boundaries (Pickett and White 1985). Disturbances such as mound building by animals create or alter the patchiness of a landscape (Allen 1988; Friese and Allen 1993; Snyder et al. 2002). These patch disturbances disrupt existing external soil mycelial networks such as those described by Finlay and Read (1986) for ECM and Friese and Allen (1991) for AM fungi (e.g., absorptive hyphal networks and hyphal bridges). Disruptions in the soil hyphal network will create openings for the colonization and spread of new fungi, thus increasing fungal biodiversity, just as occurs for colonial animals (Connell 1961) and higher plants (Allen and Forman 1976). Gophers and ants are examples of animals that are capable of overturning soil and moving mycorrhizal propagules within that soil to new patches in the soil matrix. Additionally, gophers trap spores within their fur and can transport these fungi to new areas.

Ingestion and excretion of viable propagules at new locations by large mammals are also considered small-scale disturbances which have the capacity to change the community composition of a patch. The deposition of dung containing viable mycorrhizal spores from areas adjacent to the blast zone on the tephra at Mt. St. Helens by elk, days following the blast, allowed the return of fungal propagules to a biotically sterile area (Allen 1987). Chronic small-scale disturbances can also be caused by large ungulates. Serengeti ecosystems are heavily grazed, resulting in increased nitrogen and dung applications to the soil (Seagle et al. 1992). This increases nitrogen levels and mineralizable carbon sources for the microorganisms. Studies of mycorrhizal distribution in this ecosystem demonstrated an inverse relationship between soil fertility and the presence of mycorrhizal fungi (McNaughton and Oesterheld 1990). This relationship is also associated with a smaller gradient of nutritional status associated with the vegetation. The mycorrhizae allow the plants to maintain a high nutritional status across a broad range of soil nutritional ranges, which ultimately results in better forage for the animals and a return of nutrients to the soil in the form of urine and dung.

Large-scale disturbances affect the diversity of fungal communities in different ways. Volcanoes, the most extreme of the large-scale disturbances, can completely destroy fungal communities, leaving topsoil buried under sterile tephra (Allen et al. 1984; Hendrix and Smith 1986) or lava (Gemma and Koske 1990) or even by creating new lands (Henriksson and Henriksson 1974). Fungal communities in the most severely damaged areas can be destroyed completely. The recovery of these areas is driven by wind or small animal vectors capable of bringing new propagules from surviving areas, patches, or from source areas at differing distances. Materials deposited as a consequence of the eruption, such as tephra or lava, can have different characteristics, such as altered bulk density, chemistry, or pH, which leads to the creation of new communities. On the most devastated area of Mt. St. Helens following the blast of 1980, Ascomycotina were found to colonize the tephra within the first year followed by Basidiomycotina (Carpenter et al. 1987). After 10 years, AM and ECM were reestablished on the site mediated by gophers and wind dispersal (Allen et al. 1992), although the ECM were initially poorly developed.

The rate at which reinvasion progresses on the most severely disturbed sites depends on the availability of sources of fungal inoculum. With the existence of nearby or internal source

patches of inoculum, fungal communities can begin reinvading and establishing.

The rates of reinvasion of mycorrhizal fungi onto the pumice plain of Mt. St. Helens, to Krakatau following the 1883 eruption (Allen 1991) and from Hawaii (Gemma and Koske 1990) demonstrate that reinvasion of these fungi is dependent on location and type of inocula, and upon the reinvasion of vegetation. Mt. St. Helens, by having mycorrhizae in the most damaged areas as soon as 1 year after the eruption, recovered more quickly than Krakatau, where facultatively mycorrhizal plant species were reported 3 years after the eruption. This is because the most severely damaged areas of Mt. St. Helens were surrounded by remnant vegetation patches rather than water, as in the island of Krakatau. On the Hawaiian Islands, there was a rapid invasion of mycorrhizal species from adjacent kipukas, or isolated patches of vegetation that remained untouched by disturbance (Gemma and Koske 1990). Volcanic eruptions such as the ones above occur relatively frequently and are considered predictable and therefore subject to selective evolutionary pressure. Presumably, dispersal strategies and life histories of the plants on these islands are adapted to these disturbances, and it is probable that the same is true for the fungi.

Fire presents another example of large-scale disturbance which has the ability to affect fungal communities in different ways. Following a severe fire, there is an initial drop in the number of propagules (Wright and Bollen 1961) and shift in the diversity of fungi present in an area (Wicklow 1973; Allen et al. 2003, 2005a; Bastias et al. 2006). Changes in soil pH and mineralization rates caused by fire regulate the fungi that can initially establish in the area (Gochenaur 1981). Following the initial decrease in fungal propagules, a rapid increase in fungal biomass occurs often to more than ten times the prefire value (Ahlgren and Ahlgren 1965; Wicklow 1973). These species, often referred to as pyrophilous fungi, are capable of taking advantage of the new resources made available by the fire.

Mycorrhizal species may decrease in number or diversity following fire (Vilarino and Arines 1991; Allen et al. 2003, 2005a; Tuininga and Dighton 2004) or remain unaffected within the plant root (Molina et al. 1992). However, some of these effects are relatively shallow and do not effect diversity or density lower in the profile (Pattinson et al. 1999; Bastias et al. 2006).

Fire can have a number of effects on mycorrhizal spores, depending on the maximum ground temperature reached while burning. Vilarino and Arines (1991) found that, following fire, the number and viability of AM spores decreased. They also determined that on at least one site the dominant species of mycorrhizal fungus changed from *Acaulospora laevis* to *Acaulospora scrobiculata*. Percent root colonization by AM increased over the year following the burn but did not reach the levels found before the fire. The depression in levels of soil colonization following fire in this site was detected for longer than in other similar research, such as that of Dhillion et al. (1988) on prairie soils. The authors suggested that the temperatures reached on these soils with a shrub and tree vegetation were greater than the temperatures reached with a herbaceous vegetation producing the longer-lasting effects. Treseder et al. (2004) found that fire in Alaskan boreal forests had little impact on AM fungi however recolonization by ECM appeared to be delayed up to 15 years following the disturbance. Heat may impact structures in the fungal life cycle in different manners altering growth and reproduction patterns. Peay et al. (2009) found that ECM spores were differentially impacted by heat associated with fires resulting in differences in recolonization patterns following fires. Secondary forests in the Yucatan Peninsula with a very shallow, highly organic soil, a hot fire virtually eliminated all inoculum (Allen et al. 2003) whereas following a cooler fire, where some organic matter persisted, the richness of fungi was much higher. The two types of fires can cause significantly different patterns of vegetation recovery (Allen et al. 2003, 2005a). Therefore, the frequency and intensity of a fire, which is determined largely by the structure of the plant community (e.g., forest vs. grassland), can determine both the spatial and temporal patterns of fungal community development. While, there is great heterogeneity on the effect of fire on fungal communities, it is apparent that the fungal community is active after fire and have a role in soil stabilization and remediation. (Claridge et al. 2009)

At an even larger scale are the large-scale perturbations to fungal communities resulting from global climate change. While the planet has seen changes in temperature, moisture, and atmospheric greenhouse gas concentrations historically, these have taken place over much longer time frames. Current changes have taken place over much shorter time periods, that of hundreds of years, and have resulted in significant stochasticity and variability in weather patterns leading to increased ecosystem distur-

bance (Stocker et al. 2013). The literature on the impacts of elevated CO_2 on fungi from natural enrichment sites such as volcanoes suggests that some fungi are sensitive to CO_2 certainly more so than soil bacteria and archaea, and fungi are differentially sensitive such that wood decomposing fungi may have a greater tolerance to elevated CO_2 than litter decomposers (McFarland et al. 2013). Mutualists showed changes in community structure; however, it is not possible to determine whether this is direct response to CO_2 or to changes in plant function. In terms of impacts from other disturbances such as warming, Xiong et al. (2014) found significant impacts on fungi such that Basidiomycota increased, while other groups such as Ascomycota and some of the more rare species showed variable responses. Overall, our capacity to predict the outcomes for fungi from changes to global climate change as a disturbance is currently limited which impacts our capacity to predict alterations to natural and agronomic systems.

V. Conclusions

A. The Impact of Disturbance on the Functional Role of Fungi

This chapter was designed to demonstrate that disturbance may be the single most important process regulating the structure and functioning of fungal communities. This is due to the unique physiology, morphology, and reproductive biology of fungi. While microbial ecologists are beginning to describe the importance of individual disturbance events, we know far less about the interactions of small- and large-scale perturbations set in the larger landscape of single or multiple plant communities. The patch dynamics that operate in systems in advance of disturbance create a heterogeneous landscape that often precludes simple predictions of outcomes. This distinction becomes of even greater importance in the light of the global dimensions of anthropogenic influences on ecosystems such as N fertilization, exotic species migrations, habitat fragmentation, and global climate change. We suggest that understanding the roles of disturbance in fungal communities and the feedbacks from the fungal communities to ecosystem functioning is crucial to understanding the results from these larger global concerns. Developing models for linking the range of scales that comprise disturbance dynamics depends on linking two distinct types of studies. First, we must begin to build an array of case studies from particular ecosystems in which we know the natural history of the fungi and how these natural histories contribute to the existing community composition and functioning. Second, we must develop a conceptual framework that integrates all of these various case studies into a comprehensive view of how communities work and what factors regulate them. Finally, we must continuously reevaluate those conceptual models to develop a quantitative model of the complex roles of fungi within landscapes that are undergoing anthropogenic and natural change.

While it would be easiest to evaluate disturbance as a static entity, it is unfortunately a moving target. As anthropogenic impacts alter natural ecosystems, we are also altering disturbance regimes and impacts of disturbance events. Ecosystems contain organisms that have interacted with their biotic and abiotic environments over exceedingly long time scales. The systems examined today are a consequence of organisms responding to the stresses experienced on predictable time scales. As anthropogenic disturbances have escalated only over the last 100 years, we cannot adequately predict the consequences of a given disturbance based on historical data because we do not know if the system we are examining is similar to that which existed in the past. Ecologists use the terms resistance and resilience to evaluate the degree to which a system resists change in the face of disturbance or the degree to which it returns to the pre-disturbance state. In theory, a system facing a predictable disturbance would respond in a resistant or resilient fashion. However, with added unpredictable stresses such as chronic N deposition, herbicide use, global warming, and atmospheric pollution, systems may be less resistant or resilient to disturbances that they may have easily recovered from in the past.

Loss of the evolutionary history within ecosystems not only decreases our ability to understand the complex effects of disturbance to fungal communities, but it also has great potential to irrevocably alter the ecosystems that currently exist within our biosphere.

B. Future Research Directions

As a result of the widespread interest in anthropogenic changes to the earth's atmosphere and the effects that these changes may have on the biosphere, it is important to develop a better understanding of how small-scale microbial processes (such as nutrient mobilization and immobilization) fit into the larger global picture. The diversity and biomass of microbial communities are direct indicators of the extent of the functional role that these organisms play in the dynamics of different ecosystems. The exponential increase in the use of molecular techniques to answer ecological questions has expanded our understanding of community structure at the level of the individual and at the level of specific genes. These advances will ultimately allow scientists to tie community structure to contributions to ecosystem function. This is required if researchers are to develop truly predictive models of the impacts of disturbance on ecosystem processes. If anthropogenic change alters the structure and biodiversity of microbial communities, then it is also likely that their critical functional roles in the ecosystem and global-level nutrient cycling are also impacted. As is the case with other groups of organisms, it is just as important to understand how the functional role of fungi is affected by various forms of human impact on the environment (e.g., Meyer 1993; Read 1993; Morris and Robertson 2005; Paul 2015). One approach to evaluate this impact would be to examine the fungal communities present at ecological restoration sites that are at least 10–20 years old and compare the microbial characteristics of these "established" restoration sites to their references sites. Differences in these sites would allow one to evaluate the impacts that human alteration of system characteristics has on fungal community dynamics. Future research on fungal community dynamics should also focus on linking the issues of fungal biodiversity and functionality with both natural disturbance and anthropogenic change. This research direction is critical for us to completely understand and explain the importance of fungi in ecosystem dynamics. Attempts to explore and integrate all of the above factors are crucial if we are ever to gain a comprehensive understanding of the functional role of fungi in diverse ecosystems and the biosphere as a whole.

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5 Fungi and Industrial Pollutants

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I. Introduction

Fungi may be exposed to a wide variety of organic and inorganic pollutants in the environment. Since fungi play a major role in carbon, nitrogen, phosphorus and other biogeochemical cycles (Wainwright 1988a, b; Gadd 2006, 2007, 2008a, b, 2011), impairment of fungal activity could have important consequences for ecosystem function. It is obviously desirable that more is known about the impact of pollutants on these organisms. Unfortunately, while it is easy to speculate on the likely effects of pollutants on fungi, it is often far more difficult to demonstrate such effects. Studies on pollutant effects on fungal populations are difficult, largely because of the inadequacy of many of the techniques which are available to study fungi and the complexity of mixed microbial communities (Anders and Domsch 1975; States 1981; Doelman 1985; Gadd et al. 2007). However, an appreciation of the effects which pollutants can have on fungi can be obtained by a combination of the following measurements: (1) pollutant concentration, composition and distribution, (2) pollutant bioavailability, (3) pollutant concentrations that cause a toxic or physiological response in vitro, (4) effects of the pollutant on fungal population/community size and composition and (5) secondary changes resulting from pollution effects on fungal populations, e.g. impact on leaf litter decomposition. While pollutant concentration and composition may be determined using standard analytical techniques, with varying degrees of difficulty depending on the pollutant and the environmental matrix,

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the analysis of pollutant speciation and the bioavailability remain challenging problems.

The effect of pollutants on fungal population/community size and composition is particularly difficult to assess. Many earlier studies used the dilution plate count or similar approaches to assess changes in fungal community composition. The shortcomings of this technique have been criticised at length and are well known. To overcome problems relating to the use of plate counts, biomarkers such as phospholipid fatty acid (PLFA) composition and extraction and analysis of DNA are now routinely used, though no methods are exempt from problems (Gadd et al. 2007). Another problem is that it is unlikely that a meaningful picture of how fungi respond to pollutants in the environment can be gained from determining responses to pollutants added to solid or liquid growth media in laboratory experiments. The effects of toxic metals on soil fungi growing in vitro, for example, are markedly influenced by the composition of the medium used: metals are likely to be more toxic to fungi in low-carbon media than in carbon-rich media where the production of large amounts of extracellular polysaccharides and chemical interactions with the medium will tend to reduce metal availability. Medium components may also complex or precipitate metals out of the solution, making them unavailable (Gadd and Griffiths 1978; Hughes and Poole 1991; Gadd 1992). Finally, interactions between different pollutants and their breakdown products may have a major influence on the toxicity of a pollutant in the natural environment. This chapter will outline some of the main effects of organic and inorganic pollutants on fungi and will include the discussion of effects at cellular and community levels and their applied and environmental significance.

II. Predicted Effects of Pollutants on Fungal Populations

Environmental pollution might be expected to lead to both toxic (destructive) and enrichment disturbances on fungal populations (Wainwright 1988b). Although toxic disturbance is likely to predominate, instances will occur where both types of disturbance are found together. Toxic disturbance of fungal populations is likely to be particularly damaging to ecosystem function, while the rarer enrichment disturbance may occasionally produce beneficial effects on soil processes. Toxic disturbance is likely to lead to a reduction in fungal numbers and species diversity, as well as biomass and activity changes which may detrimentally influence fundamentally important processes such as litter decomposition (Freedman and Hutchinson 1980; Hiroki 1992; Fritze and Baath 1993). The resultant degree of toxic disturbance will depend upon both toxicant concentration and its availability to the fungal population, as well as to the susceptibility of the individuals involved. Toxicants may be selective and affect only a few species, or they may have a more generalised effect. Selective inhibition may have less of an impact on overall soil fungal activity than might be imagined, since susceptible species can be replaced by more resistant fungi, some of which may be more active in a given physiological process than the original population. While concentration effects are generally emphasised, it is surprising how often the question of toxicant bioavailability is avoided in studies on the effects of pollutants on microorganisms. In soils, for example, bioavailability of a pollutant will generally depend upon factors such as (1) adsorption to organic and inorganic matter, (2) chemical speciation, (3) microbial transformation and/or degradation and (4) leaching. Another factor of importance in relation to the effects of toxicants on soil fungi concerns nutrient availability. Fungi are generally thought to be already stressed by the low levels of available carbon present in most soils and other environments (Wainwright 1992). They will grow slowly, if at all, under these conditions and may be more susceptible to pollutants than when growing in high-nutrient conditions.

Fungal populations are unlikely to remain static when confronted with a toxic agent, and resistant populations are likely to develop which will be a major factor in determining population responses to the pollutant. On the other hand, a number of studies have shown

that fungi isolated from metal-contaminated soils show less adaptation to toxic metals, such as copper, than might be expected (Yamamoto et al. 1985; Arnebrant et al. 1987). Mowll and Gadd (1985) also found no differences in the sensitivity of *Aureobasidium pullulans* to lead when isolates from either contaminated or uncontaminated phylloplanes were compared.

Enrichment disturbances may also be either selective or nonselective. Nonselective enrichment disturbance might theoretically result from the input into the ecosystem of a pollutant which is widely used as a nutrient source. Since such enrichment is rare, most examples of this form of disturbance will be selective. Reduced forms of sulphur are, for example, likely to enrich the soil for S-oxidising fungi, while phenolics and hydrocarbons may favour species capable of utilising these compounds.

III. Fungi and Xenobiotics

Some fungi have remarkable degradative properties, and lignin-degrading white rot fungi, such as *Phanerochaete chrysosporium*, can degrade several xenobiotics including aromatic hydrocarbons, chlorinated organics, polychlorinated biphenyls, nitrogen-containing aromatics, and many other pesticides, dyes, and xenobiotics (Prenafeta-Boldú et al. 2006; Pinedo-Rilla et al. 2009; Cerniglia and Sutherland 2010; Harms et al. 2011). Such activities are of bioremedial potential where ligninolytic fungi have been used to treat soil contaminated with pentachlorophenol (PCP) and polynuclear aromatic hydrocarbons (PAHs). In general, treatment involves inoculation of the contaminated soil followed by nutrient addition, irrigation and aeration and maintenance by general land farming procedures. Correct preparation of the fungal inoculum can be crucial: fungi may be grown on lignocellulosic substrates prior to introduction into the soil (Singleton 2001; Baldrian 2008). Treatment can take weeks to months or longer depending on the level of contamination and environmental factors. In many cases, xenobiotic-transforming fungi need additional utilisable carbon sources because although capable of degradation, they cannot utilise these substrates as an energy source for growth. Therefore inexpensive utilisable lignocellulosic wastes such as corn cobs, straw and sawdust can be used as nutrients for enhanced pollutant degradation (Reddy and Mathew 2001). Wood rotting and other fungi are also receiving attention for the decolorisation of dyes and industrial effluents and various agricultural wastes such as forestry, pulp and paper by-products, sugarcane bagasse, coffee pulp, sugar beet pulp, apple and tomato pulp and cyanide (Knapp et al. 2001; Barclay and Knowles 2001; Cohen and Hadar 2001).

Most pollutant degraders belong to the phyla *Ascomycota* and *Basidiomycota*, followed by the subphylum *Mucoromycotina* (Harms et al. 2011). Some genera with well-known degradative properties include species of *Cladophialophora*, *Exophiala*, *Aspergillus*, *Penicillium*, *Cordyceps*, *Fusarium*, *Pseudallescheria*, *Acremonium*, *Neurospora*, *Graphium* and *Phoma* with degradable substrates including aliphatic hydrocarbons, chlorophenols, polycyclic aromatic hydrocarbons (PAHs), pesticides, dyes, 2,4,6-trinitrotoluene (TNT), polychlorinated dibenzo-p-dioxins (PCDDs), Royal Demolition Explosive (RDX) and methyl tert-butyl ether (MTBE) (Chang 2008). Yeasts include degraders of n-alkanes, n-alkylbenzenes, crude oil, the endocrine-disrupting chemical (EDC) nonylphenol, PAHs and TNT, e.g. *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces* and *Yarrowia* spp. (Harms et al. 2011). In the *Mucoromycotina*, *Cunninghamella*, *Mucor* and *Rhizopus* spp. (*Mucorales*) include degraders of PAHs, pesticides, textile dyes and TNT (Harms et al. 2011). Mycorrhizal fungi are also able to degrade various organic pollutants, e.g. chloroaromatics, PAHs, TNT, certain herbicides and atrazine (Meharg and Cairney 2000a, b; Volante et al. 2005; Harms et al. 2011). The low specificity of many fungal degradative enzymes means that producing organisms can co-metabolise many different compounds. *Phanerochaete chrysosporium*, for example, can degrade benzene, toluene, ethylbenzene and xylene (BTEX) com-

pounds, nitroaromatic and N-heterocyclic explosives (TNT and RDX, respectively), organochlorines (chloroaliphatics, chlorolignols, chlorophenols, polychlorinated biphenyls and PCDDs), PAHs, pesticides, synthetic dyes and synthetic polymers (Asgher et al. 2008; Baldrian 2008). The range of oxidoreductases that can degrade organic compounds includes laccases, tyrosinases and peroxidases (Majeau et al. 2010). Other enzymes include mixed function cytochrome P450 oxidases, transferases, aromatic nitroreductases and quinone reductases (Harms et al. 2011).

Polycyclic aromatic hydrocarbons (PAHs) enter the environment via many routes, including fossil fuel combustion, vehicle exhaust emissions, gas and coal tar manufacture, wood preservation processes and waste incineration (Harvey 1997; Pozzoli et al. 2004). Many PAHs are toxic towards microorganisms, plants and animals, and PAHs of low molecular weight and high water solubility are the most toxic (Cerniglia and Sutherland 2006). PAHs disappear relatively slowly in the environment through physical, chemical and biological processes, some of which are mediated by bacteria and fungi. PAH recalcitrance in soils and sediments increases with molecular weight, but several other physicochemical and biological factors can contribute to this, e.g. lack of PAH-degrading microorganisms, nutrient deficiency, low bioavailability, preferential utilisation of more easily degradable substrates, the presence of other toxic pollutants or breakdown of products (Cerniglia and Sutherland 2006). Other related factors that affect PAH biodegradation in soil include soil type, pH, temperature, oxygen concentration, irradiation as well as the solubility, volatility, and sorption properties of the PAHs (Lehto et al. 2003; Huesemann et al. 2003; Rasmussen and Olsen 2004). Bioremediation by mixed communities may be enhanced by bacteria that produce degradative enzymes as well as biosurfactants (Straube et al. 1999; Cameotra and Bollag 2003). Aerobic biodegradation of PAHs by soil microorganisms uses monooxygenase, peroxidase and dioxygenase pathways; the first and third of these pathways are utilised by bacteria, while the first and second are found in fungi. The use of filamentous fungi may be advantageous when translocation of the pollutant through the mycelium is required for detoxification (Harms et al. 2011). Fungi have also been shown to stimulate organic pollutant degradation by bacteria in the soil when the hyphae act as continuous pathways for motile bacteria, bridging pore spaces and soil aggregates and thereby facilitating movement and pollutant degradation by the bacteria (Kohlmeier et al. 2005; Wick et al. 2007, 2010; Banitz et al. 2011).

Many fungi can metabolise PAHs (Cerniglia and Sutherland 2001, 2006, 2010; Sutherland 2004; Verdin et al. 2004). Since fungi cannot generally use PAHs as the sole carbon and energy source (Cerniglia and Sutherland 2001), they must be supplied with nutrients to allow co-metabolism. A small number of yeasts and filamentous fungi have been reported to use some PAHs, including anthracene, phenanthrene, pyrene and benzo[*a*]pyrene, as carbon and energy sources (Romero et al. 2002; Lahav et al. 2002; Saraswathy and Hallberg 2002; Veignie et al. 2004). Some fungi co-metabolise PAHs to *trans*-dihydrodiols, phenols, quinones, dihydrodiol epoxides and tetraols, but seldom degrade them completely to CO_2 (Casillas et al. 1996; Cajthaml et al. 2002; da Silva et al. 2003).

The transformation of PAHs by ligninolytic, wood-decaying fungi involves several different enzymes (Asgher et al. 2008). The enzymes produced by white-rot fungi that are involved in PAH degradation include lignin peroxidase, manganese peroxidase, laccase, cytochrome P450 and epoxide hydrolase (Haemmerli et al. 1986; Bezalel et al. 1996; Cerniglia and Sutherland 2006). Ligninolytic fungi metabolise PAHs via reactions involving reactive oxygen species to phenols and quinones (Pickard et al. 1999; Steffen et al. 2003), and these may be further degraded by ring-fission enzymes (Cerniglia and Sutherland 2006).

Several wood-decaying fungi, e.g. *Bjerkandera*, *Coriolopsis*, *Irpex*, *Phanerochaete*, *Pleurous* and *Trametes* spp., have been investigated for bioremediation of PAH-contaminated soils (Baldrian et al. 2000; Novotný et al. 2000; Cerniglia and Sutherland 2006; Baldrian 2008). Laboratory trials have demonstrated their abil-

ity to degrade complex mixtures of PAHs, such as those in creosote and coal tar, but actual bioremediation of contaminated soils using these fungi has met with varying success (Canet et al. 2001; Cerniglia and Sutherland 2001; Pointing 2001; Hestbjerg et al. 2003). Non-ligninolytic fungi, including *Cunninghamella*, *Mucor*, *Fusarium* and *Penicillium* spp., have also been considered for PAH bioremediation (Colombo et al. 1996; Pinto and Moore 2000; Ravelet et al. 2001; Saraswathy and Hallberg 2002).

Biodegradation may require the presence of mixed bacterial and fungal communities, although less is known about the pathways of PAH degradation by co-cultures (Juhasz and Naidu 2000). The evolution of $^{14}CO_2$ from ^{14}C-phenanthrene in soil was enhanced almost two-fold (from 19.5 % to 37.7 %) when *P. chrysosporium* was added to the indigenous soil microflora (Brodkorb and Legge 1992). Boonchan et al. (2000) combined *Penicillium janthinellum* with either *Stenotrophomonas maltophilia* or an unidentified bacterial consortium. The fungus could partially degrade pyrene and benzo[*a*]pyrene but could not use either as a carbon source; *S. maltophilia* could use pyrene as a carbon source and co-metabolise benzo[*a*]pyrene. The fungal–bacterial combinations grew on pyrene, chrysene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*ah*]anthracene, converting 25 % of the benzo[*a*]pyrene to CO_2 in 49 days. The white-rot fungus *P. ostreatus* and the brown-rot fungus *Antrodia vaillantii* enhanced the degradation of fluorene, phenanthrene, pyrene and benz[*a*]anthracene in artificially contaminated soils (Andersson et al. 2003). Unlike *P. ostreatus*, which inhibited the growth of indigenous soil microorganisms, *A. vaillantii* stimulated soil microbial activity.

Ligninolytic fungi partially oxidise PAHs by reactions involving extracellular free radicals (Majcherczyk and Johannes 2000), making the PAHs more water soluble so that they are able to serve as substrates for bacterial degradation (Meulenberg et al. 1997). Partial oxidation increases PAH bioavailability in most contaminated sites (Mueller et al. 1996; Meulenberg et al. 1997), and PAH-contaminated soils may contain large populations of PAH-transforming bacteria (Johnsen et al. 2002) and fungi (April et al. 2000; Saraswathy and Hallberg 2002). Combinations of several microorganisms are usually better able to degrade benzo[*a*]pyrene and other high-molecular-weight PAHs than pure cultures (Kanaly et al. 2000).

IV. Effects of Acid Rain and Airborne Pollutants on Fungal Populations

Although acid rain is generally regarded as a long-range pollution phenomenon, high concentrations of mineral acids will pollute ecosystems close to point source emissions (Helander et al. 1993). Acid rain effects will also impinge on the availability and effects of other pollutants such as toxic metals, which may accompany atmospheric dispersal and/or be released from soil components as a result of increased acidity (Wainwright et al. 1982; Tabatabai 1985; Francis 1986; Persson et al. 1989). Baath et al. (1984) showed that soil biological activity, as determined by respiration rate, was significantly reduced following treatment with simulated acid rain. Mycelial lengths (FDA active) were also reduced by the treatment, while plate counts showed no response. Fritze (1987), on the other hand, showed that urban air pollution had no effect on the total length of fungal hyphae in the surface horizons of soils supporting Norway spruce (*Picea abies*). Bewley and Parkinson (1985) showed that the contribution which fungi make to the total respiration of a soil was reduced by acid rain, while, in contrast, Roberts et al. (1980) concluded that the addition of acid rain to forest soils did not affect the normal 9:1 balance of fungal to bacterial respiration. These studies clearly illustrate how difficult it is to generalise about the effects of atmospheric pollutants on soil microorganisms. Among higher fungi, simulated acid rain has been shown to increase the dominance of some ectomycorrhizal fungi, while decreasing species diversity among saprophytic species (Sastad and Jensenn 1993). Shaw et al. (1992) also showed that fumigation with sulphur dioxide or ozone had no effect on

mycorrhizal populations. Acid treatments have been shown to impair the decomposition of both deciduous leaves and conifer needles (Baath et al. 1984; Prescott and Parkinson 1985). Small-scale inhibitory effects were common, although stimulatory effects were also observed. Pollution in the form of alkaline dust from iron and steel works was shown to lead to a doubling of the total length of fungal hyphae (Fritze 1987, 1991).

The measurement of leaf litter and cellulose decomposition provides a means of assessing the impact of atmospheric pollutants on soils. However, in the absence of a means of partitioning the relative impact of the toxicants on fungi, bacteria and soil animals, such methods provide only a measure of the effects of the pollutants on the total soil community. Atmospheric pollutants from coking works can, for example, reduce populations of soil microarthropods, a response which retards the rate of litter decomposition in deciduous woodland soils (Killham and Wainwright 1981).

Few examples of the effects of enrichment disturbance by air pollutants on fungal populations can be found in the literature. However, some fungi have been reported to utilise atmospheric pollution deposits from coking works as a nutrient source, as well as being able to oxidise the reduced sulphur which these particles contain (Killham and Wainwright 1982, 1984).

V. Effects of Toxic Metals on Fungi

The ability of fungi to survive in the presence of potentially toxic metals depends on a number of biochemical and structural properties, including physiological and/or genetical adaptation, morphological changes and environmental modification of the metal in relation to the speciation, availability and toxicity (Fig. 5.1) (Gadd and Griffiths 1978; Turnau 1991; Gadd 1992, 2007). Terms such as resistance and tolerance are often used interchangeably in the literature, and may be arbitrarily based on the ability to grown on a certain metal concentration in laboratory media (Tatsuyama et al. 1975; Williams and Pugh 1975; Baath 1991; Gadd 1992). 'Resistance' is probably more appropriately defined as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned, the synthesis of metallothionein and γ-glutamyl peptides in response to Cu and Cd, respectively, providing perhaps the best examples (Mehra and Winge 1991). Metal tolerance may be defined as the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity (Gadd 1992). Intrinsic properties that can determine survival include possession of impermeable pigmented cell walls, extracellular polysaccharide and metabolite excretion, especially where this leads to detoxification of the metal species by binding or precipitation (Gadd 1993a). However such distinctions are often difficult to recognise because of the involvement in fungal survival in response to metal toxicity of several direct and indirect physicochemical and biological mechanisms. Biological mechanisms implicated in fungal survival (as distinct from environmental modification of toxicity) include extracellular precipitation; complexation and crystallisation; the transformation of metal species by, for example, oxidation, reduction, methylation and dealkylation; biosorption to cell walls, pigments and extracellular polysaccharide; decreased transport or impermeability and efflux; intracellular compartmentation; and finally precipitation and/or sequestration (Fig. 5.1) (Gadd and Griffiths 1978; Gadd 1990, 1992, 2007; Mehra and Winge 1991).

A. Effects of Metals on Fungal Populations

A range of fungi from all the major groups may be found in metal-polluted habitats (Gadd 1993a, 2007, 2011). In general terms, toxic metals may affect fungal populations by reducing abundance and species diversity and selecting for a resistant/tolerant population (Jordan and Lechevalier 1975; Babich and Stotzky 1985; Arnebrant et al. 1987). However, the effect of toxic metals on microbial abundance in natural habitats varies with the metal species and the organism present and also depends on a variety

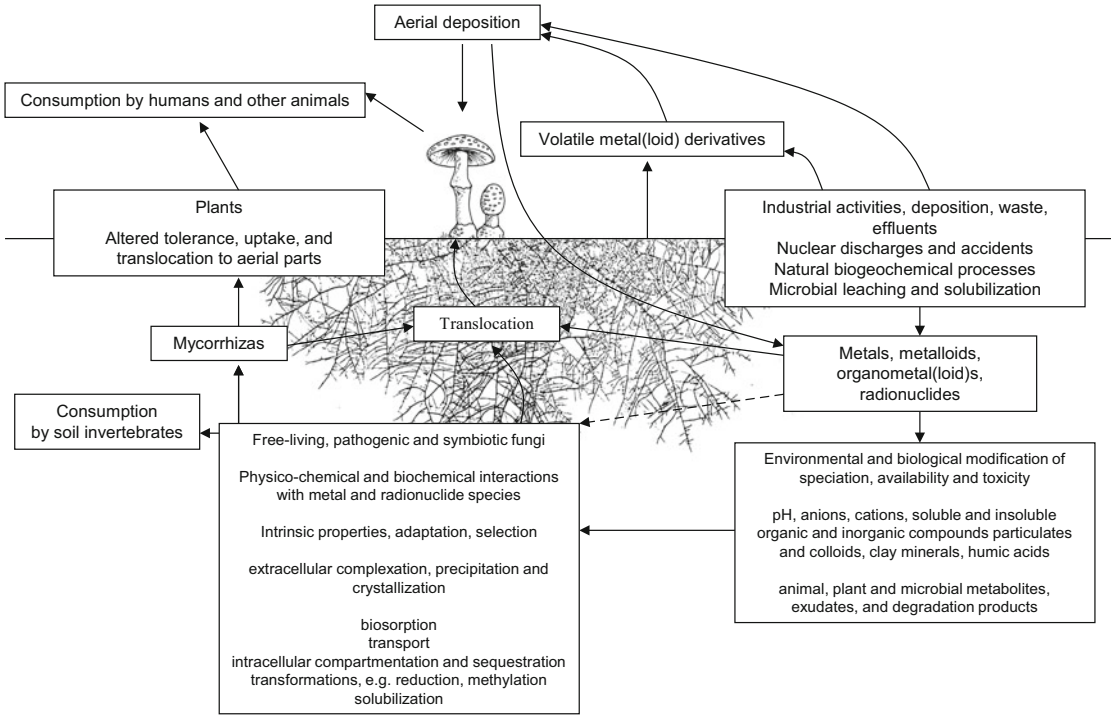

Fig. 5.1 Diagrammatic representation of the interactions of toxic metals and radionuclides with fungi in the terrestrial environment. The *dotted line* shows direct effects of metal species on fungi; this may sometimes occur and is more likely for metal species, such as Cs^+, which are highly mobile. The release of metal/radionuclide species from dead and decomposing animal and plant and microbial biomass is not shown but will be an important part of metal cycling. Fungal roles in metal solubilisation from naturally occurring substrates and/or industrial materials are indicated (see Burgstaller and Schinner 1993; Gadd 2007). For more detailed information regarding physiological and cellular interactions, see Gadd (1993a, 2007, 2010)

of environmental factors making generalisations difficult (Gadd and Griffiths 1978).

General reductions in fungal 'numbers' (as assessed by the dilution plate count in many earlier studies) have often been noted in soils polluted with Cu, Cd, Pb, As and Zn (Bewley and Stotzky 1983; Babich and Stotzky 1985). However, numerical estimates alone may provide little meaningful information unless possible changes in fungal groups and species are considered, and the problems associated with plate counting are in any case well known. Frostegard et al. (1993) analysed the phospholipid fatty acid (PLFA) composition of soil in order to detect changes in the overall composition of the microbial community and provide more reliable information on fungal populations than can be produced using plate counts. Two soils were amended with Cd, Cu, Ni, Pb and Zn and analysed after 6 months. PLFA 18:2ω6 is regarded as an indicator of fungal biomass, and this increased with increasing metal contamination for all metals except Cu, possibly reflecting the well-known mycotoxicity of Cu. However, in forest soils, such an increase in PLFA 18:2ω6 was not observed because of masking by identical PLFAs derived from plant material (Frostegard et al. 1993).

Several studies have shown that microbial population responses to toxic metals are characterised by shift from bacteria, including streptomycetes, to fungi (Mineev et al. 1999; Khan and Scullion 2002; Chander et al. 2001a, b; Kostov and Van Cleemput 2001; Olayinka and Babalola 2001). However, other studies have shown a higher metal sensitivity of the fungal component of the microbial biomass (Pennanen et al. 1996). What seems clear is

that all nutritional groups of fungi (saprotrophs, biotrophs and necrotrophs) can be affected by toxic metals. Ruhling et al. (1984) found that the soil respiration rate, fluorescein diacetate (FDA) active mycelium and mycelial standing crop were all reduced with increasing copper concentration in soils proximal to a brass mill. Nordgren et al. (1983, 1985) also showed that fungal biomass and soil respiration decreased by ~75 % along an increasing concentration gradient of metal pollution. A relative decrease in an indicator fatty acid for arbuscular mycorrhizal fungi and an increase for other fungi have been reported for zinc-polluted soil (Kelly et al. 1999). Toxic metals (Cd, Cr, Cu, Ni, Pb and Zn) led to a decrease in the number of arbuscular mycorrhizal fungi and low colonisation of plant roots and, as a result, changes in mycorrhizal species diversity (Del Val et al. 1999; Mozafar et al. 2002; Moynahan et al. 2002). Toxic metals also reduce plant root colonisation by ectomycorrhizal fungi and ectomycorrhizal species composition (Hartley et al. 1999; Markkola et al. 2002). The most frequent soil saprotrophic microfungi isolated from heavily metal-polluted habitats in Argentina, the Czech Republic and Ukraine were reported to be species of *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium*, *Rhizopus* and *Mucor*, as well as *Paecilomyces lilacinus*, *Nectria invertum*, *Cladosporium cladosporioides*, *Alternaria alternata* and *Phoma fimeti* (Kubatova et al. 2002; Massaccesi et al. 2002; Fomina, Manichev, Kadoshnikov and Nakonechnaya, unpublished). Melanised fungi, such as *Cladosporium* sp., *Alternaria alternata* and *Aureobasidium pullulans*, were often isolated from soil containing high concentrations of copper and mercury (Zhdanova et al. 1986) and can be dominant members of the mycoflora of metal-contaminated phylloplanes (Mowll and Gadd 1985). Dark septate endophytes were found to be dominant fungi among isolates from roots of *Erica herbacea* L. in Pb-, Cd-, and Zn-polluted soil (Cevnik et al. 2000).

Metal pollution of plant surfaces is widespread, but many filamentous and polymorphic fungi appear to be little affected (Smith 1977; Bewley 1979, 1980; Bewley and Campbell 1980; Mowll and Gadd 1985). On polluted oak leaves, *Aureobasidium pullulans* and *Cladosporium* species were the most numerous organisms (Bewley 1980). In fact, numbers of *A. pullulans* showed a good positive correlation with lead, whether derived from industrial or vehicular sources, and this fungus was frequently the dominant microorganism present (Bewley and Campbell 1980; Mowll and Gadd 1985).

In conclusion, elevated concentrations of toxic metals can affect both the qualitative and quantitative compositions of fungal populations although it is often extremely difficult to separate their effects from those of other environmental pollutants. It is apparent that certain fungi can exhibit considerable tolerance towards toxic metals and can become dominant microorganisms in some polluted habitats. However, while species diversity may be reduced in certain cases, resistance/tolerance can be exhibited by fungi from both polluted and nonpolluted habitats. Physicochemical properties of the environment, including changes associated with the metal pollution, may also influence metal toxicity and thereby affect species composition (Gadd 1984, 1992, 1993a; Baath 1989).

B. Morphological and Growth Responses to Toxic Metals

Effects of toxic metals on fungal growth have shown intra- and interspecific variability and dependence on the metal species present (Gadd 1993a; Plaza et al. 1998). For *T. virens* and *Clonostachys rosea* colonising spatially discrete toxic metal-containing domains, colonisation distance, hyphal extension rates and the efficacy of carbon substrate utilisation decreased with increasing concentrations of copper and cadmium (Fomina et al. 2003). A decrease in metal toxicity may be correlated with an increase in available carbon source (Ramsay et al. 1999; Fomina et al. 2003).

Several toxic metals can induce or accelerate melanin production in fungi, leading to blackening of colonies and chlamydospore development (Gadd and Griffiths 1980). Chlamydospores and other melanised forms have high biosorption capacities for metals, the majority of metal remaining within the wall

(Gadd 1984, 2009; Gadd and Mowll 1985; Gadd et al. 1987; Gadd and de Rome 1988). In rhizomorphs of an *Armillaria* sp., the highest concentrations of metals were located on the melanised outer surface (Rizzo et al. 1992).

Fungal morphology can be altered by toxic metals, and changes in mycelial density and morphology can occur (Darlington and Rauser 1988; Lilly et al. 1992; Jones and Muehlchen 1994; Gabriel et al. 1996; Baldrian and Gabriel 1997; Gardea-Torresdey et al. 1997; Ramsay et al. 1999; Fomina et al. 2000, 2005b). Biomass distribution within *Trichoderma viride* colonies was altered by toxic metals, with biomass concentrated in the periphery of the colonies in the presence of Cu and towards the interior of the colonies in the presence of Cd (Ramsay et al. 1999; Gadd et al. 2001).

During growth of fungi in metal-containing agar tiles simulating a spatially heterogeneous distribution of metal concentrations and available nutritional resources, a range of morphological changes and growth responses occurred including negative chemotropism, cessation of growth, swelling and lysis of hyphal tips (Fomina et al. 2003). Penetration of hyphae into metal-containing domains was often followed by the formation of very dense mycelia or mycelial 'bushes' (Fomina et al. 2003). Such hyphal aggregation could facilitate substrate colonisation and the production of high local concentrations of extracellular metabolites such as complexing agents (e.g. organic acids, siderophores, polyphenolic compounds), metal precipitating agents (e.g. oxalate) and polysaccharides and pigments with metal-binding abilities (Gadd 1993a; Dutton and Evans 1996; Baldrian 2003). Under poor nutritional conditions, fungi often produced long sparsely branched or branchless hyphae in toxic metal-containing domains representing an explorative growth strategy (Fomina et al. 2003). Some fungi also exhibited multiple repeated 'phase shifts' with a mixture of mycelial 'bushes' and long branchless explorative hyphae (Fomina et al. 2003). Further, microfungi-penetrating metal-contaminated domains may form mycelial cords and synnema which may be atypical for these fungi under normal conditions. The production of synnema results in a wider separation between the conidia and the substrate than in non-synnematal colonies, and this may aid dispersal as well as ensuring conidia formation away from the substrate toxicants (Newby and Gadd 1987).

C. Mycorrhizal Responses Towards Toxic Metals

Mycorrhizal fungi are involved in phosphate solubilisation, proton-promoted and ligand-promoted metal mobilisation from mineral sources, metal immobilisation within biomass and extracellular precipitation of mycogenic metal oxalates (Fomina et al. 2004, 2005a; Finlay et al. 2009; Gadd 2007, 2010, 2011). Plant symbiotic mycorrhizal fungi can therefore accumulate metals from soil components, and this may have consequences for metal nutrition of the symbiosis as well as increased or decreased toxicity (Brown and Wilkins 1985a, b; Jones and Hutchinson 1986, 1988a, b). Since plants growing on metalliferous soils are generally mycorrhizal, an important ecological role for the fungus has frequently been postulated although such a role, e.g. phytoprotection, is often difficult to confirm (Meharg and Cairney 2000a, b). Ericaceous plants appear to be entirely dependent on the presence of ericoid mycorrhizas for protection against copper, the fungus preventing metal translocation to plant shoots (Bradley et al. 1981, 1982). Arbuscular mycorrhizas (AMs) from metal-contaminated sites are often more metal tolerant to, for example, Cd and Zn, than other isolates, suggesting a benefit to the plant via increased metal resistance, nutrient uptake, etc., though in some instances, AM plants do not necessarily require fungal colonisation for survival (Griffioen 1994). It is often postulated that mycorrhizas provide a barrier to the uptake of potentially toxic metals (Bradley et al. 1981, 1982; Wilkins 1991; Hetrick et al. 1994; Wilkinson and Dickinson 1995; Leyval et al. 1997; Meharg and Cairney 2000a, b) though this has not been confirmed in every case. Further, in some instances, AM may mediate enhanced accumulation of essential metals, which, unless regulated, may lead to phytotoxicity (Killham and

Firestone 1983). It is generally concluded that local conditions in metal-contaminated sites may determine the nature of the relationship between the plant and the AM fungus, since detrimental, neutral or beneficial interactions have all been documented (Meharg and Cairney 2000a, b). For ericaceous mycorrhizas, clear host protection is observed for host plants, e.g. *Calluna* sp., *Erica* sp. and *Vaccinium* sp., growing on polluted and/or naturally metalliferous soils (Bradley et al. 1981, 1982). Further, ericaceous plants are generally found on nutrient-deficient soils, and it is likely the mycorrhiza additionally benefits the plants by enhanced nutrient uptake (Smith and Read 1997). A protective metal-binding effect of ectomycorrhizal fungi (EcM) has been postulated frequently (Denny and Wilkins 1987; Leyval et al. 1997; Dixon and Buschena 1988; Colpaert and Van Assche 1987, 1993). During growth, mycorrhizal fungi often excrete low-molecular-weight carboxylic acids and siderophores (Martino et al. 2003; Fomina et al. 2004). Ericoid mycorrhizal and ectomycorrhizal fungi can dissolve a variety of cadmium, copper, zinc and lead-bearing minerals including metal phosphates (Leyval and Joner 2001; Martino et al. 2003; Fomina et al. 2004, 2005b).

D. Metal and Metalloid Transformations by Fungi

Fungi can transform metals, metalloids (elements with properties intermediate between those of metals and non-metals: the group includes arsenic, selenium and tellurium) and organometallic compounds by reduction, methylation and dealkylation (Gadd 1993b, 2007). These are all processes of environmental importance since the transformation of a metal or metalloid may modify its mobility and toxicity. For example, methylated selenium derivatives are volatile and less toxic than inorganic forms, while the reduction of metalloid oxyanions, such as selenite or tellurite to amorphous elemental selenium or tellurium, respectively, results in immobilisation and detoxification (Thompson-Eagle and Frankenberger 1992; Morley et al. 1996). The mechanisms by which fungi (and other microorganisms) effect changes in metal speciation and mobility are important survival determinants as well as components of biogeochemical cycles for metals and many other elements including carbon, nitrogen, sulphur and phosphorus (Gadd 1999, 2004b, 2007, 2008c).

Metals and their compounds interact with fungi in various ways depending on the metal species, organism and environment, while fungal metabolism also influences metal speciation and mobility. Many metals are essential, e.g. Na, K, Cu, Zn, Co, Ca, Mg, Mn and Fe, but all can exert toxicity when present above certain threshold concentrations (Gadd 1993a, b). Other metals, e.g. Cs, Al, Cd, Hg and Pb, have no known biological function, but all can be accumulated by fungi (Gadd 1993b, 2001a, b). Metal toxicity is greatly affected by environmental conditions and the chemical behaviour of the particular metal species in question. Despite apparent toxicity, many fungi survive, grow and flourish in apparently metal-polluted locations, and a variety of mechanisms, both active and incidental, contribute to tolerance. Fungi have many properties which influence metal toxicity including the production of metal-binding peptides, organic and inorganic precipitation, active transport and intracellular compartmentalisation, while fungal cell walls have significant metal-binding abilities (Gadd and Griffiths 1978; Gadd 1993b, 2007; Fomina and Gadd 2002). All the mechanisms by which fungi (and other microorganisms) effect changes in metal speciation and mobility are survival determinants but also components of biogeochemical cycles for metals and many other associated elements including carbon, nitrogen, sulphur and phosphorus (Gadd 2004a, b, 2006, 2007, 2008a; Gadd et al. 2005, 2007). These may be simply considered in terms of metal mobilisation or immobilisation mechanisms.

1. Metal Mobilisation

Metal mobilisation from solids, e.g. rocks, minerals, soil, ash, mine spoil and other substrates, can be achieved by chelation by excreted metabolites and siderophores and methylation which can result in volatilisation.

Fungi can solubilise minerals by means of proton efflux and the production of Fe(III)-binding siderophores and as a result of respiratory carbon dioxide accumulation. In addition, other excreted metabolites with metal-complexing properties, e.g. amino acids, phenolic compounds and organic acids, may also be involved. Fungal-derived carboxylic acids provide a source of protons for solubilisation and metal-complexing anions (Gadd 1999, 2001a, 2010; Burgstaller and Schinner 1993; Gadd and Sayer 2000). Many metal citrates are highly mobile and not readily degraded. Oxalic acid can act as a leaching agent for those metals that form soluble oxalate complexes, including Al and Fe (Strasser et al. 1994). Solubilisation phenomena can have consequences for mobilisation of metals from toxic metal-containing minerals, e.g. pyromorphite ($Pb_5(PO_4)_3Cl$), contaminated soil and other solid wastes (Sayer et al. 1999). Fungi can also mobilise metals and attack mineral surfaces by redox processes. Fe(III) and Mn(IV) solubility is increased by reduction to Fe(II) and Mn(II), respectively. Reduction of Hg(II) to volatile elemental Hg(0) can also be mediated by fungi (Gadd 1993a, b).

The removal of metals from industrial wastes and by-products, contaminated soil, low-grade ores and metal-bearing minerals by fungal 'heterotrophic leaching' is relevant to metal recovery and recycling and/or bioremediation of contaminated solid wastes and perhaps the removal of unwanted phosphates (Gadd 2007, 2010). The ability of fungi, along with bacteria, to transform metalloids has also been utilised successfully in the bioremediation of contaminated land and water. Selenium methylation results in volatilisation, a process which has been used to remove selenium from the San Joaquin Valley and Kesterson Reservoir, California, using evaporation pond management and primary pond operation (Thompson-Eagle et al. 1989; Thompson-Eagle and Frankenberger 1992).

2. Metal Immobilisation

Fungal biomass provides a metal sink, either by sorption to biomass (cell walls, pigments and extracellular polysaccharides), intracellular accumulation and sequestration, or precipitation of metal compounds onto and/or around hyphae. Fungi are effective biosorbents for a variety of metals including Ni, Zn, Ag, Cu, Cd and Pb (Gadd 1990, 1993b, 2009), and this can be an important passive process in both living and dead biomass (Gadd 1990, 1993b; Sterflinger 2000). The presence of chitin, and pigments like melanin, strongly influences the ability of fungi to act as biosorbents (Gadd and Mowll 1985; Manoli et al. 1997; Fomina and Gadd 2002; Gadd 2009). In a biotechnological context, fungi and their by-products have received considerable attention as biosorbents for metals and radionuclides (Gadd and White 1992; Gadd 2002). However, attempts to commercialise biosorption have been limited, primarily due to competition with commercially produced ion exchange media of high specificity (Gadd 2009).

Fungi can precipitate a number of inorganic and organic compounds, e.g. oxalates, oxides, phosphates and carbonates (Grote and Krumbein 1992; Arnott 1995; Verrecchia 2000; Gadd 1999; Gharieb and Gadd 1999), and this can lead to the formation of biogenic minerals (mycogenic precipitates). Precipitation, including crystallisation, will immobilise metals but also leads to the release of nutrients like sulphate and phosphate (Gadd 1999). Fungi can produce a variety of metal oxalates with a variety of different metals and metal-bearing minerals, e.g. Cd, Co, Cu, Mn, Sr, Zn and Ni (Gadd 1999), which may provide a mechanism whereby fungi can tolerate toxic metal-containing environments. Fungi produce other metal oxalates besides calcium on interacting with a variety of different metals and metal-bearing minerals, including those of Ca, Cd, Co, Cu, Mg, Mn, Sr, Zn, Ni and Pb (Sayer and Gadd 1997; Gadd 1999, 2007; Sayer et al. 1999; Adeyemi and Gadd 2005; Fomina et al. 2007a, b; Wei et al. 2013). The formation of toxic metal oxalates may contribute to fungal metal tolerance (Gadd 1993a; Clausen et al. 2000; Jarosz-Wilkołazka and Gadd 2003; Green and Clausen 2003). Mycogenic oxalate minerals produced by free-living fungi include glushinskite ($MgC_2O_4{\cdot}2H_2O$) (Burford et al. 2003a, b; Kolo and Claeys 2005; Kolo et al. 2007; Gadd 2007),

moolooite ($Cu(C_2O_4)\cdot 0.4H_2O$) (Fomina et al. 2005a, 2007b) and lindbergite ($MnC_2O_4\cdot 2H_2O$) (Wei et al. 2012). A similar mechanism occurs in lichens growing on copper–sulphide-bearing rocks, where precipitation of copper oxalate occurs within the thallus (Purvis 1996). Oxalate production by *Aspergillus niger* and *Serpula himantioides* has been shown to induce the dissolution and conversion of both rhodochrosite and Mn oxides to Mn oxalate minerals (Sayer et al. 1997; Wei et al. 2012). Oxalate can act as a reductant of Mn(IV) oxides, and this can result in mobilisation of Mn(II), which can then precipitate. Both *A. niger* and *S. himantioides* were capable of solubilising the insoluble manganese oxides MnO_2 and Mn_2O_3, mycogenic manganese oxide (MnO_x) and birnessite [$(Na_{0.3}Ca_{0.1}K_{0.1})(Mn^{4+},Mn^{3+})_2O_4\cdot 1.5H_2O$]. Precipitation of insoluble manganese oxalate occurred and manganese oxalate trihydrate was detected after growth of *S. himantioides* with birnessite which subsequently was transformed to manganese oxalate dihydrate (Wei et al. 2012). Several free-living and mycorrhizal fungi can attack and transform pyromorphite ($Pb_5(PO_4)_3Cl$) to lead oxalate (Sayer et al. 1999; Fomina et al. 2004, 2005b). It has also been shown that certain fungi (e.g. *Paecilomyces javanicus*, *Metarhizium anisopliae*) were able to mediate transformation of metallic lead into pyromorphite, representing biomineralisation of mobile lead species into a very stable form (Rhee et al. 2012, 2014a, b). This might be an important process occurring in lead-containing environments and of relevance to proposed bioremedial treatments (Rhee et al. 2012). It is likely that acidolysis and complexation involving excreted organic acids play an important role in mediating precipitation of pyromorphite (Rhee et al. 2012, 2014a, b) and other metal phosphates (Fomina et al. 2007c, 2008). Fungal activity can also play an important role in the biocorrosion and transformation of lead metal into pyromorphite in the aquatic environment. The ability of fungi to immobilise mobile lead species in an insoluble form provides a further approach for the removal and detoxification of lead from aqueous solution by bioprecipitation. The principles of such a process could also be applied to other metals and insoluble biominerals for bioremediation or biorecovery of valuable elements (Rhee et al. 2014a, b). Another research has demonstrated that fungi can solubilise uranium oxides and depleted uranium and reprecipitate secondary uranium phosphate minerals of the meta-autunite group, uramphite and/or chernikovite, which can encrust fungal hyphae to high accumulation values of 300–400 mg U g dry wt^{-1} (Fomina et al. 2007c, 2008). Such minerals appear capable of long-term U retention (Fomina et al. 2008). The phosphate may arise from inorganic sources in the environment or from phosphatase-mediated hydrolysis of organic P sources, with the hyphal matrix serving to localise the resultant uranium minerals (Liang et al. 2015).

Many fungi precipitate reduced forms of metals and metalloids in and around fungal hyphae, e.g. Ag(I) can be reduced to elemental silver Ag(0), selenate [Se(VI)] and selenite [Se(IV)] to elemental selenium and tellurite [Te(IV)] to elemental tellurium [Te(0)] (Gharieb et al. 1995, 1999).

3. Organometal(loid)s

Organometals (compounds with at least one metal–carbon bond) can be attacked by fungi with the organic moieties being degraded and the metal compound undergoing changes in speciation. Degradation of organometallic compounds can be carried out by fungi, either by direct biotic action (enzymes) or by facilitating abiotic degradation, for instance, by alteration of pH and excretion of metabolites. Organotins, such as tributyltin oxide and tributyltin naphthenate, may be degraded to mono- and dibutyltins by fungal action, inorganic Sn(II) being the final degradation product. Organomercury compounds may be detoxified by conversion to Hg(II) by fungal organomercury lyase, the Hg(II) being subsequently reduced to Hg(0) by mercuric reductase, a system analogous to that found in mercury-resistant bacteria (Gadd 1993b).

E. Accumulation of Metals and Radionuclides by Macrofungi

Elevated concentrations of toxic metals and radionuclides can occur in the fruiting bodies of higher fungi sampled from polluted environments. This phenomenon is of significance in relation to the use of macrofungi as bioindicators of metal pollution and because of human toxicity resulting from the consumption of wild fungi. In general, levels of Pb, Cd, Zn and Hg found in macrofungi from urban or industrial areas are higher than from corresponding rural areas, although there are wide differences in uptake abilities between different species and different metals (Tyler 1980; Bressa et al. 1988; Lepsova and Mejstrik 1989). Cadmium is accumulated to quite high levels in macrofungi, averaging around 5 mg (kg dry wt)$^{-1}$ although levels of up to 40 mg (kg dry wt)$^{-1}$ have also been recorded (Byrne et al. 1976). *Laccaria amethystina* caps exhibited total As concentrations of 100–200 mg (kg dry wt)$^{-1}$ (Stijve and Porette 1990; Byrne et al. 1991). Accumulation of ^{110}Ag and ^{203}Hg was studied in *Agaricus bisporus*, and concentration factors (metal concentration in mushroom, metal concentration in substrate) were found to be up to 40 and 3.7, respectively, with the highest Ag and Hg contents recorded being 167 and 75 mg (kg dry wt)$^{-1}$, respectively (Byrne and Tusek-Znidaric 1990). As well as fruiting bodies, rhizomorphs (e.g. of *Armillaria* species) can concentrate metals up to 100 times the level found in soil. Concentrations of Al, Zn, Cu and Pb in rhizomorphs were 3440, 1930, 15 and 680 mg (kg dry wt)$^{-1}$, respectively, with the metals primarily located in extracellular portions (Rizzo et al. 1992).

F. Accumulation of Radiocaesium by Macrofungi

Following the Chernobyl accident in 1986, there were several studies on radiocaesium (mainly ^{137}Cs) accumulation by fungi. Free-living and mycorrhizal basidiomycetes can accumulate radiocaesium (Haselwandter 1978; Elstener et al. 1987; Byrne 1988; Dighton and Horrill 1988; Haselwandter et al. 1988; Clint et al. 1991; Dighton et al. 1991; Muramatsu et al. 1991; Heinrich 1992); these organisms appear to have a slow turnover rate for Cs and comprise a major pool of radiocaesium in soil (Clint et al. 1991). Mean activities of 25 Ukrainian, 6 Swedish and 10 North American collections were 4660, 9750 and 205 Bq (kg dry wt)$^{-1}$, respectively (Smith et al. 1993). Deviations in the ^{137}Cs:^{134}Cs ratios attributable to Chernobyl have revealed considerable accumulation of pre-Chernobyl Cs in macrofungi, probably as the result of weapon testing (Byrne 1988; Dighton and Horrill 1988). It appeared that about 20 % of the ^{137}Cs in Eastern Europe (Moscow area, Belarus, Ukraine) was of non-Chernobyl origin (Smith et al. 1993). Radiocaesium accumulation in basidiomycetes appears to be species dependent, with influences exerted by soil properties. Significantly higher activities may be found in mycorrhizal species compared to saprotrophic and parasitic fungi (Smith et al. 1993). Smith et al. (1993) found that many prized edible mycorrhizal fungi may contain unacceptably high levels of ^{137}Cs, that is, at levels of greater than 1000 Bq (kg dry wt)$^{-1}$. It has also been demonstrated that the fungal component of soil can immobilise the total Chernobyl radiocaesium fallout received in upland grasslands (Dighton et al. 1991) although grazing of fruiting bodies by animals may lead to radiocaesium transfer along the food chain (Baaken and Olson 1990).

G. Fungi as Bioindicators of Metal and Radionuclide Contamination

As mentioned above, higher fungi growing on contaminated sites can show significantly elevated concentrations of metals in their fruiting bodies, and some experiments have demonstrated a correlation between the quantities of metals in a growth substrate and the amounts subsequently found in the fruiting bodies (Wondratschek and Roder 1993). The concept of bioindicators has been usually discussed in terms of reaction indicators and accumulation

indicators. Reaction indicators may comprise individual organisms and/or communities which may decline or disappear (sensitive species) or show increases (tolerant species). For accumulation indicators, the indicator organism is analysed for the pollutant. Some organisms, in theory, can therefore serve as both reaction and accumulation indicators. As described previously, alteration of macrofungal communities by metal pollution has frequently been recorded. Ruhling et al. (1984) noted a decline from about 40 species per 100 m^2 to about 15 species near the source of metal contamination (smelter emissions), with only *Laccaria laccata* increasing in frequency at more polluted locations. Other higher fungi which are apparently tolerant of high metal pollution include *Amanita muscaria* and several species of *Boletus*; some *Russula* species, on the other hand, appear metal sensitive (Wondratschek and Roder 1993).

Fungi possess several advantages over plants as metal accumulation indicators. The fruiting bodies may accumulate greater amounts of metals than plants, while the large area of mycelium ensures contact with and translocation from a large area of soil. Furthermore, fruiting bodies may project above the ground for only a short period, thereby minimising contamination from aerial or wet deposition of metal pollutants. Sporophores are also easily harvested and amenable to rapid chemical analysis (Mejstrik and Lepsova 1993). However, it is debatable whether a sufficiently clear relationship exists between indicator species and the metal pollution under consideration. For mercury, wide variations in metal content of fruiting bodies occur in different species sampled at the same site, ranging over as much as three orders of magnitude, with some species showing extremely high Hg accumulation values. Mercury concentrations in fungi generally occur in the range 0.03–21.6 mg (kg dry wt)$^{-1}$ although concentrations greater than 100 mg (kg dry wt)$^{-1}$ have been recorded from polluted sites. Despite this, several macrofungi have been suggested as being suitable bioindicators of mercury pollution (see Mejstrik and Lepsova 1993; Wondratschek and Roder 1993) (Table 5.1).

Table 5.1 Higher fungi proposed as bioindicators for metal pollution based on metal analyses of fruiting bodies (see Mejstrik and Lepsova 1993; Wondratschek and Roder 1993)

Species	Metal(s)
Agaricus arvensis	Hg, Cd
Agaricus campestris	Hg, Cd
Agaricus edulis	Hg, Cd
Agaricus haemorrhoidarius	Hg
Agaricus xanthodermus	Hg
Agaricus sp.	Pb, Zn, Cu
Amanita rubescens	Hg
Amanita strobiliformis	Hg
Coprinus comatus	Hg
Lycoperdon perlatum	Hg
Lycoperdon sp.	Pb, Zn, Cu
Marasmius oreades	Hg
Mycena pura	Hg, Cd

A wide variation in Cd content has also been recorded in macrofungi with ranges of reported values from <0.1–229 mg (kg dry wt)$^{-1}$ (Tyler 1980). However, there is frequently a lack of correlation between the fungal Cd content and the Cd content of the soil (Wondratschek and Roder 1993). Compared to other common metal pollutants, lower concentrations of Pb tend to be found in macrofungi, with much of the Pb content being derived from aerial sources. Levels of Pb around 0.4–36 mg (kg dry wt)$^{-1}$ have been reported in sporophores, with higher levels occurring in urban areas (Tyler 1980). Zinc, an essential metal for fungal growth and metabolism, occurs at high concentrations within fungi, 50–300 mg (kg dry wt)$^{-1}$ (Tyler 1980), with a few genera apparently showing high affinities for the metal (Table 5.1). Copper may also be found at high levels (20–450 mg (kg dry wt)$^{-1}$) in higher fungi (Tyler 1980). However, with both Cu and Zn, there is a tendency for metal concentrations in fruiting bodies to be independent of soil concentrations which reduces their value as bioindicators (Gast et al. 1988).

It is clear that many factors contribute to the wide variations in recorded metal contents of macrofungal fruiting bodies, even in the same species sampled at the same site. Despite numerous studies, most investigations tend to be contradictory and provide little useful infor-

mation (Wondratschek and Roder 1993). Apart from organism-related factors, environmental factors are of paramount importance in relation to metal accumulation by higher fungi and include physicochemical soil properties like moisture and temperature, all of which influence metal availability as well as the physiological activity of the fungus. It can be concluded, therefore, that a perfect fungal bioindicator does not exist, although macrofungi may be useful in determining the extent of a polluted or unpolluted area.

H. Bioremediation, Biotechnology and Bioprocessing

Several fungal metal and mineral transformations have potential for the treatment of environmental pollution (Gadd 2004a, 2005; Pumpel and Paknikar 2001). While several fungal-based systems have received interest in the context of bioremediation of organic pollutants, there has not been so much attention given to metals. However, it should be stressed that fungi will be components of the microbiota in any metal-polluted sites where their activities may contribute to natural attenuation of the pollutants and will also be involved in many soil and waste treatment processes, revegetation strategies and effluent treatments. Fungi were clearly important in remediation of selenium-contaminated soils (Thompson-Eagle and Frankenberger 1992). In addition, fungal mineral-solubilising properties are important in plant nutrition and soil fertility especially regarding phosphates. In addition to bioremediation, metal and mineral transformations have applications in other areas of biotechnology and bioprocessing, including biosensors, biocatalysis, electricity generation and nanotechnology.

1. Bioleaching

Fungal solubilisation of metals from solid minerals and metal and mineral wastes, including contaminated soil, for metal recovery, recycling and bioremediation purposes have all been investigated, although fungal systems cannot compare with the established bacterial bioleaching processes and may be more suited to bioreactor applications. Metals can be solubilised from fly ash (originating from municipal solid waste incineration), contaminated soil, electronic scrap and other waste materials by fungal activity (Brandl 2001; Brandl and Faramarzi 2006).

2. Biosorption and Bioaccumulation

Biosorption is a physicochemical process, simply defined as 'the removal of substances from solution by biological material'. It is a property of both living and dead organisms (and their components) and has been proposed as a promising biotechnology for the removal (and/or recovery) of metals, radionuclides and organic pollutants for many years because of its simplicity, analogous operation to conventional ion exchange technology and apparent efficiency (Gadd 1986, 2001a, b, 2009; Volesky 1990; Garnham et al. 1992; Gadd and White 1990, 1993; Wang and Chen 2006, 2009). Modification of biomass has been attempted to improve efficiency or selectivity of microbial biosorbents. Fungal–clay biomineral sorbents combined the sorptive advantages of the individual counterparts, i.e. the high density of metal-binding sites per unit area and high sorption capacity of fungal biomass, high sorption affinity and the high surface area per unit weight mechanical strength and efficient sorption at high metal concentrations of the clay minerals (Fomina and Gadd 2002). *S. cerevisiae* mutants (*pmr1*Δ) hypersensitive to heavy metals due to increased metal uptake have been investigated for the ability to remove Mn^{2+}, Cu^{2+}, Co^{2+} or Cd^{2+} from synthetic effluents by a combination of biosorption and intracellular uptake (Ruta et al. 2010). Phytochelatins (PCs) are metal-binding cysteine-rich peptides, enzymatically synthesised in plants and certain fungi from glutathione in response to heavy metal stress. Overexpression of PC synthase in bacteria could be a means of improving the metal content of organisms for bioremediation (Valls et al. 2000).

3. Metalloid Bioremediation

Microbial responses to arsenic of bioremedial potential include chelation, compartmentalisation, exclusion and immobilisation. Attempts to engineer an arsenic accumulating microbe have involved modification of natural resistance mechanisms and development of novel or hybrid pathways into an easily manipulated organism. Thus, PC synthase from *S. pombe* (SpPCS) has been expressed in *E. coli*, resulting in higher As accumulation, these steps being combined in an arsenic efflux deletion *E. coli* strain to achieve the highest reported arsenic accumulation in *E. coli* of 16.8 μmol/g cells (Tsai et al. 2009). A yeast strain coexpressing AtPCS and cysteine desulphhydrase, an aminotransferase that converts cysteine into hydrogen sulphide under aerobic conditions, was used to elevate As accumulation by formation of PC–metal–sulphide complexes (Tsai et al. 2009). The ability of fungi, along with bacteria, to transform metalloids has been utilised successfully in the bioremediation of contaminated land and water. Selenium methylation results in volatilisation, a process which has been used to remove selenium from contaminated sites (Thompson-Eagle and Frankenberger 1992).

4. Mycoremediation and the Mycorrhizosphere

Mycorrhizal associations may have application in the general area of phytoremediation (Rosen et al. 2005; Gohre and Paszkowski 2006): phytoremediation is the use of plants to remove or detoxify environmental pollutants (Salt et al. 1998). Mycorrhizas may enhance phytoextraction by increasing plant biomass, and some studies have shown increased plant accumulation of metals, especially when inoculated with mycorrhizal fungi isolated from metalliferous environments. However, the potential impact of mycorrhizal fungi on bioremediation may be dependent on many factors including their metal tolerance and the nutritional status of contaminated soils (Meharg 2003). In addition, some studies have shown mycorrhizas can reduce plant metal uptake (Tullio et al. 2003). Arbuscular mycorrhizas (AMs) depressed translocation of zinc to shoots of host plants by binding of metals in mycorrhizal structures and immobilisation of metals in the mycorrhizosphere (Christie et al. 2004). Local conditions in metal-contaminated sites may determine the relationship between the plant and the AM fungus, and detrimental, neutral and beneficial interactions have all been documented (Meharg and Cairney 2000a, b). A protective metal-binding effect of ectomycorrhizal fungi (EcM) has been postulated (e.g. Leyval et al. 1997). A Cu-adapted *Suillus luteus* isolate provided protection against Cu toxicity in pine seedlings exposed to elevated Cu. Such a metal-adapted *Suillus–Pinus* combination might be suitable for large-scale land reclamation at phytotoxic metalliferous and industrial sites (Adriaensen et al. 2005). Ectomycorrhizal fungi persistently fixed Cd(II) and Pb(II) and formed an efficient biological barrier that reduced the movement of these metals in birch tissues (Krupa and Kozdroj 2004). Such mycorrhizal metal immobilisation around plant roots, including biomineral formation, may also assist soil remediation and revegetation. Naturally occurring soil organic compounds can stabilise potentially toxic metals like Cu, Cd, Pb and Mn. The insoluble glycoprotein, glomalin, produced in copious amounts on hyphae of arbuscular mycorrhizal fungi can sequester such metals and could be considered a useful stabilisation phenomenon (Gonzalez-Chavez et al. 2004). Phytostabilisation strategies may reduce the dispersion of uranium (U) and the environmental risks of U-contaminated soils. *Glomus intraradices* increased root U concentration and content, but decreased shoot U concentrations. AM fungi and root hairs improved not only P acquisition but also root uptake of U, and the mycorrhiza generally decreased U translocation from plant root to shoot (Rufyikiri et al. 2004; Chen et al. 2005a, b).

For ericaceous mycorrhizas, host protection has been observed in, for example, *Calluna*, *Erica* and *Vaccinium* spp. growing on Cu- and Zn-polluted and/or naturally metalliferous soils, the fungus preventing metal translocation to plant shoots (Bradley et al. 1981, 1982). Further, ericaceous plants are generally found on nutrient-deficient soils, and it is likely the mycorrhiza could additionally benefit the

plants by enhanced nutrient uptake (Smith and Read 1997). The development of stress-tolerant plant–mycorrhizal associations may therefore be a promising strategy for phytoremediation and soil amelioration (Schutzendubel and Polle 2002). Ericoid mycorrhizal fungal endophytes, and sometimes their plant hosts, can evolve toxic metal resistance which enables ericoid mycorrhizal plants to colonise polluted soil (Perotto et al. 2002; Martino et al. 2003). This seems to be a major factor in the success of ericoid mycorrhizal taxa in a range of harsh environments (Cairney and Meharg 2003).

The importance of mycorrhizas in plant phosphorus nutrition has been appreciated for a long time, and their ability to dissolve and transform calcium-containing insoluble compounds and minerals (calcium phosphates, carbonate and sulphate) has been widely studied (Callot et al. 1985a, b; Lapeyrie et al. 1990, 1991; Gharieb and Gadd 1999). However, toxic metal mineral solubilisation has received little attention, though this should be considered in any revegetation, natural attenuation or phytoremediation strategies. The ectomycorrhizal fungi *Suillus granulatus* and *Pisolithus tinctorius* can promote the release of cadmium and phosphorus from rock phosphate (Leyval and Joner 2001), while the ericoid mycorrhizal fungus *Oidiodendron maius* can solubilise zinc oxide and phosphate (Martino et al. 2003). Many ericoid mycorrhizal and ectomycorrhizal fungi are able to solubilise zinc, cadmium, copper phosphates and lead chlorophosphate (pyromorphite) releasing phosphate and metals (Fomina et al. 2004). Both non-mycorrhizal *Pinus sylvestris* and pines infected with the ectomycorrhizal *Paxillus involutus* could enhance zinc phosphate dissolution, withstand metal toxicity and acquire the mobilised phosphorus (Fomina et al. 2006).

5. Nanoparticle Formation and Nanobiotechnology

Metal-containing micro-/nanoparticles have applications as new ceramic–metal (cermet) or organic–metal (orgmet) composites or structured materials for a variety of applications (Hennebel et al. 2009). The use of metal-accumulating microbes for the production of nanoparticles, and their assembly, may allow control over size, morphology, composition and crystallographic orientation. The production of such biomimetic materials is relevant to the production of new advanced materials, with applications in metal and radionuclide bioremediation, antimicrobial treatments (e.g. nanosilver), solar energy and electrical battery applications and microelectronics (Dameron et al. 1989; Klaus-Joerger et al. 2001). Because of their high specific surface area and high catalytic properties, biogenic metal products also offer potential for sorption and degradation of organic contaminants, as well as a variety of other applications, e.g. electricity generation in fuel cells, novel catalysts and sensors. Biogenic Mn oxides can sequester metals like Pb, Zn, Co, Ni, As and Cr and also oxidise certain organic pollutants (Hennebel et al. 2009). In contrast to bacteria, rather less attention has been given to fungal systems in this context although fungal reductive transformations of metalloids and Ag and Au species to nano- or colloidal forms are well known, as well as metal-containing reactive crystallites (Dameron et al. 1989) and Mn oxides (Miyata et al. 2004, 2007).

6. Soil Treatment Processes

The application to soils of certain amendments that immobilise metals, e.g., lime or phosphate treatment, has demonstrated enhanced natural remediation resulting in improved vegetation growth, increased microbial activity and diversity and reduced off-site metal transport. However, while long-term stability of certain metal complexes and compounds has been shown in model systems (Adriano et al. 2004), the influence of plant roots and its microbial and mycorrhizal associations on such stability has often been neglected. For example, pyromorphite ($Pb_5(PO_4)_3Cl$), which can form in urban and industrially contaminated soils, can be solubilised by phosphate-solubilising fungi, with concomitant production of lead oxalate (Sayer et al. 1999; Fomina et al. 2004). The ability of free-living and mycorrhizal fungi to

transform pyromorphite (and other toxic metal-containing minerals) should be taken into account in risk assessments of the long-term environmental consequences of in situ chemical remediation techniques, revegetation strategies or natural attenuation of contaminated sites. The bioweathering potential of fungi has been envisaged as a possible means for the bioremediation of asbestos-rich soils. Several fungi could extract iron from asbestos mineral fibres (e.g. 7.3 % from crocidolite and 33.6 % from chrysotile by a *Verticillium* sp.), thereby removing the reactive iron ions responsible for DNA damage (Daghino et al. 2006).

VI. Conclusions

It is clear from the above that fungi are of importance in the transformation of both organic and inorganic pollutants in the natural environment. While pollutants may exhibit toxicity and cause changes in fungal community composition, fungi possess a range of mechanisms that confer resistance or tolerance, many of these resulting in pollutant transformation to less toxic forms. Such activities are part of natural biogeochemical cycles for major elements such as C, N, O, P and S but also metals, metalloids and radionuclides, as well as having applications in the bioremediation and natural attenuation of polluted habitats. However, pollutant interactions are complex and greatly influenced by environmental factors. While the theoretical response of fungi to pollutants can readily be speculated upon, some effects are difficult to demonstrate and quantify because of the inadequacy of several common techniques used to study fungal populations and their activities. Despite this, newly developed approaches using molecular biology and biomarkers are allowing a better understanding of community structure and responses to environmental factors, including pollutants. Growth media containing low and therefore more realistic concentrations of available carbon should also be used if in vitro techniques are employed to help determine the effects of pollutants on fungal growth. However, it is clear that because of the complexity of the fungal growth form and their multiplicity of biological responses and interactions with pollutants, coupled with the complexity of the terrestrial (and other) environments, a wealth of knowledge still awaits discovery.

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6 Degradation of Plant Cell Wall Polymers by Fungi

Jonas Ramoni[1], Bernhard Seiboth[1]

CONTENTS

I. Introduction

Plants are the main producers of primary biomass through carbon fixation. By photosynthesis, they convert light energy into chemical energy, thereby transforming carbon dioxide to carbohydrates. Fungi as heterotrophs depend on these organic compounds as energy and nutrient source. They are today the most important and widespread group of organisms responsible for the recycling of plant material into the ecosystem and are therefore essential components of the global carbon cycle. Saprotrophic or saprobic fungi grow on dead material and are particularly important for the enzymatic breakdown of various polymers in the cell wall of plants. This lignocellulosic plant matter is the most abundant natural material on earth and is mainly composed of the three polysaccharides cellulose, hemicellulose, and pectin and the polymer lignin. Fungi are also adapted to grow as parasites or pathogens on living plants, with biotrophic parasites feeding from living cells and necrotrophic parasites killing the host organism before they feed on the dead cells. In addition, a number of economically important plants are attacked by fungi, leading to significant crop losses either by plant decomposition or by the production of toxic substances. Hyphal fungi are not only the most efficient degraders of plant biomass but are also the main source of enzymes for commercial lignocellulose degradation. The polysaccharides of the plant cell walls have attracted the biotech industry as a source of fermentable sugars, not only for bio-ethanol production but also as raw material in the production of a wide range of other biorefinery products (Carroll and Somerville 2009; Pauly and Keegstra 2008; Youngs and Somerville 2012; Somerville et al. 2010).

The importance of fungi in global carbon cycling and the applications of these organisms and their enzymes in industry have heavily promoted research over the past decades. Applications are found amongst others in the food, animal feeding, textile, and pulp and paper industries. The fungi best studied with respect to -biopolymer-degrading enzyme formation are *Aspergillus* sp., *Neurospora crassa*, *Penicillium* sp., and *Trichoderma reesei* or—as an example of lignin degradation—the white-rot fungus *Phanerochaete chrysogenum*. In nature, most of these fungi produce considerable amounts of extracellular enzymes to get access to metabolizable sugars entrapped in the different biopolymers. Strain improvement and specific

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selection programs have resulted in industrial strains of *Aspergillus niger* and *T. reesei* which can yield more than 100 g/l of amylases or cellulases (Durand et al. 1988; Berka et al. 1991; Cherry and Fidantsef 2003), therefore making them also versatile production hosts for other enzymes and recombinant proteins. Another advantage of these fungi is that they are easy and inexpensive to grow in large bioreactors and are suitable for genetic recombination technologies. Indeed, it was not surprising that these biotechnological applied fungi were among the first filamentous fungi for which a genome sequence became available (Martinez et al. 2008; Pel et al. 2007). The recent advances in the sequencing and annotation of fungal genomes give an impression of the hidden enzymatic potential in these organisms, and transcript and proteome analysis has accelerated our current understanding of the different genes expressed on various natural substrates. However, despite the advanced technologies used today, the inability to efficiently convert the crystalline and insoluble cellulose to fermentable sugars still poses a major barrier for the commercialization of biofuel production (Himmel and Bayer 2009).

This chapter aims to summarize our current knowledge on plant cell wall degradation, giving the reader an overview about the structures of the substrates and the enzymatic strategies for their degradation. As an example for the complex deconstruction of lignocellulose, the biopolymer degradation strategy of brown- and white-rot fungi is highlighted in more detail. A complete listing of all genes and their encoded enzymes involved in the breakdown of plant cell walls is beyond the scope of this chapter. Today, a number of web-based databases are available which accomplish these tasks. One example is the Carbohydrate-Active Enzymes Database (www.CAZy.org) which is a manually curated list of enzyme classes and families known to act on carbohydrates (Cantarel et al. 2009; Levasseur et al. 2013; Lombard et al. 2014). As a complement to CAZy, the CAZypedia (www.cazypedia.org) describes the different enzyme classes and families in more detail. Initially focusing on the glycoside hydrolase (GH) families, other CAZyme classes were introduced to the CAZy database within the last years, including polysaccharide lyases (PL), glycosyltransferases, carbohydrate esterases (CE), as well as auxiliary redox enzymes and non-catalytic carbohydrate-binding modules (CBMs). A database specialized on the biochemical properties of lignocellulose-active proteins is *myco*CLAP (https://mycoclap.fungalgenomics.ca/mycoCLAP/ Murphy et al. 2011). Biochemical properties and functional annotations described in *myco*CLAP are manually curated, and the database covers fungal GHs, CEs, and PLs and enzymes with auxiliary activities.

II. Structure and Composition of Plant Cell Walls

Plant cells are enclosed by cell walls which provide rigidity to the cell for structural and mechanical support, maintain and determine cell shape, counterbalance osmotic pressure, direct growth, and, ultimately, determine the architecture and form of the plant. In addition, the plant cell wall protects against environmental factors or pathogens. The plant cell walls consist mainly of polysaccharides such as cellulose, hemicelluloses, pectins, and the aromatic polymer lignin. Together, they form a complex and rigid structure termed lignocellulose.

Typically, three regions are distinguished in plant cell walls, which are the middle lamella, the primary, and the secondary cell wall. The middle lamella is the outermost layer, composed primarily of pectin and its function is to cement the cell walls of adjacent cells together. Primary walls are synthesized during growth, whereas secondary walls are thickened structures containing lignin and surrounding specialized cells such as vessel elements or fiber cells. All differentiated cells contain walls with distinct compositions, resulting in a spectrum of specialized cell walls with primary and secondary walls as two extremes (Keegstra 2010). Primary walls are comprised of 15–40 % cellulose, 30–50 % pectins, and 20–30 % xyloglucans and minor amounts of arabinoxylans and proteins. The primary cell wall is expanded inside the middle lamella and consists of several interconnected matrices. In these matrices, cellulose microfibrils are aligned at all angles and cross-linked via hemicellulosic tethers to form the cellulose–hemicellulose network (e.g., with xyloglucan and galactoglucomannan). This network is embedded in the gelatinous pectin matrix composed of homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (O'Neill and York 2003). The pectins are particularly important for wall hydration. Two main types of primary cell walls are distinguished (Carpita and Gibeaut 1993): type I is found in all dicotyledons, non-graminaceous monocotyledons, and gymnosperms

and typically contains xyloglucan and/or glucomannan and 20–35 % pectin. Type II is found in grasses, where arabinoxylans constitute most of the matrix, and mixed-linkage glucans transiently comprise 10–20 % of wall mass (Carpita et al. 2001; Gibeaut et al. 2005). An exception are celery and sugar beet parenchyma which are rich in cellulose and pectin, but have little hemicelluloses (Thimm et al. 2002; Zykwinska et al. 2007).

In addition to primary walls, many plants synthesize a secondary wall, which is assembled between the plant cell and the primary wall. These secondary walls are found in plant tissues that have ceased growing. They provide strength and rigidity and are comprised primarily of cellulose and lignin. In addition, the hemicelluloses xylan, and glucomannan replace xyloglucan and pectins. Secondary cell walls are also less hydrated than primary cell walls. The cellulose microfibrils are generally aligned in the same direction but, with each additional layer, their orientation changes slightly. The secondary wall is altered during development by successive encrustation and deposition of cellulose fibrils and other components. Nonstructural components of the secondary wall represent generally less than 5 % of the dry weight of wood and include compounds such as phenols, tannins, fats, sterols, proteins, and ashes.

The structure of the polysaccharides found in the cell wall is highly diverse and comprises a spectrum ranging from simple linear polymers composed of a single type of glycosyl residue (e.g., cellulose is composed of 1,4-linked β-glucosyl residues), over polymers with a regular branching pattern (e.g., xyloglucan and rhamnogalacturonan II), to rhamnogalacturonan I, a polymer substituted with a diverse range of arabinosyl and galactosyl-containing oligosaccharide side chains. Understanding the structures of these polymers and determining their primary structures remain a major challenge, especially because their biosynthesis is not template driven (O'Neill and York 2003).

III. Degradation of the Plant Cell Wall

Due to the overall structural and chemical complexity of plant cell walls, a complete breakdown of the different components is brought about only by a wide range of organisms acting in a consortium. This degradation follows a characteristic decomposition sequence, which starts with the colonization of living plants and ends with the production of highly persistent soil humus. The fungi found in these decomposition sequences live in complex and diverse communities and are often specialized to degrade only certain types of polymers, reflecting their genomic capabilities. The efficient breakdown of the plant cell wall by fungi is linked, on the one hand, to their hyphal growth, which provides penetrating power, and, on the other hand, to their highly specialized extracellular plant cell wall-degrading enzyme systems. The enzymatic decomposition of plant cell walls is usually synergistic: individual, highly specialized enzymes operate as components of multi-enzyme systems to efficiently degrade the polymers. The synthesis of these enzymes is governed by a sophisticated signal transduction and gene regulation system, and a highly productive secretory machinery is available for their cellular export. These characteristics enable fungi to successfully compete with other microorganisms in their environment, and they are today the main agents of decomposition in terrestrial and aquatic ecosystems.

Degradation occurs extracellularly, since the substrates are usually large polymers which are insoluble or even crystalline. Two principal types of extracellular enzymatic systems for the degradation of the polymeric fraction have been developed: the hydrolytic system, which degrades the polysaccharides mainly by glycoside hydrolases, and a unique oxidative ligninolytic system, which depolymerizes lignin. But even for the complete degradation of the chemically simple polysaccharide cellulose, several enzymes are necessary and the number of enzymes is further increased when the polysaccharides are substituted. Historically, three classes of enzymes can be distinguished although recent advancements have revealed overlapping roles of some of the enzymes originally classified into one of these classes. These classes include (1) exo-acting enzymes, which release mainly mono- and dimers of the ends of the polymeric chain, (2) endo-acting enzymes, which cleave in the middle of the sugar chain, and (3) enzymes (often exo-acting) which are specialized in cleaving the resulting oligosaccharides into their monomers. The resulting low molecular break down

products are then readily taken up into the cell and further degraded by a wide range of specialized catabolic pathways. In contrast to polysaccharides, the complex heteropolymer lignin lacks a stereochemical regularity and is therefore degraded by a nonspecific and non-stereoselective mechanism.

GHs are the main actors in polysaccharide degradation and are represented by 135 GH families in the CAZy database. Additional families involved in plant cell wall degradation are found in the group of the polysaccharide lyases which employ β-elimination reaction mechanisms to cleave pectins and carbohydrate esterases which catalyze the de-O or de-N-acylation of substituted polysaccharides. To date, polysaccharide lyases form 23 and carbohydrate esterases 16 characterized families. "Auxiliary activities" or AAs cover redox enzymes that are currently represented by 13 families, with the majority active in lignin degradation and with four families active on polysaccharides, namely the lytic polysaccharide monooxygenases (LPMOs). The catalytic modules or functional domains of the different enzymes are often associated with other functions to improve their action on lignocellulose. The most prominent additional domain are carbohydrate-binding modules (CBMs), which are represented by 71 families in the CAZy database.

A. Cellulose Degradation

Cellulose is the most abundant biopolymer on earth, with approximately 10^{10} metric tons of CO_2 and H_2O getting transformed into this polymer every year (Field et al. 1998). It consists of a linear chain of several 100 up to 12,000 β-1,4-linked D-glucopyranose units. The glucose hydroxyl groups from one chain form hydrogen bonds with the oxygen atoms on the same, or on a neighboring chain, which leads to the formation of cellulosic microfibrils. These microfibrils provide high tensile strength in cell walls and prevent the cell wall from stretching laterally. Cellulose crystallizes shortly after its biosynthesis, but less-ordered amorphous regions can occur. A large number of fungi are able to grow on amorphous cellulose and water-soluble derivates, but relatively few are able to produce a complete enzyme system necessary to hydrolyze crystalline cellulose. For the complete hydrolysis of cellulose (Fig. 6.1) to D-glucose, a number of synergistically acting enzymes are necessary. These include cellobiohydrolases (EC 3.2.1.91; 1,4-β-D-glucan cellobiohydrolases), which attach to the cellulose chains and act processively from their ends to generate mainly the glucose disaccharide cellobiose. The cellobiohydrolases are classified in two GH families with GH6 enzymes characterized by an inverting mechanism and cleavage from the nonreducing cellulose end. In contrast, GH7 family members cleave cellobiose from the reducing end by a retaining mechanism. Endoglucanases (EC 3.2.1.4; 1,4-β-D-glucan 4-glucanohydrolase) are capable of cleaving within the amorphous regions inside the cellulose chain, thereby generating additional sites for the attack of the cellobiohydrolases. β-Glucosidases (EC 3.2.1.21) degrade the accumulating cellodextrines and cellobiose to D-glucose (Beguin 1990; Teeri 1997). Within the last years, another main player in cellulose degradation was discovered with the lytic polysaccharide monooxygenases (LPMOs). These copper dependent oxidases originally classified as fungal GH61 enzymes attack the highly crystalline regions of cellulose by an oxidative cleavage. Studies on the *T. reesei* cellulase CEL61A revealed that its addition to commercial enzyme mixes can significantly boost degradation (Harris et al. 2010). These LPMOs act on crystalline cellulose by generating both oxidized and non-oxidized chain ends. One group oxidizes the C_1 of D-glucose, thereby releasing lactones that are hydrolyzed to aldonic acids (Beeson et al. 2011). Other LPMOs act on the nonreducing end, producing ketoaldoses, or a combination thereof (Beeson et al. 2012). Therefore, these CEL61 enzymes were reclassified in auxiliary activity family 9 (AA9), which exclusively contains eukaryotic LPMOs. Originally, these LPMOS were detected in the degradation of chitin (Hemsworth et al. 2014; Vaaje-Kolstad et al. 2010) before their action on cellulose or starch was discovered (Vu et al. 2014; Levasseur et al. 2013; Lo Leggio et al. 2015; Horn et al. 2012).

By disrupting the crystalline structure of cellulose, they facilitate the enzymatic cleavage of the classical cellulases and allow other enzymes like cellobiohydrolases to attack the polymer at otherwise inaccessible sites

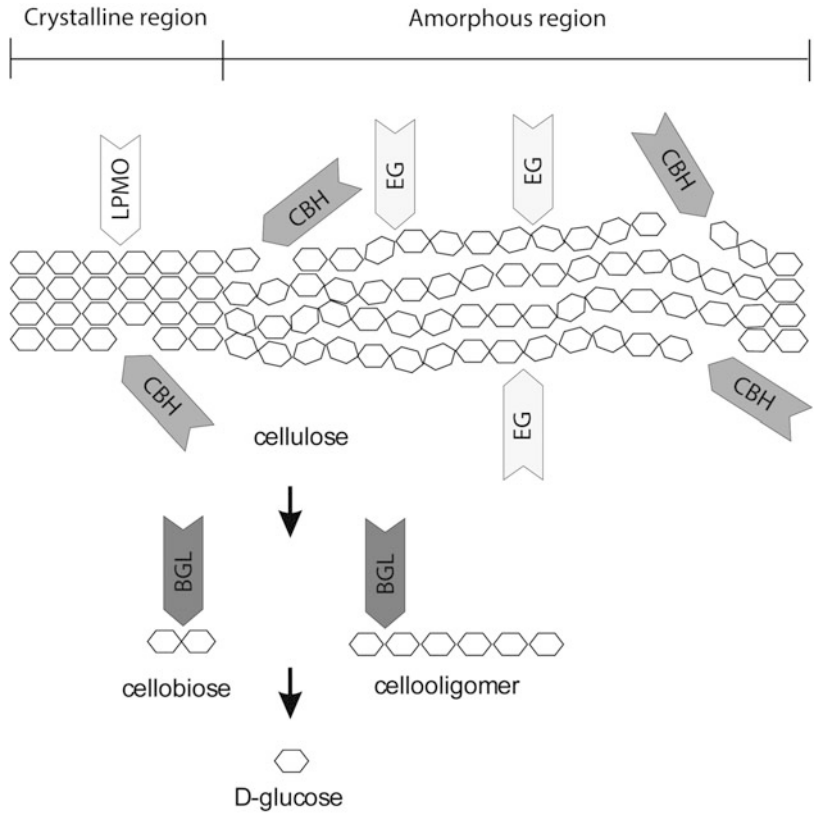

Fig. 6.1 Enzymatic degradation of cellulose. Cellobiohydrolases (CBH) act on either the reducing or non-reducing end of the glucose chain. Endoglucanases (EG) hydrolyze internal glycosidic bonds, thereby providing additional sites for the CBHs. Finally, smaller oligomers and the dimer cellobiose are cleaved by β-glucosidases (BGL) into D-glucose. The lytic polysaccharide monooxygenases (LPMO) cleave within the highly crystalline regions of cellulose in an oxidative manner

(Horn et al. 2012). In this respect, LPMOs may be the true C_1 enzyme in the C_1-C_x model proposed by Elwyn T. Reese to explain cellulose degradation (Mandels and Reese 1964; Reese et al. 1950). In this model, the non-hydrolytic C_1 enzyme is required for the initial attack on crystalline cellulose and the C_x enzymes for the hydrolysis of soluble cellulose. Later, in the 1990s, the classical endo/exo model of cellulase degradation was proposed (Wood and McCrae 1972) which consisted of EGs attacking amorphous cellulose regions of the microfibrils, thereby generating new sites for exoglucanases. The exoglucanases attack the free cellulose ends. Finally, β-glucosidases convert cellodextrines and cellobiose to glucose. Here, the dominant EG act only on amorphous cellulose and functions as the C_x activity whereas the C_1 is represented by the exoglucanases, i.e., cellobiohydrolases. But Reese suggested that C_1 was a decrystallizing protein factor which possesses the ability to swell or disrupt cellulose; the LPMOs might act like the C_1 enzyme proposed by Reese.

A structural comparison of the different cellulases shows that these proteins comprise besides the catalytic domain carbohydrate-binding modules. These modules (Várnai et al. 2014) can be found N- or C-terminally and are about 40 aa in size. They were originally described as cellulose-binding domains due to their presence in cellobiohydrolases but were later also found in other carbohydrate-degrading enzymes and therefore renamed to carbohydrate-binding modules (CBMs). Removal of this domain leads to enzymes which are still able to cleave glycosyl linkages

from smaller oligosaccharides, but their binding to cellulose and, therefore, the action on crystalline cellulose is impaired. CBMs are structurally similar, and their carbohydrate-binding capacity can be attributed to several amino acids constituting the hydrophobic surface. The CBM domain is usually spatially separated from the catalytic core domain by a flexible linker region (Sammond et al. 2012). This linker region is rich in prolines, serines, and threonines, and the latter two amino acids are highly O-glycosylated to impair proteolytic degradation (Langsford et al. 1987; Srisodsuk et al. 1993). Recent studies on this connector have shown that their length and sequences are optimized towards the type of GH and CBM domain, which indicates that also the linker is of importance for the activity of the enzyme (Sammond et al. 2012; Payne et al. 2013).

The catalytic domain of the cellobiohydrolase II (CEL6A) of *T. reesei* was the first cellulase crystal structure resolved at the atomic level (Rouvinen et al. 1990). Its structure demonstrates why the cellobiohydrolases are only able to attack cellulose chain ends. The crystal structure shows a tunnel-shaped active site which is so tight that it can incorporate only one cellulose chain. Despite a similar overall structure, endoglucanases possess a more open active site which allows the enzyme to attack the cellulose chains in the middle. The active site topology of these polymer-degrading enzymes shows that these enzymes have extended active sites which provide binding places for a number of sugar units. These subsites position the substrate tightly and correctly with respect to the catalytic amino acids.

In addition to the classical glycoside hydrolases and the new LPMOs, other types of proteins have been described to be involved in cellulose degradation. One of them is the *T. reesei* swollenin SWO1 which shows similarity to plant expansins (Saloheimo et al. 2002; Sampedro and Cosgrove 2005). Expansins induce the extension of isolated cell walls by a non-hydrolytic activity on different cell wall polymers, e.g., pectins and xyloglucans, which are tightly bound to the cellulose microfibrills (McQueen-Mason and Cosgrove 1995). A proposed model is, that swollenins are able to disrupt the cellulose microfibrils without any hydrolytic activity and thereby they make the cellulose fibers more accessible for the cellulases to act upon (Saloheimo et al. 2002). Although SWO1 has cellulose disrupting activity (Andberg et al. 2015; Jäger et al. 2011), its actual role seems to be in aiding degradation of hemicelluloses, as synergistic effects with endo-xylanases rather than endo-cellulases or cellobiohydrolases have been reported (Gourlay et al. 2013).

Numerous genes encoding cellulases have been isolated and the respective enzymes were studied in great detail. They are found in GH families 5, 6, 7, 12, and 45. One of the best studied cellulolytic fungi is *T. reesei*, which was discovered during World War II on the Solomon Islands as a severe degrader of cellulosic material of the US Army (Reese 1976) and is today the most commonly used organism for commercial production of cellulases (Gupta et al. 2014). Detailed analysis of the enzymes and gene regulation is available for, e.g., *A. niger*, *T. reesei*, and *P. chrysogenum* (Aro et al. 2005; de Vries 2003; Louise Glass et al. 2013; Kubicek et al. 2009).

B. Hemicellulose Degradation

Hemicelluloses are the second most abundant polysaccharide in plant cell walls and represent a heterogeneous group of polymers with a backbone of 1,4-linked β-D-pyranosyl residues, with the exception of arabinogalactan (O'Neill and York 2003; O'Neill and Selvendran 1985). The backbone can consist of xylosyl-, glucosyl-, galactosyl-, arabinosyl-, or mannosyl residues and, depending on the dominant sugar, they are, e.g., named xylans or arabinogalactans, if both sugars occur in near-equal amounts. This chemical diversity of hemicellulosic structures requires a larger set of enzymes which attack the main chain or side chains. The main chain is cleaved by (mainly) endo-acting enzymes whereas exo-acting enzymes liberate the respective monomers. Only a few exo-acting enzymes are known which attack the main chain (Tenkanen et al. 2013). Accessory enzymes are activities necessary to cleave off the side chains, leading to the release of various

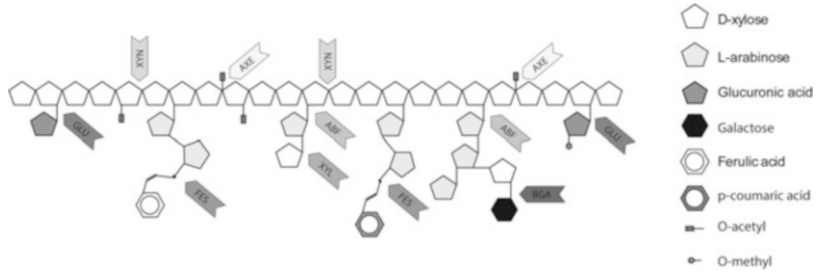

Fig. 6.2 Enzymatic degradation of hemicelluloses. The main chain of xylan is degraded by endo-1,4-β-xylanases (XYN). Accessory enzymes necessary for side group removal are β-xylosidase (XYL), α-glucuronidase (GLU), feruloyl esterase (FES), acetyl xylan esterase (AXE), 1,4-galactosidases (BGA), and α-L-arabinofuranosidase (ABF)

mono- and disaccharides. In this way, the main chain also becomes more accessible for the other group of enzymes. Most extensively studied is the enzymatic degradation of xylan, which involves exoxylanases, endoxylanases, β-xylosidases, and accessory enzymes for the side chains (Fig. 6.2).

1. Xylans

Xylans are characterized by a β-1,4-linked β-D-xylosepyranose backbone substituted by different side chains. They are the major hemicellulose in the cell walls of cereals and in hardwood and represent a minor component of the walls of dicotyledons and non-graminaceous monocotyledons.

Xylans found in cereals are highly substituted with single residues or short side chains of α-1,2- or α-1,3-linked L-arabinofuranose residues and are therefore referred to as arabinoxylans. Glucuronoxylans are typical hardwood xylans and contain large amounts of α-1,2- and α-1,3-linked 4-*O*-methyl-α-D-glucuronic acid and acetyl groups at *O*-2 or *O*-3. Glucuronoarabinoxylans are found in softwood and are substituted with a higher content of α-1,2-linked 4-*O*-methyl-α-D-glucuronic acid compared to hardwood and, in addition, contain α-L-arabinosefuranose but no acetyl groups. The L-arabinose residues may be esterified at *O*-5 with feruloyl or *p*-coumaroyl residues, and a number of other minor residues have been detected, too (Darvill et al. 1980; Ebringerova and Hienze 2000; Izydorczyk and Biliaderis 1995).

The hydrolysis of the xylan backbone involves endo-1,4-β-xylanases (endo-1,4-β-D-xylan xylanohydrolases; EC 3.2.1.8) and β-xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37). Exo-1,4-β-xylanase which cleaves xylan from the ends has received less attention but, e.g., XYN4 from *T. reesei* shows both exo- and endo-xylanase activity (Tenkanen et al. 2013). Endoxylanases cleave the main sugar chain depending on the type of xylan, the degree of branching, and the presence of different substituents (Polizeli et al. 2005). The main hydrolysis products are substituted or non-substituted oligomers which are further converted by β-xylosidases into tri-, di-, and monomers. Endoxylanases can be classified according to their end product into debranching and non-debranching enzymes, based on their ability to release L-arabinose from arabinoxylan (Wong et al. 1988). Some enzymes cut randomly between unsubstituted D-xylose residues whereas the cleavage site of some endoxylanases is dependent on the neighboring substituents of the side chains. β-Xylosidases can be classified according to their relative affinities for xylobiose or larger xylooligosaccharides and release β-D-xylopyranose by a retaining mechanism from the nonreducing end. β-Xylosidases are in general highly specific for small unsubstituted D-xylose oligosaccharides, and the activity decreases with increasing polymerization of the substrates. Accumulation of the short oligosaccharides would inhibit the action of the endoxylanases, but the hydrolysis of these products by β-xylosidases removes this possible cause of inhibition, thereby increasing the efficiency of xylan hydrolysis (Andrade et al. 2004). Similar to cellulases, most of the genes encod-

ing endoxylanases and β-xylosidases have been characterized in different *Aspergillus* spp., *T. reesei*, and *Penicillium* spp. as well as in *Agaricus bisporus* and *Magnaporthe grisea*.

2. Xyloglucan

Xyloglucan is the predominant hemicellulosic polysaccharide of dicotyledons and non-graminaceous monocotyledons, constituting up to 20 % of the plant cell wall.

Xyloglucans cross link cellulose microfibrills and support in this way the structural integrity of the cell wall (Hayashi and Kaida 2011). The backbone is composed of 1,4-linked β-D-glucopyranose residues which are substituted at *O*-6 by D-xylopyranose via an α-1,6-linkage. Depending on the number of D-glucopyranose residues attached to the main chain, they are classified as XXXG or XXGG type. In the XXXG type, three consecutive D-glucopyranose residues are substituted with D-xylopyranose followed by a fourth unbranched D-glucopyranose residue. Additional sugars found substituted to the D-xylopyranose include α-1,2-L-fucopyranose, β-1,2-D-galactopyranose, α-1,2-L-galactopyranose, or α-1,2-L-arabinose residues. Some of these residues can also contain O-linked acetyl groups (O'Neill and York 2003). XXXG-type glucans are present in numerous plants whereas the XXGG type occurs in solanaceous plants.

Some of the endoglucanases active against cellulose are also active on xyloglucans; in addition, xyloglucan hydrolases (EC 3.2.1.151) which are specific for xyloglucan have been reported (Pauly et al. 1999; Hasper et al. 2002; Grishutin et al. 2004; Baumann et al. 2007). Its degradation is not limited to glycoside hydrolases, as NcLPMO9C, a LPMO from *N. crassa*, was shown to degrade various hemicelluloses, in particular xyloglucan. It generates oxidized products and primarily acts on the xyloglucan backbone, accepting various substitutions (xylose, galactose) and helps in this way to depolymerize recalcitrant cellulose xyloglucan structures (Agger et al. 2014).

3. (Galacto-)glucomannan

Mannan consists of a β-1,4-linked β-D-mannopyranose backbone whereas in the glucomannan backbone randomly distributed β-1,4-linked β-D-glucopyranose and β-D-mannopyranose are found. Further substituents include α-1,6-linked α-D-galactopyranose residues which can be substituted further by α-1,2-linked α-D-galactopyranose. These polysaccharides are usually referred to as galactomannans and galactoglucomannans (Brett and Waldren 1996) which are the major hemicellulose structures of softwoods whereas glucomannan dominates in hardwood (Stephen 1982; Aspinall 1980). The D-glucose or D-mannose residues are partially substituted with acetyl residues linked to *O*-2 or *O*-3. The backbone is degraded by endo-1,4-β-mannanases (Mannan endo-1,4-β-mannosidase, EC 3.2.1.78) and β-mannosidases (EC 3.2.1.25). The ability of the endo-1,4-β-mannanases to degrade these polymers depends on the number and position of the side chain substituents. The enzymes releasing glucose (β-glucosidase, EC 2.1.21) and galactose (α-galactosidase, EC 3.2.1.22) residues act in synergism with endo-1,4-β-mannanases and β-mannosidases. β-Mannosidases split off the β-D-mannose residue from the nonreducing end of the manno-oligosaccharides and are characterized by a retaining mechanism.

C. Degradation of Pectins

Pectins are the most complex and heterogeneous group of polysaccharides in plant cell walls and are characterized by a significant content of α-1,4-linked D-galacturonic acids. They are found mainly in the middle lamella and in the primary cell wall where, their proportion ranges from 5–10 % in grasses to 30 % in dicotyledons. The carbohydrate composition and, hence, the structure vary depending on the species and cell type. Pectin is made up of several distinct domains which, depending on the presence of side chains, are called either "smooth" or "hairy" regions (Ridley et al. 2001; Pérez et al. 2000).

The "smooth" region or homogalacturonan (HG) is the most abundant pectin structure and consists of

linear chains of α-1,4-linked D-galacturonic acid residues which can carry methyl esters at the terminal carboxyl group and acetyl esters at the *O*-2 or *O*-3 position. Homogalacturonan with a high degree of methyl esterification is referred to as pectin whereas pectic acid (pectate) has a low degree of esterification. The esterification of the uronic acid group results in the elimination of the negative charge, which is of great significance for the gelling process of pectin, since the complexes between the carboxyl groups and Ca^{2+} ions are involved in this (Vincken et al. 2003). Additionally, the number of methyl- and acetyl esters has a strong influence on the susceptibility to cleavage by the different pectinases. Rhamnogalacturonan I+II and xylogalacturonan (XGA) are responsible for the "hairy" regions of the pectin due to their abundant and often branched side chains. The backbone of rhamnogalacturonan I consists of D-galacturonic acid and L-rhamnose in a $[1\rightarrow2)$-α-L-Rha-$(1\rightarrow4)$-α-D-GalA-$(1\rightarrow]_n$ linkage. Whereas D-galacturonic acid can be substituted with either methyl- or acetyl esters similar to those in HG, 20–80 % of the L-rhamnose residues are substituted at the *O*-4 position by L-arabinose and D-galactose with varying size from monomers up to branched, heterogeneous oligomers. They can be terminated with α-L-fucose and (4-*O*-methyl)-β-D-glucuronic acid. Arabinan chains are formed of α-1,5-linked L-arabinoses which can be further substituted with α-1,3-linked L-arabinose residues. In addition, two types of arabinogalactan side chains are present: Type I consists of a chain of β-1,4-linked D-galactopyranose whereas type II contains a backbone of β-1,3-linked D-galactopyranose residues which can be substituted with β-1,6-linked D-galactopyranose residues. Both are occasionally substituted with L-arabinose at *O*-3. In addition, ferulic acid and *p*-coumaric acids have been identified in the pectic hairy regions attached to *O*-2 of L-arabinose and *O*-6 of D-galactose. Rhamnogalacturonan II consists of a short backbone of α-1,4-linked D-galacturonic acid which is substituted either at the *O*-2 or *O*-3 position (Vidal et al. 2000). Its side chains have been found to be either dimers or branched oligomers and to contain rare sugars such as D-apiose and L-fucose in addition to L-arabinose, D-galactose, and L-rhamnose. The backbone of another substructure found in hairy regions, the xylogalacturonans, is similar to that of the HGs, but a major part of the D-galacturonic residues carry β-D-xylose substituents at the *O*-3 position. It has been found in reproductive tissues, including soybean seed, apple fruit, and pine pollen (Schols et al. 1995). Although the composition and structure of the individual subunits are well established, the manner in which they make up the pectin polymer is still under investigation. For a long time, pectin was thought to consist of linear chains of homogalacturonan interspersed with hairy regions. In another model (Vincken et al. 2003), rhamnogalacturonan I forms the backbone, substituted with homogalacturonan and the abovementioned arabinan and galactan side chains.

To efficiently degrade this complex pectin structure, fungi have developed a broad spectrum of pectinolytic enzymes. Enzymatic depolymerization of pectin weakens the cell wall and exposes the other cell wall polymers to degradation by other plant cell wall-degrading enzymes. Therefore, pectin degradation is important for plant pathogenic fungi (Kubicek et al. 2014), while other often fungi show a reduced set of these enzymes.

Pectinases can be classified in terms of their reaction mechanism into hydrolases or lyases and further according to their substrate specificity into, e.g., polygalacturonases or rhamnogalacturonases. Beside glycoside hydrolases, different lyases such as pectin- (EC 4.2.2.10), pectate- (EC 4.2.2.2), and rhamnogalacturonan lyases (EC 4.2.2.-) cleave polysaccharide chains via a β-elimination mechanism resulting in the formation of a Δ-4,5-unsaturated bond at the newly formed nonreducing end.

Most pectinases are found in GH family 28 as they share a common conserved structure which is in contrast to the cellulases and hemicellulases, which are characterized by a high diversity of protein structures. Although the overall sequence similarity is low, pectinases share a central core consisting of parallel β-strands forming a large, right-handed helix defined as parallel β-helix (Jenkins and Pickersgill 2001). Even though hydrolases and lyases differ in their catalytic mechanism, the substrate-binding sites are all found in a similar location within a cleft formed on the exterior of the parallel β-helix. This structure facilitates the binding and cleaving of the buried pectin polymers in the undamaged cell wall. The parallel β-helix fold confers the stability needed by these pectinases for efficient aggressive action in a variety of hostile extracellular environments. An exception to this rule is the rhamnogalacturonan lyase from *A. aculeatus*, which displays a unique arrangement of three distinct modular domains (McDonough et al. 2004).

The pectinolytic system has been studied in great detail in *Aspergillus* spp. including *A. niger* (de Vries and Visser 2001). About 60 genes of *A. niger* are predicted to encode pectinases for which the expression of 46 was detected (Martens-Uzunova and Schaap 2009)

and a high number was identified by mass spectrometry (Tsang et al. 2009).

Polygalacturonases (PGAs) are the most extensively studied class of pectinases and include endopolygalacturonases (1,4-α-D-galacturonan glycanohydrolase; EC 3.2.1.15) which catalyze the hydrolytic cleavage of α-1,4 D-galacturonic bonds within the chain and exopolygalacturonases (galacturan 1,4-α-galacturonidase; EC 3.2.1.67) which cleave from the nonreducing end. Both endo- and exoPGAs belong to glycoside hydrolase family 28 and have similar reaction mechanisms and substrate specificities, but their level of sequence identity is surprisingly low (Biely et al. 1996; Markovic and Janecek 2001; Henrissat and Bairoch 1993). Among the endoPGAs, some enzymes cleave only once per chain (single attack or non-processive) whereas others attack multiple times (processive behavior). Single-attack PGAs generally produce longer fragments, which are only gradually degraded into dimers, trimers, or short oligomers, providing possible sites for exoPGAs.

A factor which significantly influences the activity of PGAs is the number (and distribution) of methyl- and acetyl ester groups on the substrate. In general, most endo- and exoPGAs prefer substrates with a low degree of esterification, although some exceptions exist (Parenicova et al. 2000). In most cases, the activity of a methyl/acetyl esterase is required to prepare the pectin molecule for PGA digestion. Pectin-degrading fungi often produce multiple isozymes with a wide range of enzymatic properties, substrate specificities, and pH optima, which may reflect the complexity of the pectin molecule in plant cell walls and the need for enzymes capable of cleaving the homogalacturonan backbone in a variety of structural contexts. Another structural feature which may determine the functional diversification of these enzymes is the presence or absence and type of N-terminal extension, which has been suggested to influence their substrate specificity and to play a role in their interaction with particular regions of the pectin polymer (Gotesson et al. 2002; Parenicova et al. 2000). The degree of esterification is also important for the functional classification of lyases. Pectate lyases prefer substrates with a low degree of methyl esterification, which therefore have a more acidic character, and are strictly dependent on Ca^{2+} for catalysis. Pectin lyases, on the other hand, favor highly methyl-esterified substrates and do not require Ca^{2+} ions (Jurnak et al. 1996).

The pectinolytic enzyme system of *A. niger* serves as an example of how a microorganism can degrade a pectin molecule. *A. niger* produces seven PGAs, two of them (PgaA and PgaB) constitutively. These two are most active on pectins containing 22 % methyl esters (Parenicova et al. 2000), thus making them suitable for an initial attack on the native substrate. Enzymes subsequently induced at this early stage during growth on pectin are pectin methyl esterase PmeA, an exopolygalacturonase PgxA, and pectin lyases (PelA and PelD; (de Vries et al. 2002). The action of the methyl esterase renders the substrate accessible to PGAs, which are expressed at a later stage after removal of the methyl esters, while the pectin lyases contribute to the breakdown of the still esterified polymer. The exoPGAs cleave D-galacturonic acid monomers from the homogalacturonan poly- and oligomers, which may serve as inducers for the other pectinases. Similar to homogalacturonans, the degradation of the rhamnogalacturonan I backbone is catalyzed by hydrolases and lyases (Fig. 6.3). Endorhamnogalacturonases have been isolated from *A. aculeatus* and *A. niger* (de Vries and Visser 2001) and hydrolyze the α-1,4 glycosidic bonds in saponified hairy regions. The resulting fragments were tetra- and hexamers of the backbone, which partly were still substituted with D-galactose. This suggests that—similar to some exoPGAs ability to cleave XGA (van den Broek et al. 1996)—endorhamnogalacturonases are tolerant towards monomeric substituents. Depending on the monosaccharide cleaved from the nonreducing end of the rhamnogalacturonan, two kinds of exorhamnogalacturonases have been described: rhamnogalacturonan α-D-galactosyluron-hydrolase and rhamnogalacturonan α-L-rhamnohydrolase. All rhamnogalacturonan hydrolases were classified as members of GH family 28. Rhamnogalacturonan lyases cleave the rhamnogalacturonan backbone via β-elimination. Unlike the hydrolases, they act on the Rha-(1→4)-α-D-GalA bond, resulting in the formation of Δ4,5-unsaturated D-galacturonic acid residues at the nonreducing end.

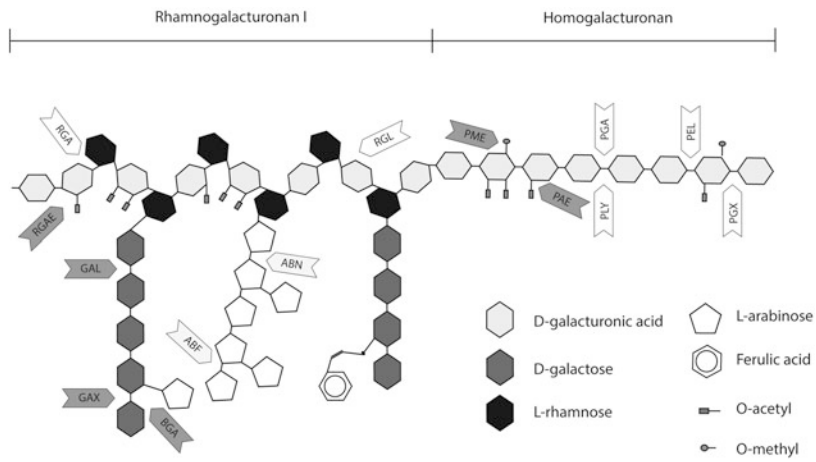

Fig. 6.3 Enzymatic degradation of rhamnogalacturonan I and homogalacturonan. The main chain of rhamonogalacturonan I is degraded by rhamnogalacturonan hydrolase (RGA) and rhamnogalacturonan lyase (RGL). The side chains are degraded by rhamnogalacturonan acetyl esterase (RGAE), endoarabinase (ABN), endo-β-1,6-galactanases (GAL), and exogalactanases (GAX). Terminal monosaccharides are removed by α-L-arabinofuranosidases (ABF) and β-galactosidases (BGA). The main chain of homogalacturonan is degraded by endopolygalacturonases (PGA), exopolygalacturonases (PGX), pectin lyases (PLY), and pectate lyases (PEL). Pectin methyl esterase (PME) and pectin acetyl esterase (PAE) act on the side groups

D. Accessory Enzymes for Plant Cell Wall Degradation

Most plant cell wall-degrading enzymes described above act on the backbone of the respective polysaccharide, but often their activity is impaired by monomeric substituents or larger side chains present in hemicelluloses and pectins. To ensure an efficient and complete breakdown of such polysaccharides, these substituents have to be removed and degraded by accessory enzymes. These accessory enzymes work in synergism with the enzymes attacking the main chain and often depend on each other for an efficient breakdown of the whole substrate to monomeric sugars. The substrate specificity of these enzymes varies; some of the enzymes can hydrolyze the intact polymer whereas others show maximum activity only in the presence of shorter breakdown products (Puls and Schuseil 1993; Tenkanen and Siika-aho 2000). A detailed list of all the enzymes involved in the degradation of hemicellulose and pectinase side chains and their mode of action has been reviewed for e.g. *Aspergillus* spp. by de Vries and Visser (2001), and only some of the major enzymes are listed here (Figs. 6.2 and 6.3).

Side groups in xylans are generally small (mono-, di-, and trimers) but can consist of several different sugars and acids (e.g., acetic acid, L-arabinose, ferulic acid, D-galactose, D-glucuronic acid), and, consequently, multiple enzymes are required to make the backbone fully accessible for the xylanases. α-L-Arabinofuranosidases (EC 3.2.1.55) remove terminal L-arabinose residues but differences in the specificity towards α-1,2-, α-1,3-, or α-1,5-arabinosidic bounds and towards the substrates themselves have been observed. Whereas several representatives are also able to release L-arabinose from pectins and xylans, arabinoxylan arabinofuranohydrolases (EC 3.2.1.99) are strictly specific for L-arabinose bound to xylan. In addition, some α-L-arabinofuranosidases are inhibited by the presence of D-glucuronic acid residues adjacent to the targeted L-arabinose. α-L-arabinofuranosidases contain also CBMs which support their binding to cellulose, xylan, or arabinofuranose side chain. The D-glucuronic acid and its 4-*O*-methyl ethers are removed by α-glucuronidases (EC

3.2.1.139) and by xylan-α-1,2-glucuronosidases (EC 3.2.1.131). In addition to these carbohydrate substituents, esters are found in xylans. Several types of feruloyl esterases (EC 3.1.1.73) have been described, their activity varying with the presence of additional methoxy or hydroxyl substituents on the ferulic acid's aromatic ring, and with the type of linkage (*O*-2, *O*-5, or *O*-6) to the carbohydrate chain. Acetyl xylan esterases (EC 3.1.1.72) participate in the breakdown of the xylan backbone by removing acetyl ester groups from *O*-2 and *O*-3 of the D-xylose chain, thereby facilitating the action of the endoxylanases (de Vries et al. 2000; Kormelink et al. 1993; Tenkanen 1998). So far, only two types of substituents have been described in galacto(gluco)mannans: D-galactose mono- and dimers and acetyl esters. The former are removed from the backbone by α-galactosidases (EC 3.2.1.22) whereas the latter are hydrolyzed by acetylglucomannan esterases (EC 3.1.1.-). Both reactions result in an increased activity of the endomannases and β-mannosidases (Tenkanen 1998; de Vries et al. 2000). For the removal of α-1-6-linked D-xylose side groups of the xyloglucan, specific α-D-xylosidases (EC 3.2.1.177) are required.

Pectin contains acetyl esters at the *O*-2 or *O*-3 and methyl esters at the carboxy group bound to the D-galacturonic acid residues in the smooth regions. Pectin methyl esterases (EC 3.1.1.11) and pectin acetyl esterases (EC 3.1.1.-) have been isolated from different fungal species, and they show a prominent synergism with polygalacturonases (de Vries et al. 2000). Similar results were achieved with rhamnogalacturonan acetyl esterases (EC 3.1.1.-) and rhamnogalacturonase or rhamnogalacturonan lyase (de Vries et al. 2000). Polymeric side chains in pectin consist mainly of L-arabinose and D-galactose. The D-galactose or L-arabinose side chains are often substituted with several other carbohydrates or acids. These chains are cleaved by endoarabinases (EC 3.2.1.99), exoarabinases (EC 3.2.1.-), endo-β-1,4-galactanases (EC 3.2.1.89), endo-β-1,6-galactanases (EC 3.2.1.-), and exogalactanases (EC 3.2.1.23). Terminal monosaccharides are removed by α-L-arabinofuranosidases (EC 3.2.1.55) and α-galactosidases (EC 3.2.1.23), but additional enzymes (e.g., α-L-fucosidases, α-glucuronidases) are required to completely degrade the side chains. α-L-rhamosidases (EC 3.2.1.40) are another group involved in pectin degradation.

IV. Degradation of Lignin

Next to cellulose, lignin is the most abundant polymer in nature and accounts for 15–36 % of the lignocellulosic material. It forms an extensive cross-linked network within the cell wall and confers structural support and decreases water permeability. It protects the other, more easily degradable, cell wall components and is therefore the main obstacle for an efficient saccharification of cellulose and hemicelluloses in bioethanol production.

The aromatic polymer is synthesized from the three substituted phenylpropanoid alcohols coniferyl (guaiacyl propanol), synapyl (syringyl propanol), and *p*-coumaryl (*p*-hydroxyphenyl propanol). The softwood of the gymnosperms contains mainly coniferyl alcohols, some *p*-coumaryl, but no sinapyl alcohol, whereas, in the hardwood of the angiosperms, coniferyl and sinapyl alcohols are found in equal amounts (46 %), with a minor proportion of *p*-coumaryl (8 %). The lignin polymer is synthesized by the generation of free phenoxy radicals, which is initiated by plant peroxidases-mediated dehydrogenation of the three precursor alcohols. The result of this polymerization is a highly insoluble, complex-branched, and amorphous heteropolymer joined together by different types of linkages such as carbon–carbon and different ether bonds. The chemical complexity and structural variability of the lignin polymer make it resistant to breakdown by conventional enzymatic hydrolysis and therefore the initial attack is oxidative, nonspecific, non-hydrolytic, and extracellular (Hatakka 1994; Higuchi 1990; Kirk and Farrell 1987).

Fungi are the most efficient group of organisms able to decompose the wood structure, and they use different mechanisms to make cellulose and heimcellulose accessible. White-rot basidiomycetes act on both hard and softwood and are the major group of wood rots. It is the only group which can completely degrade lignin into CO_2 and H_2O. They can overcome difficulties in wood decay, including the low nitrogen content of wood (a C:N ratio of about 500:1) and the presence of toxic and antibiotic

compounds. Besides these fungi, brown-rot fungi are also able to degrade wood extensively. Analysis of the genomes of different Basidiomycota suggests that the categorization into white rot or brown rot is an oversimplification because of gradations both in the expression of metabolites and the resulting patterns of decay (Riley et al. 2014; Arantes et al. 2012). Some ascomycetes also colonize wood in contact with soil but alter the lignin component only slightly. Their action leads to decrease in the mechanical properties of wood, giving rise to so-called soft rot, a process which often involves bacteria. Soft-rot fungi can degrade wood under extreme environmental conditions (extreme wetness or frequent dryness) which prohibit the activity of other wood-degrading fungi. They are relatively unspecialized (hemi-) cellulolytic ascomycetes in the genera *Chaetomium*, *Ceratocystis,* and *Phialophora*, and some basidiomycetes can also cause a soft rot-type of decay pattern. In soft rot, decay by fungi is closely associated with penetration by the fungal hyphae, because the enzymes cannot cross the plant cell wall. Two distinct types of soft rot are currently recognized. Type 1 is characterized by longitudinal cavities formed within the secondary wall of wood cells and type 2 by an erosion of the entire secondary wall (Martinez et al. 2005). Although many white rots and brown rots secrete oxidative and hydrolytic enzymes, it is generally recognized that their enzymes are unable to diffuse through healthy wood and that smaller, non-proteinaceous molecules are involved in the initiation of decay.

A. Brown-Rot Fungi

Brown-rot fungi degrade mainly cellulose and hemicellulose of coniferous softwoods and partially modify the lignin mainly by demethylation but not by oxidation (Eriksson et al. 1990). Brown-rot fungi attack cellulose in wood, which promotes rapid loss of mechanical strength leaving modified lignin behind. The term "brown rot" refers to the characteristics of this decayed wood: a reddish-brown material consisting of oxidized lignin, which cracks into characteristic brick-like pieces. Representatives of brown-rot basidiomycetes comprise *Schizophyllum commune*, *Fomes fomentarius*, *Serpula lacrimans*, *Postia placenta*, *Piptoporus betulinus*, and *Gloeophyllum trabeum*. They are also the major cause of decay of woods in commercial use and have an important role in coniferous ecosystems through their contribution to humus formation. These fungi grow mainly in the cell lumen of the woody cells, and the degradation is not localized to the fungal hyphae but found at greater distances from these. The extracellular enzymes formed are too large to penetrate healthy cell walls and therefore—as noted above—degradation of cellulose by brown-rot fungi must involve diffusible low-molecular agents. They employ small molecule reactive species to depolymerize lignin, cleave propyl side chain, and also demethoxylate the ring structures before repolymerizing the material elsewhere as a means of freeing the cellulosic components and generating greater access for deconstruction (Arantes and Goodell 2014). Brown rot fungi have evolved multiple times from the predecessors of current white rot fungi. During this evolution, the typical lignolytic enzyme systems of white rots and crucial types of cellulases have been lost (Floudas et al. 2012).

Although some brown rots possess cellobiohydrolases, they generally lack the ability to hydrolyze crystalline cellulose enzymatically. Nonenzymatic deconstruction of cellulose uses iron-dependent Fenton chemistries (Arantes and Goodell 2014). Crystalline cellulose can efficiently be degraded by a combination of classical endoglucanases and an oxidative degradation system such as extracellular reactive oxygen species (ROS). These include hydroxyl radicals (•OH) and the less reactive peroxyl (ROO•) and hydroperoxyl (•OOH) radicals (Hammel et al. 2002). There is a well-established pathway for the generation of these radicals via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \bullet OH$). In order not to destroy the fungal hyphae and to act in the lignified parts of the secondary cell wall, the •OH production has to occur at a distance

from the hyphae, and the fungal reductants should be stable enough to diffuse before they react to reduce Fe^{3+} and O_2 to Fe^{+2} and H_2O_2. The production of •OH radicals can take place in several ways including secreted hydroquinones, cellobiose dehydrogenases, low molecular-weight glycopetides, and phenolate chelators. A chelator-mediated Fenton (CMF) system has evolved in different brown rot fungi (Gloeophyllales, Polyporales, and Boletales), providing an efficient mechanism for depolymerization and modification of lignocellulolytic biomass (Arantes and Goodell 2014; Eastwood et al. 2011). The CMF system is unique as it is based on oxygen radical chemistry that permits nonenzymatic deconstruction at a considerable distance from the organism. The efficiency of the CMF system is thought to provide brown rot fungi advantages in exploiting ecological niches, and, for example, these fungi have displaced white rot predecessors in the degradation of conifer wood.

The principle of the quinone redox cycling for •OH production is that the fungus reduces the quinone extracellularly to its hydroquinone which then reacts with Fe^{3+} to Fe^{2+} and a semiquinone radical. The semiquinone reduces O_2 to •OOH, which is a source for H_2O_2, and is in this way recycled to quinone. *Gloeophyllum trabeum* produces extracellular quinones including 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone which can reduce Fe^{3+} and O_2 rapidly under physiological conditions, thereby generating both Fe^{2+} and H_2O_2. Moreover, the fungus was shown to reduce the resulting dimethoxyquinones back to hydroquinones, possibly by the action of an intracellular quinone reductase. Another nonenzymatic system includes phenolate or catechole chelators (Goodell 2003). They have a high affinity for the binding of iron and have the ability to reduce Fe^{3+} to Fe^{2+}. The two compounds 4,5-dimethoxy-1,2-benzenediol and 2,5-dimethoxy-1,4-benzenediol were identified in the Gt chelator fraction and also their oxidized benzoquinone forms (see above). •OH radicals can also be produced by the extracellular flavohaemoprotein cellobiose dehydrogenase (CDH). CDH production has been reported for all types of wood-rotting fungi (Zamocky et al. 2006), and CDH can act as cellobiose oxidase by reducing O_2 to H_2O_2. However, Fe^{3+} is a better electron acceptor than O_2 and, thus, CDHs are actually Fe^{3+} reductases. Glycopeptides, implicated in wood degradation, have been isolated from *G. trabeum* and *Tyromyces palustris*. They reduce Fe^{3+} to Fe^{2+} and bind Fe^{2+} (Goodell 2003; Enoki et al. 2003). In the presence of H_2O_2, the glycopeptide generates one-electron oxidation and possesses the ability to oxidize NADH in the presence of oxygen and thereby produces H_2O_2.

Most brown rots secrete oxalic acid, which is a strong chelator of Fe^{3+} and Fe^{2+} but also reduces the pH. The pH of wood itself is generally in the range 3–6 and is lowered to pH values between 2.5 and 1.7. The reduction of pH is important for the function of the extracellular enzymes and has been identified as a key factor in several hypotheses related to molecular weight degradation systems, as discussed in the reviews listed above.

B. White-Rot Fungi

White rots are the most frequently found wood-rotting organisms and are mainly basidiomycetes, but also some ascomycetes (*Diatrypaceae* and *Xylariaceae*) are able to cause white rot. They are characterized by their ability to completely degrade lignin, hemicelluloses, and cellulose, thereby giving rise to the name-giving cellulose-enriched white colored wood material. A typical white rot degradation is primarily enzymatic, and the attack of the wood cell wall proceeds only from lignocellulose surfaces because degradative enzymes are too large to penetrate the intact cell wall. The enzymes employed by the white rot fungi include a complete suite of cellulases, with some fungi possessing a large number of LPMO encoding genes (Busk and Lange 2015) and enzymes that can oxidize lignin components, including lignin, manganese, and versatile peroxidase or laccases (Pollegioni et al. 2015). Two different white-rot patterns have been described which are simultaneous and selective delignification. Simultaneous or nonselective delignification acts mainly on hardwood and degrades cellulose, lignin, and hemicellulose simultaneously. The cell wall is attacked progressively from the cell lumen towards the middle lamella. Degradation is associated with the fungal hyphae and substantial amounts of undecayed wood remains. Basidiomycetes (e.g., *Trametes versicolor*, *Irpex lacteus*, *P. chrysosporium*, *Heterobasidion annosum*, and *Phlebia radiata*) and some ascomycetes (e.g., *Xylaria hypoxylon*) perform this type of degradation. Selective delignification, or sequential decay, is found in hardwood and softwood. The initial attack is selective for lignin and hemicellulose, and the cellulose is attacked later. Lignin is degraded in

the middle lamella and in the secondary wall. This type of degradation is performed exclusively by basidiomycetes (e.g., *Ganoderma australe*, *Phlebia tremellosa*, *C. subvermispora*, *Pleurotus* spp., and *Phellinus pini*). Many white-rot fungi cause both types of rot, and the amount of simultaneous or selective decayed wood depends on the substrate and varies even among different strains of the same species (Martinez et al. 2005; Eriksson et al. 1990). To date, *P. chrysosporium* is the most intensively studied white-rot fungus (Martinez et al. 2004; Cullen and Kersten 2004).

White-rot fungi degrade lignin via an oxidative process involving peroxidases and laccases (phenol oxidases) which act nonspecifically by generating highly reactive, nonspecific free radicals that attack lignin causing spontaneous cleavage reactions (Hammel and Cullen 2008).

Heme peroxidases, such as the lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13), and the versatile peroxidase (VP; EC 1.11.1.16), have been described as true ligninases due to their high redox potential which enables them to oxidize non-phenolic aromatic substrates constituting up to 90 % of the lignin structure. Usually, a number of isoenzymes are produced which is either due to posttranslational modifications or the presence of multiple genes in the genome. Peroxidases require the presence of H_2O_2 as oxidizing substrate and oxidize phenolic and non-phenolic compounds (Hatakka 1994; Kersten 1990; Hammel et al. 1986; Eggert et al. 1997). LiP and MnPs were firstly described for *P. chrysosporium* (Glenn and Gold 1985; Kirk and Farrell 1987; Paszczyński et al. 1985).

The catalytic, oxidative cycle of LiP is similar to those of other peroxidases. A heme group (Fe^{3+}) in the active center oxidizes H_2O_2 while forming an intermediate compound I known as LiP oxyferryl. Compound I is reduced by veratryl alcohol via one-electron transfer forming compound II before it is reduced to its peroxidase resting state via a second one-electron transfer (Hofrichter et al. 2010; Liu et al. 2003). Veratryl alcohol is oxidized to short-lived cation radicals which oxidize lignin directly or pass on the charge to other, more stable carriers which can act as diffusible mediators. Since enzymes such as LiPs are too large to enter the plant cell, direct degradation is carried out only in exposed regions of the cell lumen (simultaneous delignification). But microscopic studies of selective lignin biodegradation revealed that white-rot fungi remove the polymer from inside the cell wall, which can be performed only by indirect oxidation mediated by low molecular-weight diffusible compounds capable of penetrating the cell wall.

MnPs are closely related to LiPs and have an iron protoporphyrin IX (heme) prosthetic group. They have the same catalytic cycle involving a two-electron oxidation of the heme by H_2O_2, followed by two subsequent one-electron reductions. But instead of veratryl alcohol, MnPs oxidize Mn^{+2} to Mn^{+3}, which is stabilized by organic acids such as oxalate, fumarate, and malate.

Chelated Mn^{+3} ions can act as diffusible oxidizer on phenolic substrates and oxidize non-phenolic substrates via lipid peroxidation reactions (Martinez et al. 2005; Hofrichter et al. 2010). The reaction is similar to LiPs and initiated by the enzyme and H_2O_2 to form MnP compound I, a Fe^{4+}-oxo-porphyrin-radical complex. A monochelated Mn^{+2} ion transfers one electron to the porphyrin intermediate to form compound II and is oxidized to Mn^{3+} before the resting state is reached by one electron transfer to form Mn^{+3} by an electron transfer from Mn^{+2}. The more recently discovered VP combines the enzymatic properties of both LiP and MnP and oxidizes Mn^{+2} and veratryl alcohol (Hofrichter et al. 2010). A VP was firstly described for *Pleurotus eryngii* (Martinez et al. 1996). The catalytic cycle of VP combines both LiP and MnP, but this cycle differs from the classical MnPs by catalyzing the Mn^{+2}-independent oxidation of simple amines and phenolic monomers (Perez-Boada et al. 2005). The catalytic versatility of VP permits its application in Mn^{+3}-mediated or Mn-independent reactions in both low- and high-redox potential aromatic substrates. Although VP from *P. eryngii* catalyzes the oxidation of Mn^{+2} to Mn^{+3} with H_2O_2, it differs from classical MnPs in its manganese-independent activity, thereby enabling it to oxidize substituted phenols and the veratryl alcohol (Camarero et al. 1999).

The crystal structures have been resolved for both LiP and MnP (Piontek et al. 1993; Poulos et al. 1993; Sundaramoorthy et al. 1994). The prosthetic group (iron protoporphyrin IX) of LiPs is accessible only through a narrow pore (Piontek et al. 2001). Although its catalytic cycle is common to peroxidases, it is noted that the position of the iron-binding histidine residue in ligninolytic peroxidases is located further away from the heme iron which increases the redox potential. Also the existence of specific binding sites for substrate oxidation are unique (Martinez 2002). The substrate-

binding sites have been identified for LiP, MnP, and VP, and explain the dual catalytic properties of VP. Mn^{2+} oxidation occurs at a binding site near the cofactor which enables direct electron transfer. By contrast, veratryl alcohol is oxidized at the surface of the protein by a long-range electron transfer mechanism. The rationale of the existence of this electron transfer mechanism is related to the fact that many of the aromatic substrates cannot penetrate inside the LiP/VP and, therefore, these substrata are oxidized at the enzyme surface, and electrons are transferred to the heme (Doyle et al. 1998; Gold et al. 2000; Sundaramoorthy et al. 1997).

Laccases (EC 1.10.3.2) are multicopper phenoloxidases and generally larger than peroxidases. They directly oxidize phenols and aromatic amines and catalyze a one-electron oxidation combined with a four electron reduction of O_2 to H_2O. Laccase catalysis requires four copper atoms, which are held in place at the catalytic center by four histidine-rich, copper-binding regions (Claus 2004). The phenolic nucleus is oxidized by removal of one electron, generating phenoxy-free-radical products, which can lead to polymer cleavage. Due to their low redox potential, non-phenolic substrates have to be oxidized by other mediators. Metabolites such as 3-hydroxyanthranilate can mediate oxidation in *Pycnoporus cinnabarinus* (Eggert et al. 1997), and also lignin degradation products can act as redox charge transfer molecules (ten Have and Teunissen 2001).

H_2O_2-generating enzymes are essential for the function of the peroxidases and include glyoxal oxidase, glucose 1-oxidase, methanol-oxidase, aryl-alcohol oxidase, and oxalate decarboxylase oxidases which reduce O_2 to H_2O_2 (Zhao and Janse 1996). Flavin is often used as cofactor, with the exception of, e.g., copper-containing glyoxal oxidase from *P. chrysosporium*. Cellobiose dehydrogenase oxidizes soluble cello- and mannodextrine and uses a wide spectrum of electronacceptors (Fe^{3+}, Cu^{2+}, quinone, and phenoxy radicals). Proposed roles of CDH in the ligninolytic system comprise (1) the reduction of aromatic radicals formed by ligninolytic enzymes, thereby preventing repolymerization and supporting lignin degradation, (2) the production of •OH radicals via a Fenton-type reaction to modify cellulose hemicellulose and lignin, and (3) a cooperation with the manganese peroxidases to make the abundant non-phenolic components of lignin accessible for MnP and laccases. The role of ROS in the initial attack of lignin has also been discussed (Hammel et al. 2002) and was reviewed already in the brown-rot section.

V. Conclusions

The recycling of polymers by fungi is an essential process for life on earth which is today exploited in the biotech industry to produce fermentable sugars for different biorefinery products including bioethanol. Fungi produce a plethora of plant cell wall-degrading enzymes and efficiently degrade this highly complex carbon source. Although most of these enzymes are known for decades, the recent discovery of a new group, the lytic polysaccharide monooxygenases, as main actors in polysaccharide degradation shows that our understanding of polysaccharide and lignocellulose deconstruction is still limited. Comparative genomics, transcriptomics, and proteomics have provided us over the past years with a number of potentially interesting enzymes and accessory proteins, which will reveal new insights into how fungi use plant cell walls for their metabolism. Still, a major challenge for future research is to understand the complex multienzyme process of lignocellulose decomposition and to transfer this knowledge for the development of novel enzyme formulations to increase the efficiency of plant cell wall saccharification in the bio-based economy.

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Biotic Interactions of Fungi

7 Evolution in Heritable Bacterial–Fungal Endosymbioses

Teresa E. Pawlowska[1]

CONTENTS

I. Introduction

Bacterial–fungal associations are increasingly recognized as important components of various biological systems (Kobayashi and Crouch 2009; Peleg et al. 2010; Frey-Klett et al. 2011; Scherlach et al. 2013). Such recognition challenges the traditional view that fungi do not engage in intimate interactions with bacteria (Soanes and Talbot 2010) and underscores the significance of these symbioses. Recent acceleration in the pace of discovery of new bacterial–fungal associations is accompanied by rapid accumulation of insights into how these alliances function and how they evolve. It is expected that some of the mechanisms of bacterial–fungal interactions will be distinct from those that govern relations of bacteria with other eukaryotes, such as protists, animals, and plants. This expectation is justified by life history features that set fungi apart from other major groups of eukaryotes and include absorptive nutrition, filamentous somatic structures, and spore-based reproduction. Because of these distinctions, it is anticipated that studying bacterial–fungal interactions will yield novel insights into the coevolution between bacteria and eukaryotes, leading, in turn, to the enrichment and expansion of the existing evolutionary models. Most of the insights that have been generated thus far come from interactions between fungi and heritable endobacteria, i.e., bacteria that are transmitted from one host generation to the next. Remarkably, these heritable associations involve representatives of early divergent lineages of Glomeromycota and Mucoromycotina that partner with *Burkholderia*-related beta-proteobacteria and mycoplasma-related Mollicutes (Bianciotto et al. 1996; Partida-Martinez and Hertweck 2005; Naumann et al. 2010; Sato et al. 2010; Desirò et al. 2014a). Whether such concentration of bacterial–fungal symbioses among coenocytic early divergent fungi reflects a detection bias or a true predisposition of this group of fungi to form symbioses with bacteria remains to be elucidated.

While heritable bacterial–fungal symbioses tend to involve closely related fungal hosts and bacterial symbionts, there are few biological features that are shared among these associations. In fact, it is not entirely clear whether all of these associations are mutualisms rather than antagonisms. For example, in the symbiosis formed by *Rhizopus microsporus* (Mucoro-

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mycotina) with *Burkholderia rhizoxinica* and *B. endofungorum* (beta-proteobacteria), the symbionts reside directly in the host cytoplasm (Partida-Martinez and Hertweck 2005). The bacteria control fungal ability to reproduce asexually (Partida-Martinez et al. 2007b) and provision the host with potent toxins, rhizoxin and rhizonin, which may be important for fungal pathogenesis of plants (Partida-Martinez and Hertweck 2005; Partida-Martinez et al. 2007a). Despite fungal dependence on bacteria for reproduction, both partners can be separated and cultivated independently of each other (Partida-Martinez and Hertweck 2005). In contrast, in the symbiosis formed by the representatives of the Gigasporaceae family of Glomeromycota, also known as arbuscular mycorrhizal fungi (AMF), with *Candidatus* Glomeribacter gigasporarum (a beta-proteobacterium hereafter referred to as *Glomeribacter gigasporarum*), the endobacteria are contained in vesicles (Bianciotto et al. 1996). The symbionts improve fungal ability to elongate germ tubes emerging from asexual spores (Lumini et al. 2007). This feature may facilitate AMF colonization of plant roots important for the completion of the fungal host life cycle, as Glomeromycota are obligate biotrophs reliant on plants for assimilated carbon (Lumini et al. 2007). While for AMF the association with *Glomeribacter* is facultative (Lumini et al. 2007; Mondo et al. 2012), the endobacteria are uncultivable (Jargeat et al. 2004) and metabolically dependent on the fungus (Ghignone et al. 2012). Finally, in the association between Glomeromycota and the recently discovered novel lineage of Mollicutes, referred to mycoplasma-related endobacteria (MRE), the endobacteria reside directly in the host cytoplasm (Naumann et al. 2010). Unlike *Glomeribacter*, whose distribution is limited to the Gigasporaceae family of AMF, MRE were detected not only in all major lineages of Glomeromycota (Naumann et al. 2010) but also in some *Endogone* representatives of Mucoromycotina (Desirò et al. 2014a). The role of MRE in the biology of their fungal hosts is unknown.

Despite the differences among symbioses formed by early diverging fungi with bacteria and uncertainties surrounding their biology, these associations have already generated and will continue to provide important insights into the evolution of heritable endosymbioses, including: (A) the role of symbiont vertical transmission in evolution of mutualisms from antagonisms, (B) the form of evolutionary trajectories in mutualisms, and (C) the mechanisms of genome evolution in heritable symbionts.

II. Evolution in Bacterial–Fungal Symbioses

A. The Role of Symbiont Vertical Transmission in Evolution of Mutualisms from Antagonisms

Vertical transmission allows for coupling of partner reproductive interests and, over time, is expected to lead to a transformation of antagonistic interspecific interactions into mutualisms (Yamamura 1993). Evolutionary theory suggests that a symbiotic system will transition from antagonism to mutualism if a parasite is able to dominate the co-evolutionary race with the host and achieve a rate of vertical transmission that enables efficient reciprocal selection between the partners (Yamamura 1993) (Fig. 7.1). In other words, when an interaction is dominated by the host, resulting in low rates of parasite vertical transmission, it will continue to function as an antagonism. However, once the parasite dominates the interaction and is able to increase the rate of vertical transmission, its reproductive success will eventually become bound to the host reproductive success. If the increase in the rate of symbiont vertical transmission is accompanied by the development of host abilities to complement its metabolism with symbiont metabolites, a mutualism is expected to evolve (Yamamura 1993).

While the model that explains the evolution of mutualisms from antagonisms through changes in the rates of symbiont transmission is rather straightforward (Yamamura 1993), the actual mechanisms that permit symbiont vertical transmission remain elusive as nearly all known heritable endosymbionts are uncultivable (Moran et al. 2008), and many hosts are

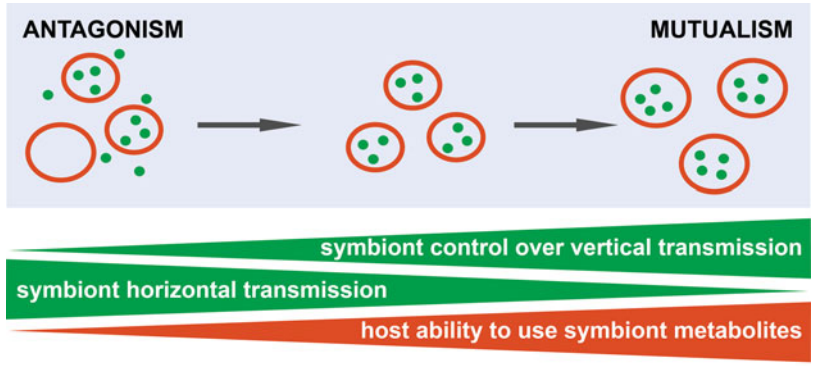

Fig. 7.1 Evolutionary theory predictions concerning the role of vertical transmission in evolution of mutualism from antagonism. Hosts are depicted as red ovals; endosymbionts are shown as green dots. Relative host fitness is reflected by the size of ovals

unable to survive without their endobacteria. Therefore, the *Rhizopus–Burkholderia* symbiosis (Partida-Martinez and Hertweck 2005), in which the symbionts control host asexual reproduction (Partida-Martinez et al. 2007b), offers an unprecedented opportunity to understand the role of symbiont vertical transmission in evolution of mutualisms from antagonisms.

In the *Rhizopus–Burkholderia* symbiosis, removal of the endobacteria abolishes the ability of the fungus to form asexual sporangia and sporangiospores (Partida-Martinez et al. 2007b). Moreover, reconstructions of the host evolutionary history suggest that the interaction between bacteria and fungi was initially an antagonistic one, with the bacteria using a rhizoxin toxin to parasitize rhizoxin-sensitive fungi (Schmitt et al. 2008). Based on this pattern, it can be speculated that an encounter with the rhizoxin-resistant *Rhizopus* hosts, followed by bacteria gaining control of the host reproductive biology, created conditions favorable to the evolution of a mutualism, in which the fungus benefits from the endosymbiont-derived toxin to infect plant hosts and use them as energy sources.

Because the *Rhizopus-Burkholderia* association is an ideal model to study the evolution of symbioses, it can be anticipated that in the future this system will be a source of rich insights into not only the genetic mechanisms of symbiont vertical transmission but also into other facets of partner coevolution.

B. The Form of Evolutionary Trajectories in Mutualisms

Evolutionary models predict that reciprocal selection and close spatial association of the partners will over time lead to coupling of partner metabolic and reproductive interests through endosymbiont vertical transmission, eventually producing a relationship in which the partners cannot live without each other (Yamamura 1993; Frank 1995; Law and Dieckmann 1998). This prediction implies that facultative mutualisms with heritable bacteria, in which one of the partners is not essential to the other, represent short-lived transitory stages along the evolutionary trajectory leading to reciprocal partner dependence (Fig. 7.2a). Support for the transitory nature of facultative endosymbioses comes from the observation that many obligate endosymbioses, including endobacteria essential to nutrition of insects (Moran et al. 2008), are of great evolutionary age. For example, the oldest obligate nutritional mutualisms include the ~260-million-year-old symbiosis of *Candidatus* Sulcia muelleri with auchenorrhynchous insects (Moran et al. 2005) and the 180 million-year-old symbiosis of *Buchnera aphidicola* with aphids (Moran et al. 1993). In contrast, examples of ancient facultative endosymbioses are difficult to find, which may be related to problems with establishing their antiquity. Dating the origin of a symbiosis requires fossil record for at least one of the

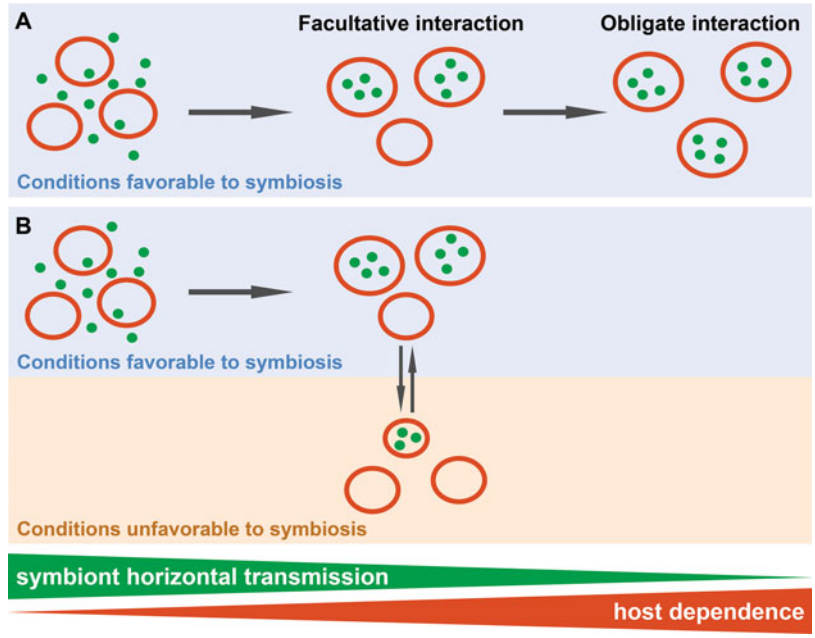

Fig. 7.2 Hypothetical evolutionary trajectories in heritable mutualisms. Hosts are depicted as red ovals; endosymbionts are shown as green dots. Relative host fitness is reflected by the size of ovals. (**a**) Evolutionary trajectory leading to obligate reciprocal partner dependence. (**b**) Shifting environmental conditions are expected to arrest an association at the facultative dependence stage. If conditions remain unfavorable for prolonged periods of time, host populations would be expected to completely lose endosymbionts. Image modified from Mondo et al. (2012)

partners combined with evidence of phylogenetic codivergence between the partners. However, evolutionary histories of facultative endosymbioses are difficult to reconstruct because phylogenies of the partners are often incongruent with each other (Bright and Bulgheresi 2010). Consequently, the apparent shortage of old facultative endosymbioses may be related to impediments in estimating the age of these associations rather than to their ephemeral nature.

Because fossil record is available for many fungal lineages, fungi offer excellent models for studying coevolution in symbiotic associations (Taylor et al. 2015). In particular, the hypothesis of the ephemeral nature of facultative symbioses was tested using the Gigasporaceae–*Glomeribacter* symbiosis (Mondo et al. 2012). To achieve this goal, the phylogenetic histories of both the Gigasporaceae hosts and their *Glomeribacter* symbionts were reconstructed and compared using a statistical tool ParaFit (Legendre et al. 2002) (Fig. 7.3). ParaFit tests the null hypothesis that symbiotic partners evolve independently of each other and, in addition to calculating a test statistic for the entire dataset, allows for examining individual partner pairs to detect evidence of codivergence. In the case of the Gigasporaceae–*Glomeribacter* symbiosis, the ParaFit test rejected the hypothesis of independent partner evolution and identified partner pairs that contributed to the overall significant signal of codivergence (Fig. 7.3). These partner pairs included representatives of several populations of *Gigaspora margarita*, *Cetraspora pellucida*, *Racocetra castanea*, and *R. verrucosa.* Based on this pattern of codivergence, the origin of the Gigasporaceae–*Glomeribacter* symbiosis appears to predate the divergence of *Gigaspora* from *Cetraspora* and *Racocetra* (former *Scutellospora*). The existence of *Scutellospora* fossil record from the Rhynie chert dated to 396 ±12 mya (Dotzler et al. 2006) allows ascribing the origin of the Gigasporaceae–*Glomeribacter* symbiosis to at least 400 million years ago.

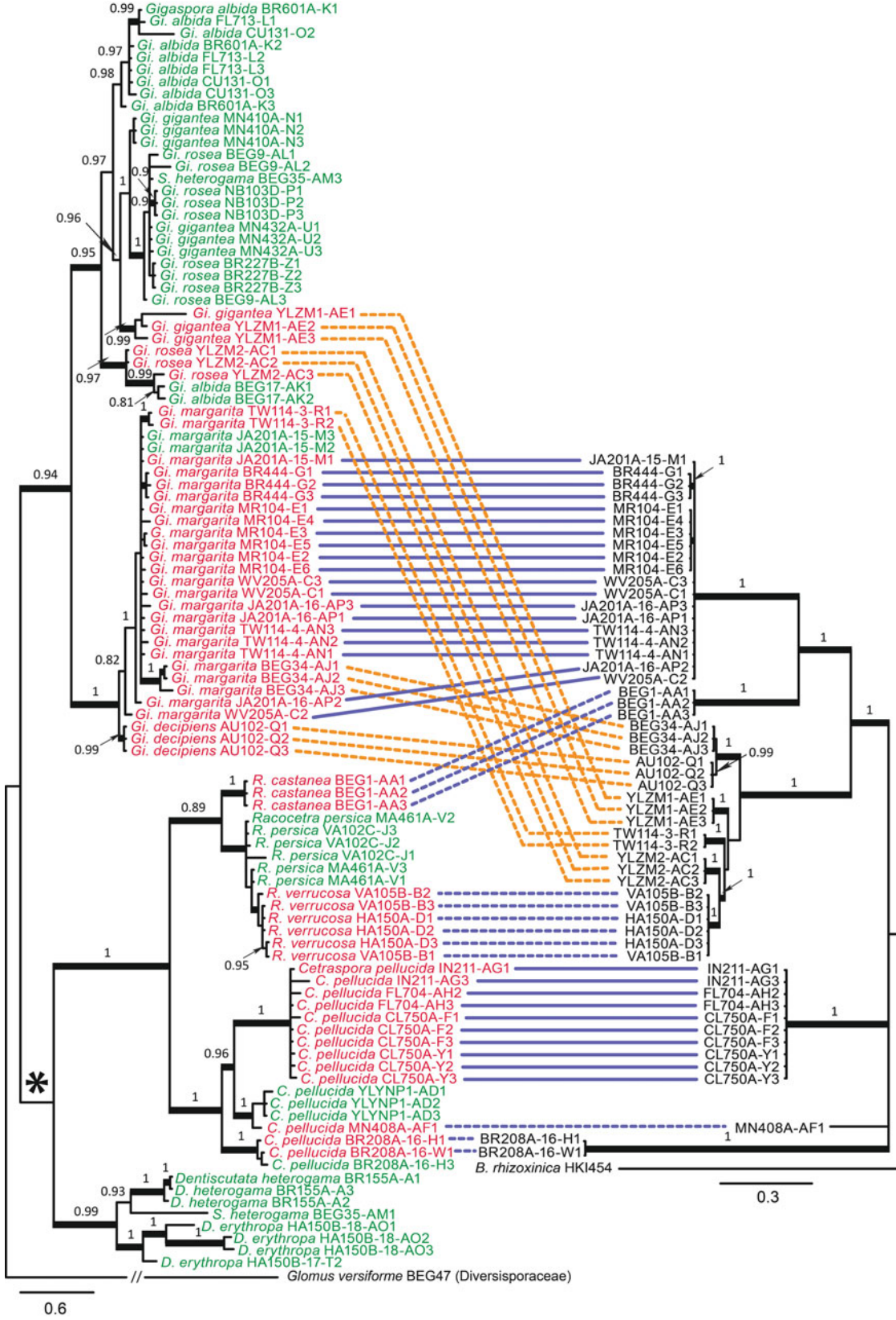

Fig. 7.3 Patterns of coevolution between the Gigasporaceae fungal hosts (*left*) and the *Ca.* Glomeribacter gigasporarum bacterial endosymbionts (*right*). The fungal phylogeny was reconstructed using 18S rRNA,

Older than 400 million years, the facultative mutualism between Gigasporaceae and *Glomeribacter* predates several obligate nutritional mutualisms of insects (Moran et al. 2008). The existence of an ancient facultative mutualism suggests that not all facultative mutualisms are transitional stages along the evolutionary trajectory leading to reciprocal partner dependence (Fig. 7.2b). A discovery that facultative mutualisms can be ancient and stable in their facultative state rises a question concerning the mechanisms that prevent the host from evolving toward obligate dependence on the endosymbiont. While other heritable nonessential mutualisms, such as defensive symbioses of insects (Oliver et al. 2003; Scarborough et al. 2005), might also be arrested in a facultative state, the Gigasporaceae–*Glomeribacter* symbiosis is uniquely suited to addressing this question. Because AMF depend on plant-assimilated carbon, their interaction with *Glomeribacter* is likely to be tightly regulated to avoid unnecessary costs of supporting symbionts under conditions when they become a burden to the host fitness. Since the Gigasporaceae hosts can be readily sampled from nature, as they are commonly found in dune habitats (Bergen and Koske 1984; Koske 1987; Gemma et al. 1989), the distribution of *Glomeribacter* across the AMF hosts could be correlated with environmental conditions to reveal factors responsible for the facultative nature of the Gigasporaceae–*Glomeribacter* symbiosis.

C. Mechanisms of Genome Evolution in Heritable Endosymbionts

Genome reduction is one of the hallmarks of endosymbiont reproductive dependence on the host (McCutcheon and Moran 2012). In maternally transmitted essential mutualists of insects, genome reduction is a consequence of a degenerative process. Endosymbionts are propagated exclusively through host reproductive structures, leading to transmission bottlenecks in every host generation (Mira and Moran 2002). In addition, endosymbiont populations associated with individual host lineages are reproductively isolated from each other, resulting in endosymbiont clonality and population subdivision. All these phenomena reduce endosymbiont effective population size and magnify the impact of genetic drift relative to natural selection (Charlesworth 2009). As a consequence, slightly deleterious mutations undergo rapid fixation (Ohta 1973), leading to the loss of gene functions. Due to the strong deletional bias prevailing in the bacterial genomes (Mira et al. 2001; Kuo and Ochman 2009), mutation-compromised genes are eliminated, resulting in genome contraction (Moran et al. 2009). Another consequence of rapid fixation of slightly deleterious mutations is acceleration of molecular evolution rate relative to free-living taxa (Ohta 1973). While genome size reduction is apparent in several bacterial endosymbionts of fungi examined thus far, including (1) *Burkholderia rhizoxinica* (Lackner et al. 2011b), (2) *Glomeribacter gigasporarum* (Ghignone et al. 2012), and (3) MRE (Naito et al. 2015), none of these genomes appears to be a product of exclusively degenerative evolution (Castillo and Pawlowska 2010; Naito et al. 2015). Consequently, examination of genome evolution in bacterial endosymbionts of fungi is likely to reveal novel mechanisms responsible for genome contraction.

Fig. 7.3 (continued) 28S rRNA, and beta-tubulin gene sequences; the bacterial phylogeny is based on 16S rRNA, 23S rRNA, *ftsZ*, and *pstA* genes. Bayesian posterior probabilities greater than 0.80 are shown above branches. Branches with Maximum Likelihood bootstrap support over 70 % are thickened. *Solid blue lines* connecting host and symbiont pairs indicate significant evidence of codivergence detected by ParaFit. *Dashed blue lines* link partners showing evidence of codivergence after recombination was accounted for in the bacterial dataset. *Dashed orange lines* indicate partner pairs with no evidence of codivergence, likely due to host switch events. Fungal isolates harboring endobacteria are colored red; individuals with no endobacteria are shown in green. The node likely associated with the *Scutellospora* Rhynie chert fossil record (Dotzler et al. 2006) is marked by an *asterisk*. Image modified from Mondo et al. (2012)

1. *Burkholderia rhizoxinica*

Compared to free-living *Burkholderia* relatives with genomes of 8–9 Mb (Winsor et al. 2008), the 3.75 Mb size of the *Burkholderia rhizoxinica* genome (HKI 0454) is noticeably reduced (Lackner et al. 2011b). The endosymbiont primary metabolism is specialized for the uptake of fungal metabolites. In addition to the rhizoxin biosynthesis gene cluster, *B. rhizoxinica* harbors 14 nonribosomal peptide synthetase gene clusters, which may be important for the fungal host ability to pathogenize plants. Furthermore, the genome encodes elements of type II, type III, and type IV secretion systems of which type II and type III are essential for the symbiosis establishment (Lackner et al. 2011a; Moebius et al. 2014).

Comparisons of molecular evolution patterns indicative of accumulation of slightly deleterious mutations between symbiotic and free-living *Burkholderia* did not reveal any substantial differences (Castillo and Pawlowska 2010). In particular, thermodynamic stabilities of rRNA secondary structures as well as total numbers of mutations in rRNA genes were comparable between them. Similarly, the ratios of the rate of nonsynonymous substitutions to the rate of synonymous substitutions, d_N/d_S, in protein coding loci as well as evolution rates were equivalent (Castillo and Pawlowska 2010). Such absence of obvious disparities in the ability to purge slightly deleterious mutations between symbiotic and free-living *Burkholderia* suggests that the mechanisms of *B. rhizoxinica* genome evolution, and of size reduction in particular, are similar to those in free-living bacteria. In free-living bacteria, the loss of abilities to synthesize costly metabolites that are readily available in the environment is generally associated with fitness advantages over genotypes that retain such biosynthetic functions (Zamenhof and Eichhorn 1967; D'Souza et al. 2014). Consequently, it is likely that in *B. rhizoxinica*, genome evolution is driven by adaptation to the host cellular environment rather than by degenerative processes that dominate genome evolution in essential mutualists of insects.

2. *Glomeribacter gigasporarum*

Glomeribacter endosymbionts of AMF are a sister lineage of the *Burkholderia* endobacteria of *R. microsporus* (Ghignone et al. 2012; Mondo et al. 2012).

The ~1.72 Mb genome of *G. gigasporarum* BEG34 is considerably reduced compared to the *B. rhizoxinica* genome and substantially smaller than the genomes of free-living *Burkholderia* (Ghignone et al. 2012). The *Glomeribacter* gene content confirms that these endobacteria depend on the AMF host for carbon, phosphorus, and nitrogen supply. In addition, their genomes encode type II and type III secretion systems as well as biosynthetic pathways for vitamin B12, antibiotics- and toxin-resistance molecules (Ghignone et al. 2012).

Molecular evolution patterns apparent in *Glomeribacter*, including thermodynamic stability of rRNA molecules, types and numbers of mutations in rRNA genes, as well as d_N/d_S values in protein coding loci, are substantially different from those in essential mutualists of insects *Candidatus* Tramblaya princeps (Castillo and Pawlowska 2010), which are close relatives of *Glomeribacter* and are characterized by highly eroded genomes (McCutcheon and von Dohlen 2011; McCutcheon and Moran 2012). Instead, *Glomeribacter* shares some of the mutation accumulation patterns with symbiotic and free-living *Burkholderia* (Castillo and Pawlowska 2010). This is not unexpected for several reasons. First, the *Glomeribacter* genome contains a nearly full complement of DNA repair genes vital to successful elimination of mutations (Ghignone et al. 2012). Second, examination of codivergence patterns between *Glomeribacter* and AMF revealed a history of horizontal transmission (Mondo et al. 2012) (Fig. 7.3). Finally, *Glomeribacter* population structure is indicative of infrequent recombination (Mondo et al. 2012). Both horizontal transmission and recombination are important for maintenance of effective population size that enables efficient selection against deleterious mutations. However, unlike symbiotic and

free-living *Burkholderia*, *Glomeribacter* exhibits a moderate acceleration of the molecular evolution rate, a feature attributed to degenerative evolution (Castillo and Pawlowska 2010). Consequently, a reconciliation between the free-living-like mutation accumulation patterns and molecular evolution rate acceleration suggestive of degenerative evolution is required to understand the prevailing mode of *Glomeribacter* evolution.

3. Mycoplasma-related endobacteria

In contrast to symbiotic *Burkholderia* and *Glomeribacter*, which appear to exist as genetically uniform populations within their fungal hosts (Lackner et al. 2011b; Mondo et al. 2012), some of MRE populations associated with individual AMF spores are highly diverse, with rRNA gene sequences exhibiting as little as 88 % similarity (Naito et al. 2015). Consistent with such intrahost rRNA gene diversity, sequencing of MRE metagenomes associated with three different AMF host species, *Claroideoglomus etunicatum* (family Claroideoglomeraceae), *Racocetra verrucosa* (Gigasporaceae), and *Rhizophagus clarus* (Glomeraceae), revealed extensive chromosomal rearrangements, which could be attributed to genetic recombination, activity of mobile elements, and a history of plectroviral invasion (Naito et al. 2015). The MRE genomes are small in size, ranging from 0.7 to 1.2 Mb. They also exhibit a reduced gene content, indicating limited metabolic capabilities and dependence on the fungal host. Several MRE genes encode proteins with domains involved in protein–protein interactions with eukaryotic hosts. In addition, the MRE genomes harbor genes horizontally acquired from AMF. The extent of MRE genome plasticity and reduction, along with the large number of horizontally acquired host genes, suggests a high degree of adaptation to the fungal host (Naito et al. 2015).

The minimal genomes of vertically transmitted MRE represent a marked departure from the small genomes typical for heritable mutualists (Naito et al. 2015). MRE evolved from animal-associated mycoplasmas of the Mycoplasmataceae family (Naito et al. 2015). Consistent with this origin, MRE genomes have retained traits shared by members of Mycoplasmataceae, including propensity for mutation accumulation and gene loss as well as features contributing to genome plasticity (Marenda 2014; Naito et al. 2015). Rapid mutation accumulation in mycoplasmas is related to the loss of proofreading ability of DNA polymerase (Kelman and O'Donnell 1995). Genes encoding these proteins are missing in the genomes of mycoplasmas and also from the MRE genomes (Naito et al. 2015). The features responsible for genome plasticity include the presence of recombination genes as well as mobile DNA elements that facilitate recombination (Marenda 2014). Consistent with the existence of these features in the MRE genomes is evidence of recombination detected in MRE populations using population genetics tools (Desirò et al. 2014b; Naito 2014). The role of recombination in the restoration of high fitness genotypes (Muller 1964) as well as in facilitation of adaptation (Fisher 1930) is well established. It is, therefore, apparent that the minimal genomes of MRE are not exclusively products of degenerative evolution.

Remarkably, MRE can share fungal hosts with *Glomeribacter* (Desirò et al. 2014b). However, in contrast to *Glomeribacter*, MRE intrahost populations accumulate diversity (Mondo et al. 2012; Desirò et al. 2014b). Clearly, despite their coexistence in the same hosts, the lifestyles and genetic mechanisms are distinctly different between MRE and the *Glomeribacter* endobacteria. Consequently, like in the case of *Glomeribacter*, identifying specific mechanisms that are responsible for MRE genome evolution requires further work.

Conclusion

The recently discovered heritable bacterial–fungal associations represent a diverse array of biological functionalities and evolutionary histories. Thus far, they revealed that, contrary to evolutionary models, not all facultative mutualisms are ephemeral and that adaptive processes play an important role in genome evolution of heritable endosymbionts. Further understanding of the mechanisms that drive

reciprocal selection and coevolution in bacterial–fungal symbioses is expected to be a critical source of novel findings needed to advance evolutionary theory.

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8 An Emerging Interdisciplinary Field: Fungal–Bacterial Interactions

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CONTENTS

I. Introduction

Fungi and bacteria coexist in various environments and often share niches. The formation of physical associations between them is frequent and depends on various modes of molecular communication (reviewed in Tarkka et al. 2009). The established fungal–bacterial associations affect the composition of the surrounding substrate via physical, biological, and biochemical processes (reviewed in de Boer et al. 2005; Barea et al. 2005; Wargo and Hogan 2006). In recent years, we have acquired conclusive evidence that the classical separation of microbiological research to bacteriology and mycology in pure culture axenic settings cannot deeply characterize the processes occurring in natural systems (reviewed in Frey-Klett et al. 2007, 2011; Harriott and Noverr 2011; Scherlach et al. 2013).

The formation of fungal mycelium facilitates the growth of specialized bacteria and affects bacterial community structures (Kurth et al. 2013; Folman et al. 2008). The activity of bacteria is stimulated by fungus-provided energy sources, including both exudates and fungus storage sugars (Warmink et al. 2009; Deveau et al. 2010). Bacteria may, in turn, stimulate the hyphae by growth and cofactors (Riedlinger et al. 2006; Ghignone et al. 2012), detoxify fungus inhibitory compounds (Tsukamoto et al. 2002), and protect the mycelium against parasitic microorganisms (Cafaro et al. 2011). Mycelial network may serve as transport vector (Kohlmeier et al. 2005), thereby improving the access of bacteria to non-diffusible substrates at sites separated by, e.g., air gaps, and enabling chemotactic bacterial dispersal toward the source of nutrition. In addition, fungal–bacterial interaction (FBI) may result in unique contributions to biogeochemical cycles: with joint degradation capacities of bacteria and fungi, transport of degrading bacteria to the metabolites, or support of easily consumable fungal carbohydrates, the degradation of recalcitrant substrates or xenobiotics is supported (Schamfuss et al. 2013). The co-occurrence of

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fungi and bacteria leads to changes in pH and to enrichment of certain metabolites, which induces the production of some but suppresses that of other compounds (reviewed in Tarkka et al. 2009; Scherlach et al. 2013). Beneficial effects of microbial cocultures for food production include cheese (Corsetti et al. 2001) and vinegar (Valera et al. 2015) production or suppression of mold growth and mycotoxin production in foodstuffs (Dalie et al. 2010), but FBI may also cause food spoilage by toxin production (Garcia et al. 1999). The activation of "silent" secondary metabolite gene clusters by bacteria during FBI (Schroeckh et al. 2009) has emerged as a novel strategy to produce therapeutical agents, but the FBI may either suppress or activate secondary metabolite production (Scherlach et al. 2013).

Pathogenicity or nutritional influence of plant and animal symbionts may be affected by FBI. Bacteria may interfere with fungi by a multitude of mechanisms, including competitive tissue colonization; synthesis of antibiotics, organic acids, and hydrogen peroxide; production of lytic enzymes; detoxification of toxins; and degradation of virulence factors (reviewed in Compant et al. 2005; Harriott and Noverr 2011). Stimulatory interaction exists between nitrogen-fixing and phosphate-solubilizing bacteria and plant beneficial mycorrhizal fungi (Requena et al. 1997). Some bacteria stimulate the virulence of a plant pathogen by and Hertweck 2005 producing virulence factors (Partida-Martinez and Hertweck 2005), promote the growth of the pathogen (Xu et al. 2008) or anchor the mycelia of a fungal pathogen to the target tissue, and stimulate the formation of mixed FB biofilms (Silverman et al. 2010), which are less responsive to antimicrobials than free-living cells.

The yearly growing number of studies published on the FBI points to an exciting and emerging area of research with increasing numbers of researchers involved (Table 8.1). Niches for fungal–bacterial networks are just beginning to be described, and in the following review, we will focus on how the targeting of the "fungus microbiome" in more detail will help us understand fundamental biological processes. The review highlights recent contributions to the understanding of bacterium–fungus interactions and focuses on the rapid methodological development in this area. On the basis of this introductory review, we propose that the reader subsequently consults the review of Pawlowska (chapter "Evolution in Heritable Bacterial-Fungal Endosymbioses"), to learn more about the fascinating bacterium endosymbiosis in the hyphae of the oldest fungal mutualists, the Glomeromycota.

II. FBI Theater Takes Place Anytime, Anywhere, Every Way

The following section focuses on fungus–bacterium interactions taking place in simple in vitro cultures and different environments. Mediation of the FBI by metabolites, enzymes, and physical associations is described; examples from simple two-partner model systems to more complex dynamic consortia are included; and implications for medicine, biotechnology, agriculture, and forestry are mentioned.

A. Simple Models Drive the Analysis of FBI Evolutionary Ecology

Evolution and ecology of FBI has been targeted perhaps most elegantly with the simple yeast–bacterium interaction. For instance, by using an artificial community composed of baker's yeast *Saccharomyces cerevisiae* and nitrogen-fixing bacterium *Rhizobium etli*, Andrade-Domínguez et al. (2014) investigated what happens when two species with no known history of previous interaction meet for the first time. At the beginning of the interaction, yeast produced growth inhibitor orotic acid (OA) and growth-promoting C4-dicarboxylates, and since the dicarboxylates dominated over the impact of OA, the early interaction was commensal, bacterium favoring. When the growth promoter OA levels later became depleted, the interaction developed to a harmful one for the bacterium, and as a result, OA-resistant bacterial variants arose that allowed bacterial growth. The growth of the bacterium created a stage of nutrient competition with the yeast,

Table 8.1 Same functional properties occur in FBI-based processes taking place in different niches

Functional property	Process 1	Process 2
Suppression of antifungal substance production	Competition in rhizosphere Van Rij et al. (2005)	Competition on mucus Coleman et al. (2008)
Suppression of fungal pathogens	Plant pathogen suppression de Boer et al. (2003)	Human pathogen suppression Hogan and Kolter (2002)
Antibiotics production	Button mushroom disease Lo Cantore et al. (2006)	Spoilage of milk Reybroeck et al. (2014)
Stimulated migration of bacteria	Oral polymicrobial biofilm formation Sztajer et al. (2014)	Transport of remediating bacteria Schamfuss et al. (2013)
Metabolic cooperation	Plant disease development Partida-Martinez and Hertweck (2005)	Cheese production Irlinger et al. (2012)

and as an ecological consequence of this diversification of bacteria, a new change in the community appeared: the interaction became antagonistic. This was due to bacterium-caused alkalinization of pH and consequent starvation of yeast cells. It led to the extinction of the yeast partner.

Elsewhere, Romano and Kolter (2005) discovered a beneficial interaction, reminiscent to the commensal phase of the last report, between *Saccharomyces cerevisiae* and *Pseudomonas putida*. When the bacteria were incubated alone glucose-rich environment, they lost viability rapidly during stationary phase: the bacteria seemed unable to control the activity of their glucose dehydrogenase, and the conversion of glucose to gluconate led to a pH drop and bacterial growth arrest. The pseudomonad's stationary phase survival improved dramatically by the presence of the yeast, since yeast rapidly consumed part of the glucose, practically eliminating the pH drop. The interactions between bacteria and fungi in the extramatrical mycelium may also express mutualistic features. For instance, the mycorrhizal fungus *Morchella crassipes* can be termed "bacterial farmer," since the bacteria can migrate on the fungal mycelium and consume nutrition from fungal exudates. The fungus then harvests and translocates bacterial carbon (Pion et al. 2013). Warmink et al. (2009) showed that there is a decrease of the diversity of the total bacterial communities in the soil from beneath fruiting bodies, which also argues for selection or "farming" of bacteria by the host fungal mycelium. Both universal and fungus-specific bacterial groups could be distinguished, and among the *Pseudomonas* communities, there was an increase in diversity in most of the fruiting bodies tested. The selection of the bacteria was strongly related to their capacities to use characteristically fungus-enriched carbohydrates, arabitol, mannitol, and trehalose.

B. Coevolution in Ant Fungus Gardens: Antagonistic Warfare Keeps the Balance on the Side of Good Fungi

The mutualism between "fungus-gardening" insects and their fungal associates is an excellent example of specialized networks of bacteria and fungi, focused on insect nutrition. Most attention has been given to tripartite animal–bacterium–fungus interaction between fungus-growing attine ants, their bacterial companions, and fungal associates. The ants are dependent on their fungal associates, since the cultivars serve as the sole food source for the ant larvae and queen. The fungus gardens are under attack from parasites, in particular from the ascomycete genus *Escovopsis*, and the ants keep their gardens free of microbial pathogens, thanks in part to beneficial bacteria (Currie et al. 1999, 2006). These Actinobacteria symbionts of the leaf-cutting ants, often belonging to *Pseudonocardia* and *Streptomyces* genera, support the ants in defending their fungus gardens against infections by providing antimicrobial and antifungal compounds.

Antibiotic assays suggest that despite *Escovopsis* being generally susceptible to inhibition by diverse Actinobacteria, the ant-derived *Pseudonocardia* isolates inhibit the parasite more strongly than they inhibit other fungi and are better at inhibiting this pathogen than most environmental strains, indicating that fungus-growing ants may maintain specialized symbionts that help with garden defense (Cafaro et al. 2011). Some antibiotics produced by Actinobacteria associated with attine ants selectively target *Escovopsis* but not the fungal garden, such as candicidin and dentigerumycin (Oh et al. 2009; Haeder et al. 2009), and some of the secondary metabolites express strong synergistic effects against *Escovopsis*, but some of the antifungal substances are not only active against pathogenic fungi but also the garden fungus itself, e.g., the antifungal antimycins (Schoenian et al. 2011). In conclusion, secondary metabolites of microbial symbionts of leaf-cutting ants contribute to shaping the microbial communities within the nests of leaf-cutting ants. Parasitic yeast affects the ant fungus gardens as well. *Phialophora*, a genus of ascomycete black yeasts, also grow on the ants' cuticle and appear to be localized to sites where mutualistic, *Escovopsis*-killing bacteria occur. The basis for this localization is unknown, but the yeasts appear to obtain nutrients from the bacteria and have a negative effect on their growth, which reduces their antibiotic effects on *Escovopsis* (Little and Currie 2008). In conclusion, the microbial symbionts of leaf-cutting ants contribute to shaping the microbial communities within the nests of leaf-cutting ants. As the common patterns of garden fungus and parasites occur in the fungal gardens of ants, beetles, and termites, all insects that have independently evolved nutritional symbioses with fungi, it is an important question whether the gardens harbor similar microbiomes. Aylwards et al. (2014) showed that this is indeed the case; the microbiota, their fungal gardens, are remarkably similar, comprising primarily of the bacterial genera *Enterobacter*, *Rahnella*, and *Pseudomonas*. Future work assessing the roles of these bacteria in fungal gardens will give us further insight to this fascinating FBI network.

C. Polymicrobial Biofilms as Virulence Factors

Traditional microbiological research emphasized the study of single isolates of microorganisms in liquid culture, but the past decades have witnessed a growing interest on the structure and function of the dominant mode of microbial life, consortia between different species, and strains of microorganisms. **Wherever the submerged microbial cells encounter surfaces, they can switch from a planktonic lifestyle to form sessile communities that are enclosed by a slimy matrix termed biofilms** (Battin et al. 2007). The biofilms are heterogeneous communities encased in an extracellular matrix and from their form often complex three-dimensional architectures (Xiao et al. 2012; Ramírez Granillo et al. 2015). The chemical properties of biofilms are distinct from the planktonic cultures, as these tightly packed cells share resources, compete, or cooperate in order to survive.

The biofilms are complex and dynamic systems, and as they grow and reproduce, they may possess novel or amplified biochemical activities, as has been, e.g., suggested for petroleum-degrading microorganisms (White et al. 1995; Charlesworth et al. 2012). For pathogenic ones, the protection of fungi and bacteria by biofilm formation is often manifested through increased tolerance against host defenses and high-level resistance to antimicrobial drugs. There is considerable evidence to suggest that choosing antibiotics based on biofilm rather than conventional antimicrobial susceptibility testing could potentially improve response to antimicrobial treatments. For these reasons, *Candida albicans* and *Aspergillus fumigatus*, common fungal participants of pathogen polymicrobial biofilms in mammals, have centered a wealth of pioneering FBI research, which has been extensively reviewed in recent years (e.g., Wargo and Hogan 2006; Harriott and Noverr 2011). For this reason, the following includes just a few examples of inter-

kingdom signaling and recent microbiome approaches.

C. albicans is a resident of mucosal surfaces. It can overgrow under favorable conditions, particularly when the immune system is compromised, leading to a wide range of diseases, referred to as candidiasis. *C. albicans* infections often involve more than one cellular morphology; yeast, pseudohyphal, and hyphal growth forms have all been associated with virulence (Wargo and Hogan 2006). Numerous studies have described its interactions with bacteria, and *C. albicans*–bacterium interactions can promote or prevent human diseases (Morales and Hogan 2010). *C. albicans* infections are associated with the formation of mixed biofilms, where *C. albicans* and bacteria adhere to one another through the action of surface adhesins and extracellular polymers. Another fungal pathogen, *Aspergillus fumigatus*, causes severe lung diseases such as aspergillosis in immunocompromised patients, and biofilm formation by *A. fumigatus* is an important virulence factor in these diseases.

C. albicans interactions with bacteria are modulated by farnesol, a fungal quorum-sensing molecule (Hogan et al. 2004). Farnesol represses hyphal growth and early biofilm formation and is required for virulence during scattered infection and affects bacterial pathogens, inhibiting biofilm formation and bacterial virulence. For instance, farnesol production by *C. albicans* leads to decreased antibiosis by *P. aeruginosa*, and this takes place by decreased production of *P. aeruginosa* quinolone signal. The bacterial quinolone signal acts in concert with other bacterial quorum-sensing regulators and controls the production of *P. aeruginosa* extracellular proteases, hydrogen cyanine, and phenazines (Cugini et al. 2007). Farnesol usage may be a strategy of *C. albicans* growing in biofilms to compete under conditions of stress, and due to the inhibition of sessile biofilm formation, it may also lead to dispersal of bacteria from the polymicrobial biofilm (Harriott and Noverr 2011). *P. aeruginosa* also suppresses *Aspergillus fumigatus* biofilm formation in vitro, perhaps leading to comparably low mortality in the lungs of cystic fibrosis patients with *P. aeruginosa* as an inhabitant (Mowat et al. 2010). Whereas *A. fumigatus* monoculture biofilms show organized structures with extensive hyphal growth and extracellular matrices, *P. aeruginosa* antibiosis leads to disorganized fungal structures and limited hyphal growth (Ramírez Granillo et al. 2015). *P. aeruginosa* inhibits *A. fumigatus*, just like *C albicans*, by the production of phenazines, and at higher dozes, these antibiotics block fungal growth. But lower concentrations of phenazines allow slow extension of mycelium and thereby affect the development of *A. fumigatus* by means of oxidative stress regulation (Zheng et al. 2015). Wrinkled colony biofilms with higher oxygen concentrations develop (Morales et al. 2013) and fungal yeast-to-filament transition is inhibited, but also asexual sporulation is stimulated and an iron-uptake system is induced (Briard et al. 2015; Zheng et al. 2015). These data give insight to the interkingdom signaling and show that antifungal substances can be assessed not only as growth inhibitors but also as interspecies signaling compounds.

Human serum induces hyphal development of *Candida albicans*, and given the abundance of bacterial cell wall fragments in the intestine, a natural habitat and invasion site for *C. albicans*, one would expect that the fungus responds to bacterial cell wall fragments. Xu et al. (2008) showed that this is the case: *C. albicans* virulence is induced by the perception of bacterial cell wall fragments, muramyl dipeptides that are present in low concentrations in the human serum. And *C. albicans* and bacteria may promote coaggregation and formation of mixed-species biofilms.

Co-occurring in the oral cavity, *C. albicans* and the caries-promoting bacterium *Streptococcus mutans* express a synergistic interaction and a most aggressive onset of caries. Mixed biofilms reach higher biomass and cell numbers than mono-species biofilms. The resulting three-dimensional biofilm architecture displays small *S. mutans* colonies surrounded by fungal cells, which are embedded in a dense extracellular polysaccharide matrix (Falsetta et al. 2014). Coexistence with *C. albicans* induces the expression of virulence genes in *S. mutans*, and *C. albicans* achieves this by including the complete quorum-sensing system of *S. mutans*

(Sztajer et al. 2014). *C. albicans* forms biofilm communities also with *Streptococcus gordonii*, a ubiquitous oral bacterium. The molecular nature of bacterial-fungal contact has been examined in this association, and the formation of dual-species *S. gordonii–C. albicans* biofilm communities involves interaction of the bacterial cell wall-anchored SspB protein and hyphal filament surface Als3 protein (Bamford et al. 2009; Silverman et al. 2010). The attachment is mediated by *O*-mannosylation in *Candida albicans* surface (Dutton et al. 2014).

In addition to cross-kingdom adhesion and quorum sensing, metabolic changes in the environment (pH, metabolic substrate production, inhibitor production, nutrient sensing/sequestering, etc.) and indirect activity on the host response are mechanisms by which *Candida* and other fungi interact with bacteria in the microbiome to regulate fungal levels and host responses to fungal colonization (Wargo and Hogan 2006). These changes lead to enrichment of certain bacteria. When Fox et al. (2014) investigated mixed biofilms composed of the major fungal species of the gut microbiome, *C. albicans*, five prevalent bacterial gastrointestinal inhabitants, *Bacteroides fragilis*, *Clostridium perfringens*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*, they observed that biofilms formed by *C. albicans* provided a hypoxic microenvironment that supported the growth of anaerobic bacteria, even when cultured in ambient toxic conditions that are normally toxic to the bacteria. In suspension cultures, *C. perfringens* induced aggregation of *C. albicans* into "mini-biofilms," which allowed *C. perfringens* cells to survive in a normally toxic environment.

D. An Intracellular Bacterial Symbiont in a Plant Pathogenic Fungus

Beyond the most commonly observed FB cell-to-cell interactions, there is a growing number of known endosymbioses and endobacteria living inside the fungal cells. In these intracellular associations, bacteria grow and multiply within fungal hyphae, and the two partners may have developed strategies to interact with each other, to the mutual success of the interaction (Frey-Klett et al. 2011). Plant-associated fungal systems have become complementary models of endobacterial symbioses. Hoffmann and Arnold (2010) recovered phylogenetically diverse endohyphal bacteria occur in living hyphae of diverse foliar endophytes, but the presence of intracellular bacteria is particularly well established among the species of mycorrhizal fungi (Bianciotto et al. 2004; Bertaux et al. 2003). Reviewed in the paragraph X of this volume, the endobacteria of Glomeromycota were already discovered in the early 1970s on the basis of electron microscope observations (Mosse 1970).

The bacterial endosymbionts of the Glomeromycota fungi provide ammonium and thiamine for the fungus (Minerdi et al. 2001; Ghignone et al. 2012) and stimulate spore formation and growth of germinating mycelium (Lumini et al. 2007). But another in-detail investigated model of an intracellular symbiosis in fungi (Lackner et al. 2011) exists between the plant pathogen, rice seedling blight fungus *Rhizopus microsporus*, and an endosymbiont bacterium, *Burkholderia rhizoxinica*. The key virulence factor of rice seedling blight is the phytotoxin rhizoxin, a potent cell cycle inhibitor that kills plant and animal cells. Rhizoxin is not a fungal metabolite but in fact produced by *B. rhizoxinica* (Partida-Martinez and Hertweck 2005). Endobacteria are often very difficult to get in culture, but Partida-Martinez and Hertweck (2005) were not only able to culture the endosymbiont in vitro but also to produce bacterium-free *Rhizopus* mycelium. This enabled them to examine the roles of the partners of this intriguing relationship. A specialized mechanism has evolved during evolution that guarantees the persistence of the *Rhizopus-Burkholderia* endosymbiosis. The bacteria invade the *Rhizopus* cells by excreting fungal cell wall-degrading chitinolytic enzymes and chitin-binding proteins through type 2 secretion system (T2SS). The T2SS is required for the formation of the endosymbiosis (Moebius et al. 2014). The presence of *Burkholderia* endosymbiont is necessary for vegetative reproduction of *Rhizopus*, in particular for spore production (Partida-Martinez and

Hertweck 2007), and this control measure against the host fungus takes place by another, Hrp T3SS. Interestingly, Lackner et al. (2011) showed that mutants defective in T3SS not only failed to elicit sporulation of the host but also showed reduced intracellular survival of the bacterium, underlining the intimacy and interdependency of this endosymbiotic association. General importance of the secretion systems as delivery systems of effectors in FBI is hinted by the role of T3SS in mycorrhiza symbiosis stimulation expressed by the mycorrhiza helper bacterium *Pseudomonas fluorescens* BBc6R8 (Deveau et al. 2010). The respective bacterial effectors warrant further examination.

E. Soil and Wood as Arenas of FBI Affecting Nutrient Cycling and Microbe–Plant Interactions

Soil represents a very heterogeneous environment for the available organic and mineral nutrients, for water, and for its microbiota. Both fungi and bacteria are involved in biogeochemical cycling processes of the soil, and a main energy source driving the soil system is formed by plants through the provision of assimilated carbon as rhizodeposits. The microorganisms, for their part, mobilize soil nitrogen and phosphorus and transfer them from the soil back to the plant, with intensive fungus–bacterium interactions (Nazir et al. 2010), which can be in many instances seen as a cooperative system, with fungi, bacteria, and plants interacting with each other (Barea et al. 2005; de Boer et al. 2005).

In the carbon-limited soil, the emergence of plant roots and the formation of fungus-root mutualism termed mycorrhiza create nutritional hot spots for soil bacteria providing an extensive and enduring energy source. The majority of plants form mycorrhizas, and the mycorrhizal symbiosis improves plant nutrient and water uptake, while the fungal partner gains carbohydrates from its host plant. The mycorrhiza-associated organisms are known to influence each other, the outcome of which is described as the "mycorrhizosphere" (Foster and Marks 1967), comprising mycorrhizas, extramatrical mycelium, bacteria, fungal saprophytes, and protists. In the same way the rhizospheres exert a pressure on microbial populations (Barea et al. 2005), the mycorrhizal roots and hyphae of mycorrhizal fungi (MF) shape the bacterial species composition due to root and hyphal exudation and turnover. Bacteria have been visualized inside mycorrhizas, as colonies on soil-colonizing fungal hyphae and inside the fungal mycelium (Bertaux et al. 2003). Mycorrhizal fungi and bacteria undergo complex interactions that influence the biology of the fungus and the nutrition of the plant (Frey-Klett et al. 2007), with the plant partner selecting for bacterial strains beneficial for the symbiosis and for the plant (Frey-Klett et al. 2005). Although the mycorrhizal fungi interact with bacterial strains which can have beneficial, neutral, or harmful effects on fungal physiology, this "mycorrhizosphere effect" may lead to improved plant nutrition, growth, and disease resistance (Frey-Klett et al. 2005). There is a body of evidence that points to the influence of fungi on bacterial community structure at both the taxonomic and functional levels, notably in the mycorrhizosphere (Frey-Klett et al. 2011). Determining the functional significance of the mycorrhizosphere organisms for plant productivity presents a major challenge for sustainable agriculture (Artursson et al. 2006). The intimate relationship between the oldest of all symbioses, arbuscular mycorrhiza, and fungus endophytic bacteria is described in chapter "Mycorrhizal Fungi and the Soil Carbon and Nutrient Cycling." In the following we focus on another level of FBI with importance to mycorrhizal symbiosis, bacteria which stimulate mycorrhiza formation.

The presence of bacteria that are directly involved in mycorrhiza formation was first indicated by the studies of Bowen and Theodorou (1979), which showed that some bacterial isolates promoted and others inhibited the colonization of *Pinus radiata* roots by *Rhizopogon luteolus*. Confirmed in other mycorrhizal forms (Garbaye and Bowen 1987; Meyer and Linderman 1986), the bacteria able to promote mycorrhizal development have been collectively named as MHB: mycorrhiza helper bacteria (Garbaye 1994). The presence of MHB as

an ubiquitous group of microorganisms and important for mycorrhizal symbiosis is suggested by the following findings: (1) MHB have been found whenever they have been looked for, (2) they are present in very different habitats, (3) many of these bacteria seem to be closely associated with MF, and (4) MHB can be found from taxonomically diverse bacterial groups (Frey-Klett et al. 2007). There exists a multitude of interaction mechanisms promoting mycorrhiza formation, and some directly affect fungal growth.

Mechanisms by which ectomycorrhizal fungi perceive and react to surrounding bacteria have been explored in ectomycorrhizal (mycorrhiza of forest trees and shrubs) model systems. The most studied one concerns *Laccaria bicolor* S238N, the first genome-sequenced mycorrhizal fungus (Martin et al. 2008), which is commercially used in France for controlled mycorrhization of Douglas fir seedlings, because it significantly improves plant growth and survival. As the bacterium, the MHB *P. fluorescens* strain BBc6R8, significantly improves the pre-symbiotic survival and growth of *L. bicolor* in the soil (Brule et al. 2001) as well as in vitro (Deveau et al. 2007).

P. fluorescens is chemoattracted by fungal extracts but also by trehalose, a disaccharide in *L. bicolor* mycelium. Conversely, BBc6R8 secretes thiamine which promotes fungal growth (Deveau et al. 2010). Thiamine (vitamin B1) is an essential cofactor of several enzymes of the central carbon metabolism, and it has been previously associated with growth promotion of the yeasts *Debaryomyces vanrijiae* by *Bacillus* sp. TB-1 (Rikhvanov et al. 1999). Coculture with BBc6R8 led to altered growth pattern of *L. bicolor* mycelium; angles and numbers of hyphal branches, as well as numbers of hyphal apices, changed. Simultaneously, pleiotropic alterations in fungal transcriptome were observed, which varied in time (Deveau et al. 2007). An early-stage BBc6R8-responsive fungal gene sharing 54 % similarity at the protein level to tectonin II of *Physarum polycephalum* was identified, and later shown to be induced when *L. bicolor* interacts with other bacteria, as well (Deveau et al. 2015). In *P. polycephalum*, tectonins may be involved in the aggregation of bacteria during the phagocytosis process (Huh et al. 1998). In *L. bicolor*, the tectonin orthologue could thus play a role in cell recognition and/or fungal cell interaction with *P. fluorescens* BBc6R8.

Second central MHB model is *Streptomyces* sp. AcH 505, which promotes the extension of fungal mycelium and mycorrhiza formation by the symbiotic fungus fly agaric *Amanita muscaria* and Norway spruce (Maier et al. 2004). Streptomyces–Amanita interaction has a strong influence on the growth pattern of fungal hyphae. The hyphal diameter and the mycelial density decreased in cocultures with AcH 505. The dense and polarized actin cap in hyphal tips of pure culture *A. muscaria* changed to a loosened and dispersed structures in coculture (Schrey et al. 2007). Apart from changes in cell biology, the interaction with AcH 505 has a strong impact on gene expression levels in *A. muscaria*, suggesting that the fungal physiology is strongly altered due to the contact with the bacterium (Schrey et al. 2005). Whereas Streptomyces AcH 505 promotes the extension of *A. muscaria*, it inhibits the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Riedlinger et al. (2006) found that AcH 505 produced the fungal growth-promoter auxofuran, and two antifungal substances, WS-5995 B and C. Fungi that were sensitive against WS-5995 B, were found to be inhibited in cocultures with AcH 505 (Lehr et al. 2007). *A. muscaria* induced conditions that are favorable to fungal growth, promoted auxofuran, and suppressed WS-5995 B production by AcH 505 (Riedlinger et al. 2006). Lehr et al. (2007) characterized 11 WS-5995 B-sensitive and 1 WS-5995 B-tolerant strains among a collection of fungal plant pathogen *Heterobasidion abietinum* isolates. Sensitivity against the antibiotic is reflected by decreased expression levels of cell stress-related genes (Lehr et al. 2007). Mycorrhizal fungus enhanced the growth of AcH 505 (Kurth et al. 2013), and AcH 505 affected the structure of microbial community and improved plant resistance against nematode colonization (Caravaca et al. 2015). Furthermore, AcH 505 elicited a systemic defense response in oak, which was associated in reduced powdery mildew symptoms in leaves, indicative of priming

of plant defenses by the bacterium (Kurth et al. 2014). The defense elicitation process was attenuated by Piloderma croceum (Kurth et al. 2015), presumably to faciliate root colonisation by the mycorrhizal fungus.

FBI take place also at the degradation of recalcitrant organic matter, e.g., lignocellulose in litter or wood (de Boer et al. 2005). Wood decomposition is an important process for the forest biogeochemical cycle that is driven by microorganisms such as white-rot fungi which naturally coexist with bacteria. Soils are usually not saturated in water, but in order to find water-saturated microaggregates and to degrade organic compounds, the bacteria may have to move larger distances. Wick et al. have identified a role of mycelia for the translocation of organic compounds and microorganisms. Briefly, contaminant biodegradation in soil is frequently limited by hindered physical access of bacteria to the contaminants, and, e.g., Schamfuß et al. (2013) showed that water-insoluble hydrophobic organic compounds, polyaromatic hydrocarbons, were transported on the hyphae of mycelial oomycete *Pythium ultimum* along air-water interfaces. Mycelia-dispersed hydrocarbons were degraded by the bacterium *Burkholderia sartisoli* and the bacterium enriched on hyphal surfaces. This suggests that both bacteria and their substrates can migrate and confront each other on fungal hyphae and that this interaction is beneficial for the bacterium. This could in particular stimulate the degradation of organic compounds with patchy distribution and low solubility in water (Schamfuß et al. 2013). The ability of fungi to colonize and decompose woody resources may be strongly influenced by wood-inhabiting bacteria that grow on easily utilizable compounds released by the fungal enzymes. Since fungi acidify the substrate at decomposition, coexisting bacteria have to also be acid tolerant (Valásková et al. 2009; Frey-Klett et al. 2011). Decay of wood by white-rot fungi can be either inhibited or promoted by associated bacteria, depending on the relative timing of the fungal and bacterial inoculations (Murray and Woodward 2003). Indicated by the study of Folman et al. (2008), fungal presence may suppress certain bacterial taxa, as the numbers of cultivable wood-inhabiting bacteria were considerably lower in wood blocks that became colonized by the white-rot fungi *Hypholoma fasciculare* and *Resinicium bicolor* than control blocks. Analysis of the bacterial community structure in soil adhering to the mycelial cords of the fungi revealed fungal species-specific effects on bacterial community composition. Bacteria can cause changes in wood chemistry, permeability, and structure, which may favor subsequent succession by fungi (Clausen 1996). Nitrogen is limited in wood, and associations with N-fixing bacteria may enable wood-decaying fungi to meet their nitrogen requirements for vegetative and generative growth (Hoppe et al. 2014; Herve et al. 2014). Nitrogen-fixing bacteria have been isolated from mycorrhizal (including truffle) fungi and in the fungus gardens of leaf cutter ants, further suggesting positive inputs of bacteria to fungal nutrition (Minerdi et al. 2001; Pinto-Tomas et al. 2009). Bacteria may also provide nutritional benefits in the surrounding soil while assisting fungal degradation of preserved woods through the sequestration or detoxification of preserving agents (Wallace et al. 2008).

The description of these associations demonstrates the functional diversity of bacterium–fungus interactions. Treatment of the literature implies that there are yet-to-be discovered potentials to unravel among the FBI, and the following paragraphs shall devote to the methodologies which allow us to dig even deeper to this challenging subject.

III. How to Study the FBIs?

A wide spectrum of techniques can be applied to explore FBIs. They mainly fall into two categories: cultivation-dependent and cultivation-independent approaches (Fig. 8.1). Cultivation-dependent approaches have been for long time the main way used to describe and analyze FBIs, but the recent fast expansion of -omics techniques and their relatively easy use allow now to get inventories of microorganisms colonizing almost any environment and to list potential functions that they could detain.

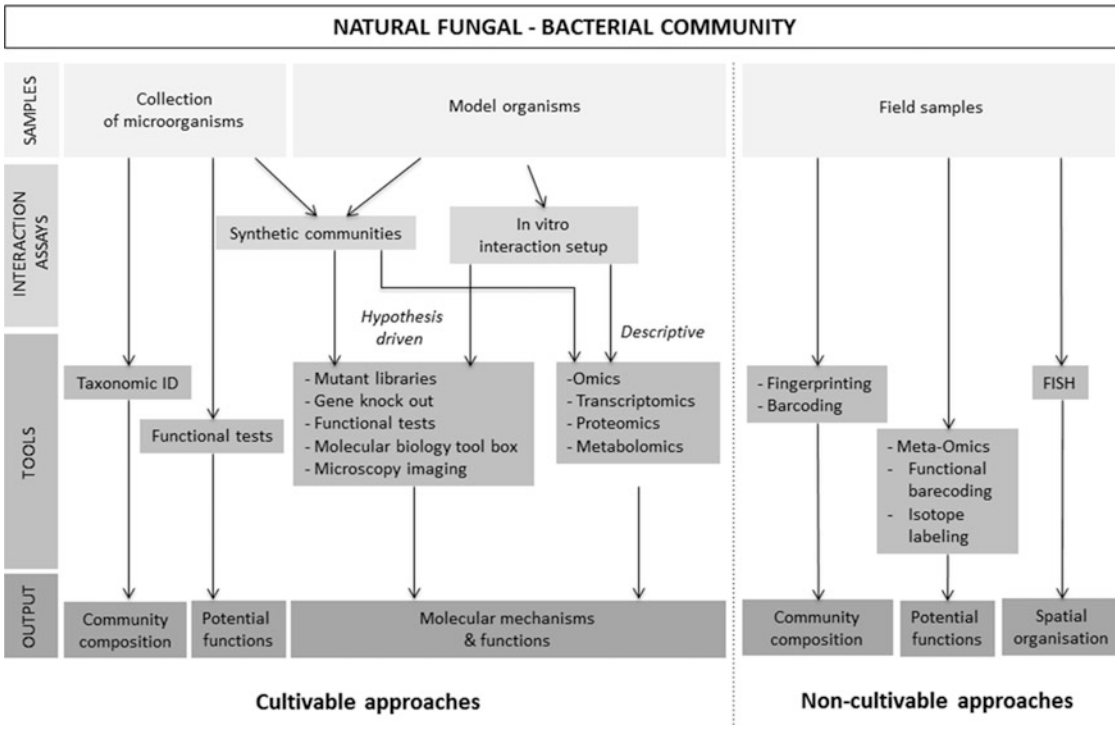

Fig. 8.1 Methods which have been applied to explore fungal–bacterial interactions

Thus, these new tools offer great opportunities to better describe the world of FBIs. The two approaches can bring complementary information and both have important pitfalls, and the best means is probably to combine them.

A. Cultivation-Dependent Approaches

One of the methods the most frequently used by microbial ecologists consists in isolating microorganisms from specific environments. Collections of pure microorganisms are obtained by plating on selective or nonselective media. Microorganisms are then most often identified by the sequencing of specific DNA regions such as 16S ribosomal DNA region for bacteria and internal transcribed spacer (ITS) or 18S rRNA for fungi. Microbial collections can further be screened for numerous phenotypes, metabolic properties, and functions, thus generating hypotheses that can be further tested in vitro or in situ. It is important to take into account that the choice of medium composition used to isolate microorganisms will strongly influence the type of microorganisms that will be recovered. Combination of different media and/or creation of media that mimic the environment from which the microorganisms are isolated can help broadening the diversity of microorganisms that are recovered (Vieira and Nahas 2005).

Such process was used by Frey-Klett et al. (2005) to isolate 220 bacterial strains from ectomycorrhizae of *Laccaria bicolor*, Douglas fir and surrounding soil, and permitted to show that ectomycorrhizosphere—the zone of influence of ectomycorrhizae—selects for specific *Pseudomonas fluorescens* strains that are potentially beneficial for the symbiosis and the plant. Using similar plate media isolation methods, Warmink and van Elsas 2008 also demonstrated that the mycosphere of *Laccaria proxima* offered a niche to specific bacterial communities and that it was particularly enriched in bacterial strains possessing type III secretion systems (T3SS). This observation led them to hypothesize that T3SS could have

new unknown functions and play a role in the interaction of those bacteria with their fungal host.

One of the main drawbacks of cultivation-based analyses is due to the fact that a very small percentage of microorganisms can be cultivated. Therefore one shall keep in mind that cultivable approaches will provide a very biased view of the microbial community composition. Thus it is most relevant to analyze, in parallel, the community composition using fingerprinting or bar coding methods. A second limitation of cultivation is that functional capacities obtained by in vitro bioassays only provide an image of the functional potential of the microorganisms. Whether these functionalities are expressed in the natural environment is hypothetical. Despite these drawbacks, cultivation approach has the immense advantage to generate hypotheses which can be tested in controlled environments since the microorganisms of interest can be grown and tracked. Specific microorganisms can then be used as model systems to decipher molecular mechanisms of interactions. Almost any molecular biology tool can be applied to study FBIs and a large number of them have been successfully used to decipher mechanisms of FBIs (Fig. 8.1). This requires the design of a bioassay setup in which the interaction can be tracked.

Depending on the microorganisms studied, liquid cultures (Hogan and Kolter 2002; Balbontín et al. 2014), Petri dish agar-based bioassay (Schrey et al. 2005; Morales et al. 2010; Mela et al. 2011; Pion et al. 2013), or more complex systems containing natural substrates such as soil (Nazir et al. 2014; Aspray et al. 2006; Kurth et al. 2013) or wood (Hervé et al. 2014) or including hosts (Peleg et al. 2008; Nash et al. 2014) have been designed. Microfluidic systems that allow one to probe FBIs at the single cell level were also recently developed (Stanley et al. 2014). These devices provide a platform to follow in vivo interactions by microscopy and to perform time-lapse analyses that are otherwise particularly challenging to obtain. As an example of the different tools that can be combined to analyze FBIs, we describe here the process we used to study the mechanisms by which the soil-born bacteria *Pseudomonas fluorescens* BBc6R8 stimulates the growth of the ectomycorrhizal fungus *Laccaria bicolor* and promotes the establishment of symbiosis between the fungus and tree roots. We first developed an in vitro assay in which the bacterium stimulates the fungal growth and then analyzed the transcriptomic responses of the fungus and the bacterial strain during their interaction (Deveau et al. 2007, 2014). These responses were compared to those induce by and in other bacterial strains to determine the degree of specificity of the interaction. In parallel, we acquired the genome sequence of strain BBc6R8. Genome mining and comparative genomics to other *P. fluorescens* strains pointed to a peculiarity of strain BBc6R8: while being a nonpathogenic bacterium, it possesses a complete cluster encoding for a potential type III secretion system (Cusano et al. 2010). These systems serve as molecular syringe to inject effectors in eukaryotic host cells, but their function is unknown in commensal bacteria. However, bacteria harboring T3SS-encoding genes are enriched in the vicinity of some soil fungi (Warmink and van Elsas 2008; Viollet et al. 2011), suggesting a potential role in the interaction with fungi. We thus hypothesized that T3SS could be involved in the helper effect of strain BBc6R8 and we carried out a targeted mutagenesis of the T3SS genes. We further showed that T3SS was essential to the helper ability of strain BBc6R8 as T3SS mutants were not anymore able to promote symbiosis. The creation of a library of random inserted transposon mutants of strain BBc6R8 and its screen allowed us to identify four additional mutants that lost their ability to promote symbiosis (Deveau et al. unpublished). Further analyses are on going to identify the role of the mutated gene products in the interaction. This combination of descriptive transcriptomic (Deveau et al. 2007, 2014; Mela et al. 2011), proteomic (Melin et al. 2002), or metabolomic (Phelan et al. 2014) methods together with targeted analyses permit to obtain an overview of the interaction and to identify mechanisms. Since all these experiments are performed in vitro, one may ask how much they tell about what is really happening in natural settings. An interesting approach consists in going back to

(semi)complex environments using synthetic communities in controlled environments. It requires the development of ways to track down the microorganisms such as quantitative PCR, fluorescent labeling of microorganisms, or fluorescent in situ hybridization. In this purpose, a system based on gamma sterilized soil-perlite substrate and amended with a defined microbial starting community, using pedunculate oak as the plant host, was developed (www.trophinoak.de; Tarkka et al. 2013). In this system they could co-inoculate MHB bacteria, ectomycorrhizal fungi, soil bacterial communities, and host plant and follow the impact plant and bacterial native communities on the MHB-ectomycorrhizal fungi interaction (Kurth et al. 2013). This type of top-bottom bottom-top approach has the advantage of providing experimental settings that are closer to the reality of complex communities while allowing one to test mechanistic hypotheses.

B. Methods Which Are Independent of Cultivation

Research has focused for a long time on very specific organisms that have been identified to be either the good or the bad guys. The concept of microbiome is very ancient and we know since decades that microbes live and behave within and as complex communities. However, most of the tools that allow to dissect and to question deeply these community behaviors were developed only very recently. The concepts of keystone species, functional redundancies, community resilience, etc. that have been intensely analyzed for macroorganisms remain understudied in microbial ecology, particularly in the field of FBIs. Three different approaches are commonly used to address these questions: taxonomic analyses of FBIs communities, functional analyses, and spatial organization of the FBIs in communities. A first glance of the diversity of a microbial community can be given by fingerprinting techniques. Fingerprinting methods (Zhou et al. 2015) differentiate groups of microorganisms based on unique characteristics of biomolecular markers such as DNA sequence (DGGE, TGGE, ARISA, etc.) or phospholipid (PLFA) composition. Identification of the main microorganisms from DNA fragments can be implemented through gel extraction followed by sequencing.

However, one needs to keep in mind that the fingerprinting methods can only provide a partial image containing the major representatives of a microbial community. Nevertheless, they represent powerful tools to getting an overall profile of microbial communities, to compare community patterns and to analyze very large sets of samples for a reasonable cost. For example, DNA fingerprinting was used to test whether diversity of microorganisms and macroorganisms are driven by similar processes – dispersal and selection (Ranjard et al. 2013). The comparison of the bacterial diversity between 2085 soil samples covering 5.3×10^5 km^2 of French landscape by DNA fingerprinting demonstrated that the turnover rate of bacterial diversity in soils on a wide scale is very high and strongly correlated to the turnover rate of soil habitats.

DNA bar code sequencing of short genetic markers such as bacterial 16S ribosomal RNA or fungal internal transcribed spacer between the 5.8S and 18S rRNA sequences, often referred to as monogenic rRNA metagenomic studies, are a powerful alternative to fingerprinting. Similarly to fingerprinting, they permit to measure the richness of a community—alpha diversity—and to compare community profiles, but they also give access to the taxonomic composition of community members. The level of precision in the taxonomic identification depends on the marker used and on the class of microorganisms studied. For example, fungal ITS and rRNA 18S, and bacterial 16S markers generally permit to assign sequences up to genera but will not allow to distinguish between species (Bruno et al. 2015). These tools can be applied to describe bacterial communities associated to fungi or to analyze the response of mixed fungal–bacterial communities to environmental changes.

They have been applied to many different types of ecosystems as, for example, cystic fibrosis lung patients (Willger et al. 2014), cheeses (Wolfe et al. 2014), decomposing wood (Hervé et al. 2014), and truffle mushrooms (Antony-Babu et al. 2014). Bar code sequencing has fantastically enhanced our

capacity to describe the composition of microbial community and to analyze how they can be impacted by biotic and abiotic factors and how much these microbial communities influence their host physiologies. Large catalogs of the microorganisms that colonize many environments are now available. Despite this knowledge, most environments colonized by fungi and bacteria remain a black box because we have a poor understanding of the actual activities performed by these microorganisms in situ. In addition, little is known on how organized these communities are. Indeed, most microorganisms behave differently when they are growing alone or in interaction with others (Frey-Klett et al. 2011; Scherlach et al. 2013). Therefore the spatial organization of complex microbial communities most likely influences the activity of each organism. Network analyses of metagenomic datasets can provide a first glimpse of this structure by inferring cooccurrence between microorganisms (Faust and Raes 2012; Hoppe et al. 2014). FBI community structures can also be assessed by microscopy using fluorescence in situ hybridization (FISH). Recent progresses made in confocal microscopy and the expansion of fluorescent probes now allow to visualize simultaneously up to 15 different microbial phylotypes in a single sample (Valm et al. 2011). Additionally, visualization of 3D spatial distributions of microbial communities can now be obtained as illustrated by Erlacher et al. (2015) with lichen-associated microbial communities. The next question one will ask is what are the activities these microorganisms are expressing in situ and how are these activities altered by biotic and abiotic parameters. This is probably the most important but also the most challenging question to answer. Zhou et al. (2015) recently proposed an extended review of both open and close format technologies to analyze the complex microbial communities at the functional level. "Close format techniques" refer to technologies using known biomarkers such as GeoChip (He et al. 2010) by contrast to the "open format" ones which do not require prior knowledge of the community. Until now, these techniques have been mainly applied to study separately either bacterial or fungal communities. They now need to be applied to the FBI field.

IV. Perspectives

To date, the evidence for the FBI argues for common themes governing the physical and biochemical characteristics of these associations, whatever the ecological niches investigated. The development of bacterial-fungal model systems (Hogan et al. 2004; Deveau et al. 2007: Partida-Martinez and Hertweck 2005) has been crucial for unraveling the physical interactions and communication patterns between the hyphae and bacterial cells. The observation of growth substrate as well as bacterial migration on hyphal surfaces has revolutionized our idea on the migration of bacteria and mobility of substrates over air gaps. Increased number of model systems investigated by functional genomics, microscopy, and metabolite analyses, in a way that has been implemented for the *Burkholderia–Rhizopus* endosymbiosis, is necessary for a deeper understanding of the FBI. Confrontation studies have been implemented mostly in simple, artificial systems which are suitable for the identification of interaction mechanisms (Andrade-Domínguez et al. 2014), but the use of more complex substrates (Kurth et al. 2013) is necessary when the relevance of the in vitro data is questioned. Bacterial enrichment by fungi (Fox et al. 2014) and fungal enrichment by bacteria (Caravaca et al. 2015) are emerging themes and clearly worthy of further consideration as potential targets for investigation are microbial community-based questions. Started by in situ analysis of microbial community structure, subsequent reconstruction of artificial microbial communities by adding different combinations of widely distributed and cultivable genera of bacteria and fungi on seminatural substrate, and monitoring the establishment and FBI (Wolfe et al. 2014), surely is a powerful way to address FBI in a community context. A growing number of examples suggest that the FBI serve as a framework or even facilitates fungal symbioses with animals and plants (Frey-Klett et al. 2007, 2011; Morales and

Hogan 2010), and the future research should dissect the direct FBI effect from direct impacts of bacteria and fungi on the host immunity or nutrition. The data reviewed here strongly support the postulate (Frey-Klett et al. 2011) that fungus–bacterium interactions are an emerging research theme which binds different scientific disciplines together.

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9 Lichen–Bacterial Interactions

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CONTENTS

I. Introduction

Lichen symbioses are composed of a dominating fungal partner, which hosts green algal and/or cyanobacterial photosynthetic partners. The algal partners provide fixed carbon as the main energy source to build up the holobiont structure. The formation of a distinct symbiotic phenotype, also known as "lichen thallus," is so unique in the fungal kingdom that it was long considered a separate organismal group. The light-exposed lichen thallus represents a self-sustaining structure, which generally grows as slow rate, and usually without a predetermined lifetime. Unless the preferred ecological settings change, it can therefore persist for many years and occasionally even reach ages of several 1000 years. Periodic desiccation in the natural habitat is survived by cryptobiosis. Lichens can therefore dominate the landscape in hostile habitats characterized by drought, high and low temperatures, and excessive light intensities, where higher plants are outcompeted.

With their long-persisting fungal structures, lichens provide a habitat for other microorganisms. About 1800 host-specific lichenicolous fungi are known by their phenotypes (Lawrey and Diederich 2003). Most of these are commensals or weak parasites, while the biological relations of other, inconspicuous eukaryotic associates are less known (Bates et al. 2012). In this chapter, however, we spend the focus on the associations of lichens with bacteria, which raised more scientific interest recently. We will not particularly discuss cyanobacteria, as these are well known as photosynthetic and nitrogen-fixing partners ("blue green algae") in estimated 10 % of lichenized fungi. In addition to green algal partners, some lichens ("tripartite" lichens) also host cyanobacteria in specialized organs ("cephalodia") for nitrogen fixation (Millbank and Kershaw 1969; Hyvärinen et al. 2002). Others, primarily crust-like lichens, grow preferentially in the vicinity of cyanobacterial mats ("cyanotrophy," Poelt and Mayrhofer 1988).

Lenova and Blum (1983) already suggested bacteria as a "third component" in lichens, but it was only research of the past 10 years which contributed substantial new information about their diversity and abundance of lichen-associated bacteria (Bates et al. 2011; Bjelland et al. 2011; Cardinale et al. 2006, 2008, 2012a, b; Grube et al. 2009, 2015; Grube and Berg 2009; Hodkinson and Lutzoni 2009; Hodkinson et al. 2012; Selbmann et al. 2010; Mushegian et al. 2011). According to these results, lichen thalli

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provide unique niches for bacteria, suggesting that bacteria are an integral component of lichen symbioses. The bacterial communities are host specific and reach densities of up to 10^{10} bacteria per gram of lichen dry weight. Moreover, they are able to form biofilm-like communities on the thallus surfaces (Grube et al. 2009; Cardinale et al. 2012b). The associations of lichens with bacteria seem to be very old, as they were demonstrated in fossilized lichens already from the lower Devonian period (Honegger et al. 2013).

The present data, which mainly come from lichens with green algal photobionts, reveal a predominance *of Alphaproteobacteria* as well as presence of other bacterial phyla, such as *Acidobacteria* or *Actinobacteria*. Many of these likely represent novel species (Selbmann et al. 2010; Lee et al. 2014; Sigurbjörnsdóttir et al. 2014), but only few of the lichen-associated strains were so far described as new taxa (Li et al. 2007; Lang et al. 2007; An et al. 2008, 2009; Hamada et al. 2012; Yamamura et al. 2011a, b; Cardinale et al. 2011). Since only a minor fraction of lichen-associated bacteria is culturable and because fungal symbionts grow too slow for efficient experimental resynthesis of bacterial associations, the analysis of interactions is a challenging topic. In the first section, we will therefore present an outline of the different methods that have so far been used to study these hardly culturable symbioses.

II. Methodological Approaches

Lichen-associated bacteria have been studied using a range of techniques. Culture-dependent studies date back to the first half of the last century and were used to verify the presence of nitrogen-fixing bacteria (e.g., Henckel and Yuzhakova 1936; Iskina 1938). While Cardinale et al. (2006) confirmed the growth of isolated lichen-associated bacteria on N-free media, N-free enrichment media were used to selectively sample nitrogen-fixing bacteria from tropical green algal lichens (Liba et al. 2006). Molecular data in these studies already revealed that phylogenetically diverse bacteria colonize lichens and that the isolated bacteria also may contribute auxiliary functions to the symbiosis. Although the cultivable fraction in most of the cases represents a very low proportion of the whole microbiome, antagonistic bacteria are now well presented in the culture collections (Grube et al. 2009; Cernava et al. 2015). Altogether, 24.5 % of all isolates were shown to display antagonistic properties against plant and lichen pathogens in vitro. Cultured isolates are amenable to further experimental approaches, which may show their interaction with fungal hyphae in greater detail (Senevirratne and Indrasena 2006). Cultivation-independent methods, originally accomplished by DNA community fingerprints and clone libraries, were used to gain more inside in the diversity of the total bacterial community or of specific groups, such as *Actinobacteria*. For example, Gonzáles et al. (2005) showed differences of actinobacterial associations with lichens between tropical and cold climatic regions using fingerprinting methods.

Grube et al. (2009) provided first evidence for host specificity of bacterial communities. They compared DNA fingerprints of bacterial communities from three lichen species occurring in the same subalpine habitat. Host specificity was confirmed by subsequent studies, either using gene clone library sequencing or amplicon sequencing (Bjelland et al. 2011; Bates et al. 2012). Amplicon sequencing meanwhile became the gold standard to assess microbial community structure, and this technique also helped in exploration of ecological influence on bacterial community structure (Cardinale et al. 2012a, b; Grube et al. 2012).

In parallel with sequencing approaches, lichen-associated bacteria were also studied by advanced microscopic approaches. While previous analyses with light or electron microscopy revealed the presence of bacteria, it is only possible with fluorescence in situ hybridization (FISH) to study the structure of bacterial colonization in more detail. For this purpose, Cardinale et al. (2008) introduced a method to directly make all steps of FISH using fragments of lichens instead of fixed sections. It was then

possible to visualize the total abundance of bacteria on lichen surfaces and also to evaluate the relative abundance of certain bacterial lineages, by the use of specific DNA probes. Visualization usually requires a confocal laser scanning microscopy (CLSM), by which the blur of fluorescence signals is avoided (in comparison with epifluorescence wide field microscopy). Using sophisticated image analysis tools, it is also possible to create three-dimensional representations of the bacterial colonization (Fig. 9.1). Cardinale (2014) discussed technical challenges of FISH-CLSM and gives valuable comments on possible misinterpretation of results. Confocal laser scanning microscopy proved useful to assess the effect of thallus age and variation of habitat conditions on bacterial composition (Cardinale et al. 2012a) or to demonstrate the endohyphal occurrence of bacteria (Cardinale et al. 2008; Erlacher et al. 2015). FISH-CLSM can therefore be used to complement and validate results from sequencing approaches and to precisely localize interactions with the lichen host.

In the meantime, meta-omics methods have become popular to further explore lichen–

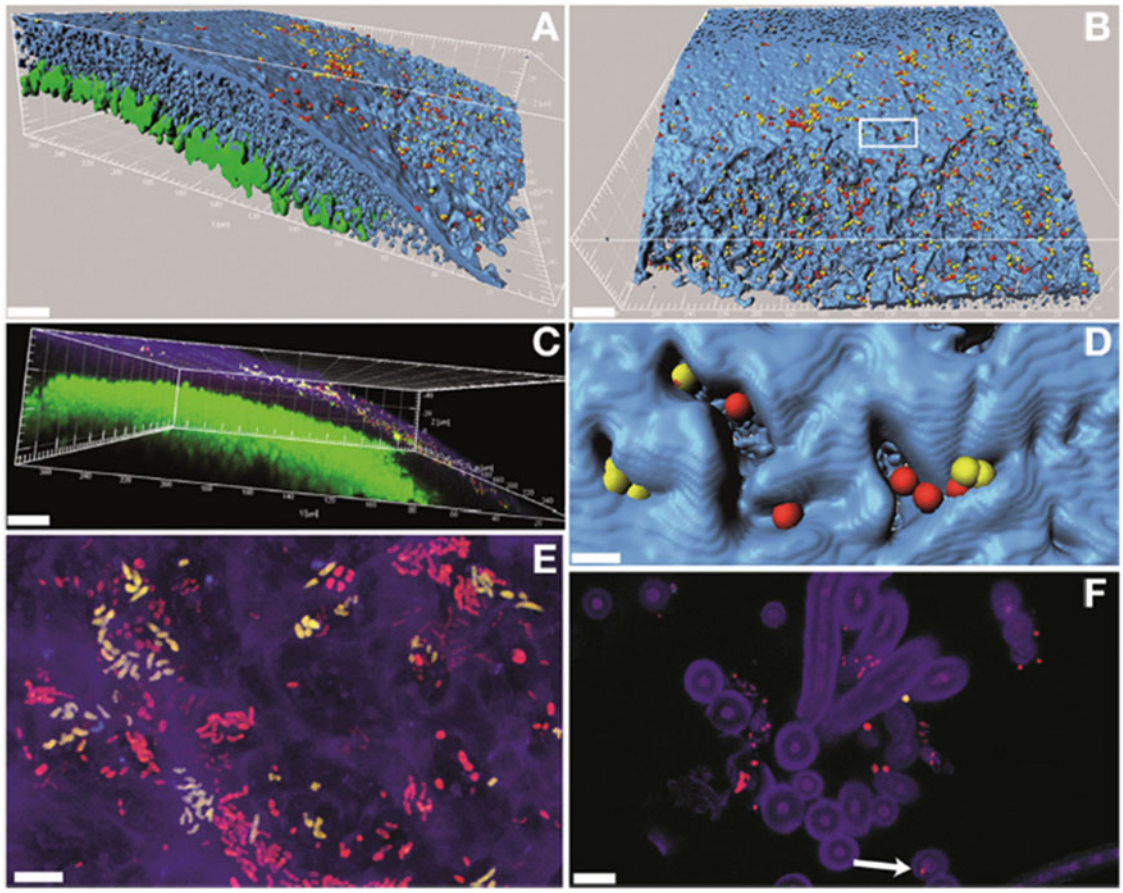

Fig. 9.1 Confocal laser scanning microscopy (CLSM) of bacteria associated with *L. pulmonaria*. *Bacteria* are stained by fluorescence in situ hybridization (FISH). *Green*, algae; *blue/purple*, fungi; *yellow*, *Rhizobiales*; *red*, other bacteria. (**a**, **b**, **d**) Three-dimensional models made of isosurfaces and spheres; (**c**) volume rendering; (**e**) maximum projection; (**f**) single optical slice. In (**b**) *white box* indicates the region shown in (**d**). *Arrow* in (**f**) indicates an endophytic bacterial cell. Scale bars, (**a**–**c**) 30 μm, (**d**–**f**) 5 μm. (Reproduced from Erlacher et al. 2015; ©Frontiers Media S.A.)

bacterial interactions. By including the bacterial fraction, Schneider et al. (2011) provided a first environmental proteomics insight into the functional complexity of lichen holobionts. This work, conducted with the lung lichen *Lobaria pulmonaria*, was recently complemented by a metagenomic dataset (Grube et al. 2015). The comparison of metagenomic and metaproteomic data revealed that bacteria take part in several functions, which potentially contribute to the stability of the lichen symbioses. We will discuss the results of these studies in more detail further below.

One of the greater challenges is to understand the exchange and signaling of compounds between bacteria with their host thallus in the lichen symbiosis, which requires a refined and localized chemical analysis of metabolite patterns. As the primary metabolism dominates the overall happening, analyses need to be targeted for secondary metabolites. So far, most studies have focused on the fungal secondary metabolites with a few exceptions (Boustie et al. 2011), including recent spatial analyses of compound patterns (Le Pogam et al. 2015). A stable symbiotic association is most likely maintained by a set of regulatory compounds, which may represent a complex chemical signature of symbiotic relations, for which we here introduce the term symbiotic molecular patterns (SYMPs). So far SYMPs are rather a hypothetical concept than a revealed known set of compounds. SYMPs may comprise exudates from the lichen holobiont, small molecules, which might include volatiles and other secondary metabolites (the parvome according to Davies and Ryan 2012). We also suppose that the compositions of SYMPs differ among species of lichens.

We argue that symbioses in general are valuable objects for finding new regulatory compounds and mechanisms, which can be exploited for other purposes. Lichens, with their high phylogenetic age, might offer particularly rich biological resources in this respect. It is already well known that lichens host a tremendous richness of secondary metabolites produced by the fungal partner (Elix 2014), but recent results suggest that bacteria associated with lichens may also diverse with respect to their biosynthetic potential.

III. Bioactive Secondary Metabolites from Lichen-Associated Bacteria

One of the most productive bacterial lineages regarding the biosynthesis of complex secondary metabolites is represented by *Actinobacteria*. Apparently, many *Actinobacteria* occurring in lichens are also culturable, and preliminary data also suggest that closely related strains may occur in the same host lichens (Cardinale, unpublished data). However, only very few lichen-associated *Actinobacteria* have been characterized in more detail so far, but already these data are very promising for more future studies (Suzuki et al. 2016). *Streptomyces uncialis*, isolated from reindeer lichen *Cladonia uncialis*, was shown to be a first potent species for finding new products. *Streptomyces uncialis* produces the enediyne uncialamycin (Davies et al. 2005), which shows strong antibacterial activity against human pathogens *Burkholderia cepacia* (MIC, 0.001 μg/ml) and *Staphylococcus aureus* (MIC, 0.0064 μg/ml), as well as strong cytotoxic activities against various cancer cell lines. Further investigation of that isolate revealed new alkaloids called cladoniamides A–G. Cladoniamide G showed significant in vitro cytotoxicity against human breast cancer MCF-7 cells (Williams et al. 2008). Two other *Streptomyces* species have been isolated from lichen species and produced novel cytotoxic compounds: chlorinated anthraquinonic angucyclines (Motohashi et al. 2010) and aminocoumarins structurally closed to novobiocin (Cheenpracha et al. 2010).

Streptomyces cyanofuscatus was isolated by Parrot et al. (2015) from coastal lichens and is also found in marine organisms. Surprisingly, the lichen-derived strain (MOLA1488) of this species is able to produce usnic acid (Parrot 2014), a dibenzofuran compound that was until now known to be produced only by lichen-forming fungi (but not by the lichen from which the strain was isolated). Other isolates of *S. cyanofuscatus* may have identical 16S rRNA genes but differ substantially in their biosynthetic properties. Considering the generally high conservation of 16S rRNA genes in *Actinobacteria*, these strains may either repre-

sent different species or their chemical variation is driven by other factors. In addition, one new compound, cyaneodimycin, an acrylate derivative, was isolated from the lichen-inhabiting *S. cyanofuscatus*. Six other known compounds were also characterized (diketopiperazines, actinomycin derivatives, and indole derivatives). Total EtOAc extract of *S. cyanofuscatus* showed antibacterial activities against *Staphylococcus epidermidis* and antiproliferative properties against B16 and HaCaT cell lines (IC_{50} 0.33 μg/ml and 0.25 μg/ml, respectively). Moreover, cynomycin exhibited antiproliferative properties against Jurkat cell lines after 72 h of incubation with IC_{50} value of 18.5 μM.

IV. Omics Technologies as Indicators of Interactions

Grube et al. (2015) explored the metabolic potentials of the bacterial microbiome of the lung lichen *Lobaria pulmonaria*. Metagenomic and proteomic data were compared and visualized by Voronoi treemaps. The study was further complemented by molecular, microscopic, and physiological assays. It was found that more than 800 bacterial species grow on the lung lichen. This diverse collective may contribute multiple aspects to the symbiotic system, including essential functions such as (1) nutrient supply, especially nitrogen, phosphorous, and sulfur, (2) resistance against biotic stress factors (i.e., pathogen defense), (3) resistance against abiotic factors, (4) support of photosynthesis by provision of vitamin B12, (5) fungal and algal growth support by provision of hormones, (6) detoxification of metabolites, and (7) degradation of older parts of the lichen thallus. These findings showed the considerable potential of lichen-associated bacteria to interact with the fungal as well as algal partner to support health, growth, and fitness of their hosts.

Contributing to one third (32.2 %) of the overall bacterial community, *Rhizobiales* (*Alphaproteobacteria*) are the most common partners in the symbiosis of *Lobaria pulmonaria*, and most of the *Rhizobiales* belonged to the families Methylobacteriaceae, *Bradyrhizobiaceae*, and *Rhizobiaceae*. Erlacher et al. (2015) studied this order in more detail using the available metagenomic dataset. About 20 % of our metagenomic assignments could not be placed in any of the *Rhizobiales* lineages, which indicates the incomplete knowledge of this order. Focused on *Rhizobiales*, the SEED-based functional analysis revealed again functions supporting the symbiosis, including auxin and vitamin production, nitrogen fixation, and stress protection.

Similar results were found meanwhile also in other lichens, e.g., in an analysis of metagenomically derived 454 sequences from *Peltigera membranacea* after subtraction of sequences attributed to the primary fungal and cyanobacterial symbionts (Sigurbjörnsdóttir et al. 2015). The dominant groups in *P. membranacea* are *Proteobacteria* (*Alphaproteobacteria* 59 %, *Betaproteobacteria* 29 %), while *Actinobacteria* and *Bacteroidetes* represent minor fractions. This metagenomic data agrees largely with microscopic and amplicon sequencing studies (e.g., Cardinale et al. 2008; Mushegian et al. 2011; Hodkinson et al. 2012). Also *P. membranacea* hosts bacteria capable of synthesizing indole acetic acid, albeit in a small number according to BLASTX hits to indoleacetimide hydrolase (most similar to those from *Actinobacteria* and *Betaproteobacteria*). The few hits for chitinase A were nearly exclusively actinobacterial. This is in accordance with observations that *Actinobacteria* are particularly associated with senescing thalli (Cardinale et al. 2012a). Several further glycosyl hydrolases were representative for other bacterial classes, including *Alphaproteobacteria*. The use of AppA phytase and AcpA acid phosphatase genes as query sequences for *Peltigera* metagenome data yielded diverse hits, with alphaproteobacterial *appA* and betaproteobacterial *acpA* homologs being particularly prominent. This supports the hypothesis that inorganic phosphate solubilization may be among the roles of these abundant members (Sigurbjörnsdóttir et al. 2015; Grube and Berg 2009; Grube et al. 2015).

V. Localization of Bacteria

Microscopic analyses complement the results from amplicon sequencing and other metaomics approaches by visualizing the bacterial colonization. Cardinale et al. (2008) originally demonstrated that bacteria colonized lichens in a biofilm-like manner but also provided further insights. Because the hyphae of lichenized fungi conglutinate and are embedded in a common matrix of polysaccharides, they also provide colonizable surfaces for bacteria. Generally, hydrophilic surfaces of lichens are the preferred environment for dense bacterial colonization. In contrast, hydrophobic hyphal surfaces (due to a self-assembled layer of hydrophobins or crystallized lichen metabolites) are often less abundantly colonized or colonized only by small colonies of bacteria.

Recently, Erlacher et al. (2015) developed a specific oligonucleotide probe to localize *Rhizobiales* by confocal laser scanning microscopy and fluorescence in situ hybridization (FISH-CLSM). The bulk of *Rhizobiales* again preferred the thallus surfaces, but there was also clear evidence that members of the *Rhizobiales* are also able to intrude at varying depths into the gelatinous matrix of the upper lichen cortical layer (Fig. 9.1). At least occasionally, some bacteria also are capable to colonize the interior of fungal hyphae. The penetration of fungal polysaccharide matrix agrees well with the presence of lytic function of bacteria (Grube et al. 2009).

So far, the microscopic data could not directly support the potential metabolic functions of the lichen-associated bacteria. Also, experimental data from coculture experiments of lichen symbionts with bacteria are still rare. Seneviratne and Indrasena (2006) presented results from coculture of lichen fungi with *Rhizobiales*. They could demonstrate that growth of rock-inhabiting lichenized fungi is enhanced in the presence of *Bradyrhizobium*. While evidence from cocultivation studies is valuable to reveal effects of associated bacteria on fungi, we are aware that the bacteria of lichens act on the whole symbiotic system of the lichen thallus. Future experiments need thus be carried out with the fully differentiated thalli of lichens, comparable to mesocosm experiments in microbial ecology.

VI. Functional Model of the Lichen Symbiosis

On the basis of the recent evidence, we outline a revised model of the lichen symbiosis depicting the functional multiplayer network of the participants (Fig. 9.2; Grube et al. 2015). In addition to the newly gained knowledge about bacteria, the metaproteomic approach by Schneider et al. (2011) provided a holistic view of the lichen symbiosis together with the fungal and algal partner. The vast majority of proteins retrieved from the algal partner belong to the functional complex of energy production and conversion as well as carbohydrate transport and metabolism, which agrees well with their function as primary producers in the system. Fungi on the other hand have a much richer spectrum of functions (including secretion and vesicular transport), which can be attributed to their function in guiding the entire system by its architectural shape. A similarly rich spectrum of functions can be attributed to the bacterial fraction, with a significant number of hits also involved in stress responses (posttranslational modifications, protein turnover, chaperones). This is not very surprising, since the surfaces of lichens are stressful habitats. Bacterial degradative functions may help to mobilize carbon in lichens, which may be reincorporated in growing parts (Ellis et al. 2005). We suppose that the functional diversification of lichen-associated bacteria support the longevity and persistence of lichens under extreme and fluctuating ecological conditions. Internalization in the lichen structure of the algae might be correlated with functional specialization (either carbohydrate fixation by algae or cyanobacterial N-fixation in specialized organs, cephalodia, present in c. 2 % of lichens), whereas the functionally more diverse bacteria are external colonizers that may vary in composition with the age state of the lichen structure (Cardinale et al. 2012a; Mushegian et al. 2011).

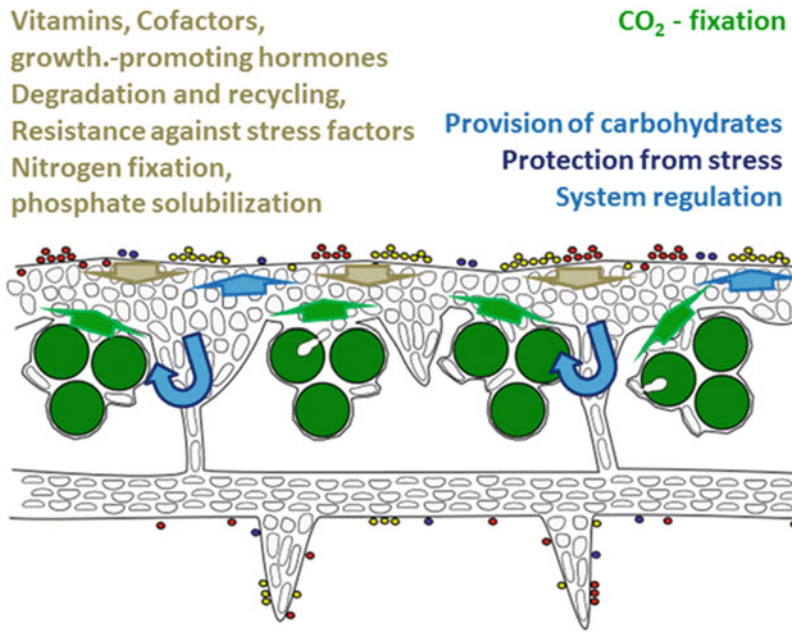

Fig. 9.2 Functional model of the lichen symbiosis, including bacteria as third interacting partner on fungal surfaces. Bacterial functions, *light brown*; algal functions, *green*; fungal functions, *blue*. Notes: (1) nitrogen fixation of lichens with cyanobacterial partners, either in the algal layer or in cephalodia, is not considered here; (2) the *green* algal photobionts form an apoplastic continuum with the mycobiont due to a surface layer with hydrophobins (Honegger 1998)

VII. Applications

The study of fungal–bacterial interactions in lichens could be of considerable interest for a range of biotechnological applications (Suzuki et al. 2016). Since the early work of Burkholder et al. (1944), a large number of publications only studied the effects of fungal secondary metabolites on bacteria (reviewed in Boustie and Grube 2005). For the most part, the assays in these studies demonstrated antibiotic effects of lichen compounds. Only exceptionally, the activities of these compounds were analyzed in greater detail. For example, Francolini et al. (2004) reported the inhibition of biofilm formation of bacteria using the lichen compound usnic acid. Even less known is about any positive effect of fungal lichen metabolites on bacteria, but those activities seem to exist as well. Gaikwad et al. (2014) discovered the positive effects of lichen compounds on probiotic lactobacilli.

So far, the biotechnological potential of lichen-associated bacteria has been little exploited, but certainly goes beyond the search for bioactive small metabolites. One third of the lichen-associated bacteria have the potential to produce PHA biopolymers (Gasser et al. 2012). Interestingly, the strains isolated also showed a remarkable high antagonistic potential against plant pathogens, including the common plant pathogen *Alternaria alternata* (Gasser et al. 2012). The antimicrobial properties of lichen-associated bacteria were meanwhile confirmed by other studies (Kim et al. 2013, 2014), and Cernava et al. (2015) studied the activity against several model bacteria and fungi by an integrative approach combining isolate screening, omics techniques, and high-resolution mass spectrometry. To efficiently select bacteria as stress-protecting agents for plants, a screening assay was developed which uses plants as "baits" for the lichen-associated fraction (Zachow et al. 2013).

VIII. Conclusions and Outlook

In this chapter, we characterized lichens as a more complex system than previously known, by involving their relations with bacteria. These new insights may stimulate further research, especially to find out what regulative mechanisms help to keep the entire symbiotic systems in balance and regulate the partnering physiologies. Lichen–bacterial interactions are apparently as old as the lichen lifestyle and eventually have also contributed to the fungal genome evolution. There are already indications for horizontal gene transfer events from bacterial to fungal genomes, for example, in the diverse polyketide synthase genes (Schmitt and Lumbsch 2009). Yet, there are still many other new questions for research. For example, little is known so far about the diversity of lichen-associated *Archaea*. Lichens are a highly interesting case of interkingdom interactions (Berg 2015), and they are specifically designed for persistence and longevity under diverse ecological conditions. We think this symbiosis deserves more study in the future, since understanding and exploiting of the functional principles may be of considerable interest for biotechnological applications.

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10 Mycorrhizal Fungi and the Soil Carbon and Nutrient Cycling

Roland Marmeisse[1], Mariangela Girlanda[2]

CONTENTS

I. Introduction

"Mycorrhiza" is a generic word used to designate usually beneficial, mutualistic associations between plant roots and fungi. All mycorrhizae share the presence of fungal hyphae forming characteristic symbiotic structures within plant roots, which are connected to external hyphae that grow outwards, exploring the surrounding soil matrix (Smith and Read 2008). Besides these shared characteristics, it has long been recognized that mycorrhizae encompass plant-fungal associations of distinct evolutionary origins (Smith and Read 2008; van der Heijden et al. 2015). However, despite these distinct origins, a classical and unified vision of mycorrhiza describes this association as a "balanced" plant-fungus partnership where each of the two associates provides essential nutrients to the other one at the plant-fungal interface (either a Hartig net, arbuscules, or pelotons) (Brundrett 2004). In such a classical model, plants allocate to their fungal partners photosynthesis-derived simple sugars (mono- and/or disaccharides) in exchange for soil-derived macronutrients (e.g., nitrogen, phosphorus, potassium) provided by the fungi.

In terms of interaction with the environment, mycorrhizal fungi thus represent a hub for the bidirectional transit of major carbon, nitrogen, and phosphorus elements between the plant biosphere and the pedosphere. The active role of mycorrhizal fungi in these transfers of nutrients and the consequences for the global terrestrial ecosystem budget are now being revisited by a combination of genomic and ecosystemic approaches that are summarized in the present chapter. Recent advances in our understanding of different aspects of the mycorrhizal symbiosis, such as biodiversity and community ecology of mycorrhizal fungi, mycorrhizal mycelial and ecological networks, mechanisms underlying partner selection, and the molecular crosstalk between plants and mycobionts, have been extensively covered in other reviews (e.g., Bonfante and Genre 2015; van der Heijden et al. 2015; Johnson and Gilbert 2015; Raudaskoski and Kothe 2015; Smith and Smith 2015; Werner and Kiers 2015; Bahram et al. 2014; Bucher et al.

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2014; Chagnon et al. 2014; Schmitz and Harrison 2014; Wyatt et al. 2014).

II. Mycorrhizae, a Diversity of Plant-Fungal Associations

The four main types that will be discussed in this chapter are the so-called ectomycorrhizae (EcM), arbuscular mycorrhizae (AM), ericoid mycorrhizae (ErM), and orchid mycorrhizae (OrM).

The AM symbiosis can be well defined by the fungal partners, which all belong to a single monophyletic fungal lineage, the Glomeromycota clade (Schüßler et al. 2001). With only one known exception (*Geosiphon pyriformis* associated to *Nostoc* cyanobacteria), all Glomeromycota species are obligate biotrophs that cannot be grown in vitro in the absence of a host plant. Both fossil records (Redecker et al. 2000; Strullu-Derrien et al. 2014) and molecular phylogeny (Wang et al. 2010) suggest that the AM symbiosis could be the most ancient mycorrhizal association, dating back to the Devonian period (at least 400 Myrs ago), where it could have been instrumental in the colonization of nonaquatic terrestrial habitats by green plants (Bonfante and Selosse 2010). As a matter of fact, the AM symbiosis is found in most Embryophyta lineages including early diverging ones such as liverworts and hornworts (Selosse et al. 2015). A majority of present seed plant families, including families of agricultural importance (e.g., the Fabaceae, Poaceae, and Solanaceae), are concerned by the AM symbiosis, and absence of AM associations in specific families (e.g., the Brassicaceae, Caryophyllaceae, Chenopodiaceae, Juncaceae, Polygonaceae, and Proteaceae) should be seen as a secondary loss, not as an ancestral character (Bonfante and Selosse 2010). The name arbuscular mycorrhiza derives from the arbuscule, a highly branched and metabolically active fungal hypha that grows within a living plant rhizodermal or cortical root cell. Despite growing intracellularly, the arbuscule does not disrupt the host plant cell integrity. The arbuscule is indeed entirely surrounded by an invaginated plant cell plasmalemma, and most of the apoplastic space between the fungal and plant plasmalemma is filled by a matrix of both plant and fungal origin (Genre and Bonfante 2012; Gutjahr and Parniske 2013; Balestrini and Bonfante 2014). This AM symbiosis-specific structure is thought to be the place where the bidirectional exchanges of nutrients between the plant and the fungus take place (Smith and Read 2008).

Regarding the AM symbiosis, the large number and diversity of host plants contrasts with the "relatively low" number of fungal species described thus far, despite the ever-increasing number of studies dedicated to the molecular diversity of AM fungi (i.e., Glomeromycota) in a wide range of geographically distant "undisturbed" and disturbed soils (Öpik et al. 2013). Current estimates of global species numbers range from ca. 300 to 1600 (van der Heijden et al. 2015). Such values reflect the fact that many individual Glomeromycota species seem to have a large, potentially global, geographic distribution; support a wide range of environmental conditions; and can associate to numerous, taxonomically unrelated, plants (Öpik et al. 2013).

According to an abandoned classification scheme, AM, ErM, and OrM were all qualified as "endomycorrhizae" as in all three cases the fungal hyphae grow intracellularly in living host plant cells. Besides this similarity, these mycorrhizae are evolutionary and potentially functionally distinct plant-fungal associations. First, hyper-ramified arbuscules are absent from ErM and OrM where intracellular hyphae form so-called pelotons. Secondly, as suggested by their names, ErM and OrM concern a restricted number of plant species belonging, respectively, to the families Ericaceae and Orchidaceae (Smith and Read 2008; Brundrett 2009). Finally, ErM and OrM also differ from each other with respect to their fungal partners. ErM involve a restricted number of fungal taxa (ca. 150 according to van der Heijden et al. 2015, commonly assigned to the *Rhizoscyphus ericae* (syn. *Hymenoscyphus ericae*) aggregate (Helotiales, Ascomycota), *Oidiodendron* species (Onygenales, Ascomycota), or the Sebacinales (Basidiomycota) (Smith and Read 2008). However, there is evidence that the spectrum of mycobionts detected in the Ericaceae may be considerably larger (Walker et al. 2011; Gorzelak et al. 2012; Vohnik et al. 2012; Bruzone et al. 2015). As for OrM, they are mostly formed by fungal species belonging to different clades in the Agaricomycetes and Basidiomycota (such as fungi in the "rhizoctonia" complex, encompassing Tulasnellaceae, Ceratobasidiaceae, and Sebacinaceae), although Ascomycota in the Tuberaceae and Pezizaceae can also be involved (Dearnaley 2007; Dearnaley et al. 2012).

Both ErM and OrM hosts can associate with fungi simultaneously forming EcM on neighboring trees (Villarreal-Ruiz et al. 2004; Selosse and Roy 2009; Grelet et al. 2009,

2010; Hynson and Bruns 2010; Dearnaley et al. 2012). Although ErM and OrM could be regarded, at first sight, as marginal cases of plant-fungal associations, they have been the focus of numerous research works for two distinct reasons. Regarding ErM, a significant fraction of land at high latitudes and altitudes and in specific geographic zones (such as in the south of the African continent) is covered by a vegetation dominated by Ericaceae plants (e.g., heathland), where it is suspected that ericoid mycorrhizal symbionts play essential roles in plant nutrient acquisition (Smith and Read 2008). Orchids feature a peculiar mycorrhizal relationship, since association with symbiotic fungi providing organic carbon is essential for germination of the minute, reserveless seeds and the development and survival of the heterotrophic protocorm lifestage. At adulthood, achlorophyllous orchids or orchids with reduced photosynthetic efficiency (such as forest species, growing in deeply shaded habitats) still depend on mycorrhizal fungi for organic carbon supply. By contrast, green orchids thriving in exposed, grassland habitats are believed to emancipate themselves from fungal-derived organic carbon as soon as they form fully developed leaves, although they are thought to be still heavily reliant on their fungal symbionts for mineral nutrition (Waterman and Bidartondo 2008; Selosse and Roy 2009; Rasmussen and Rasmussen 2014; Selosse 2014).

The last type of mycorrhizal association that will be discussed in this chapter is the ectomycorrhizal (EcM) one. As suggested by its name and as opposed to "endomycorrhizae," in ectomycorrhizae, the fungal hyphae do not penetrate plant cells but rather entirely surround rhizodermal and/or external root cortical cells to form the so-called Hartig net where the nutrient exchanges between the plant and fungal partners take place. Additionally, the fungal hyphae can entirely surround the plant absorbing rootlets to form a plectenchyma-like tissue called fungal mantle or fungal sheath that "isolates" the plant root from the surrounding soil (Smith and Read 2008). In plants, the evolutionary origin of the EcM symbiosis is not fully resolved as it concerns essentially woody plants (shrubs and trees) belonging to the Gymnosperms (most of the Pinaceae) and the Angiosperms. While most, if not all, species in, e.g., the Fagaceae (oak, beech, chestnut), Betulaceae (birch, alder, hazelnut), Salicaceae (salix, poplar), or Myrtaceae (eucalyptus) are ectomycorrhizal, a few woody species in otherwise overwhelmingly AM plant families can also form EcM (Smith and Read 2008; Brundrett 2009). From a geographic perspective, forests in the temperate, alpine, and arctic biomes are dominated by EcM forest trees, while this symbiosis is less frequent, but not absent in the tropics, with EcM trees in, e.g., families Fabaceae, Caesalpiniaceae, or Dipterocarpaceae. From a fungal perspective, EcM fungi belong essentially to the Pezizomycotina (Ascomycota) and the Basidiomycota (Smith and Read 2008). EcM fungi are "numerous" with an estimated number of species exceeding 20,000 (van der Heijden et al. 2015) but do not share a common ancestor. Indeed, phylogenetic studies demonstrate that the "aptitude" at forming EcM has appeared at least 80 times in different fungal lineages (Hibbett et al. 2000; James et al. 2006; Tedersoo and Smith 2013). Although EcM could be dated back to 100–200 M years ago (Brundrett 2002), transition from saprotrophy to symbiosis could still be an ongoing process in some fungal lineages as suggested by the occurrence of both saprotrophic and EcM species in single fungal genera such as *Amanita* (Wolfe et al. 2012).

Categorization of mycorrhizal associations does not however reflect the diversity of these associations under natural conditions. For example, many EcM plants can also host AM symbionts (Smith and Read 2008) and most if not all plant individuals simultaneously accommodate different mycorrhizal symbiotic fungal species and genotypes on a single root system. For instance, Bahram and coworkers (2011) could identify up to 122 different EcM fungal operational taxonomic units (OTUs, potentially as many different species) and tens of genets of the species *Cenococcum geophilum* on the root system of a single *Populus tremula* plant.

III. Mycorrhizae, a Hot Spot of Nutrient Exchange Between Plants and Fungi

A classical and unified vision of mycorrhizae describes this association as a "balanced" plant-fungus partnership where each of the two associates provides essential nutrients to the other one at the plant-fungal interface (either arbuscules, pelotons, or Hartig net). In a classical model, plants allocate to their fungal partners photosynthesis-derived simple sugars (mono-

and/or disaccharides) in exchange for soil-derived macronutrients (e.g., nitrogen, phosphorus, potassium) provided by the fungi. Therefore, this model implicitly envisions the extraradical mycelium, which explores the soil matrix, as a substitute to the root system itself for the function of primary nutrient acquisition from the soil solution (Girlanda et al. 2007). This model hypothesizes that the "beneficial effects" of the mycorrhizal association recurrently observed on host plants' growth and nutrition result from a combination of at least two main fungal traits. The first trait is the capacity of the extraradical mycelium to efficiently explore a larger volume of soil than the root system itself in the quest of low concentration and/or poorly mobile (e.g., phosphate ions) soil nutrients. The second trait is that fungi display a wider metabolic versatility than plants and can access soil nutrient sources not readily available for plant direct uptake such as the organic forms of nitrogen and phosphorus.

Historically, the main predictions of this global model of functioning of the mycorrhizal symbiosis have received experimental support in a series of classical studies performed on different plant-fungus associations. For example, labeled $^{14}CO_2$ applied to plant leaves has been used to visualize the transfer of ^{14}C from plant leaves to roots and then to mycorrhizae and to the soil-colonizing extraradical mycelium (Ho and Trappe 1973; Bending and Read 1995). Conversely, the use of ^{32}P-enriched poorly mobile phosphorus sources added to soil compartments colonized by fungal hyphae, but from which associated plant roots were excluded, allowed visualizing the transfer of phosphorus from mycorrhizal hyphae to the host plant (Finlay and Read 1986). More recently, the use of nonradioactive ^{13}C and ^{15}N sources fed to the plant and/or fungal hyphae has also been used to visualize at the cellular level by NanoSIMS the transfer of carbon and nitrogen at the fungal-plant interface in both AM (Kaiser et al. 2015) and OrM (Kuga et al. 2014). In the AM symbiosis, the bidirectional transfer of nutrients at the plant-fungus interface has also received direct molecular support by the characterization of essential plasma-membrane nutrient transporters. From the plant side, this is the case of a phosphate transporter (MtPT4 in the legume *Medicago truncatula*), present in most studied plant species, which is specifically expressed in arbuscule-containing root cells and whose suppression leads to premature arbuscule degeneration and a drop in P transfer from the fungus to the plant (Harrison et al. 2002; Javot et al. 2007; Breuillin-Sessoms et al. 2015). From the fungal side, *Glomus* monosaccharide transporter genes expressed in arbuscules were also shown to be necessary to arbuscule formation (Helber et al. 2011).

Nutrient versatility has essentially been tested for EcM and ErM fungi, which can be grown in vitro on controlled N and P sources. It is now well established that, for example, EM fungal species can efficiently use proteins as sole N sources, thanks to secreted proteases (Abuzinadah and Read 1986a, b; Bajwa et al. 1985). As a result, these fungi can provide N derived from these macromolecules to their host plants (Abuzinadah and Read 1986a, b). Thus, at the ecosystem scale, the mycorrhizal association "shortcuts" the N and P mineralization process of N- and/or P-containing organic molecules, normally necessary to the release of inorganic forms of P (PO_4^{2-}) or N (NH_4^+ or NO_3^-) that can be assimilated by nonsymbiotic plants. "Unexpected" soil constituents have thus been demonstrated to represent direct and significant sources of P and/or N for EcM plants. This is the case of pollen grains (Perez-Moreno and Read 2001a) or of the soil animal necromass (Perez-Moreno and Read 2001b; Klironomos and Hart 2001).

IV. Fungal "Omics," Global Approaches to Probe the Mycorrhizal Symbiosis Lifestyle

Most of earlier studies on the mycorrhizal symbiosis have addressed specific aspects of this association such as the contribution of a particular fungal metabolic pathway to the plant mineral nutrition. Understanding this complex and diverse plant-fungus interaction may benefit from a more holistic approach as provided by the so-called "omics" research field (genomics, transcriptomics, proteomics, but also environmental genomics). Besides cataloging genes and proteins from organisms, through their functional annotation and cross-species comparisons, "omic" approaches are also susceptible to decipher the ecological roles of microbial species.

A. The Saprotrophic-Mycorrhizal Continuum and Debate

One of the most debated issue in the field of mycorrhizal research and on the roles of mycor-

rhizal fungi in soil nutrient cycling concerns certainly the putative role of these species as soil saprotrophs (Baldrian 2009; Cullings and Courty 2009; Lindahl and Tunlid 2015). Considering that soil is one of the largest reservoirs of organic carbon on earth (Lal 2004) and that the mycelia of EcM fungal species can represent up to one third of the microbial biomass in some forest soils (Högberg and Högberg 2002), the debate over saprotrophy and mutualism is not anecdotic. Indeed any significant contribution of mycorrhizal fungi to soil organic matter degradation could in this context significantly affect the whole carbon budget of the entire ecosystem. This debate over saprotrophy may have been partially promoted by different interpretations of the word itself. In this chapter, we define a fungal saprotroph as a fungal species capable at living autonomously in its natural habitat by deriving all of its carbon and other elements needed for its metabolism and growth from organic carbon and other nutrients present in dead organic matter. According to this definition, all Glomeromycota AM species, which have thus far failed to be maintained in pure culture in the absence of a host plant, are obligate symbionts and not saprotrophs. Still according to this definition, one of the many EcM fungal species whose mycelium can be grown in vitro on a defined medium is a symbiotic species with potential saprotrophic capacity. In this latter case, it remains however to demonstrate that this species is indeed capable of surviving autonomously in soil, outcompeting soil saprotrophs for the use of complex soil carbon sources.

In this context, arguments in favor of saprotrophy essentially include the observation that ectomycorrhizae formed by specific fungal species are often found in close association with coarse woody debris and that numerous EcM root tips secrete hydrolytic enzymes (e.g., laccase, cellobiohydrolase, xylosidase) susceptible to attack constituents of the plant cell wall (Courty et al. 2005). As estimated by Talbot et al. (2013), enzymes secreted by mycorrhizal root tips may however account for a small fraction of the total bulk soil enzyme activity. However, by using an experimental setup allowing the specific colonization of an organic soil compartment by hyphae from EcM fungal species, Phillips and colleagues (2014) showed that this soil compartment presented levels of hydrolytic activities as similar to those assayed in control soils colonized by hyphae from both EcM and saprotrophic species. In most cases, results from enzyme assays performed on environmental samples (root tips, soil) must however be interpreted cautiously as the substrate used in these assay measures the activities of so-called exoenzymes hydrolyzing either the end of plant cell wall (PCW) polysaccharides (e.g., cellobiohydrolase, xylosidase), disaccharides, or trisaccharides (e.g., β-glucosidase). None of these enzymes is probably susceptible to significantly degrade a long polysaccharide in the absence of cooccurring endoenzymes attacking the internal links between monomers and whose activities are seldom assayed in field studies.

Arguments against saprotrophy include the theoretical one that the secretion of large amounts of plant cell wall (PCW)-degrading enzymes by a mycorrhizal species would be damageable for the integrity of its host plant. Among factual observations, no study convincingly reported the use of major PCW polymers (e.g., cellulose or hemicelluloses) as sole C sources by EcM fungal species. EcM fungal species may however use “minor” PCW polymers such as pectins as in the case of *Laccaria bicolor* (Veneault-Fourrey et al. 2014) or polysaccharides absent from the PCW such as starch or β-1,3 glucans as in the case of *Hebeloma cylindrosporum* (Doré et al. 2015). Along this line, a selected set of 15 EcM species produced several orders of magnitude less hydrolytic enzymes (exoenzymes) active on PCW polysaccharides than saprotrophic species when inoculated to sterilized leaf litter (Talbot et al. 2015). Oxidative enzymes (e.g., peroxidases) did not follow this rule as they can be produced in similar amounts by EcM and saprotrophs. Current biochemical models (see below) suggest however that, in EcM fungi, these latter enzymes do not directly participate to fungal carbon provision, but rather to the chemical modification of lignin and other plant phenolics. Another argument used against saprotrophy is the observation that sporocarps from EcM fungi display a significantly lower ^{13}C natural abundance ($\delta^{13}C$) by comparison to cooccurring sporocarps from saprotrophic species (Mayor et al. 2009). In this respect, $\delta^{13}C$ values of EcM sporocarps are closer to the $\delta^{13}C$ of the surrounding fully autotrophic plants suggesting that they derive most of their carbon from recently fixed atmospheric CO_2 provided by their host plants (Högberg et al. 1999).

Finally, in undisturbed forest soils, there is usually a strong physical separation between the distribution of saprotrophic fungal species present in the uppermost litter layer where most of the litter mass loss takes place and the lower soil layers where EcM fungi predominate (Lindahl et al. 2007; Voříškov et al. 2014).

B. "Omics" and the Saprotrophic-Mycorrhizal Continuum

Genome sequencing of several saprotrophic fungal species (degrading soil litter or producing white or brown rot on wood) demonstrated that almost all of them can produce a wide range of hydrolytic enzymes susceptible to completely hydrolyze most of the polymers constitutive of the PCW (e.g., Martinez et al. 2004, 2009; Stajich et al. 2010; Eastwood et al. 2011; Floudas et al. 2012). Based on sequence homology, these enzymes fall into different protein families indexed in specialized databases such as CAZy (Lombard et al. 2014). According to this database, these enzymes can be, for example, glycoside hydrolases (GH families), polysaccharide lyases (PL families), or auxiliary enzymes (AA families). Most auxiliary enzymes are not directly involved in polysaccharide hydrolysis, but rather participate to the removal of lignin whose presence in PCW represents an "obstacle" to the actual enzymatic hydrolysis of PCW polysaccharides. Laccases, fungal "Class II peroxidases," and other enzymes responsible for the production of H_2O_2 necessary for peroxidase activity represent different auxiliary enzymes.

In most saprotrophic fungal genomes, each PCW-degrading enzyme family is also often represented by several active copies. For example, the wood-degrading oyster mushroom (*Pleurotus ostreatus*) has 16 copies and the coprophilic *Coprinopsis cinerea* has 6 copies of glycoside hydrolase 7 (GH7)-encoding genes involved in cellulose degradation. Similarly, these two species have also, respectively, 3 and 5 copies of GH10-encoding genes involved in xylan degradation (Rytioja et al. 2014). Saprotrophic fungal species do not however represent a homogeneous functional group with respect to their capacity at producing PCW-degrading enzymes. For example, striking differences have been observed between so-called "white-rot" and "brown-rot" wood-degrading basidiomycete species. White-rot species, as exemplified by *Phanerochaete chrysosporium*, initially fully hydrolyse lignin, thanks to a complex repertoire of class II peroxidases (16 gene copies) and peroxide-producing enzymes. Exposed polysaccharides, freed of lignin protection, are then fully degraded by a complex array of GH enzymes active on cellulose and hemicelluloses (Martinez et al. 2004; Eastwood et al. 2011). By contrast, brown-rot species as exemplified by *Postia placenta* that presumably evolved from white-rot ancestors leave lignin almost untouched, as reflected by a lack or reduced number of both class II peroxidases and peroxide-producing enzymes encoding genes. To access lignin-protected polysaccharides, brown-rot fungal species have developed a specific strategy involving the production of highly diffusible reactive oxygen species that perform the chemical attack of PCW polysaccharides (Martinez et al. 2009; Eastwood et al. 2011).

In this context, the sequencing of the genomes of different mycorrhizal fungal species set as a major objective the inventory of PCW-degrading enzymes in their genomes to address both the evolutionary transition from saprotrophism to mutualism and the potential implication of these enzymes in symbiosis establishment and functioning. Genome sequences are now available for at least one species forming AM (Lin et al. 2014; Tisserant et al. 2014), EcM (Martin et al. 2008, 2010; Kohler et al. 2015), ErM (Kohler et al. 2015) and OrM (Kohler et al. 2015). Both AM and EcM genomes share one relevant genomic feature, which is a strikingly low number or absence of genes encoding PCW-degrading enzymes. For example, the GH6 family encoding endo-cellulases is absent in all AM and EcM genomes sequenced thus far. The GH7 family encoding either exo- or endo-cellulases is also absent from all species with the exception of the basidiomycetes *Piloderma croceum* and *Hebeloma cylindrosporum* (Kohler et al. 2015). Regarding this latter species, it cannot use cellulose as only C source in vitro and its unique GH7 gene copy is transcribed at very low levels in both free-living mycelia and mycorrhizae (Doré et al. 2015). Sequencing of both AM and EcM species, which originated independently from unrelated fungal lineages, thus demonstrate that mutualism is associated with the parallel loss of unrelated genes involved in the hydrolysis of structural PCW constituents.

Partial or total loss of PCW-degrading enzymes in AM and EcM genomes does not however seem to be part of a global reduction in the proteome repertoire of these species as reported for the proteomes of several obligate pathogenic or symbiotic bacteria (McCutcheon and Moran 2011). Indeed the gene content of the genomes of all mycorrhizal species sequenced thus far is almost as diversified and of the same order of magnitude to the gene content of phylogenetically related saprotrophic species (Kohler et al. 2015). Gene loss seemed to have specifically affected few gene families, among which those encoding enzymes active on structural constituents of the PCW. As already mentioned, the EcM fungus *H. cylindrosporum*, for example, retained a full repertoire of enzymes that allows it to grow on starch or β,1-3 glucans absent from PCW. As for some of the putative PCW-degrading genes that remained in the genomes of several EcM species, it can be hypothesized that they have gone through a process of neo-functionalization and/or that they are the last witnesses of a, not fully achieved, evolutionary transition from saprotrophy to mutualism. Regarding the first hypothesis, in a global transcriptomic analysis of *L. bicolor* CAZyme genes, Veneault-Fourrey et al. (2014) showed that several of the few remaining PCW active genes in the genome of this species were upregulated at either early of late stages of EcM formation, thus suggesting a role in host plant cell wall remodeling during this ontogenic process. Regarding the second hypothesis, Wolfe et al. (2012) showed that in the genus *Amanita* in which coexists saprotrophic and EM species, all symbiotic species had lost both the ability to grow on sterilized litter and genes encoding GH6 and GH7 exo-(=cellobiohydrolases) and endo-cellulases active on cellulose polymer. Many, but not all, of these symbiotic *Amanita* species have however retained β-glucosidases active on cellobiose but not on the original cellulose polymer. The specific conservation of the final metabolic gene in the pathway of cellulose degradation was also observed in *L. bicolor* (Veneault-Fourrey et al. 2014) and *H. cylindrosporum*, a species which retained a seemingly inactive GH7 endo-cellulase gene (Doré et al. 2015).

While the genome sequences of all AM and EcM published so far are consistent with a loss of saprotrophic capacities and a strong dependency to a host plant for carbon supply and survival in soil, this is not the case for both ErM and OrM symbionts. Both *Oidiodendron maius* (Ascomycota, ErM symbiont) and *Tulasnella calospora* (Basidiomycota, OrM symbiont) possess in their genome a diversified set of PCW-degrading genes (Kohler et al. 2015) consistent with earlier studies demonstrating their capacity to use plant-derived substrates as C sources (Smith and Read 2008). These observations illustrate the diversity and lack of unicity of the different forms of mycorrhizal associations and plead for further research to understand how these symbionts associate to a host plant without damaging its tissues.

Besides a specific loss in the capacity of using soil carbon sources, a strict dependency toward a host plant could also be explained by a partial or total loss of essential metabolic pathways leading to the production of key metabolites that would need to be provided by the host plant. Such loss has not been reported for any of the EcM fungal genomes. In the case of the AM fungus *Rhizophagus irregularis*, the absence from its genome of genes encoding thiamin biosynthesis enzymes could be one of the reasons of its seemingly obligate symbiotic status (Tisserant et al. 2014).

C. Saprotrophy and the Soil Organic Nitrogen Lead

In natural unmanaged ecosystems, a significant fraction of nitrogen enters the soil as complex organic N forms (e.g., proteins, nucleic acids) trapped in plant litter. In the global N budget of a forest ecosystem, the total N that returns back annually to the soil in litter can represent two thirds of the N assimilated by the plants the year before (Ranger and Bonneau 1984). According to a classical scheme of N cycling in terrestrial habitats, complex N forms are first hydrolyzed as oligopeptides, amino acids, and nucleotides by microbial proteases and nucleases and further mineralized by microbes into ammonium and/or nitrate, which can be assimilated back by plants. As mentioned before, this classical view has been challenged and it has been proposed that mycorrhizal (at least EcM and ErM) plants shortcut this mineralization process and directly tap, via the associated fungus, into the organic N pool containing oligopeptides, amino acids, or nucleotides (Read and Perez-Moreno 2003). Preferential acquisition of amino acids (up to 80 % of N supply) over ammonium and nitrate by plants in boreal soils has indeed been demonstrated in situ using a nondestructive microdialysis technique (Inselsbacher and Näsholm 2012).

Most of the N present in dead and partially decomposed plant biomass becomes complexed with polyphe-

nolic compounds that partially or totally protect it from enzymatic hydrolysis. A series of studies proposed that EcM mycorrhizal fungi would have retained different pathways, originally used by their saprotrophic ancestors to modify organic matter including lignin, in order to specifically access this complexed N pool.

Using the *Boletales Paxillus involutus*, that may have evolved from a brown-rot saprotrophic ancestor (Kohler et al. 2015), and a diverse set of analytical approaches, including spectroscopy, Rineau et al. (2012) demonstrated that in pure culture *P. involutus* could partially degrade and/or modify the chemical structure of polysaccharides and phenolic compounds present in the organic matter extract. The observed chemical modifications are reminiscent of the modifications performed by saprotrophic brown-rot fungi on wood. These modifications involved the Fenton reaction, which leads to the production of hydroxyl (HO•) radicals through the interaction between Fe^{2+} ions and hydrogen peroxide. These diffusible highly reactive radicals in turn oxidize and/or hydrolyze the different constituents of the organic matter. Oxidative modification of complex organic matter by *P. involutus* was further supported by the upregulation of a series of genes implicated in oxidative metabolism and iron homeostasis. Rineau et al. (2013) also showed that in vitro mycelium of this species could remove more than 50 % of the organic N bound to complex organic matter. Significant N removal only occurred when the culture was amended with glucose, a readily available C source that could be provided by an associated host plant in a forest ecosystem. Altogether, these observations suggest that *P. involutus* would have inherited from its saprotrophic ancestors the capacity at modifying complex organic matter in order to extract selectively bound organic nitrogen. As opposed to brown-rot saprotrophic species, in *P. involutus*, this capacity needs however to be primed by simple sugars provided by an associated host plant.

While *P. involutus* likely derives from a saprotrophic brown-rot species, many other EcM fungi seemingly derive from white-rot ancestors (Kohler et al. 2015), which use secreted class II peroxidases for the oxidation and/or degradation of lignin and phenolic compounds. Indirect correlative evidences suggest that several Basidiomycota EM fungi could indeed use manganese (Mn)-dependent class II peroxidases to obtain nitrogen bound to phenolic compounds in soil. First of all, several species in the genera *Hebeloma*, *Laccaria*, *Piloderma* (Kohler et al. 2015), and *Cortinarius* (Bödeker et al. 2014) possess one or several copies of genes encoding secreted class II peroxidases. Secondly, in a field study, Bödeker et al. (2014) observed a positive correlation between the abundance of *Cortinarius* ITS rDNA amplicons from Swedish pine and birch forest soil samples and the level of peroxidase activity measured in these samples. Talbot et al. (2013) also reported a similar positive correlation between EcM fungi, but not saprotrophic fungi, community richness in Californian pine forests and soil peroxidase and protease activity levels. Finally Bödeker et al. (2014) also recorded lower levels of soil peroxidase activities in ammonium-amended forest soils, a readily assimilated N source for both plants and fungi which become less dependent upon soil protein hydrolysis.

All these different genomic, laboratory, and field observations suggest that while most, if not all, EcM fungi have lost the ancestral capacity of degrading plant organic matter for their provision in carbon sources, several of these fungi have retained limited degradative activities for an efficient use of soil organic nitrogen protected from degradation by polyphenolic molecules.

V. Global Assessment of the Impact of the Mycorrhizal Symbiosis on Soil Element Cycling

The different models of mycorrhiza functioning and the breakthrough studies presented above, most of them performed in microcosms in the laboratory, suggest that mycorrhizae and mycorrhizal fungi could represent major players in global carbon and nutrient cycling in terrestrial ecosystems. This role is now being appreciated by a combination of field measurements and of ecosystem manipulation experiments that are described in this section.

These studies led to the formulation of two main hypotheses. The first one is that mycorrhizae represent one of the main route by which recently photosynthetically fixed carbon enters the soil ecosystem, explaining a significant frac-

tion of soil respiration and metabolic activity which cannot be attributable to saprotrophs living on dead plant litter. The second one is that mycorrhizae, and more specifically EcM, could represent essential drivers of long-term carbon storage in soils. Altogether, these two hypotheses plead for incorporation of mycorrhizae in models of terrestrial carbon cycling, especially in a context of global change.

A. Fast Nutrient Transfer Between Plant and Fungal Partners

The proportion of fixed carbon transferred by a host plant to its mycorrhizal partners varies widely according to the type of association, identity of the host plant, and type of study. However, values of 10–20 % and 20 %(-50 %) been repeatedly reported for the AM and EcM associations, respectively (van der Heijden et al. 2015). Similarly, it has also been estimated that a large proportion of mineral nutrients such as phosphorus (up to 90 % for AM plants) assimilated by a mycorrhizal plant has been transferred from the mycorrhizal fungi to the plant (van der Heijden et al. 2015). Although these figures have been reported for some time, the consequences of these massive transfers of primary nutrients between symbiotic partners on the global functioning of the soil ecosystem have long remained elusive.

Two main experimental approaches are used to follow the fate and redistribution of assimilated nutrients in plants. The first one is the use of isotopically labeled nutrients such as ^{14}C or ^{13}C-labeled CO_2 supplied to the leaves of ^{15}N-labeled NO_3 of NH_4 supplied to the roots. Although initially used in laboratory-grown plants, this approach is now also being used in the field including on trees. In the case of trees, the impact of recently assimilated CO_2 on the soil microbiota can also be appreciated by either root severing or tree girdling (the annular sectioning of the tree bark and associated phloem tissues), which both result in the termination of the descending flow of phloem sap from the plant aerial parts to the root tips and the mycorrhizal symbionts.

Experiments measuring the fate of labeled C supplied to the leaves consistently show that its transfer to fine roots is fast, occurring within hours for an herbaceous plant (Kaiser et al. 2015) to a few 3–4 days for a 1–4 m tall tree (Högberg et al. 2008, 2010; Warren et al. 2012; Albarracín et al. 2013). Detection of labeled C in roots is immediately (within less than 1 day) followed by its detection in CO_2 resulting from "soil respiration" which combines the respiration of tree roots and of all other components of the soil biota, including soil fungi and bacteria. By combining NanoSIMS imaging to follow the fate of ^{13}C at the cellular level in mycorrhizal *Triticum* roots and the ^{13}C enrichment of bacterial- or fungal-specific fatty acids extracted from the rhizosphere, Kaiser et al. (2015) concluded that a significant fraction of recently fixed C entered the soil compartment through extraradical AM hyphae. In a high-resolution follow-up of ^{13}C in the soil biota following pulse labeling of *Pinus* foliage, Högberg et al. (2010) monitored a rapid (maximum at 14 days postlabeling) labeling of fungal-specific fatty acids as well as of newly formed sporocarps from EM species, thus demonstrating that not only basal metabolism, but also developmental processes of these fungi are sustained by immediate host plant photosynthetic activity. Considering that mycelia from EcM species can represent *ca* one third of forest soil microbial biomass in boreal forests (Högberg et al. 2001; Högberg and Högberg 2002), their belowground mycelial networks are therefore likely to represent, from a quantitative point of view, one of the main entry point of recently fixed carbon in soil. This is indeed supported by a large-scale girdling experiment of *Pinus sylvestris* in Northern Sweden, which resulted in a sharp and immediate drop (within days) in soil respiration which reached ca −50 % after 2 months (Högberg et al. 2001), thus suggesting that, in this case, the global soil biological activity was largely driven by recently fixed carbon and its translocation to EcM fungi.

Dependency toward immediate carbon supply from the plant may however vary between EcM species as indicated by Högberg et al. (2008) and Albarracín et al. (2013) who both observed significant differences in the levels of ^{13}C enrichment in EcM root tips formed by different fungal species. Heterogeneity between EcM fungal species was also observed by Pena et al. (2010) who showed that girdling led to the selective disappearance of the less frequent EcM fungal species on

beech tree root systems. In term of fungal community structure, Lindahl et al. (2010) showed that, as expected, local root severing resulted in a rapid decline in EM species abundance and diversity mirrored by an increase in abundance of opportunistic saprotrophic species, which in the short term may use senescing hyphae from EcM fungi as nutrient source.

While field measurements suggest that increased plant photosynthetic carbon fixation results in increased carbon allocation belowground and to associated mycorrhizal symbionts (Druebert et al. 2009; Näsholm et al. 2013), this does not always results in an anticipated increase in N allocation from the fungi to their host plants. Albarracín et al. (2013) reported that while N fertilization led to increased C flow belowground and to increased N concentration in *Pinus* EcM mycorrhizae, most of this assimilated N became blocked in the symbiotic tissues and was not transferred to the aerial parts of the plant. A similar N retention in *Pinus* ectomycorrhizae was independently reported by Näsholm et al. (2013) who concluded that in N-limited soils the ectomycorrhizal association could increase the N-starvation status of their associated plants. This "cheating" behavior of mycorrhizal fungi, which derive plant carbohydrates for the acquisition of soil N to their exclusive profit, could be canceled by external N fertilization that led to effective N transfer from the fungus to the plant (Näsholm et al. 2013).

B. Mycorrhizal Fungi and Soil Carbon Storage

Besides being the recipient of recently fixed atmospheric CO_2, EcM and ErM fungi could also drive directly or indirectly the long-term carbon storage in soil which represents one of the major sink of C on earth (Lal 2004). In a global meta-analysis of soil carbon pools across biomes, Averill et al. (2014) showed that EcM and ericoid-dominated ecosystems stored 70 % more carbon per unit nitrogen than soils in ecosystems dominated by AM-associated plants. Using a model-based approach, Orwin et al. (2011) suggested that increased C storage in EcM and ericoid-dominated soils could result from either higher C input to soils and/or from slower litter decomposition rates due to strong competition for N between saprotrophs and mycorrhizal fungi. The latter hypothesis may marginally apply for well-stratified forest soils in which there is a strong physical separation between the uppermost litter and fermentation soil horizons colonized by mainly saprotrophic fungal species (characterized by high PCW degrading enzymatic activities) and the horizons underneath where mycorrhizal fungi predominate (Lindahl et al. 2007; Voříšková et al. 2014). Besides this physical separation between these two functional groups, which limits direct competition between them, it has also been recorded that litter degradation by saprotrophic species results in an increase of the C/N ratio, which rather decreases in the lower soil horizons, presumably as a result from N foraging activity by mycorrhizal fungi and subsequent N transfer to host plants (Lindahl et al. 2007).

By using ^{14}C atomic bomb modeling, Clemmensen et al. (2013) suggested that a large fraction of the recently stored soil carbon in late successional boreal forests could actually directly derive from root and EcM fungal biomass instead of aboveground litter input. From a mechanistic point of view, Clemmensen et al. (2015) observed that in the course of boreal forest aging, the mycorrhizal fungal community shifted from a predominance of "cord-forming" fungal species in the earlier stages to a predominance of ericoid fungi producing melanized hyphae. Melanized cell walls may become recalcitrant to degradation (Fernandez and Koide 2014) and therefore contribute to the long-term C storage in these forests.

VI. Conclusions and Future Perspectives

In this chapter, we have summarized recent advances in the field of mycorrhizal ecology prompted by "omic" approaches, which have offered insights in the genome signatures of different fungal trophic strategies and the roles of mycorrhizal fungi in the functioning of terrestrial ecosystems.

While the latter paragraphs clearly demonstrate the importance of the mycorrhizal association in terrestrial carbon cycling, it remains however to be seen if several of the hypotheses formulated in recent studies can be generalized or on the contrary modulated according to the

type of ecosystem. Indeed, many studies on carbon cycling refer to forests, in boreal zones, with well-defined soil horizons. However, other forests are characterized by, for example, mull-type humus in which newly deposited litter is rapidly incorporated in the soil column by the microfauna, thus possibly leading to a cooccurrence and direct competition between saprotrophic and mycorrhizal soil fungi.

In the next years, we should also observe a better integration of "omic" data in functional ecology through metagenomics and metatranscriptomic studies targeting mycorrhizal fungi. In this respect, the metatranscriptomic study by Liao et al. (2014), in which soil transcripts were attributed to *Piloderma croceum*, whose genome has been entirely sequenced (Kohler et al. 2015), offers a first illustration of this approach. Such studies will allow identifying in situ what is the contribution of single mycorrhizal species to complex soil processes.

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11 Understanding the Biodiversity and Functions of Root Fungal Endophytes: The Ascomycete *Harpophora oryzae* as a Model Case

Zhi-lin Yuan[1], Zhen-zhu Su[2], Chu-long Zhang[2]

CONTENTS

I. Introduction

Fungi are the important regulators of ecosystem functioning by decomposing litter, soil nutrient cycling, and contributing to plant growth. Fungi can interact with plants in various ways and they are able to colonize all types of host tissues and organs. Most often, fungal symbionts express flexible lifestyles ranging from parasitism to mutualism (Rodriguez and Redman 2008). The impact of mycorrhizal fungi, mostly arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (ECM), on individual plants fitness as well as the entire plant community productivity is already well documented. The current knowledge and the hypotheses of the benefits of root–fungal interactions are derived from the insights into mycorrhizal symbiosis.

Advanced environmental PCR-based molecular methods produce unambiguous evidence that plant tissues—besides mycorrhizal symbionts—harbor a diverse array of fungal endophytes both below and above ground (Arnold et al. 2007). Endophytes thereby defines a group of fungi that colonize living internal tissues of plants without causing overt symptoms.

Historically, the grass clavicipitaceous endophytes (mainly referred to *Epichloë* species) received earliest attentions as they produced many kinds of alkaloids toxic to grazing animals (Guerre 2015). The functions of other fungal endophytes, however, are too often neglected or underestimated until several significant milestones in the history of endophyte research have been achieved. For instance, *Taxomyces andreanae* as the first taxol-producing endophytic fungus was isolated from inner bark of Pacific Yew (Stierle et al. 1993). Varma et al. (1999) discovered the root endophytic basidiomycete *Piriformospora indica* strongly favors plant performance. Redman et al. (2002) stressed that habitat-adaptive endophytic fungal symbiosis can raise the thermotolerance of plants. The potential of foliar endophytes to efficiently control pathogens was also emphasized (Arnold et al. 2003). More recently, some endophytes were shown to produce volatile organic components that can be used for mycodiesel production and mycofumigation to control postharvest diseases (Yuan et al. 2012).

Yet there is still a big gap in our knowledge of biodiversity of fungal endophytes. Submit-

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ting sequences of the internal transcribed spacer (ITS), small-subunit (SSU), and large subunit (LSU) of the rRNA genes of a newly found endophyte to BLAST analysis at public databases sequences often leads to no hits, indicating their potentially taxonomic novelty. In addition, frequently neither sexual nor asexual sporulation can be seen, precluding the possibility of morphological descriptions. Recently, a new class (Xolonomyectes) and a new order (Phaeomoniellales) within the Ascomycota were established during a survey of tree fungal endophytes (Gazis et al. 2012; Chen et al. 2015). These findings further highlight that fungal endophytes comprise the important component of fungal biodiversity on a global scale.

From an evolutionary perspective, the ability of filamentous fungi to colonize roots guaranteed the viability of earliest land plants as evidenced by fossil records (Bidartondo et al. 2011). Root infecting fungi are usually detected by staining methods, traditional culture-based methods, and contemporary molecular tools (such as pyrosequencing-based community profiling; cf. Lindahl et al. 2013; Toju et al. 2013). On the other hand, detection of non-mycorrhizal fungal root symbionts received also strong attention in the past decades, because (a) individual plant roots generally harbor a highly diverse array of fungal root endophytic species. Among them, dark septate endophytes (DSEs) are frequently found in a wide range of host species and seem even more predominant than mycorrhizal fungi at least in some cases (Green et al. 2008; Mandyam and Jumpponen 2015); and (b) some root endophytes confer multiple beneficial services to plants, including substantial stimulation of plant biomass accumulation and improving stress tolerance. A novel microbial inoculant called BioEnsure (formulated a Class 2 endophytic *Fusarium culmorum* FcRed1) has entered commercial applications for achieving a sustainable agriculture (www.adaptivesymbiotictechnologies.com, see more details in the section "An Overview of Diversity and Function of Root Fungal Endophytes"), exemplifying the importance of root endophyte-mediated plant ecophysiology.

In this chapter, we will provide an overview of current progress, future directions, and challenges regarding the diversity, binary root–fungal interaction, and field applications of root endophytes. To provide a thorough understanding of the molecular basis of root–fungal symbiosis, we will specially focus on a novel DSE, *Harpophora oryzae*, and highlight its importance as a model organism to study fungal biology and evolution.

II. An Overview of Diversity and Function of Root Fungal Endophytes

A. Diversity of Root Fungal Endophytes

Due to the ancient coevolutionary processes that occurred between plant roots and fungi, it is not surprising that today a diverse array of fungi are able to penetrate root tissues and finally establish a symbiotic relationship with hosts. Based on the pattern of symbiosis (e.g., host range, colonization pattern, transmission model, and *in planta* biodiversity), Rodriguez et al. (2009) distinguished four classes of fungal endophytes, among which Class 2 and Class 4 endophytes are the primary root associates. Taxonomically, most of them belong to the Ascomycota, with a minority belonging to Basidiomycota.

Class 2 endophytes appear to show less diversity compared to the other three groups, but they exclusively express an extensive colonization pattern within the whole plant and are not only limited in roots. One of the most striking characters observed in Class 2 endophytes is their habitat-adapted benefits (see below).

Among the class 4 endophytes, DSEs are the representative members. It is important to bear in mind that some root endophytes do not fall strictly into either category, but their roles should not be neglected.

For instance, non-mycorrhizal root endophytes in orchids are often emphasized in recent studies (Oliveira et al. 2014): *Fusarium semitectum* isolated from an

orchid (*Cypripedium reginae*) was proven to be effective in enhancing seed germination and seedling establishment (Vujanovic and Vujanovic 2007). Nematophagous and entomopathogenic fungi infecting nematodes and insects also endophytically colonize roots (Lopez-Llorca et al. 2006). The entomopathogenic fungal endophyte–plant–insect tripartite interaction model sheds light on the soil nitrogen cycling (Behie et al. 2012); these fungi are found to have the ability to colonize roots and transfer the insect-derived nitrogen to plants. Some soil-borne *Trichoderma* species have also been described as endophytic root symbionts (Harman et al. 2004).

The traditional way of detecting root infecting fungi makes use of both staining and culturing approaches: a large-scale survey of root fungal endophytes in Mediterranean environments was performed by Maciá-Vicente et al. (2008) using a culture-dependent method. Maciá-Vicente et al. (2012) further examined the culturable root endophytes in halophytic and non-halophytic plants. With the advent of ribosomal DNA-based methodology, molecular profiling of the fungal communities gained extensive popularity. In a pioneering example, Porras-Alfaro et al. (2008) adopted a cloning library method to identify a novel group of DSEs within the order Pleosporales. With the advent of reasonable priced genome sequencing methods, however, e.g., the pyrosequencing technology enables a holistic view of biodiversity of root microbiota. Gottel et al. (2011) examined the fungal community in endosphere of *Populus deltoids* using the barcoded pyrosequencing targeting D1/D2 region for fungal 28S rRNA genes. They found that 25 % LSU sequences remained unclassified even at phylum level, indicating the existence of potentially novel fungal lineages contributing to the plants physiology. Similarly, Toju et al. (2013) used a nested-PCR targeting internal transcribed spacer 2 (ITS2) to construct the amplicon libraries of root samples of oak species (*Quercus serrata*), which showed that diverse ascomycetous fungi, in particular DSEs, dominated the roots.

B. Common Properties of DSEs

DSEs represent a phylogenetically diverse group featured with melanized and septate hyphae. They frequently fail to sporulate on artificial media and colonize roots asymptomatically accompanied by formation of microsclerotia-like structures. Taxonomically, they belong to different orders across the Ascomycota (e.g., Pleosporales, Helotiales, Hypocreales, Eurotiales, Xylariales, and Magnaporthales). In some cases, taxonomic affinities of DSEs are still unresolved (e.g., *Scolecobasidium humicola* and *Pseudosigmoidea ibarakiensis*) (Mahmoud and Narisawa 2013; Diene et al. 2013). *Chloridium paucisporum*, *Leptodontidium orchidicola*, *Phialocephala dimorphosphora*, *Phialocephala fortinii*, *Phialophora finlandia,* and *Harpophora oryzae* are the well-known classic representatives of DSEs species (Jumponen and Trappe 1998; Rodriguez et al. 2009).

The current knowledge of biodiversity of DSEs remains largely incomplete. In a semiarid ecosystem, 60 % of root associated fungal isolates were identified as DSEs using in vitro inoculation system (Knapp et al. 2012). The authors hypothesized that plants of (semi)arid grasslands share common DSE communities. It becomes more and more clear that a large proportion of DSEs fall into the order Pleosporales,. and even many more taxa new to science still await discovery (Knapp et al. 2015). The endophytic pleosporalean genera are often recorded from roots in stressful (arid, saline) environments (Porras-Alfaro et al. 2008; Maciá-Vicente et al. 2012; Knapp et al. 2015).

C. Ecological Functions of Class 2 and Class 4 Endophytes

Based on an exhaustive characterization of root fungal colonization pattern in different adverse environments and related functions, Rodriguez and coworkers introduced the concept of "habitat-adapted symbiosis" for characterizing the ecological significances of Class 2 endophytes (Rodriguez et al. 2008).

As an example, they found that fungal endophytes isolated from geothermal habitats conferred survival of plants at high temperature, whereas salt tolerance was not increased (Rodriguez et al. 2008). In another case, endophytes recovered from crops conferred resistance to fungal diseases but not heat or salt resistance. However, there are also contrary reports: the class 2 endophyte *Piriformospora indica*, as a basidiomycete of the order Sebacinales, confers a multiple beneficial effects on a variety of plants (i.e., growth promotion, yield increase, salt and drought tolerance, and pathogen protection) (Waller et al. 2005; Sherameti et al. 2008). This supports earlier evidence that fungi from the Sebacinales often benefit plants (Riess et al. 2014). Environmental PCR (DNA-based) approaches indicated the ubiquity and diversity of sebacinalean endophytes in roots (Weiss et al. 2011), yet their isolation in pure cultures was so far unsuccessful, probably owing to

their low quantity in roots or need of special nutrient (Riess et al. 2014). In addition, even in the same location, a single plant species would host different Class 2 endophytes along a salinity gradient (Rodriguez RJ and Redman RS, personal communication). Therefore, selection of the plant part and methods for isolation (e.g., surface sterilization and medium) have a significant impact on finding these cryptic microorganisms.

Positive effects of DSEs on plant growth and nutrition acquisition are also illustrated by a meta-analysis approach (Newsham 2011). Under controlled conditions, most DSEs significantly increase plant biomass, particularly when organic nitrogen sources were present (Mahmoud and Narisawa 2013). There is growing evidence that DSEs are capable of synthesizing proteolytic enzymes like gelatinase guaranteeing the mineralization of organic *N*-containing compounds in the rhizosphere (Caldwell et al. 2000; Mandyam et al. 2010). Organic nitrogen uptake enhancement in plants achieved by colonizing them with DSEs may highlight a larger role of DSEs in soil nitrogen cycling or nitrogen-related ecological process than previously thought (Zijlstra et al. 2005). In addition, the ability of phosphorus solubilization in a DSE fungus (*Aspergillus ustus*) has been verified as, e.g., *A. ustus* improves plant biomass when using unavailable P (rock phosphate and tricalcium phosphate) source (Barrow and Osuna 2002). Thus, it is evident that DSEs actively participate in host nutrient acquisition from organic and recalcitrant sources (Mandyam and Jumpponen 2005). With regard to DSEs-mediated abiotic stress response, it is often hypothesized that the high melanin levels in hyphae is responsible for alleviating host abiotic stresses, despite experimental data is relatively limited. It is now well recognized that melanin plays an important role in the ability of melanized fungi to survive under stressful environments. Li et al. (2011) showed that a DSE fungus (*Exophiala pisciphila*) improved the tolerance of maize to heavy metals (HM). The authors provided evidence that *E. pisciphila* restricted the translocation of HM from roots to shoots. In essence, *E. pisciphila* is a HM tolerant strain. Glutathione S-transferases (GSTs) gene family and high melanin content are of great importance for HM detoxification in *E. pisciphila* (Ban et al. 2012; Shen et al. 2015). Beyond this, some work provides some microscopic evidence that DSEs have branched mucilaginous hyphae containing lipid vesicles and finally form biofilms on the surface of roots, which prevent direct exposure of roots to the adverse environments. This is thought to be a primary interpretation for DSEs conferring drought tolerance (Barrow 2003; Barrow et al. 2004).

III. *Harpophora oryzae*, a Novel DSE Recovered from Wild Rice Roots and Model System

A. Morphology of *H. oryzae*

H. oryzae has originally been isolated from wild rice roots in Yunnan province, China (Yuan et al. 2010b), during isolation of DSE fungi in 2007 and 2008.

It shows a moderate growth rate, with a frequent production of rope-like strands around the colony edge. It sporulates readily on artificial media and produces a mass of sticky, single-celled, and hyaline conidia (microconidia) from phialides (Yuan et al. 2010a), and most conidia are very falcate or strongly curved. Recently, it has been shown that *H. oryzae* also produces macroconidia (Fig. 11.1). Size and shape of macroconidia is variable (unpublished data), often fusiform, ellipsoidal, or amygdaliform and slightly inflated at either end. Only macroconidia are able to germinate. In fact, two types of conidia produced by its close relatives have been observed previously (Deacon 1974; Sivasithamparam 1975; Wong and Walker 1975; Zhang et al. 2014). It is necessary to bear in mind that both microconidia and macroconidia can germinate and are infectious in *Magnaporthe oryzae* (Zhang et al. 2014).

B. Molecular Phylogeny of *H. oryzae* and Related Close Members

Gams (2000) erected the genus *Harpophora* for anamorphs of *Gaeumannomyces* and *Magnaporthe* within the Magnaporthaceae. In addition to *H. oryzae*, four species including *H. radicicola*, *H. maydis*, *H. graminicola*, and *H. zeicola* have so far been described, *H. radicicola* thereby serving as the type species.

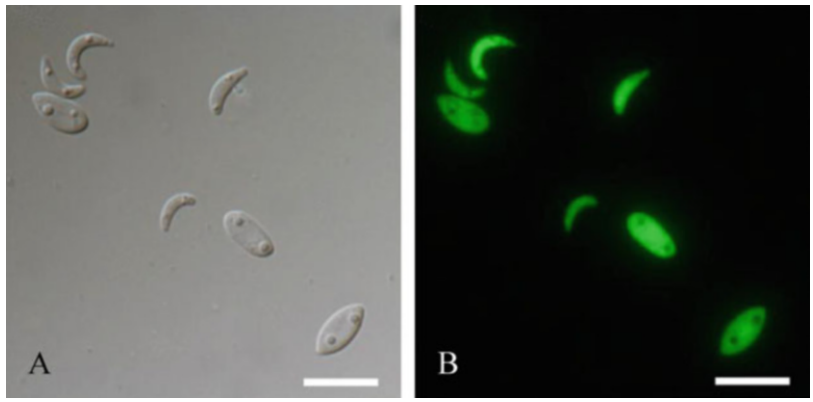

Fig. 11.1 Non-germinating phialospores (microconida) and germinating phialidic conidia (macroconidia) produced by GFP (green fluorescent protein)-tagged *H. oryzae*. (**a**) Conventional light microscopy; (**b**) fluorescence microscopy. Bar = 10 μm

H. graminicola also plays as a beneficial DSE in grass roots (Newsham 1999), while the remaining members are thought to be potential pathogens. A phylogenetic tree inferred from ITS-5.8S rRNA gene regions reveals that *H. oryzae* forms a distinctive subclade within the genus *Harpophora* and is not genetically close to other species of *Harpophora*, although it shows phylogenetic affinity to two unidentified *Harpophora* sp. (Yuan et al. 2010a).

IV. Effects of *H. oryzae* Colonization on Plant Growth and Disease Resistance

Using an in vitro co-culture system, the response of the plants to *H. oryzae* was examined (Yuan et al. 2010a). This showed that *H. oryzae* contributes to growth promotion in several plant taxa (e.g., rice, tobacco, and *Arabidopsis*) (Fig. 11.2), but has no obvious beneficial effects on barley growth, indicating that *H. oryzae* may have a relaxed host specificity. Unlike *P. indica* or other beneficial root fungus, *H. oryzae* does not induce the elongation of roots. In contrast, *H. oryzae* inhibits root growth to some degree, probably owing to an extensive colonization on the root surface under laboratory conditions. Nevertheless, *H. oryzae* efficiently stimulates the accumulation of plant biomass above the ground, especially in shoots (Fig. 11.2). The mechanisms have not yet been addressed in detail. A complete genome analysis of *H. oryzae* may yield important clues as genes involved in the synthesis of auxin and gibberellin are highly enriched in *H. oryzae* (Xu et al. 2014). In addition, *H. oryzae* can protect rice roots from invasion by its pathogen *Magnaporthe oryzae*, which makes it an attractive candidate for biocontrol (Su et al. 2013). *H. oryzae* causes local resistance by forming reactive oxygen species (ROS) and highly antioxidative compounds and also induces the OsWRKY45-dependent salicylic acid (SA)-mediated systemic resistance against this disease (Su et al. 2013).

V. The Colonization Patterns of Mutualistic and Pathogenic Interactions

Considering the close phylogenetic relationship between *H. oryzae* and other members (e.g., *Gaeumannomyces* and *Magnaporthe*) in the family Magnaporthaceae, Su et al. (2013) compared the commonalities and differences with regard to the colonization patterns of mutualistic and pathogenic interactions (Fig. 11.3). Using the fluorescent proteins DsRed2 and eGFP as markers in mycelia of *H. oryzae* and *M. oryzae* (Su et al. 2013), it was found that rice root infection by *H. oryzae* was initiated by

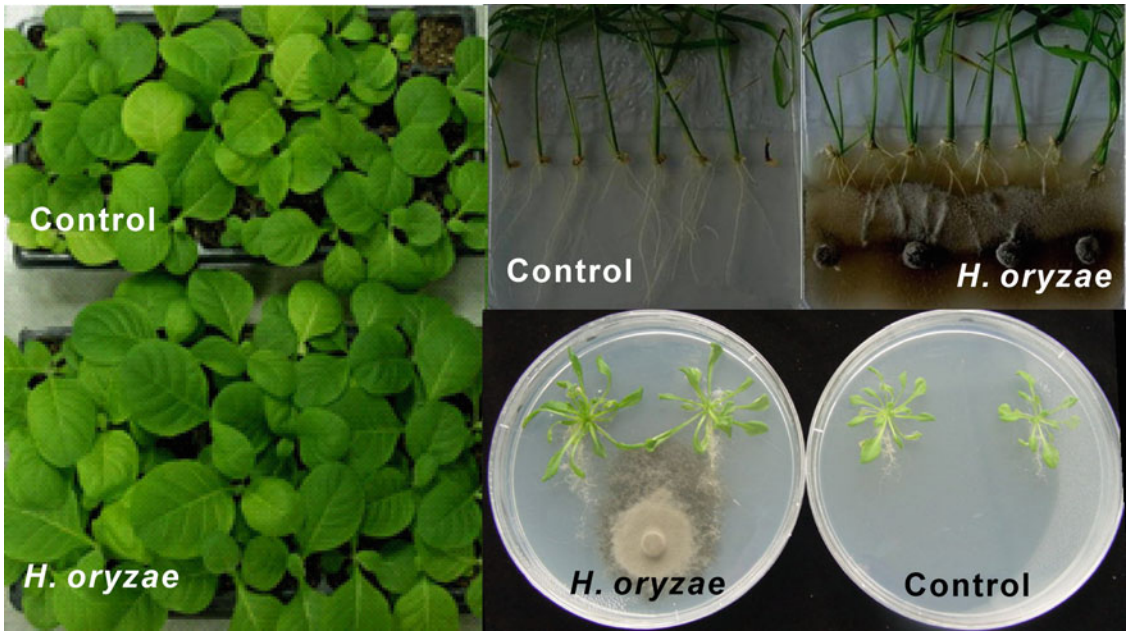

Fig. 11.2 *Harpophora oryzae* colonization confers growth-promoting activity to tobacco (*left*), rice (*upper right*), and *Arabidopsis* (*bottom right*) seedlings

runner hyphae, which extended to weave a loose fungal network on the root surface. This subsequently progressed to a biotrophic proliferation phase with inter- and intracellular hyphae growing along the main axis of the root. This was accompanied by centripetal branching and subsequent reproduction of extracellular and intracellular chlamydospores, while most of the colonized epidermal and cortical cells remained alive. Finally, most epidermal and partial cortical cells were colonized by hyphae and microsclerotia which led the plant to initiate cell death, while less infected adjacent cells remained alive. *H. oryzae* invaded the roots from the epidermis to the inner cortex, but not into the root stele. It can heavily colonize the root differentiation zone, but is mostly absent from the meristematic or the elongation zone. Unlike *H. oryzae*, *M. oryzae* readily propagated in the stele and systemically spread from root to leaf through the vascular tissue (Marcel et al. 2010). Both *M. oryzae* and *H. oryzae* would initiate the formation of appressoria or hyphopodia. The appressoria developed by *H. oryzae*, however, lost the ability to penetrate into the leaf cells.

VI. Friend or Foe: Differentiation of Pathogenic or Mutualistic Fungi

Comparative genomic analysis can enable a holistic view of both pathogenic and beneficial plant–fungal interactions (Bonfante and Genre 2010). In the *H. oryzae* genome, one of the most striking features observed was its relatively high number of transposon-like elements, which essentially enhanced the gain or loss of orphan genes, gene duplications, and gene family expansions (Xu et al. 2014; Fig. 11.3). Evidence for the development of a surveillance pathway for counteraction of the strong transposon activity was found.

A remarkable differentiation relating to the infection patterns, triggering of host defense responses, signal transduction, and nutritional preferences was verified between the two fungi (Fig. 11.3) (Xu et al. 2014). For example, as a root endophyte, *H. oryzae* lost the ability to infect rice leaves by loss of genes, such as *mpg1*, *moact*, and *morgs7*, which are important for interactions with the leaf surface or the penetration ability of appressoria (Talbot et al. 1996; Guo et al. 2011; Zhang et al. 2011). *H. oryzae* also encodes an arsenal of small secreted cysteine-rich proteins (SSCRPs), which are

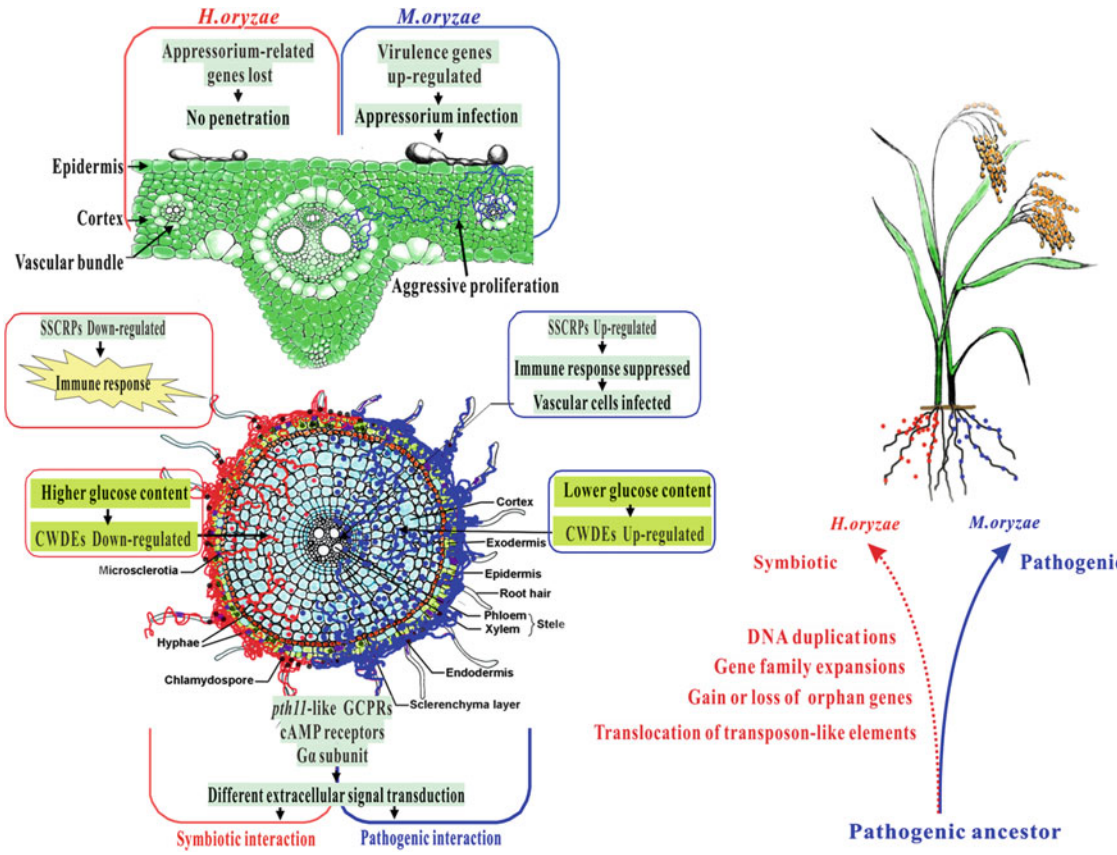

Fig. 11.3 The differentiation and evolution of mutualistic or pathogenic interactions between rice and endophytic fungus *Harpophora oryzae* or pathogenic fungus *Magnaporthe oryzae*

considered to facilitate infection by reprogramming host cells and modulating plant immune responses (Jones and Dangl 2006). Although a total of 173 orthologs of virulence-associated genes are shared by *M. oryzae* and *H. oryzae*, it has been shown that most of these genes are upregulated in *M. oryzae*, but suppressed in *H. oryzae* (Xu et al. 2014). Furthermore, the expressions of plant cell wall-degrading enzyme (CWDE) genes differ greatly between *M. oryzae* and *H. oryzae*. Most of them are downregulated in *H. oryzae*, but upregulated in *M. oryzae* (Xu et al. 2014). To maintain a biotrophic life style, weak CWDE expressions in *H. oryzae* will avoid destruction of the plant cell wall. In addition, *M. oryzae* and *H. oryzae* may respond differently to extracellular signals from the host. In *H. oryzae*, the number of G-protein-coupled receptors (GPCRs) is dramatically less than in *M. oryzae*. The expression pattern of pth11-like GPCRs and cAMP receptor-like GPCRs is also clearly different between them. Surprisingly, the orthologous gene of the *M. oryzae* G protein α subunit (magC) was strongly expressed in *H. oryzae* (Xu et al. 2014).

VII. Conclusions

There is no doubt that root fungal endophytes cooperate with mycorrhizal fungi and other microbes to influence host fitness. On one hand, plant abiotic stress tolerance and nutrient acquisition (especially nitrogen source) strengthened by endophytes, in particular Class 2 and Class 4 endophytes, suggest the pivotal role of root fungal endophytes for altering plant response to global change (e.g., drought and *N* deposition). On the other hand, from the view of application, a handful of root fungal endophytes have biotechnological interest for their use in agriculture and forestry. This may raise the question that what's the best strategy that facilitating the successful commercialization. Failure to get desir-

able outcomes in field trials is the major limitation for applying microbial inoculants. Thus, combination of different microbial groups with functional complementarity is believed to be a good alternative. What is more, recent rapid progress in rhizosphere microbiome research would allow us to design and reconstruct the powerful "synthetic microbial community" that providing more benefits to plants than individual isolates.

Several types of plant–fungal symbiosis have been defined. Genome analysis of the ectomycorrhizal fungus (*Laccaria bicolor*), the arbuscular mycorhizal fungus (*Glomus intraradices*), the grass endophytic *Epichloë* species, and the basidiomycetous endophytic *P. indica* contributes significantly to the knowledge of evolution of fungal symbionts (Martin et al. 2008; Tisserant et al. 2012; Zuccaro et al. 2011; Schardl et al. 2013). *H. oryzae* is an additional model organism to study root–fungal mutualism and is also of great importance to fully elucidate the evolutionary events in Ascomycota lineages. Further attempts such as a robust combination analysis of metabolome and transcriptome in the two binary fungal–root systems will result in uncovering more details about the key elements leading to either mutualistic or pathogenic interactions.

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12 Ecological Genomics of Mycotrophic Fungi

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CONTENTS

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I. Introduction

A key feature of fungal communities—their interdependence with other organisms—is explained by their inability of primary production (heterotrophy). In consequence, fungi cannot form separate self-sustaining communities, and their occurrence is irrevocably linked with that of organisms on which they depend for their nutrition (Hawksworth and Mueller 2005). Contemporary interactions of fungi with plants derived from initially saprotrophic living of early fungi on dead algal material in periodic dry, limnetic ecosystems. It is conceivable that some of these fungi may have formed mutualistic associations with early terrestrial algae, which later gave rise to complex symbioses between high fungi and vascular plant modern algae. A phylogenomic study of pectinase gene expansions demonstrated that the early group of true fungi Chytridiomycota diverged from its sister clade and thus leading to the high fungi Dikarya only after pectin evolved in plant cell walls that happened not earlier than 750 million years ago (Mya) (Chang et al. 2015).

The establishment of interactions between fungi and other opisthokonts (nucleariids, other fungi and animals) is definitely more ancient than their relationships with plants and dates back to the origin of fungi as an entire monophyletic group. The divergence of the plant–animal–fungal lineages occurred likely 820–1200 Mya. Recent recalibrations of the most important fungal fossils and the construction of molecular clock phylogenetic trees allowed to put fungal evolution on a right

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track with the origin and diversification of other major lineages of multicellular eukaryotes (Lücking et al. 2009).

II. Obligate Intracellular Mycoparasitism in Cryptomycota

The availability of genomic and phylogenomic techniques shed light on the interactions between lineages of early fungi. Such studies included microsporidia (single-celled spore-forming endopathogens of animals, *Microspora*) with the ancient and relatively newly recognized group of Cryptomycota including *Rozella allomycis* (Fig. 12.1), the endoparasite of water mold (James et al. 2013; Jones et al. 2011). The latter fungus serves as the most important source of information as it is the only clade member that grows in culture. *R. allomycis* is an obligate endoparasite of the water mold fungus *Allomyces* (Blastocladiomycota) that grows as a naked mitochondriate protoplast capable of phagocytosis to devour the cytoplasm of its host. (Held 1980; James and Berbee 2012; Powell 1984) showed that *R. allomycis* has a fungal-specific chitin synthase and its resting sporangia contain chitin in cell walls. They thus conclude that Cryptomycota and *Rozella* are not evolutionary intermediates as it was previously assumed but are rather the divergent fungi that evolved from an ancestor that already had a complete suite of classical fungal characteristics.

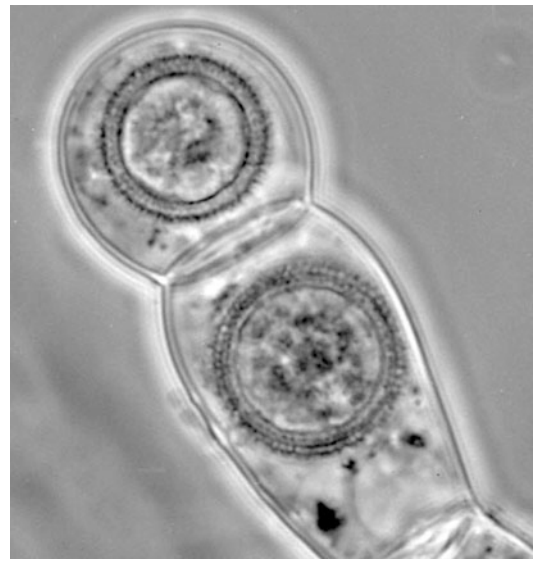

Fig. 12.1 *Rozella allomycis* parasitizing the chytrid *Allomyces*. Permission obtained from (Bruns 2006)

Genome sequencing of *R. allomycis* revealed insights into the previously unfeasible nature of its interactions with its host (James et al. 2013): it is diploid and contains 6350 predicted gene models. It includes four chitin synthases, one of which (division II chitin synthase) is specific for fungi and microsporidia (Ruiz-Herrera and Ortiz-Castellanos 2010). Interestingly, among the division II chitin synthases of *R. allomycis*, one contains a myosin domain, a feature that may be required for the polarized growth during invasion of *Allomyces*, a mechanism similar to the development of the penetration tube in corn smut Ustilaginales (Basidiomycota) (Schuster et al. 2012). James et al. (2013) used Oregon Green 488 conjugate of wheat germ agglutinin fluorescent stain that binds to *N*-acetylglucosamine residues and demonstrated that the infective cyst of *R. allomycis* contains this chitin precursor and that the chitin stain is most intense at those points where it penetrates the hyphae of *Allomyces*. A comparison of Cryptomycota including microsporidia with aphelids (Aphelidea, Opisthokonta) provided further insights into the interaction between the parasites and their hosts. Microsporidia and aphelids were previously considered as endoparasitic protozoans, and their placement within fungi only recently proposed (James et al. 2006; Karpov et al. 2013) and finally confirmed by phylogenomic analysis of a concatenated matrix of 200 gene sequences (James et al. 2013). Moreover, they found that *R. allomycis* genome contains orthologs of the three genes that were previously considered to be only present in microsporidian genomes and were thus interpreted as incidences of horizontal gene transfer (HGT) to serve the needs of intracellular parasitism (Cuomo et al. 2012). These are genes encoding a nucleotide phosphate transporter (NTTs; Pfam PF03219), a nucleoside H^+ symporters (PANDIT PF03825), and a chitinase class I genes (Pfam PF00182). The identification of these genes in *Rozella* represents an independent line of evidence for a close evolutionary link between Cryptomycota and microsporidia and indicates shared signatures of energy parasitism in the form of nucleotide and nucleoside transporters and genes for chitin degradation. Importantly, NTP transporters are involved in a specific theft of ATP from the host in microsporidia and the intracellular parasitic prokaryotes *Chlamydia* (*chlamydiae*) from which the genes were originally obtained by HGT (James et al. 2013; Tsaousis et al. 2008). Interestingly, the mitochondrial genome of *Rozella* showed features of degeneration that supports the hypothesis that the capacity to import ATP results in drastic genome changes for the mitochondrion. Sim-

ilar findings were also made in microsporidia, in which the capacity to retrieve ATP from their hosts by the HGT-derived bacterial NTTs is linked with a severe degeneration of their mitochondrion to a vestigial, genome-less organelle called a mitosome (Williams et al. 2002). Analysis of *Rozella*'s proteome and secretome, respectively, mainly revealed adaptations to endoparasitism that are convergent to those in other lineages of single-celled eukaryotes with a similar lifestyle. As expected for an obligate intracellular pathogen, the *Rozella* proteome is missing key components of primary metabolism. Overall, the portion of the proteome responsible for primary metabolism of *Rozella* is more similar to that of the apicomplexan parasites, *Plasmodium* and *Toxoplasma*, than that of microsporidia or other fungi. On the other hand, the amino acid metabolism of *R. allomycis* is more similar to that of Metazoa and Amoebozoa, perhaps suggestive of a phagotrophic mode of protein consumption and amino acid extraction. However, proteins involved in protein–protein interactions (e.g., signal transduction, protein folding, protein kinases, and proteins with WD40 domains) are all enriched in the *R. allomycis* proteome. James et al. (2013) hypothesized that some of the protein–protein interaction domains are actually involved in the direct manipulation of host signaling or recycling of host proteins. In support of this argument, they identified 22 genes of the Crinkler family of effector proteins. Crinkler proteins are found in many symbiotic, microbial eukaryotes but are best known in oomycete plant pathogens as secreted proteins that translocate into the host cytoplasm or nucleus to induce plant cell death. Thus, these new and most advanced genomic studies clearly demonstrate the ancient nature of intimate interactions between fungal lineages. The existence of mainly obligate and endocellular parasites in Cryptomycota sensu lato and the obvious lack of less specialized facultative associations between organisms from early fungal lineages is probably best explained by the long evolutionary history in aquatic ecosystem. Consequently, such interactions are rare among high fungi, which, however, interact in a great diversity of ways.

III. Diversity of Interactions Between High Fungi

Even before the inclusion of Cryptomycota in true fungi, this kingdom was considered as one of the most diverse members of the eukaryotic domain being probably only second after Arthropoda (Animalia). Consequently the ecology of these organisms in general and the structures of fungal communities in particular are very complex. Unfortunately fungal ecosystems and interactions are frequently described by using the better established botanical (and rarely zoological) terminology that creates considerable confusion. The review of several inherent problems and ambiguities associated with terminologies used in general ecology to describe fungal interactions is made by Tuininga (2005). Based on the way how fungi receive nutrients, she proposed to divide interfungal interactions in nutritive and nonnutritive. So-called nutritive fungal interactions can then be further be differentiated into biotrophy (deriving nutrients from the cytoplasm of a living host) and necrotrophy or predation, i.e., rapid utilization of nutrients from an organism after killing it (Jeffries and Young 1994; Dighton et al. 2005; Atanasova et al. 2013). While necrotrophy is ultimately beneficial for one partner only (the host or the predator), biotrophic interactions may vary in their importance for the two fungi from mutualism (hypothetically assumed but almost not documented) to commensalism and classical parasitism.

A. Fungi that "Stick Together"

To the best of our knowledge, cases of interfungal mutualism are not well documented. Commensalism between fungi has been demonstrated in vitro although explanations for such observations are still insufficient. Deacon (2005), working with thermophilic fungi *Chaetomium* (Sordariales, Ascomycota) and *Thermomyces* (Eurotiales, Ascomycota), showed that the latter non-cellulolytic fungus clearly benefited from the ability of *Chaetomium* to degrade cellulose in the compost. When inoculated together, the two fungi could degrade more of the cellulose filter paper sample compared to *Chaetomium* alone. The advantages of *Chaetomium* from the presence of *Thermomyces* remain to be explained. Beneficial interactions between fungi were also shown by Friedl and Druzhinina (2012), studying infrageneric communities of *Trichoderma* (Hypocreales, Ascomycota) in vertical profiles of the two undisturbed soils in the Danube valley. They detected up to a dozen of *Trichoderma* species to coexist in a soil sample of not more than 200 mg.

Pairwise in vitro modeling of *Trichoderma* communities by cultivating one species on the culture filtrate of the other species and measuring the resulting fitness (growth rate and conidiation efficiency) revealed that many of such interactions provided a benefit, but cases of no effect or even inhibition of growth and/or conidiation were observed too. Our studies of *Trichoderma* molecular evolution and diversity in different habitats demonstrate frequent cases of sympatric speciation and cohabitation of sibling species that remains to be explained (Atanasova et al. 2010; Friedl and Druzhinina 2012; Hoyos-Carvajal et al. 2009; López-Quintero et al. 2013; Migheli et al. 2009). Besides these few examples, the absolute majority of described nutritional interactions between fungi are neither mutualistic nor based on commensalism. The aggressive behavior of fungi against each other is widely used in agriculture to suppress plant pathogenic fungi, but it may also cause adverse effects on mushroom farms and on fungal bioeffectors used for plant growth promotion such as arbuscular mycorrhizal fungi. We therefore first describe the types of such hostile interactions between fungi and then focus on several best studied cases.

B. Types of Hostile Interactions Between Fungi

"The term mycoparasitism applies strictly to those relationships in which one living *fungus* [*underlined by the authors*] acts as a nutrient source for another." This definition by Peter Jeffries (Jeffries 1995) that limits the term to the fungal kingdom is only one of numerous similar clear statements commonly present in books and articles (Barnett 1963; Deacon 2005; Gupta et al. 2014). Ideally, the strictness of the definition should limit the use of a term to appropriate cases. Unfortunately it is not always the case in fungal ecology as numerous interactions between fungi and fungi-like protozoans (e.g., Oomycota) are also referred as mycoparasitic (Ait Barka and Clément 2008; Benhamou et al. 1999; Gaderer et al. 2015; Rey et al. 2005; Vallance et al. 2009) due to the similar impact made by these and the true mycoparasitic interactions to plant pathology. Below we describe terms related to non-mutualistic interactions between fungi:

- **Fungivory, mycophagy, or mycotrophy**—the use of fungi for food. All three terms are synonymous and may be applied (1) to grazing on fungal hyphae (e.g., by mites or ants) or fruiting bodies (e.g., by deer or humans), (2) to various biotrophic interactions with fungi ranging from mutualism through commensalism and parasitism to predation, and (3) to saprotrophic nutrition on all types of dead fungal biomass. Fungivory nutrition is known for fungi, bacteria, plants, vertebrates (particularly for birds and mammals), invertebrates (gastropods, nematodes, and insects), and protozoans including fungi-like oomycetes and amoeba. Thus, when *Pythium* (Pythiales, Oomycota) attacks a fungus, this interaction may be referred as **mycophagy** (or fungivory or mycotrophy); in contrast, when a fungus attacks *Pythium*, another term should be used (e.g., parasitism).
- **Mycoparasitism**—the case of **mycophagy** when one fungus feeds on another fungus. It includes the true cases of parasitism when parasite does not kill its host. Such interactions are biotrophic are beneficial for a parasite (or a pathogen) and are harmful for the host.
  Necrotrophic mycoparasitism—the case of **mycophagy** that is best described as predation when the feeding fungus aims to kill its prey and then feed on its dead biomass. Some authors prefer to use "prey" and "predator," respectively, for simplicity and clarity (Atanasova et al. 2013; Barnett and Binder 1973; Druzhinina et al. 2011; Seidl et al. 2009). Necrotrophic mycoparasites tend to be more aggressive and unspecialized (Chet and Viterbo 2007). Biotrophic mycoparasites, on the other hand, are usually restricted to a certain host range and may also develop specialized structures to adsorb nutrients from their hosts. Some fungi may behave as biotrophic mycoparasites of some hosts, while in interactions with others they behave rather as predators (Zhang et al. 2015).
- **Hyperparasitism**—parasitism on a parasite. The term is not limited to fungi and may be used for any group of organisms. Different cases of mycophagy including mycoparasitism may belong to this category. For example, when the host of a mycoparasitic fungus is a plant pathogen, mycoparasite may be consid-

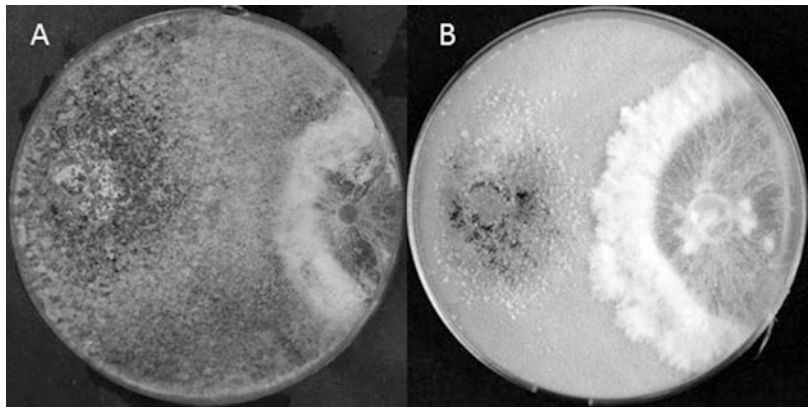

Fig. 12.2 Nutritive and nonnutritive interactions between fungi. (**a**) Mycoparasitism of *Trichoderma harzianum* (*left*) on *Athelia rolfsii*. (**b**) Agonism between *T. reesei* and *A. rolfsii*. *Trichoderma* (*left*) is overgrown by aerial hyphae of *A. rolfsii* (*right*) that does not parasitize on *Trichoderma*

ered as hyperparasite. It is important to note that for any hyperparasitic interactions at least two hosts and two parasites should be present. The use of this term in the absence of the primary host is not correct. For example, *Trichoderma* may be considered as a hyperparasite when it grows on sclerotia of *Athelia rolfsii* (Agaricales, Basidiomycota) that are formed on tomato plants. In this case, both *Trichoderma* and *Athelia* are parasites (and pathogens), respectively, while *Athelia* and tomato are the hosts, respectively. When the same *Trichoderma* is in vitro confronted with the same *Athelia* and it is performed in the absence of tomato, the term pathogen (or parasite) is applicable to *Trichoderma*, not to *Athelia*, and no organisms may be called as a hyperparasite.

- **Antagonism**—a type of **nonnutritive interactions** where one fungus inhibits the growth of other fungi, while continuing to grow uninhibited itself. Similar interactions include **coantagonism** with negative outcome for both fungi and **agonism** when one fungus is harmed and the other receives benefit. The latter interaction is similar to mycoparasitism, but it should not be confused with it as the benefitting fungus does not feed on the one that is harmed. An example for such interaction is *A. rolfsii* that is capable of overgrowing some strains of *Trichoderma* but does not feed on them. The benefit for *A. rolfsii* from this behavior is the reduced competition pressure for space and resources (Fig. 12.2). Tuininga (2005) notes that the term "coantagonism" is preferable to the frequently used term "competition," because the latter term describes only one possible mechanism of antagonistic interactions.

Other theoretically possible and nonnutritive interfungal interactions are bilaterally neutral **cohabitation**, neutral/beneficial **commensalism**, and **mutualism**, but they are very rare in fungi (vide supra) because of the usually present antagonism.

High fungi from many taxonomic groups that are able to either parasitize on plant pathogenic fungi or to antagonize them have been proposed for use in plant protection. For example, in the late 1970s, *Teratosperma sclerotivorum* (syn. *Sporidesmium sclerotivorum*, Ascomycota) that is an obligate pathogen on sclerotia of *Sclerotinia* spp. (Helotiales, Ascomycota) was suggested for use in biological control of the latter plant pathogen (see Fravel 2006 for the review). However, this technology likely did not get commercialized as the recent literature on the topic is limited: public databases contain no gene sequences for this hyperparasitic fungus (NCBI, November 22, 2015), and there is also no recent descriptions of the mechanisms of respective mycoparasitic interactions. Most of the modern antifungal biocontrol formulations use mycotrophic fungi from the order Hypocreales (Sordariomycetes, Pezizomycotina, Dikarya) that are also best studied at molecular biological, ecological, and taxonomic levels, respectively.

IV. Mycotrophic Hypocrealean Fungi

The order Hypocreales from the class of Sordariomycetes contains the best studied mycoparasitic fungi such as *Trichoderma*, *Escovopsis*, and *Clonostachys*, and genome sequences have been obtained for at least one but often several of their species (Fravel 2006; Gruber et al. 2011; Karlsson et al. 2015; Kubicek et al. 2011; de Man et al. 2015; Martinez et al. 2008; Studholme et al. 2013; Xie et al. 2014). Fungi from this order show widely diverse symbiotic associations with plants, animals, and other fungi and are also capable to saprotrophic growth (Sung et al. 2008). The most common animal hosts for hypocrealean fungi are the arthropods from the orders Coleoptera, Hemiptera, and Lepidoptera (Kobayasi 1941; Mains 1958; Sung et al. 2008). Respective arthropod pathogenic hypocrealean fungi consist of several genera mainly from three families: Clavicipitaceae, Cordycipitaceae, and Ophiocordycipitaceae. Nectriaceae and Bionectriaceae mainly feed on plants (Sung et al. 2007, 2008). Although the latter authors marked the family Hypocreaceae as a mixture of fungicolous and plant-associated fungi, recent studies suggest that it is dominated by mycotrophs, of which many taxa may also grow in the rhizosphere or become endophytes (Druzhinina et al. 2011).

Sung et al. (2008) reported and described fossils of the ancient *Paleoophiocordyceps coccophagus*, a fungus belonging to the genus *Ophiocordyceps*, which represents the eldest evidence of animal parasitism by a fungus. This finding allows an estimation of the divergence times of major lineages of Hypocreales which revealed that the hypocrealean fungi were at least present since the Early Jurassic, i.e.; 193 Mya. The authors proposed that the ancestral nutritional state of hypocrealean fungi was plant based, followed by shifts first to animal and then to fungal hosts (Sung et al. 2008). According to this study, the evolution of fungal–animal symbioses of the hypocrealean fungi is characterized by the origin and diversification of three families, Clavicipitaceae, Cordycipitaceae, and Ophiocordycipitaceae, that happened 173 or 158 Mya. The family Hypocreaceae that includes such mycotrophic genera as *Trichoderma* and *Hypomyces* is inferred to have arisen at least 145 Mya (Sung et al. 2008). Their analysis also showed that shifts to fungicolous nutrition occurred several times during the evolution of hypocrealean fungi. It is likely that mycoparasitic *Clonostachys* (Bionectriaceae) that are closely related to plant pathogenic *Fusarium* (Nectriaceae) obtained this possibility diverging from a plant-feeding host, while ancestors of *Trichoderma*, *Verticillium*, and *Escovopsis* likely evolved from animal pathogens. This is nicely illustrated by species of *Elaphocordyceps* (anamorph *Tolypocladium*, Ophiocordycipitaceae) that are mostly parasites of the ectomycorrhizal truffle genus *Elaphomyces* (Eurotiales, Ascomycota), but their next phylogenetic neighbors are all pathogens of insects.

A. *Escovopsis*: The Devastating Pest in Gardens of Leaf-Cutting Ants

The mycoparasitic hypocrealean genus *Escovopsis* is isolated from the nests of fungi-growing leaf-cutting ants, which belong to the tribe Attini (Hymenoptera, Insecta), namely, leaf-cutting ants (*Atta* and *Acromyrmex*), that share an obligate mutualism with Lepiotaceaous fungi of the genus *Leucoagaricus* such as *L. weberi* and *L. gongylophorus* (Agaricales, Basidiomycota) (Currie et al. 2006; Muchovej and Della Lucia 1990) or with pterulaceous fungi (Chapela et al. 1994; Villesen et al. 2004). These fungal cultivars have been acquired by the ants for their gardens from the environment multiple times in the course of evolution (Aylward et al. 2012; Chapela et al. 1994; Mikheyev et al. 2010). The basidiomycetes thereby form specialized hyphae called gongylidia, which serves as the main food supply for the ants (Seifert et al. 1995). In return, the ants provide the fungus with substrate for growth, means of dispersal to new locations, and protection from competitors and parasites (Muchovej and Della Lucia 1990). *Atta* colonies are one of the predominant herbivores in the Neotropics and therefore are frequently considered important agricultural pests in these areas (Hölldobler and Wilson 1990; Wallace et al. 2014). Colonies of these ants exhibit a rapid growth rate, consume hundreds of kilograms of leaves per year (Wirth et al. 2002), and cause the destruction of plantations and gardens in tropical areas of Central and South America and Costa Rica (Reynolds and Currie 2004; Wallace et al. 2014). *Escovopsis weberi* was isolated from nests of leaf-cutting ants as a natural pathogen

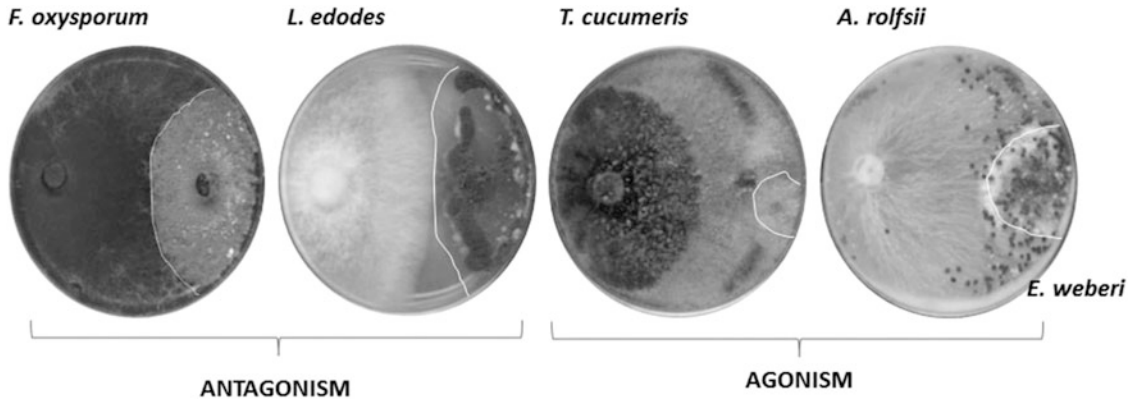

Fig. 12.3 Dual confrontations of *Escovopsis weberi* (*left*) with other fungi. The *white line* indicates the growth of *E. weberi* as detectable from the back side of the plate

of *Leucoagaricus* and was also proposed as a potential bioeffector against these ants (Reynolds and Currie 2004). According to the above explained terminology, *E. weberi* should not be assigned as a hyperparasite because *Leucoagaricus*—its host—is not a parasite but a saprotroph.

Until recently it remained unclear whether the primary nutrient source for *E. weberi* was the mushroom itself or the vegetative substrate placed on the gardens by ants, in other words whether the interaction was nutritive or rather nonnutritive. Reynolds and Currie (2004) demonstrated the true mycoparasitic nature of *E. weberi* by showing its rapid growth on pure culture of *Leucoagaricus* and negligible development on sterilized leaf fragments. Consequently, these authors described *E. weberi* as a necrotrophic mycoparasite of *Leucoagaricus* (Reynolds and Currie 2004). More recently, Marfetán et al. (2015) based on the microscopic analysis of interactions between *E. weberi* and *Leucoagaricus* spp. revealed hooklike structures and the penetration of the host hyphae and thus described *E. weberi* as a true mycoparasite. Furthermore, the most virulent *E. weberi* isolates were those which developed hooks involved in capturing *Leucoagaricus* sp. (Marfetán et al. 2015). The formation of these structures and growth rates positively correlated with virulence of individual *E. weberi* isolates, while the formation of hyphal traps did not show any correlation with virulence. Traps formed by *E. weberi* were also not able to generate pressure over their target nor degrade the *Leucoagaricus* sp. hyphae (Marfetán et al. 2015). Mycoparasitism of *E. weberi* is accompanied by secretion of enzymes and chemotropism toward *Leucoagaricus* (Marfetán et al. 2015; Reynolds and Currie 2004). Moreover water-soluble metabolites secreted by the latter fungus stimulate growth of *E. weberi* and induce its conidiation (Marfetán et al. 2015). Our own results suggest that parasitism of *E. weberi* is specialized on the attack of *Leucoagaricus* spp. (K. Chenthamara and I.S. Druzhinina, unpublished data) We investigated the antifungal potential of *E. weberi* in dual confrontation assays with a standard range of plant pathogenic fungi that are used to estimate the biocontrol potential of *Trichoderma* and *Clonostachys* (Fig. 12.3). We thereby found that *E. weberi* is generally not an aggressive fungus as it is hardly able to attack *Fusarium oxysporum* (Hypocreales, Ascomycota) and is completely agonized by *Thanatephorus* sp. (*Rhizoctonia solani*, Cantharellales, Basidiomycota) and *A. rolfsii* (Agaricales, Basidiomycota). The interaction of *E. weberi* with wood-rotting fungus *Lentinula edodes* (shiitake, Agaricales, Basidiomycota) was more complex as growth of the latter one was somewhat stimulated by the presence of *E. weberi* (data not shown). Our attempts to cultivate *E. weberi* on plates that were pre-colonized by such fungi as *Trichoderma atroviride*, *Alternaria alternata* (Pleosporales, Ascomycota), *A. rolfsii*, and *L. edodes* failed, which suggested that *E. weberi* is not able to parasitize on them (data not shown).

Despite becoming a model system for the study of coevolution and host–parasite dynamics (Currie et al. 2003, 2006; Gerardo et al. 2004; Little and Currie 2008; Mendes et al. 2012; Reynolds and Currie 2004; Rodrigues et al. 2008; Seifert et al. 1995; Taerum et al. 2007, 2010), little attention has been paid to the taxonomy of *Escovopsis* until recently. In the 1990s, when the genus *Escovopsis* was proposed, only two species were known: *E. weberi* (Muchovej and Della Lucia 1990) and *E. aspergilloides* (Seifert et al. 1995). In 2013, three additional *Escovopsis* species—*E. microspora*, *E. moelleri*, and *E. lentecres-*

cens—were described, and a new genus, *Escovopsioides*, was proposed (Augustin et al. 2013). Later on, Meirelles et al. (2015) performed a survey for *Escovopsis* species in gardens of the lower attine ant *Mycetophylax morschi* in Brazil and found four strains belonging to the pink-colored *Escovopsis* clade. The examination of these strains revealed significant morphological differences when compared to previously described species of *Escovopsis* and related *Escovopsioides*. Based on sympodial type of conidiogenesis, percurrent morphology of conidiogenous cells and non-vesiculated conidiophores, Meirelles et al. (2015) described the four new strains as a new species *E. kreiselii*. Phylogenetic analyses using three nuclear markers (28S and ITS1 and 2 or the rRNA operon and the partial sequence of the translation elongation factor 1-alpha, *tef1*) from the new strains and sequences retrieved from public databases confirmed that all known fungi infecting attine ant gardens comprise a monophyletic group within the Hypocreaceae family. Specifically, *E. kreiselii* is likely associated with gardens of lower attine ants, but the mode of its pathogenicity remains uncertain. Even more interestingly, a further new species of *Escovopsis*, *E. trichodermoides*, isolated from a fungus garden of the lower attine ant *Mycocepurus goeldii*, which has highly branched, *Trichoderma*-like conidiophores lacking swollen vesicles, with reduced conidiogenous cells and distinctive conidia morphology, was described by Masiulionis et al. (2015). We compared *tef1* sequences of the two almost simultaneously described and therefore not compared *Escovopsis* species and found that they are only 90 % similar (see NCBI accession numbers KF033128 and KJ808766 for *E. trichodermoides* and *E. kreiselii*, respectively). Thus, in November 2015, there are seven species of *Escovopsis* recorded in the Index Fungorum database (http://www.indexfungorum.org/): *E. aspergilloides*, *E. kreiselii*, *E. lentecrescens*, *E. microspore*, *E. moelleri*, *E. trichodermoides*, and the oldest *E. weberi*. All these taxa are only known from gardens of leaf-cutting ants.

The genome of *E. weberi* was sequenced by de Man et al. (2015) and shown to have a significantly reduced size and gene content compared to closely related but less specialized mycotrophic fungi from the genus *Trichoderma* (Kubicek et al. 2011; Martinez et al. 2008), which emphasizes the specialized nature of the interaction between *Escovopsis* and ant agriculture. While genes for primary metabolism have been retained, the *E. weberi* genome is depleted in carbohydrate-active enzymes, which may represent a reliance on a host capable to perform these functions. *E. weberi* has also lost genes necessary for sexual reproduction. Contrasting these losses, the genome encodes unique secondary metabolite biosynthesis clusters, some of which exhibit upregulated expression during host attack. The availability of the whole genome sequences of *E. weberi* and several species of *Trichoderma* makes the detailed comparison of ecophysiology of these fungi a challenging task.

B. Versatile Mycoparasites from the Genus *Trichoderma*

Of all mycoparasites and/or mycotrophs, the hypocrealean genus *Trichoderma* is probably the best studied and the most frequently applied bioeffector with the widest host/prey range (Atanasova et al. 2013; Baek et al. 1999; Brunner et al. 2005; Druzhinina et al. 2011; Elad et al. 1980; Kotasthane et al. 2015; Kubicek et al. 2011; Mukherjee et al. 2013; Studholme et al. 2013; Zhang et al. 2015). One of the many important qualities that makes *Trichoderma* outstanding as a biological control agent for plant pathogenic fungi (biocontrol; see below) is its high opportunistic potential (Jaklitsch 2011; Jaklitsch 2009) and adaptability to various ecological niches (Atanasova 2014). It has been well documented that *Trichoderma* spp. used for biocontrol can act through a diversity of mechanisms and combinations of them. Despite of the fact that these fungi are mycoparasites, necrotrophic mycoparasites, and nonspecific mycotrophs (Kubicek et al. 2011; see also Druzhinina and Kubicek 2013, for more references), they can establish themselves in the rhizosphere and stimulate plant growth and thus elicit a general plant defense reactions against pathogens (Druzhinina et al. 2011; Galletti et al. 2015; Harman 2011; Kotasthane et al. 2015). Some *Trichoderma* spp. have been also

isolated as endophytes too (Bae et al. 2009; Bongiorno et al. 2015; Chaverri et al. 2015; Gazis and Chaverri 2010; Rosmana et al. 2015). All of these characteristics make *Trichoderma* a genus of particular interest for application in agriculture as biofungicide and biofertilizer.

The genomic properties of *Trichoderma* spp. that add to their ability for biocontrol have been discussed (Martinez et al. 2008; Kubicek et al. 2011). In general these properties can be divided into such related to interactions with other fungi (Fig. 12.4) and such related to the interactions with plants and nonfungal pathogens of plants (nematodes, bacteria). As the latter topic is behind the scope of this review, the following description will only consider interfungal interactions with participation of *Trichoderma*. Druzhinina et al. (2011) and Druzhinina and Kubicek (2013) provided detailed reviews of *Trichoderma*'s ability to interact with living fungi as both mycoparasites and predators (necrotrophic mycoparasites) and also to their ability to saprotrophically feed on dead fungal biomass. The targeted biotrophic interaction of *Trichoderma* with other fungi includes such steps as sensing the presence of the host and optional coiling around their hyphae. host cell wall degradation and penetration of the host hyphae, repair of damages caused by hosts, and production of toxic secondary metabolites that may eventually kill the host and thus transforming it to a prey. In this chapter we will focus on those studies that functionally characterized genes involved in the interactions between *Trichoderma* and other fungi (Table 12.1). Most of them are involved in signal transduction during mycoparasitism, in fungal cell wall degradation, and in the production of antifungal secondary metabolites. Fewer studies focused on general and specific regulator genes such as *nox1*, *noxR*, *laeA*, *vel1*, and *xyr1* and the role of proteases.

Table 12.1 demonstrates that the absolute majority of functional genetic investigations were performed on two species of *Trichoderma* only, i.e. *T. atroviride* and *T. virens*. Atanasova et al. (2013) used DNA microarrays to compare the transcriptional response of the latter two species in comparison to *T. reesei* to the presence of *Thanatephorus cucumeris* (*Rhizoctonia solani*). They found that the three *Trichoderma* spp. exhibited a strikingly different transcriptomic response already before physical contact with alien hyphae. *T. atroviride* expressed an array of genes involved in the production of secondary metabolites, GH16 β-glucanases, various proteases, and small secreted cysteine-rich proteins. *T. virens*, on the other hand, expressed mainly the genes for biosynthesis of gliotoxin, respective precursors, and also glutathione, which is necessary for gliotoxin biosynthesis. In contrast, *T. reesei* increased the expression of genes encoding cellulases and hemicellulases and of the genes involved in solute transport. The majority of differentially regulated genes were orthologs present in all three species or both in *T. atroviride* and *T. virens*, indicating that the regulation of expression of these genes is different in the three *Trichoderma* spp. The genes expressed in all three fungi exhibited a nonrandom genomic distribution, indicating a possibility for their regulation via chromatin modification. The authors concluded that the initial *Trichoderma* mycotrophy demonstrated earlier by Kubicek et al. (2011) has differentiated into several alternative ecological strategies. In the context of their study, when *T. cucumeris* was used as an opponent for *Trichoderma*, the interactions ranged from parasitism of *T. atroviride* to predation of *T. virens* and competitive cohabitation of *T. reesei*. The neutral response of the latter species is best explained by the fact that the exclusively tropical *T. reesei* has never been isolated from soil so far and is not able to recognize temperate soil-borne *T. cucumeris* as its host or prey (Druzhinina et al. 2010). But it is important to note here that the assumption that *T. reesei* is merely a saprotrophic fungus that is not capable to mycotrophy is contradicted by numerous studies that demonstrated the ability of this fungus to attack a variety of fungi (Druzhinina et al. 2010; Atanasova et al. 2013), as also shown in Fig. 12.4.

The other conclusion that can be drawn from Table 12.1 is that the majority of the studies made were based on only a limited number of opponent fungi. In most studies either *T. cucumeris*, *A. rolfsii*, or *Botrytis cinerea* (Helotiales, Ascomycota) was used for confrontations with *Trichoderma*. As most *Trichoderma* species are capable of biotrophic and necrotrophic types of mycoparasitism and may also efficiently feed on dead fungal biomass, the conclusions of these studies therefore demonstrated only partial reduction of either one or another mycotrophic strategy employed by the respective *Trichoderma* species in given interactions. The

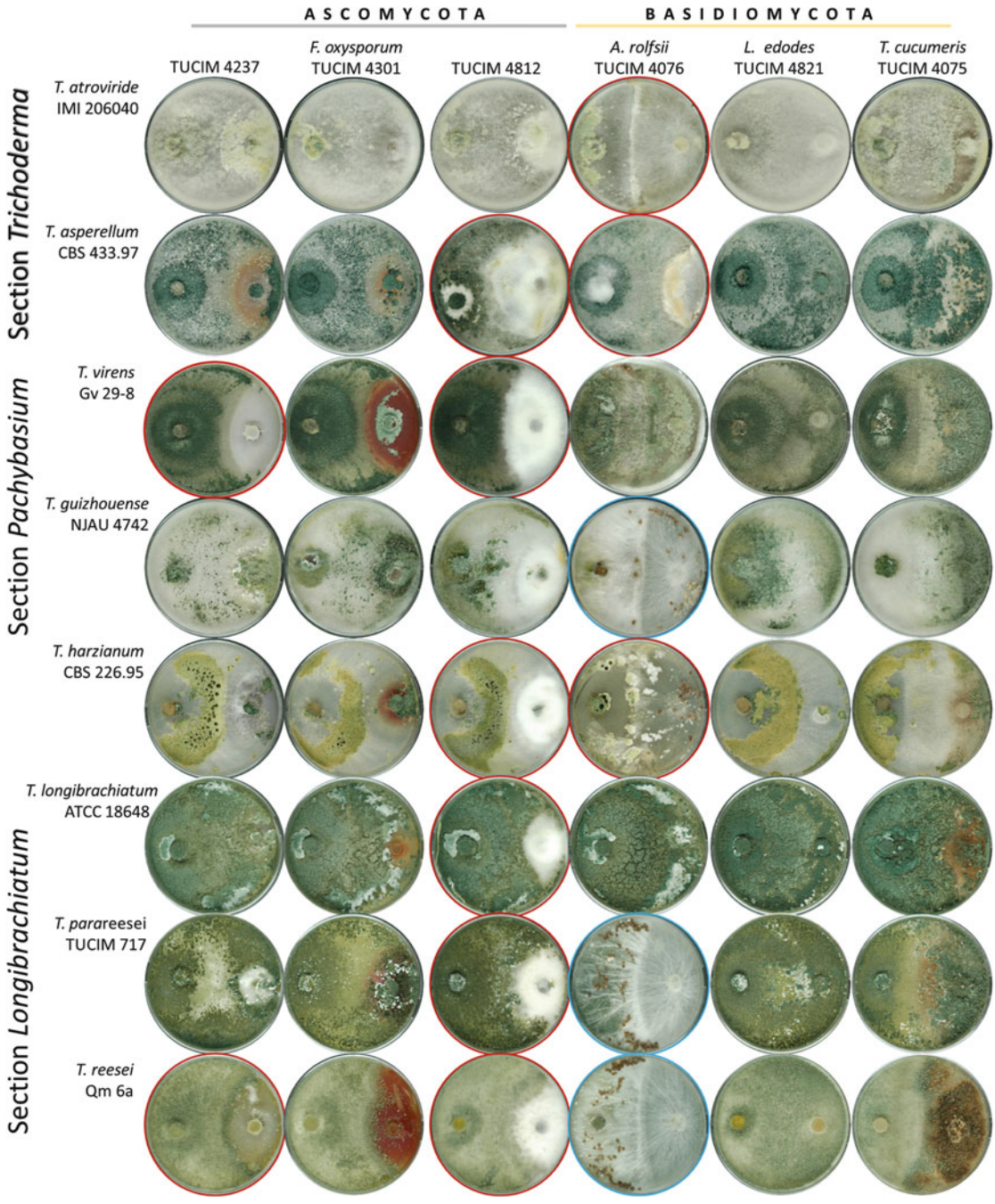

Fig. 12.4 In vitro interactions between *Trichoderma* (*left*) and other fungi in dual confrontation assays as observed after 10 days of incubation on PDA medium at 25° and 12 h cyclic illumination. Cases of non-mycoparasitic interactions are marked by *dark red* (antagonism) and *blue* (agonism) background

recent study by Zhang et al. (2015) demonstrated a clear role of *nmp1* gene encoding a secreted neutral deuterolysin metallopeptidase in the predation by *T. guizhouense* [former *T. harzianum* species complex (Chaverri et al. 2015; Li et al. 2012)] on *F. oxysporum*, *A. rolfsii*, and *A. alternata*. However, NMP1 was also found to be involved in mycoparasitism on *B. cinerea* and *S. sclerotiorum* and did not have any role in the

Table 12.1 Genes of *Trichoderma* that have been studied for their role in fungal–fungal interactions

Mycoparasitism-related process	Gene	General function	Predator/parasite	Prey/host	Phenotype due to the deletion or silencing	Phenotype caused by the gene overexpression	Study	Year
Signal transduction	*tga1*	G-protein α-subunit; takes part in G-protein-mediated signaling pathway; involved in conidiation, sexual reproduction; important for sensing mating partners/preys/hosts	*T. atroviride*	*Thanatephorus cucumeris* (*Rhizoctonia solani*)	Light-independent hyper-sporulation; retarded mycoparasitic-related coiling against *R. solani*; loss of GTPase activity, which is stimulated by the peptide toxin, Mas-7	An increase of coiling; inhibited sporulation; increased mycoparasitism on *R. solani*	Reithner et al.	2005
Signal transduction	*tga3*	G-protein α-subunit; takes part in G-protein-mediated signaling pathway; involved in conidiation, sexual reproduction; important for sensing mating partners/preys/hosts	*T. atroviride*	*T. cucumeris* (*R. solani*); *Botrytis cinerea*	Reduced growth; defect in chitinase secretion; loss of infection structure formation; avirulence against *R. solani* and *Botrytis cinerea*	n.a.	Zeilinger et al.	2005
Signal transduction	*tac1*	Adenylate cyclase, a signal regulator in cAMP signaling pathway	*T. virens*	*Athelia rolfsii* (*Sclerotium rolfsii*); *T. cucumeris* (*R. solani*); *Pythium* sp.[a]	Retarded morphology; retained only 5–6 % of the wild-type growth rate on agar; lowered intracellular cAMP levels below the detection limit; loss of virulence against *Athelia rolfsii*, *R. solani*, and *Pythium* sp.; negatively affected production of secondary metabolites	n.a.	Mukherjee et al.	2007
Signal transduction	*tmkA*	Mitogen-activated protein kinase (MAPK) signaling pathway gene, involved in transmitting signals for mating, filamentous growth, cell integrity, response to osmotic stress, and ascospore formation	*T. virens*, *T. atroviride*	*A. rolfsii* (*S. rolfsii*); *T. cucumeris* (*R. solani*)	Reduced ability to parasitize *R. solani* and *S. rolfsii* and the ability to induce systemic resistance in plant; in another strain of *T. virens*, improved mycoparasitism against *R. solani*; in *T. atroviride*, it resulted in improved mycoparasitism	n.a.	Mukherjee et al. Viterbo et al. Mendoza-Mendoza et al. Reithner et al.	2003 2005 2003 2007
Signal transduction	*tmkB*	Mitogen-activated protein kinase (MAPK) signaling pathway gene, involved in transmitting signals for mating, filamentous growth, cell integrity, response to osmotic stress, and ascospore formation	*T. virens*	*A. rolfsii* (*S. rolfsii*); *T. cucumeris* (*R. solani*); *Pythium* sp.[a]	Reduced radial growth; constitutive conidiation in darkness; defects in cell wall integrity (autolysis of the mycelia and increased sensitivity to cell wall-degrading enzymes); attenuated ability to overgrow the plant pathogen *A. rolfsii*	n.a.	Kumar et al.	2010
Signal transduction	*hog1*	High-osmolarity glycerol response, along with other proteins is required to develop stress resistance	*T. harzianum*	*Phoma betae* (Pleosporales, Ascomycota); *Colletotrichum acutatum* (Glomerellales, Ascomycota)	Reduced osmotic and oxidative stress tolerance; reduced antagonistic activity against the tested plant pathogens	n.a.	Delgado-Jarana	2006

(continued)

Table 12.1 (continued)

Mycoparasitism-related process	Gene	General function	Predator/parasite	Prey/host	Phenotype due to the deletion or silencing	Phenotype caused by the gene overexpression	Study	Year
Cell wall degradation	*ech42* (*chi18-5*)	Endochitinase, secreted	*T. atroviride*, *T. harzianum*, *T. virens*	*A. rolfsii* (*S. rolfsii*); *T. cucumeris* (*R. solani*)	In *T. harzianum*: unaffected mycoparasitism of *A. rolfsii* and *T. cucumeris*. In *T. virens*: reduced mycoparasitism of *T. cucumeris*	*T. atroviride*: improved mycoparasitism	Carsolio et al. Deng et al. Baek et al.	1999 2007 1999
Cell wall degradation	*nag1*	*N*-acetyl-β-D-glucosaminidase, secreted	*T. atroviride*, *T. harzianum*	*T. cucumeris* (*R. solani*); *B. cinerea*	In *T. atroviride*: resulted in reduced ability to protect bean seedlings against *R. solani*. In *T. harzianum*: resulted in reduced antagonistic activity against *B. cinerea*	n.a.	Brunner et al. Dubey et al.	2003 2012
Cell wall degradation	*bgn3*	β-1,6-glucanase, secreted	*T. virens*	*Rhizopus oryzae* (Mucorales, Mucoromycotina); *T. cucumeris* (*R. solani*); *P. ultimum*[a]	No effect on growth and development; reduced ability to inhibit growth of *P. ultimum*	Improved antagonism against *P. ultimum*, *Rhizopus oryzae*, and *T. cucumeris*	Djonović et al.	2006b
Cell wall degradation	*thpg1*	Endopolygalacturonase, secreted	*T. harzianum*	*T. cucumeris* (*R. solani*); *B. cinerea*; *P. ultimum*[a]	Reduced PG activity, ability to grow on pectin medium, and ability to colonize *Solanum lycopersicum* (tomato) (Solanales, Streptophyta) roots	n.a.	Morán-Diez et al.	2009
Regulator	*nox1*	NADPH oxidase	*T. atroviride*, *T. harzianum*	*P. ultimum*[a]	Severely affected ability to form conidia in response to injury; uncompromised hyphal regeneration; loss in ability to produce reactive oxygen species (ROS) in response to injury	In *T. harzianum*: resulted in improved biocontrol potential against *P. ultimum*	Montero-Barrientos et al. Hernández-Oñate et al.	2011 2012
Regulator	noxR	Regulator of NADPH oxidases involved in reactive oxygen species formation	*T. atroviride*	n.a.	Affected ability to form conidia in response to injury; hyphal regeneration was not compromised	n.a.	Hernández-Oñate et al.	2012
Regulator	*laeA*	Methyltransferase. Global regulator that affects the expression of secondary metabolite gene clusters and controls sexual and asexual development	*T. atroviride*	*Alternaria alternata*; *T. cucumeris* (*R. solani*); *B. cinerea*	Decrease in conidiation by 50 % in light; no conidiation in darkness; abolishment of sporulation in response to injury; increased sensitivity to oxidative stress; affected expression of genes encoding several proteases, GH16 β-glucanases, PKSes, and SSCPs; decrease in antagonism against *A. alternata*, *T. cucumeris*, and *B. cinerea*; decrease in production of known antifungal metabolites including 6PP (6-pentyl-2H-pyran-2-one)	Increased conidiation by 30–50 % in light; enhanced mycoparasitic vigor; resistance to oxidative stress (H_2O_2, 5 mM); increased production of a known antifungal metabolite, 6PP (6-pentyl-2H-pyran-2-one)	Aghcheh et al.	2013

General regulator	*xyr1*	Regulator protein for cellulase and hemicellulase gene expression in *Trichoderma*	*T. atroviride*	*Phytophthora capsici* (Peronosporales)[a]; *B. cinerea*; *T. cucumeris* (*R. solani*)	Reduced transcript levels of *axe1* and *swo1*, which encode accessory cell wall-degrading enzymes; delayed response of *Arabidopsis thaliana* during *Trichoderma–Arabidopsis* interaction; upregulation of *prb1* expression; overall enhanced competition with studied plant pathogens probably due to overexpression of *prb1*	n.a.	Reithner et al.	2014
Secondary metabolites	*tri4*	Cytochrome P450 monooxygenase that oxygenates trichodiene to give rise to isotrichodiol	*T. arundinaceum*, *T. harzianum*	*B. cinerea*; *T. cucumeris* (*R. solani*)	In *T. arundinaceum*: reduced antifungal activity against *B. cinerea* and *T. cucumeris*; reduced ability to induce the expression of *S. lycopersicum* defense-related genes belonging to the salicylic acid (SA) and jasmonate (JA) when attacked by *B. cinerea*	In *T. harzianum*: reduced ability to colonize roots; downregulation of defense-related genes in *S. lycopersicum*	Malmierca et al. Cardoza et al.	2012 2015
Secondary metabolites	*tri5*	Terpene synthase	*T. arundinaceum*, *T. brevicompactum*	*B. cinerea*, *T. cucumeris* (*R. solani*)	In *T. arundinaceum*: no production of HA (a non-phytotoxic trichothecene), which has role in antagonistic activity against fungal plant pathogens and induction of plant genes involved in defense responses; altered the expression of other tri genes involved in HA biosynthesis; altered the expression of hmgR, dpp1, erg9, erg1, and erg7, all genes involved in terpene biosynthetic pathways	In *T. brevicompactum*: increase of the trichodermin; increase in the antibiotic activity against a large panel of yeasts; affected *S. lycopersicum* growth and the lesions caused by *B. cinerea*	Malmierca et al. Tijerino et al.	2013 2011
Secondary metabolites	*gliP*	Involved in the production of gliotoxin, one of the most studied secondary metabolite in fungi that is known for its strong antimicrobial and cytotoxic activity. It is also successfully investigated as an antibiotic	*T. virens*	*Sclerotinia sclerotiorum*; *T. cucumeris* (*R. solani*); *P. ultimum*[a]; *Galleria mellonella* (Lepidoptera, Arthropoda)[b]	Abolition of gliotoxin production; reduced growth; dispersed and less dense mycelium; less branched hyphae; increased sensitivity to oxidative stress (H_2O_2, 10 mM); ineffective as mycoparasites against *P. ultimum*, *S. sclerotiorum*, but retained mycoparasitic ability against *T. cucumeris*. Reduced entomopathogenic activity against *G. mellonella*	n.a.	Vargas et al.	2014

(continued)

Table 12.1 (continued)

Mycoparasitism-related process	Gene	General function	Predator/parasite	Prey/host	Phenotype due to the deletion or silencing	Phenotype caused by the gene overexpression	Study	Year
Regulator	*vel1*	Velum formation protein 1; known to be one of the regulators of morphogenesis and secondary metabolism in some filamentous fungi	*T. virens*	*R. solani*, *P. ultimum*[a]	Defective gliotoxin production; no conidiation; early chlamydospore formation under nutrient stress conditions; delayed or eliminated chlamydospore formation in nutrient-rich media; absence of mycelial and extracellular pigments; defects in the regulation of many other secondary metabolism-related genes; decrease in mycoparasitism against *T. cucumeris* and *P. ultimum*	n.a.	Mukherjee and Kenerley	2010
Secondary metabolites	*pks4*	Polyketide synthase 4, involved in the production of the characteristic green pigment and the non-melanized structures of fruiting bodies in *Trichoderma*	*T. reesei*	*R. solani*; *S. sclerotiorum*; *A. alternate*	Loss of green pigmentation in their conidia; reduced resistance to UV; reduced stability of the conidial wall and the antagonistic abilities against *R. solani*, *S. sclerotiorum*, and *A. alternate*; reduced formation of water-soluble antifungal metabolites; altered expression of other PKS-encoding genes	n.a.	Atanasova et al.	2013
Proteases	*prb1*	Alkaline serine protease, secreted	*T. atroviride*[c]	*T. cucumeris* (*R. solani*)	n.a.	Improved mycoparasitism	Flores et al.	1997
Protease	*sp1*	Serine protease, secreted	*T. virens*	*T. cucumeris* (*R. solani*)	No effect on growth rate, conidiation, extracellular protein accumulation, antibiotic profiles, or the ability to induce phytoalexins in *Gossypium* (Malvales, Streptophyta) seedlings	Increase the ability of to protect *Gossypium* seedlings	Pozo et al.	2004
Protease	*nmp1*	Neutral deuterolysin metallopeptidase, secreted	*T. guizhouense*	*A. rolfsii*; *T. cucumeris*; *B. cinerea*; *S. sclerotiorum*; *A. alternata*; *Fusarium oxysporum*; *F. fujikuroi*	Reduced mycoparasitism; no coiling around hyphae of *F. oxysporum* f. sp. *cubense* 4; reduced ability to produce antifungal secondary metabolites; reduced ability to defend against other fungi	Increased mycoparasitism; self-toxicity	Zhang et al.	2015

Accessory proteins	*sm1*	Cerato-platanin, SSCPs	*T. virens*	n.a.	Unaffected growth or development, conidial germination, production of gliotoxin, hyphal coiling, hydrophobicity, or the ability to colonize *Zea mays* roots; same levels of systemic protection as in plants that have not been treated with *Trichoderma*; reduced level of protection in plants against diseases (*Colletotrichum graminicola* was used as a plant pathogen)	Unaffected growth or development, conidial germination, production of gliotoxin, hyphal coiling, hydrophobicity, or the ability to colonize *Z. mays* roots; enhanced levels of protection against plant diseases	Djonović et al. Djonović et al. (2006b) Salas-Marina et al.	2007 2006 2015
Accessory proteins	*sm2*	Cerato-platanin, SSCPs	*T. virens*	n.a.	Dramatic decrease (more in compared to *sm1*) in the ability of the fungus to induce resistance against disease caused by *Cochliobolus heterostrophus* in *Zea mays* (maize) (Poales, Streptophyta)	n.a.	Gaderer et al.	2015
Accessory proteins	*epl1*	Cerato-platanin, SSCPs	*T. atroviride*, *T. virens*	*Alternaria solani*; *B. cinerea*	In *T. atroviride*: resulted in diminished systemic protection of tomato plants (*S. lycopersicum*) against *A. solani* and *B. cinerea*, whereas in *T. virens* was less effective in protecting tomato against *Pseudomonas syringae* pv. tomato (Pseudomonadales, Proteobacteria) and *B. cinerea*	An increase in disease resistance against all tested pathogens	Salas-Marina et al.	2015

[a, b]Indicate nonfungal hosts from Oomycota and Insecta, respectively

[c]The strain IMI 206040 was initially published as *T. harzianum*

efficient attack of this fungus on *T. cucumeris* at all. Moreover, the secretion of the protein was induced when the fungus was confronted with itself on dead fungal biomass as the carbon source and was not activated when *T. guizhouense* was grown on glucose or potato dextrose agar. Besides the role of the exact protease that is definitely only one of the numerous other proteases that likely act synergistically in different *Trichoderma* species (Druzhinina et al. 2012), this study demonstrates the diversity of types of interaction that may be formed by one individual *Trichoderma* strain against a broad range of opponent fungi. It is thus impossible to assign *Trichoderma* to either exclusively biotrophic mycoparasitic fungi or describe them as necrotrophic mycoparasites or saprotrophs. Figure 12.4 illustrates the diversity of interactions between eight *Trichoderma* strains from eight species representing the three major infrageneric clades and six opponent fungi including three closely related strains of *Fusarium oxysporum* and three unrelated Basidiomycota fungi. For this reason, Druzhinina et al. (2011) proposed the more general term mycotroph as the best ecological identifier for *Trichoderma* spp. Results of Zhang et al. (2015) also demonstrate the need to study the role of individual genes in at least several possible interactions including at least parasitism and predation.

C. *Clonostachys rosea* Demonstrates an Alternative Toolkit for Successful Mycoparasitism

The mechanisms of interfungal interactions with the participation of still another hypocrealean mycotrophic fungus—*Clonostachys rosea*—have only recently attracted researchers' interest. Schroers et al. (1999) classified the mycoparasite *Gliocladium roseum* as *Clonostachys rosea* because it differed from the type species of *Gliocladium*, *G. penicillioides*, in morphology, ecology, teleomorph, and DNA sequence data. Jensen et al. (2004) used an ecological approach to present *C. rosea* as an effective mycoparasite against *Alternaria dauci* (Pleosporales, Ascomycota) and *A. radicina* on carrots (*Daucus carota* subsp. *sativus*). *C. rosea* showed a similar efficiency against these pathogens as the fungicide iprodione. A *C. rosea* strain, *C. rosea* IK726, was transformed with GFP (green fluorescent protein) and was used in biopriming of carrot seeds. Microscopy after 7 days of this biopriming showed seeds covered with a fine web of sporulating mycelium of *C. rosea*. Rodríguez et al. (2011) demonstrated the antagonism of *C. rosea* BAFC3874 against *Sclerotinia sclerotiorum* (Helotiales, Ascomycota) in pot-grown lettuce (*Lactuca sativa*) and soybean (*Glycine max*) plants and established that the strain produced antifungal compounds. They comprised a microheterogeneous mixture of peptaibols. These are short linear peptides that are rich in α-aminoisobutyric acid and bear an acetylated N-terminus and an amino alcohol at the C-terminus (Kubicek et al. 2007). They form helices that are inserted into the plasma membrane of the host causing alterations in the osmotic balance of the cell (Degenkolb et al. 2006) and inhibit membrane-bound enzymes such as cell wall polysaccharide synthases (Lorito et al. 1996). Such effects may explain some of the changes observed in the mycelium of the pathogen, including cell lysis of the hyphae and melanization (Rodríguez et al. 2011).

Recently, Karlsson et al. (2015) sequenced the whole genome of *C. rosea* IK726. A comparative phylogenetic analysis between *C. rosea*, *Trichoderma* spp., and *Fusarium* spp. suggested that *C. rosea* are sister taxa to *Fusarium* spp. (frequent plant pathogens), which belongs to family Bionectriaceae. In their study *Trichoderma* spp., which belongs to family Hypocreaceae, appeared in basal position to *C. rosea*. A comparative analysis of gene family evolution under the hypothesis that evolution of mycoparasitism in Bionectriaceae and Hypocreaceae results in selection for converging interaction mechanisms, revealed several differences between the studied mycoparasites. In comparison to *Trichoderma* spp., *C. rosea* showed expansion in several gene families such as those involved in plant cell wall degradation (polysaccharide lyase family 1 (pectin lyase), auxiliary activity family 3 (glucose–methanol–choline oxidoreductases), and auxiliary activity family 9 (lytic polysaccharide monooxygenase)); secondary metabolite synthesis (PKS, cytochrome P450 monooxygenases, PKS, and NRPS genes), likely attributing to production of antifungal components; ABC transporter and major facility superfamily membrane transporters, attributing to the fungi's high tolerance to toxins like boscalid, ZEN, and other microbial metabolites; and several ankyrin repeat proteins. In contrast, the genome of *C. rosea* contains significantly fewer carbohydrate-binding family 18 (CBM18, chitin binding) module containing genes (only two B group GH18 chitinases, only two C group GH18 chitinases, eight A group GH18 chitinases), suggesting that cell wall degradation of the fungal prey may not be a prominent strategy for interactions of *C. rosea* with other fungi.

Table 12.2 summarizes the results of functional characterization of *C. rosea* genes

Table 12.2 Genes of *Clonostachys rosea* studied for their role in fungal–fungal interactions

Mycoparasitism-related process	Genes	General function	Prey/host	Phenotype due to the deletion or silencing	Expression analysis	Study	Year
Secondary metabolites	*zhd101*	Zearalenone hydrolase	*F. graminearum*	Lowered in vitro ability to inhibit growth of the ZEA-producing *F. graminearum*. Failed to protect wheat seedlings against foot rot caused by the ZEA-producing *F. graminearum*	n.a.	Kosawang et al.	2014
Accessory proteins	*hyd1*, *hyd2*, *hyd3*	Hydrophobins	*F. graminearum*; *B. cinerea*; *T. cucumeris* (*R. solani*)	Higher growth rate of *Δhyd1Δhyd3* in high salinity. Faster overgrowth of *Δhyd1Δhyd3*, *Δhyd1*, and *Δhyd3* on prey/hosts. Improved protection of plants against fungal pathogens. Increased colonization ability by *Δhyd1Δhyd3* on *Arabidopsis thaliana* roots	Repressed expression of *hyd1*, *hyd2*, and *hyd3* in interactions with *B. cinerea* and *F. graminearum*. Upregulation of *hyd1*, *hyd2*, and *hyd3* during self-interaction. High expression of *hyd1* in germinating conidia	Dubey et al.	2014
Transporter	*abcG29*	ATP-binding cassette (ABC) transporter, induced by zearalenone and the fungicides Cantus, Chipco Green, and Apron	*F. graminearum*, *F. oxysporum* f. sp. *radicis-lycopersici*; *B. cinerea*	Delay in conidial germination and subsequent reduction in total germ tube length when subjected to H_2O_2; increase in necrotic lesion area caused by *B. cinerea* measured on *A. thaliana* leaves pre-inoculated with *ΔabcG29* strain spores compared to the necrotic lesion area on leaves pre-inoculated with *C. rosea* WT spores; increase of *F. graminearum* foot rot disease severity in barley seedlings	n.a.	Dubey et al.	2015
Transporter	*abcG5*	ATP-binding cassette (ABC) transporter, induced by zearalenone, secondary metabolites secreted by *F. graminearum* and different classes of fungicides	*F. graminearum*	Reduced antagonism toward *F. graminearum*; reduced biocontrol efficiency to protect barley seedlings from foot rot disease caused by *F. graminearum*; decreased tolerance to xenobiotics secreted by *F. graminearum* and toward ZEN; iprodione-and mefenoxam-based fungicides	n.a	Dubey et al. (2014b)	2014b

required for its interaction with other fungi. The availability of the genome sequence and its comparative analysis now provides the basis for studying of those gene families that are overrepresented in the genome of this fungus (*vide supra*).

D. Further Candidates for Whole Genome Sequencing of Mycoparasitic Fungi

A number of other hypocrealean fungi are mycotrophic with different degrees of specialization. However, most of them remain poorly investigated. These are such genera as, for example, *Hypomyces* (Põldmaa et al. 1997), *Cosmospora*, and *Verticillium*, but none of them have been investigated on the levels of genes and/or genomes. *Hypomyces*, with about 50 species recognized in recent studies (Poldmaa 1996; Rogerson and Samuels 1985, 1989, 1993, 1994), among which more than 30 are listed in NCBI taxonomy browser (November 2015), is the largest genus of almost exclusively fungicolous fungi.

Hypomyces species occur mainly on discomycetes, boletes, agarics, or polypores. The polyporicolous *Hypomyces* are more numerous than any other group, with 19 species accepted by Rogerson and Samuels (1993). The genome of *Hypomyces chrysospermus* CBS 394.52, a bolete mold that grows on *Boletus* (Polyporales, Basidiomycota) mushrooms, turning the host a whitish, golden yellow, or tan color and making it not edible, is currently sequenced by JGI DOE in collaboration with Joe Spatafora (https://gold.jgi.doe.gov/project?id=36363). The same group has sequenced the whole genome sequence of *Tolypocladium inflatum* (Bushley et al. 2013), which is a pathogen of beetle larvae but is closely related to fungicolous species of *Elaphocordyceps*. The comparative analysis of *H. chrysospermus* with already sequenced fungicolous hypocrealean fungi and *T. inflatum* may give insights in convergent evolution of this lifestyle.

Sphaerodes quadrangularis (Ceratostomataceae, Hypocreales) is another example of a facultative (i.e., able to grow saprotrophically in vitro, even when the host is absent) contact biotrophic mycoparasite. It establishes an intimate relationship with its host *Fusarium avenaceum* (Nectriaceae, Hypocreales) by producing hook-shaped and clamp-like attachment structures that appeared to derive nutrients and essential growth factors from living host cells (Vujanovic and Goh 2009).

Vujanovic and Goh (2009) demonstrated that *S. quadrangularis* produces hook-shaped structures within four days of subjection with *F. avenaceum*. Although *S. quadrangularis* also produces clamp-like or other contact structures, hook-shaped contact cells are more prominent. It is interesting that the diameter of hyphae parasitizing *F. avenaceum* is much smaller compared to its saprotrophic hyphae. *S. quadrangularis* also shows host specificity, as it did not form any specialized attachment structures when confronted with other *Fusarium* species. *S. mycoparasitica*, another biotrophic mycoparasite from the same genus, has however proven to be effective against a broad range of *Fusarium* species, showing positive effect on wheat (*Triticum* spp.) seed germination and seedlings growth (Vujanovic and Goh 2012). This fungus is not affected by the mycotoxins produced by *F. graminearum* such as deoxynivalenol (DON), trichothecene, and its acetyl derivatives 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (ADON), and zearalenone (ZEA) (Shinha and Bhatnagar 1998), probably because of the presence of similar defense genes as found in *C. rosea* (Karlsson et al. (2015).

An interesting further target for more detailed investigations may be *Calcarisporium arbuscula* (Watson 1955) that is only putatively related to Hypocreales based on the similarity of its nucleotide sequence encoding *rpb2* gene for the RNA polymerase II large subunit 2 (NCBI accession number LN714633). Although the morphology of interaction of these fungi with their hosts that belong to Xylariales (cf. *Physalospora*) has been interpreted by Barnett (1958) as intimate balanced mycoparasitism, no advanced recent studies on this fungus have been performed. The low attention to *Calcarisporium* is demonstrated by only 18 nucleotide sequences deposited in public databases for the entire genus (NCBI, November 2015), none of which were obtained in relation to studies of interfungal interactions.

Numerous other fungicolous fungi are only investigated on the level of their taxonomy that also frequently yields unexpected results. For example, Hawksworth et al. (2010) studied *Roselliniella*, a pyrenocarpous fungi growing on lichens and forming single-celled brown ascospores and persistent interascal filaments that were previously assigned to Sordariales. The molecular phylogeny showed them to belong to Hypocreales. Jaklitsch and Voglmayr (2014) investigated and

reinstated the fungicolous genus *Thyronectria* as also belonging to Hypocreales.

V. First Transcriptomic Insight into Mycoparasitism of *Ampelomyces quisqualis*

Mycoparasitic fungi from other groups than Hypocreales are studied less intensively. One exception is the mycotrophic fungus *Ampelomyces quisqualis* (Pleosporales, Ascomycota) that is a hyperparasite of *Erysiphe*, *Podosphaera*, *Sphaerotheca*, *Uncinula*, and others that all belong to the order Erysiphales (Ascomycota) and cause powdery mildew disease of wine grapes, cucumber, carrots, mango, and other plants (Sztejnberg et al. 1989; Takamatsu 2004). In total *Ampelomyces* has been described to be associated with more than 60 species from eight different genera of the order Erysiphales and is thus the most widespread and oldest known natural enemy of powdery mildews (Kiss 2008). It is therefore frequently used for biological control of this disease (Kiss 2003, 2008; Kiss et al. 2004; Sundheim 1982).

The biology and life cycle of *A. quisqualis* has been extensively studied (Kiss et al. 2004; Kiss 2008). Conidia of *A. quisqualis* are produced in pycnidia, which develop intracellularly in the parasitized mycelia of the powdery mildew host. In the presence of water, conidia become released and form hyphae that then penetrate the nearby hyphae of powdery mildew. *A. quisqualis* can withstand cold periods in the form of pycnidia that are saprotrophically produced in the killed plant tissues, but the fungus is not an efficient saprotroph. *A. quisqualis* is able to infect and form pycnidia within powdery mildew hyphae, conidiophores, and chasmothecia that causes reduced growth and death of the parasitic mildew (Kiss 2008).

Although ecological aspects of the mycoparasitic activity in *A. quisqualis* have been widely investigated (Angeli et al. 2011, 2012; Hashioka and Nakai 1980; Kiss 2008; Kiss et al. 2004), its molecular physiology remained largely unstudied. It is known that conidia of *A. quisqualis* poorly germinate in water or in the presence of glucose but their germination is stimulated by the presence of a water-soluble substance from the host whose chemical structure is yet unknown (Gu and Ko 1997; Sundheim 1982). Penetration of the host hyphae is made through either mechanical (Sundheim and Krekling 1982) or enzymatic processes. Rotem et al. (1999) reported the isolation of an exo-β-1,3-glucanase from *A. quisqualis*, and in vitro production of lytic enzymes has been reported for different isolates of *A. quisqualis* (Angeli et al. 2012). Siozios et al. (2015), using a high-throughput sequencing approach, established a catalog of transcripts that are formed by *A. quisqualis* during mycoparasitic interactions with *Podosphaera xanthii* (Erysiphales, Ascomycota). This catalog was then used to manufacture oligonucleotide microarrays for large-scale genome-wide analysis of transcriptional changes that occur during the early germination phase of *A. quisqualis*. They retrieved 1536 putative genes showing significant changes in transcription during the germination of *A. quisqualis*, documenting an extensive transcriptional reprogramming of *A. quisqualis* induced by the presence of the host. Genes encoding secreted proteases, virulence factors, and enzymes related to toxin biosynthesis were fund to be upregulated and interpreted as putative mycoparasitism related. They also found that a rapid activation of the transcription and translation machinery in the early stages of conidial germination is crucial for the successful transition from a dormant state to vegetative growth of *A. quisqualis*. The later phase of hyphal germination is hallmarked by upregulation of the genes involved in proteasomal and vacuolar protein degradation, protein secretion, transport, and localization, and genes related to the Snf7 family of proteins, which is involved in protein sorting and transport to lysosomal compartments (Peck et al. 2004). An involvement of these proteolytic genes in mycoparasitism has also been suggested for other fungi (Grinyer et al. 2005; Monod et al. 2002; Muthumeenakshi et al. 2007; Olmedo-Monfil et al. 2002; Zhang et al. 2015). Furthermore, the authors detected homologues of secreted proteases such as dipeptidyl-peptidase 5 and the tripeptidyl-peptidase SED3 and two putative genes with homology to the M6 family of metalloprotease domain-containing proteins which all may facilitate the penetration of the host mycelium. They also identified a small secreted protein related to the ceratoplatanin family (Chen et al. 2013; Gaderer et al. 2014; Skinner et al. 2001). They are widespread among fungi and believed to be involved in fungus–host interaction phytotoxicity in different plant pathogens (Jeong et al. 2007; Pazzagli et al. 1999) or elicitors of the plant defense response in mycoparasitic *Trichoderma* spp. (Djonović et al. 2006a; Seidl et al. 2006). The actual role of this protein in the mycoparasitic action of *A. quisqualis* remains therefore to be determined.

Siozios et al. (2015) identified genes encoding proteins involved in toxin biosynthesis among the upregulated genes: a homologue of a trichodiene oxygenase, which has a key role in

the trichothecene biosynthesis pathway (Cardoza et al. 2011), and a homologue of the sterigmatocystin biosynthesis P450 monooxygenase. Finally, two of the upregulated genes encoded multidrug transporters and the major facilitator superfamily to that resembled in *C. rosea* (Karlsson et al. 2015).

Several genes reported for their role in mycoparasitism have been found in dormant conidia of *A. quisqualis*. These were cell wall-degrading enzymes, including different glycosyl hydrolases and homologues of MAPK 1 such as *Pmk1* of *Magnaporthe grisea* (Magnaporthales, Ascomycota) and the *Tmk1* of *T. atroviride*. In fungi, MAPK signaling pathways are involved in the transduction of a wide variety of extracellular signals and play an important role in the regulation of different developmental processes, including those related to pathogenicity (Table 12.1). The authors also noted two lectin-related proteins that are well known for carbohydrate-binding properties and are widely distributed in animals, plants, and microorganisms (Lam and Ng 2010). *A. quisqualis*-related lectins could potentially be involved in the mycoparasitic process by recognizing the powdery mildew host and facilitating penetration. This study revealed several convergent strategies deployed by mycoparasites from different taxonomic groups. Future studies, including the sequencing of the *A. quisqualis* genome, could aid our understanding of the biology and evolution of the mycoparasitic lifestyle in general.

VI. Genomic Properties of *Pseudozyma flocculosa*, a Mycotrophic Basidiomycete That Evolved from an Advanced Plant Pathogenic Ancestor

Another hyperparasitic fungus that may be used to control powdery mildews is *Pseudozyma flocculosa* (Ustilaginales, Ustilaginomycotina, Basidiomycota) that is closely related to the model plant pathogen *Ustilago maydis* yet not capable to attack plants (Kemen and Jones 2012). Lefebvre et al. (2013) presented the comparative genomics of *P. flocculosa* and plant pathogenic smut fungi *U. maydis* (Kaemper et al. 2006), *U. hordei* (Laurie et al. 2012), and *Sporisorium reilianum* (Schirawski et al. 2010) (all from Ustilaginales). Several Ustilaginomycetes smut fungi share common features that are essential for pathogenicity. *U. maydis* interaction with maize (*Zea mays*) became the model system in phytopathology for investigation of factors essential for the establishment of the biotrophic parasitism. The genome sequence of *U. maydis* has revealed previously unknown genes that play key roles during such pathogenicity (Kaemper et al. 2006). Among these was a distinctive set of genes that coded for small secreted proteins referred to as effector proteins (or effectors), of which many had unknown functions. However, some were essential for infection and several counteracted plant defense responses, thus facilitating infection by the smut fungus (Brefort et al. 2009; Doehlemann et al. 2011).

In the case of *U. maydis*, the secreted effectors were found to be arranged in clusters and were upregulated upon recognition of the host plant, upon invasion, and in developing tumor tissue. Cluster deletion analysis proved their importance in pathogenicity (Kämper et al. 2006; Schirawski et al. 2010). The *P. flocculosa* genome comprises 6877 predicted protein coding genes and exhibited genomic features, including hallmarks of plant pathogenicity, that were very similar to the plant pathogens *U. maydis*, *Sporisorium reilianum*, and *Ustilago hordei* (Lefebvre et al. 2013). These findings and phylogenomic analysis suggested that *P. flocculosa* diverged from a plant pathogenic ancestor. Interestingly, however, Lefebvre et al. (2013) observed a loss of a specific subset of the secreted effector proteins (CSEP) reported to influence virulence in *U. maydis*. Although 345 CSEP-encoding genes were encoded by the *P. flocculosa* genome, which is a similar number as those found in the plant pathogenic Ustilaginales, orthologs for 51 out of 55 genes encoding secreted proteins that influence plant pathogenicity and virulence were absent in *P. flocculosa*. Since otherwise *P. flocculosa* has a high level of conservation of all other pathogenicity-related genes, e.g., encoding for enzymes in cell wall degradation and biosynthesis of secondary metabolites, this suggests that the loss of above described effectors represents the crucial factor which explains the not plant pathogenic lifestyle of *P. flocculosa*.

Yet the interaction between *P. flocculosa* and its fungal host might be dictated by other effector proteins. For example, the secretome of *P. flocculosa* includes two NPP1-containing proteins that are absent from plant pathogenic Ustilaginales (Kämper et al. 2006; Schirawski et al. 2010; Laurie et al. 2012) and also from other basidiomycetes and which are involved in the formation of necrosis and ethylene. They have so far only been identified in *Moniliophthora perniciosa* (Agaricales), the causal agent of witches' broom disease of *Theobroma cacao* (Meinhardt et al. 2008). Interestingly, the NPP1-containing proteins exhibit structural similarities to actinoporins, which form transmembrane pores (Ottmann et al. 2009), which fits well to previous observations that the collapse of powdery mildew colonies caused by *P. flocculosa* could be due to alteration of the plasma membrane and cytoplasmic leaking (Hajlaoui and Belanger 1991; Hajlaou et al. 1994; Mimee et al. 2009). Thus, NPP1-containing proteins could be key elements explaining the antagonism of *P. flocculosa* toward powdery mildews.

Other species-specific genes also provided further insights into how *P. flocculosa* acquired its potential to antagonize powdery mildews. For instance, two divergent GDSL lipases/esterases (Akoh et al. 2004) that contain a CE16 carbohydrate esterase motif that is exclusive to *P. flocculosa* have been identified that may be of relevance to its activity as an epiphytic competitor.

Another interesting observation differentiating *P. flocculosa* from the plant pathogens was the identification of a gene encoding a subgroup C GH18 chitinase adjacent to another gene encoding a chitin-binding LysM protein. The same genomic arrangement has also been found in mycoparasitic *Trichoderma* species (Kubicek et al. 2011). Interestingly, the LysM protein TAL6 of *T. atroviride* inhibited its own spore germination, while it had no effect on *Aspergillus niger* or *Neurospora crassa* (Ascomycota, Sordariales) (Seidl-Seiboth et al. 2013), suggesting a self-regulatory role in fungal growth and development. TAL6 could also act to protect the fungus against self-degradation by its other chitinases during mycoparasitism. Such a protective function for LysM chitinases against wheat (*Triticum aestivum*) was described during infection by for *Mycosphaerella graminicola* (Capnodiales, Ascomycota) (Marshall et al. 2011). While there is no evidence for a role of chitinases in the biocontrol activity of *P. flocculosa* (Bélanger et al. 2012), these finding suggests that a feature is shared with the mycoparasites, which requires further investigation.

VII. Conclusive Remarks on the Use of Mycotrophic Fungi in Agriculture

The biological control of plant diseases, or biocontrol, is an agricultural technique that is based on the use of natural hyperparasites and/or antagonists of plant pathogenic organisms to prevent or combat disease; in a broad sense, biocontrol may also include the application of plant stimulating (micro)organisms that help crops sustain abiotic stresses such as drought or salinity. It is very important to note that not all organisms but only humans[1] are capable to do biocontrol. The success of biocontrol is best defined by its result—reduced disease index for crops, but not by the mechanism of action and the type of interactions involved. Thus, efficient bioeffectors (organisms used in biocontrol) may (1) stimulate plants to induce their resistance, (2) compete with plant pathogens, (3) antagonize plant pathogens by means of secondary metabolite production, or (4) directly attack such pathogens as parasites or predators. Figure 12.5 gives an overview of biocontrol relevant interfungal interactions. Nonnutritive antagonistic interactions are depicted in pane a, while b–d demonstrate cases of parasitism among which b and c are beneficial for the plant as the "good" fungus or bioeffector attacks either plant pathogenic nematodes (b) or plant pathogenic and therefore "bad" fungi. The nature of the interaction showed in e is disputable and may be considered as either nonnutritive mutualism (plant gets stimulated while mycoparasitic fungus may find a greater diversity of host organisms) or commensalism when only plant benefits.

[1] The cases of natural agriculture as that of leaf-cutting ants are briefly discussed above.

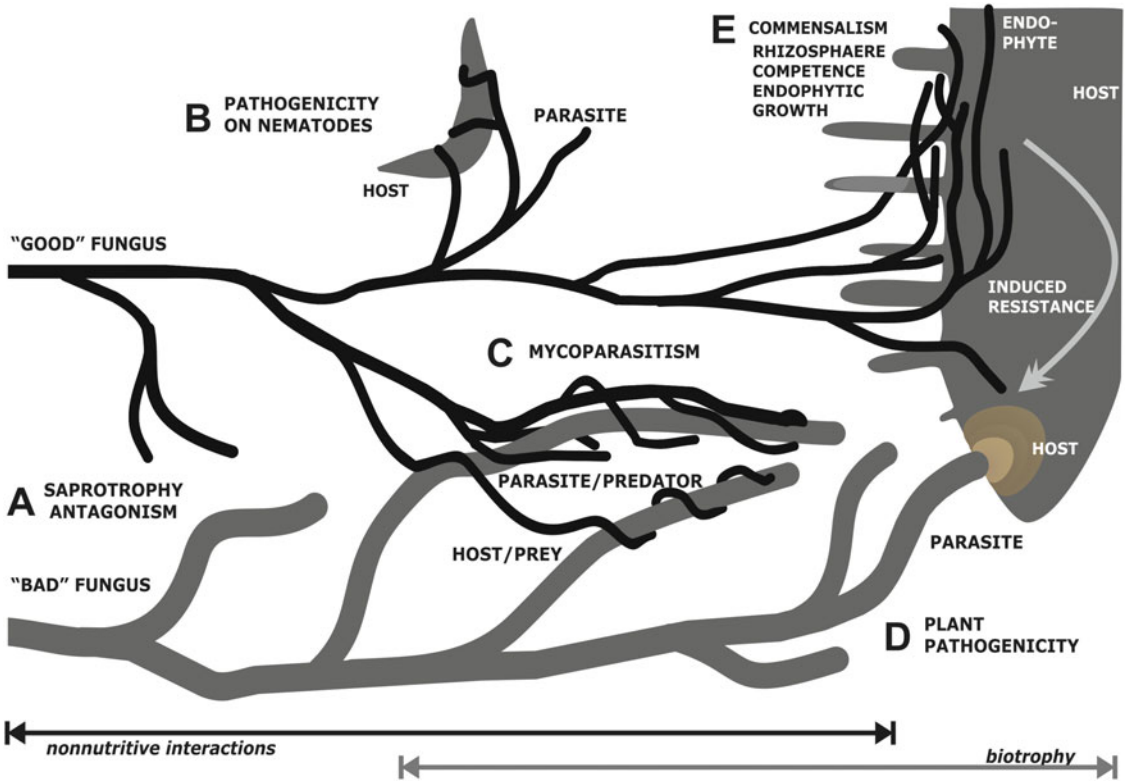

Fig. 12.5 A simplified overview of interfungal interactions that are relevant for biological control of plant pathogenic fungi and nematodes. Exclusively nonnutritive antagonistic interactions are depicted on pane (**a**), while (**b**–**d**) demonstrate cases of parasitism among which (**b**) and (**c**) are beneficial for the plant as the "good" fungus or bioeffector attacks either plant pathogenic nematodes (**b**) or plant pathogenic and therefore "bad" fungi (**c**). However, the cases of nutritive mycoparasitism (biotrophic and necrotrophic) may also be accompanied by nonnutritive interactions such as antagonism or agonism. Due to the potential applications of mycoparasitic fungi for crop protection, the so-called "bad" fungi are frequently labeled as pathogens even in the absence of their host plants when solely fungal–fungal interactions are investigated. However, in such studies, these "pathogens" serve as hosts for "good" fungi that parasitize on them; therefore, the latter ones—the "good" fungi—should rather be named as pathogens

A "good" label for a bioeffector organism is conditional and may only be applied in respect of exact interactions and an exact crop plant (Fig. 12.6). The application of mycoparasitic and antagonistic fungi for biocontrol allows to reduce the use of chemical pesticides which is usually strongly supported by the general public, and therefore respective research will likely attract more attention and funding. The Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 "establishing a framework for Community action to achieve the sustainable use of pesticides" (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32009L0128) contains the respective statement: "Appropriate risk management measures shall be taken and the use of low-risk plant protection products as defined in Regulation (EC) No 1107/2009 and biological control measures shall be considered in the first place" that illustrates the future trend toward reduced use of chemical pesticides under the need to increase crop production for the growing population. However, despite the generally accepted low risk, the release of bioeffectors in the environment may also have adverse effects on both agricultural and natural ecosystems. It appears to be conceivable that introduced biocontrol fungi in case of either importation or augmentation practices will increase competition pressure for naturally present plant-beneficial microorganisms including other fungi and bacteria. For instance, the most prominent and widely accepted as "good" fungus *Trichoderma* may parasitize on arbuscular mycorrhizal fungi *Gigaspora* (Diversisporales, Glomeromycota) that are used to enhance plant nutrition and stress resistance (Lace et al. 2015) or even affect the plant as demonstrated by the colonization of

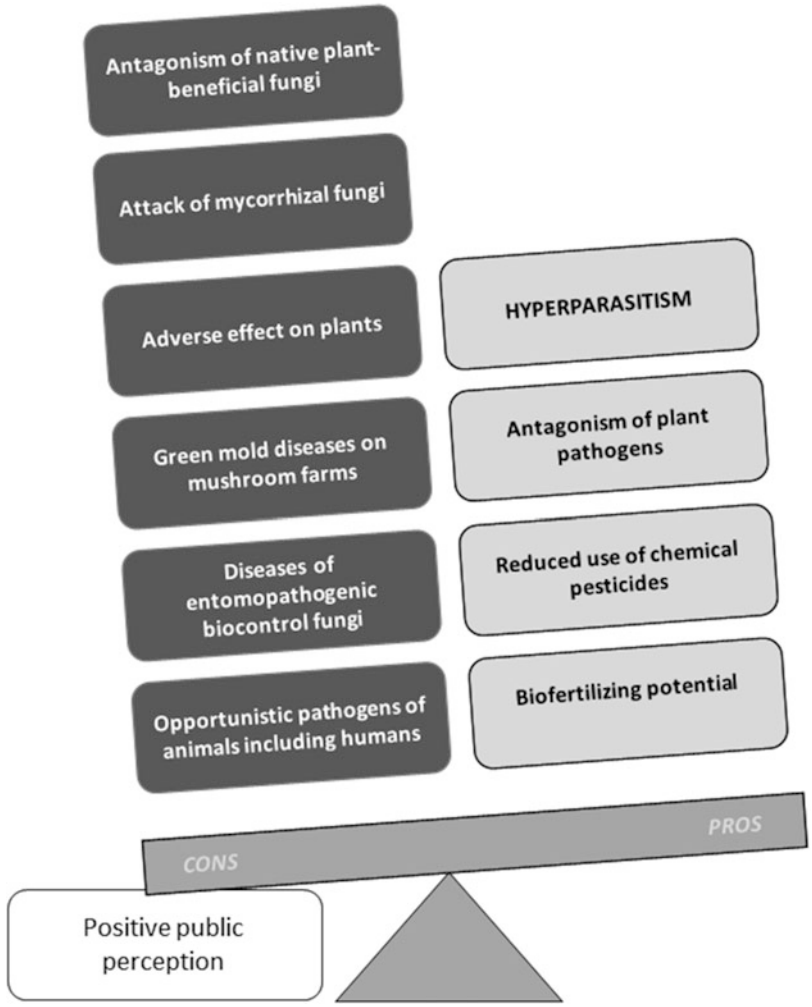

Fig. 12.6 Positive and negative arguments for the use of mycoparasitic fungi in biological control of plant pathogenic fungi

broad areas of the root epidermis of *Medicago truncatula* (Fabaceae, Angiosperms, Plantae) by *T. atroviride* leading to localized death. However, reports on direct adverse effects of biocontrol fungi on plants are rare: *T. viride* was diagnosed as a causative agent of dieback of *Pinus nigra* (Pinales, Plantae) seedling in Italy (Li Destri Nicosia et al. 2015) and different species of *Tilletiopsis* (Entylomatales, Basidiomycota) that are well-known antagonists of powdery mildews caused by Erysiphales fungi (Hijwegen 1986, 1989; Hoch and Provvidenti 1979; Klecan 1990; Knudsen and Skou 1993; Urquhart 1994). Smut fungi belonging to genus *Tilletiopsis* were demonstrated to cause "white haze" on the apple surface by Boekhout et al. (2006), in particular under conditions of ultralow oxygen storage. Clearly these fungi are able to reduce the growth of other fungi that contributes to their success as apple colonizers. The extensive colonization of harvested apples by *T. minor* and *T. pallescens* may diminish the prospects for their commercial application as biocontrol agents, as registration as a biocontrol agent will become more complicated (Baric et al. 2010).

Several studies also document the adverse effect of fungal hyperparasites on fungi used to control insect pests. It has been shown that the mycoparasitic *Syspastospora parasitica* (Hypocreales, Ascomycota) attacks *Beauveria bassiana* (Hypocreales, Ascomycota) growing on a Colorado potato beetle (*Leptinotarsa decemlineata*) cadaver (Klinger et al. 2006). Our own data indicate that this action that may also be

performed by almost any *Trichoderma* species (Druzhinina, Atanasova, unpublished) and thus the application of *Trichoderma* may counteract the positive role of *B. bassiana* on the control of the disease. Similar to this, the chytrid fungus *Gaertneriomyces semiglobifer* (Spizellomycetales, Chytridiomycota) is capable to parasitism of entomophthoralean gypsy moth *Lymantria dispar* pathogen *Entomophaga maimaiga* (Entomophthorales, Entomophthoromycota) in soil (Hajek et al. 2013). The authors propose that mycoparasitism, whether by *G. semiglobifer* or other mycoparasitic fungi, might be partially responsible for declines in azygospore reservoirs, especially under wet conditions where the motile zoospores of chytrids would have better access to susceptible fungal host spores.

Besides the direct impact on plants and plant-interacting microorganisms, fungi used in biocontrol may also have adverse effects on mushroom production (Castle et al. 1998; Hajek et al. 2013; Hermosa et al. 1999; Kim et al. 2012; Komon-Zelazowska et al. 2007; Kredics et al. 2010; Park et al. 2006) and animals including humans as opportunistic pathogens (Komon-Zelazowska 2014). Interestingly *T. longibrachiatum* that is the most frequently detected *Trichoderma* species capable to attack even immunocompetent humans (Kredics et al. 2003; Molnár-Gábor et al. 2013; Park et al. 2006; Sandoval-Denis et al. 2014) is still referred as a "good" biocontrol fungus (Ruocco et al. 2015). Moreover, the recent broad survey of clinically relevant *Trichoderma* species that was based on the detailed DNA barcoding demonstrated that almost all most prominent plant-beneficial *Trichoderma* species such as *T. harzianum*, *T. asperellum*, *T. atroviride*, *T. gamsii*, *T. koningiopsis*, and others are capable to attack immunocompromised humans (Sandoval-Denis et al. 2014). Last but not least, the materials presented in other chapters of this book on multiple and complex interactions between fungi and bacteria allow to assume the severe impact of introduced "good" but environmentally aggressive fungi on these communities, which may cause both positive and negative consequences for soil microbiome in general and consequently on plants.

Interestingly, to the best of our knowledge, up to now there are no reports published on adverse effects of *Clonostachys rosea* on humans, cultivated mushroom, or biocontrol insects. It could be possible that the mycoparasitic ability derived from herbivorous ancestors may possess fewer number of possible adverse effects compared to mycoparasites that evolved from an entomopathogenic-like organisms. No detailed ecological risk assessment analyses on the use of mycotrophic fungi have been performed. However, the newest genome-wide mechanistic and evolutionary studies would provide sufficient background for such research.

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13 Nematophagous Fungi

Alfredo Herrera-Estrella[1], Sergio Casas-Flores[2], Christian P. Kubicek[3]

CONTENTS

I. Introduction

The nematodes or roundworms constitute the diverse animal phylum Nematoda. Over 25,000 species have been described of which more than half are parasitic (Zhang 2013). They have successfully established in nearly all-ecological niches. Plant parasitic nematodes, which obtain their food from plant foliage and roots, cause a total global agricultural damage of more than $100 billion USD per year (Nordmeyer 1992). The most important nematodes thereby are the so-called "root-knot nematodes" (e.g., *Meloidogyne* spp.), which are globally distributed and infect more than 2000 plant species and thus reduce global crop yields by about 5 % (Park et al. 2014). Many nematodes that directly or indirectly affect plant growth have developed parasitic strategies to more efficiently exploit their source of food (Sijmons et al. 1994). Plant parasitic nematodes affect the plant directly by altering the morphology of the root system as a result of their feeding activities or by invasion of the plant tissue. The most specialized level has been reached by the sedentary endoparasitic nematodes that invade the root and partially reorganize the root function to satisfy their demand of nutrients (Jung and Wyss 1999). The nematode penetrates root tissues by means of its stylet and injects secretory fluids, produced in esophageal glands; these fluids modify the plant cytoplasm prior to food removal. Some species feed on the root tips of their host plants, which thereby become transformed into terminal galls, which contain necrotic cells and enlarged multinucleated cells that are essential for nematode development, growth, and reproduction. The most drastic alterations in root architecture are generated by cyst and root-knot nematodes. Root-knot nematodes are generally polyphagous, and each species can infect a large variety of plants species, from grasses to trees, by generating galls in the root. Due to their broad host range, these nematodes cannot be controlled by crop rotation. In contrast, cyst nematodes are highly host-specific parasites and can be effectively controlled by crop rotation using non-host plants (Timper 2014).

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Since the origins of agriculture, men have used diverse strategies to eliminate pests that attack crop plants. Today the most common plant parasitic nematodes are controlled with chemical nematicides, cultural practices, and by the use of resistant cultivars (Timper 2014). In spite of the "successful" use of chemicals to efficiently control plant pests, it has been determined that these compounds are highly hazardous to human health and the environment. Another disadvantage of chemical pesticides is their persistence in the environment, which favors the selection of resistant pests, leading to the use of more aggressive chemicals. These actions have generated concerns around the world. Consequently, there is a heightened scientific interest on the establishment of integrated pest management strategies in order to reduce the application of chemical pesticides, that should be more effective, and less pollution, such as traps, and other means of biological control.

Yet there are still other means, such as the direct introduction of a biological control agent, i.e., a specific organism that will rapidly reduce nematode populations and/or protect the growing seedling from damage (Flint and Dreistadt 1998). Nematophagous fungi are on the top of the list of natural enemies against nematodes, because they are found in diverse environments and have been shown to be very effective as biocontrol agents in early and recent studies (cf. Kerry 2000). They have therefore attracted the attention of scientists as model organisms for "carnivorous and/or eaters" of nematodes and the deciphering of the mechanism used by them to hunt their prey (Van Ooij 2011).

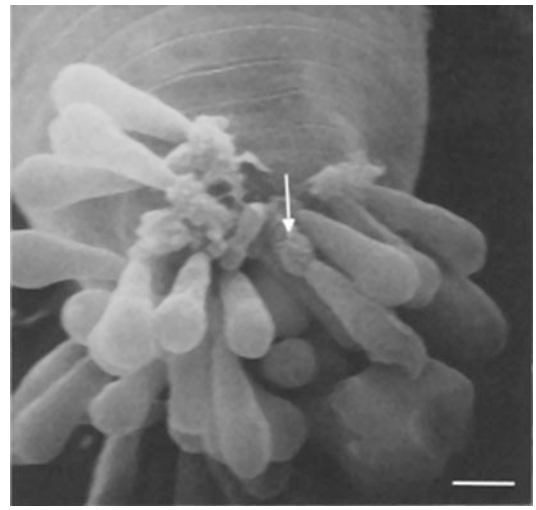

Fig. 13.1 Scanning electron micrograph of the head region of a nematode, fixed in glutaraldehyde, heavily infected with conidia. *Arrow*: adhesive bud of conidia. Bar: 2

II. Biology of Nematophagous Fungi

Nematophagous fungi are a diverse group of fungal species that use refined mycelial structures or their conidia to capture their prey. In addition, a large group of opportunistic fungi can parasitize the eggs and cysts of these worms (Niu et al. 2010). More than 200 species of parasitic fungi known differ in saprophytic/parasitic ability (Nordbring-Hertz et al. 2006). Therefore, they are classified in three main groups on the basis of their mechanism of interaction with the animal:

- Endoparasites: They are mostly obligate parasites and in most cases have a restricted host range. Their use for biocontrol is therefore limited (Stirling 1992). They infect nematodes by their spores (Fig. 13.1), either through ingestion or their attachment to the cuticle (Morton et al. 2004). Some endoparasites produce zoospores that are attracted to the nematodes before adhesion and encystment on the cuticle surface. *Esteya vermicola*, an endoparasite of the pine wood nematode *Bursaphelenchus xylophilus* (Wang et al. 2010), produces α-pinene, β-pinene, and camphor—volatile compounds that are also emitted by the pine—for attraction of *B. xylophilus* (Lin et al. 2013).
- Predators or trap-forming fungi: This group of predatory fungi is the most studied and comprises the most widely used species for the biological control of nematodes. They effectively reduce the respective nematode population both under laboratory and field conditions. The trap-forming fungi include the genera *Arthrobotrys*, *Duddingtonia*, and *Monacrosporium*. To catch nematodes, they produce special extracellular structures of adhesive networks and tridimensional traps, which can be differentiated into six types: (a) non-differentiated adhesive hyphae; (b) ramifications of hyphae that undergoes anastomosis, forming three-dimensional adhesive networks; (c) adhesive ramifications, which, at times, can join together forming simple two-dimensional adhesive networks; (d) adhesive nodules; (e) constricting rings; and (f) non-constricting rings (Yang et al. 2007a,

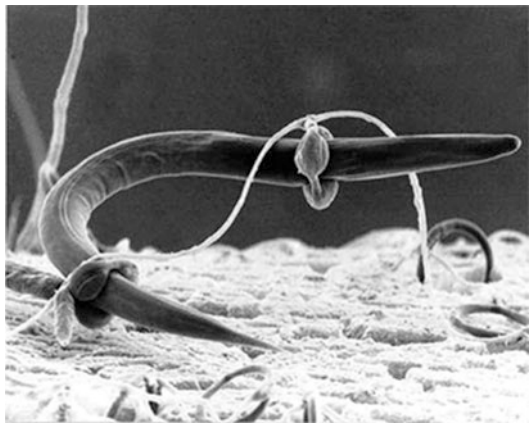

Fig. 13.2 Nematode captured by the constricting rings of the predatory fungus *Arthrobotrys anchonia*. Note that the ring cells "cushion" around the body of the victim but have not yet constricted the body. This is a very early stage after capture. Scanning Electron Micrograph N. Allin and G.L. Barron

b). The constricting ring is the only one that actively captures nematodes (Fig. 13.2). The most common trap in predatory fungi is the one composed of adhesive networks, which has undergone significant specialization during evolution (Yang et al. 2007a, b). Despite the fact that the morphology of the traps can vary extensively, nematode-trapping fungi are generalists, and they can infect many different nematode species (Nordbring-Hertz et al. 2011).

- Opportunistic fungi that parasitize eggs, cysts, and female nematodes: this group of fungi has been studied for a long time, as they were considered the most promising agents for the reduction of nematode and helminth populations because they reduce levels of viable eggs in the soil (Frassy et al. 2010; Mello et al. 2013; Braga and de Araújo 2014). They are opportunistic saprophytes and do not depend on the presence of the parasite in the soil for their survival. Their hyphae penetrate the eggshell through the small pores in the vitelline layer, causing changes in permeability of the shell and expanding its volume. The hyphae then move through the adjacent layer of chitin and lipid. They thus colonize the interior of the egg and also the developing larvae (Frassy et al. 2010; Dallemole-Giaretta et al. 2012; Araujo et al. 2013). Fungi, for which this ability has been shown, include *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium* Goddard), several *Trichoderma* spp. (particularly *T. longibrachiatum* and *T. harzianum*), *Paecilomyces lilacinus*, and *Dactyella ovoparasitica*. Some of them, like *P. chamydosporia*, grow much better on nematode infested roots than on healthy roots or in the soil (Kerry 2000) and show a genetic variability closely related to the host from which they were isolated (Morton et al. 2003), suggesting that the nematodes may be more important to these fungi, than just an eventual source of nutrients.

Trichoderma spp. significantly reduce nematode infection in plants as well as the number of egg masses per plant. In vitro experiments have shown a significant effect on nematode hatching due to egg weakness and mortality due to penetration of the eggs. Additionally, nematode eggs induced growth and chitinase production in *Trichoderma* (Sahebani and Hadavi 2008). The reduction in nematode infection in plants could also be attributed to the induction of defense-related genes and priming by *Trichoderma* spp. (Sahebani and Hadavi 2008; Salas-Marina et al. 2011; Velazquez-Robledo et al. 2011; Contreras-Cornejo et al. 2011).

Although successful biological control of plant pathogens has been reported, a single fungal or bacterial biological control agent often results insufficient for effective control. Thus, the combination of two or more biological control agents could enhance their activity to protect plants; however, this combination could result in incompatibility or even antagonism. In this regard, recently, 18 strains belonging to five species of the genus *Trichoderma* (*T. harzianum*, *T. virens*, *T. atroviride*, *T. rossicum*, *and T. tomentosum*) were tested against six strains of four nematode-trapping fungal species (*Arthrobotrys oligospora*, *A. tortor*, *Monacrosporium haptotylum*, and *M. cionopagum*). Interestingly, *T. harzianum* and *M cionopagum* showed nearly identical growth rate, and no coiling around the hyphae of the nematode-trapping fungi by *Trichoderma* was observed (Szabó et al. 2012). The antagonism assays for each *Trichoderma* species against *Caenorhabditis elegans* revealed that *T. harzianum* parasitized the most eggs during the time course of the examination.

Taxonomically, the nematode-trapping fungi are exclusively found in the lineage of

the Orbiliomycetes, which consists of a single order (Orbiliales) and one family (Orbiliaceae). Phylogenetic analysis placed Orbiliomycetes as a basal branch among the Pezizomycotina (James et al. 2006), the largest subphylum of the fungi that includes the vast majority of filamentous growing and fruiting-body-producing species. The time of divergence of nematode-trapping fungi from the other Pezizomycotina species is not completely clear: Yang et al. (2007a, b, 2012) estimated 400–520 Mya, whereas Meerupati et al. (2013) arrived at a much more recent time frame (198–208 Mya). The reason for this difference could be that the latter authors used 9632 orthologous genes (in contrast to five used by Yang et al. 2012). Also the calibration of the time scale differs: Meerupati et al. (2013) used the split between ascomycetes and basidiomycetes (500–650 Mya; Lücking et al. 2009), whereas Yang et al. (2007a, b) used two fossil records of carnivorous fungi, dated to 100 Mya and 24 Mya, respectively, which however is complicated by the uncertainties in the identification of the trap structures and the assignment of the taxa in fossils (Meerupati et al. 2013). Constricting rings are probably the most ancient device for nematode trapping because the fungi displaying this type of structure form a basal branch in the tree of nematode-trapping fungi (Ahrén et al. 1998).

Genotypification of 228 isolates from the nematode-trapping fungus *A. oligospora* from different ecological niches and geographical locations, by means of 12 single nucleotide polymorphic loci located at eight random DNA fragments, showed that ecological niche separation contributed significantly, whilst geographic separation contributed relatively little to genetic variation. The differences found between strains isolated from polluted zones versus those from non-contaminated locations suggested that environmental stress might have contributed to ecological divergence for populations. Thus, these data confirm the relevance of local adaptation and ecological niche specialization. Additionally, the lower level of differentiation among geographical populations suggested a long-distance dispersal and frequent gene flow because of the predominant clonal reproduction between populations. It was also found that those strains isolated from stressful niches presented more variability, an unambiguous evidence for recombination at the same geographic areas (Zhang et al. 2012).

To investigate the genetic variation over the geographic and ecological contexts, two virulence associated genes and two housekeeping gene fragments of 80 natural *Paecilomyces lilacinus* strains were analyzed. Using this approach, it was found that 32 and 19 multilocus genotypes were represented by a single isolate. Several multilocus genotypes were shared by multiple isolates, all of them from different geographical locations. Various degrees of polymorphisms and haplotypes were determined among the six genes analyzed. The analysis showed that *P. lilacinus* has a clonal reproduction mode in natural populations. These results suggest that these fungal isolates have limited geographic distribution and might have undergone strong genetic differentiation during their adaptation to environments in different geographic regions (Li et al. 2013).

III. Biology of Trap Formation and Nematode Infection

There are distinct differences in the mechanism of trapping with adhesive cells and the constricting ring. Fungi that feature adhesive traps capture nematodes by secretion of extracellular polymers that accumulate at the site of infection (Tunlid et al. 1992), whereas those with constricting rings ensnare the nematode by rapid swelling of a ring formed by three cells (Meerupati et al. 2013). When the nematode enters the ring, the cells inflate and the nematode is trapped. This closure occurs very rapidly (0.1 s) and is triggered by pressure of the nematode on the constricting-ring cells (Higgins and Pramer 1967). Ultrastructural examinations revealed that the cell wall of the constricting-ring cells is folded; when the cells inflate, the folded cell wall balloons out and forms the new cell wall (Heintz and Pramer 1972; Liu et al. 2012). Interestingly, nematodes may still be able to escape trap formation: the constricting rings of *Drechslerella doedycoides* catch early larval stages with a diameter, which is similar to the trap opening. Yet there is a short delay between the ring entry and ring closure, which allows the animal to withdraw from the trap before being caught. Mutants that fail to suppress head movements in response to touch are caught more efficiently than the wild-

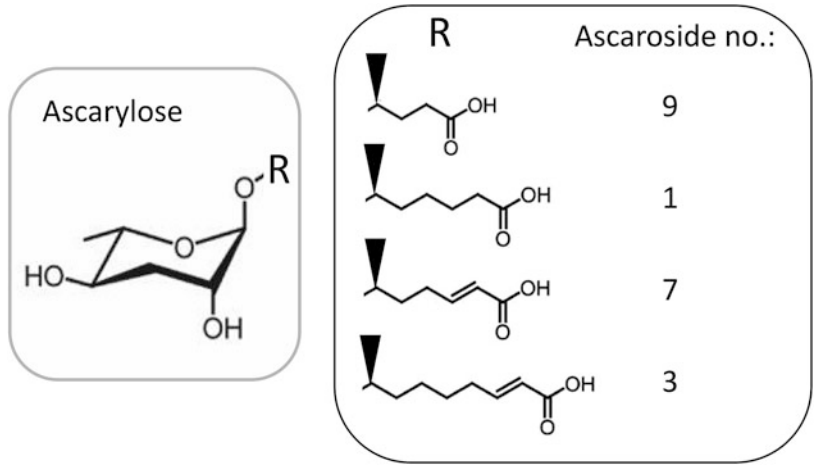

Fig. 13.3 Chemical structure of the ascaroside hormones from nematodes, according to Hsueh et al. (2013)

type. This demonstrates that the coordination of motor programs allows *C. elegans* to smoothly retract from a fungal noose and evade capture (Maguire et al. 2011).

In contrast, the adhesive trap is surrounded by a layer of fibrillar, extracellular polymers, which becomes reorganized during the attachment of the traps to the nematode cuticle (Tunlid et al. 1992). Following the trapping of nematodes, the infection mechanisms appear to be rather similar in the species with constricting rings and adhesive traps: the fungus forms a penetration tube that pierces the nematode cuticle and paralyzes the nematode. Subsequently, the internal tissues are rapidly colonized and digested by fungal hyphae (Nordbring-Hertz et al. 1995).

One striking feature of the nematode-trapping fungi is that they can sense the presence of their prey and only then form the traps. Earlier work demonstrated that nematodes secrete a morphogenic substance that induces trap formation in the fungi (Pramer and Stoll 1959). Hsueh et al. (2013) have recently shown that these substances are in fact ascarosides, nematode pheromones that are composed of the dideoxy-sugar ascarylose linked to a fatty acid-like side chain (Fig. 13.3). More than 100 different ascarosides have meanwhile been identified from nematodes and function as inter-organismal signals that play a central role in regulating nematode development and behavior (Butcher et al. 2007; Srinivasan et al. 2008, 2012). In both adhesive and constricting-ring types, the cuticles of the captured nematodes are then penetrated and finally an infection bulb is formed inside the nematode. After the nematode is killed, the fungus grows inside and feeds on it (Nordbring-Hertz et al. 1995). Interestingly, Li et al. (2011) showed that attachment of the soil bacterium *Chryseobacterium* spp. to the hyphae of *A. oligospora* also induced trap formation in the absence of a nematode host. However, the mechanism has not been determined.

Andersson et al. (2014) used comparative transcriptomics to investigate the molecular events during the infection process of two nematodes (the root-knot nematode *M. hapla* and the sugar beet cyst nematode *H. schachtii*) and of three nematode-trapping fungi that display different trapping mechanisms, i.e., adhesive nets (*A. oligospora*), adhesive branches (*M. cionopagum*), and constricting rings (*A. dactyloides*). They studied the phases of adhesion, penetration, and digestion stages. Their data showed that the divergence in interspecific gene expression between the three fungi was significantly larger than that inferred by the nematode host used. The core set of genes, identified by the Pfam domains of the encoded proteins, that was significantly expressed by all three fungi included serine endoproteases belonging to the subtilisin family, aspartyl peptidases, proteins containing a CFEM domain (a fungal-specific cysteine-rich domain that is found in some proteins with proposed roles in fungal pathogenesis), proteins involved in fungal stress response, cell signaling, organization of the cytoskeleton, vesicular transport and

membrane transport, as well as several families of calcium-binding proteins and transcription factors.

Among the species-specific transcripts were those encoding proteins of metallopeptidase families M1 and M24, lectins, tyrosinase, as well as some transcription factors and cell-signaling components, and proteins containing the WSC (cell *w*all integrity and *s*tress response *c*omponent) domain and the DUF3129 domain. The latter is a domain of unknown function that is found in the GAS1 protein of *Magnaporthe grisea*, which participates in appressorial penetration and lesion formation (Xue et al. 2002). The DUF3129 domain protein-encoding gene was highly expressed during infection among the species that forms adhesive branches and adhesive knobs. As for lectins, a fruiting-body lectin and a D-mannose-binding lectin were only highly expressed in *A. oligospora* and not in the other two fungi. Also, the WSC domain proteins, which are one of those gene families that are expanded in nematode-trapping fungi, are highly—yet differently—expressed during pathogenesis in different nematode-trapping fungi, suggesting that they contribute to the specialization of the trapping mechanisms. Two DUF3129-domain proteins, whose orthologs in other fungi were proven as virulence genes, were differently expressed by the three fungi (Andersson et al. 2014). Finally, a hydrophobin-like protein (AOL_s00006g570) was upregulated more than 12-fold in *A. oligospora*. Hydrophobins are able to assemble spontaneously into amphipathic monolayers at hydrophobic–hydrophilic interfaces (Linder et al. 2005). In *Beauveria bassiana*, a nonspecific hydrophobic interaction between the fungal spore coat hydrophobin and the insect epicuticle was found to be essential for the pathogenicity of the fungus (Zheng et al. 2011).

Trap formation has been shown to be favored under poor nutrient conditions. Chen et al. (2013) showed that the presence of the nematodes induces autophagy—a process characterized by the degradation of unnecessary or dysfunctional cellular components that are involved in morphogenesis and morphology in fungi—by nematodes during the early stage of trap formation in *A. oligospora*. This is illustrated by the high expression of the *atg8* gene, which encodes an essential protein in the autophagic pathway (Nakatogawa et al. 2007). Disruption of a homolog of this gene in *A. oligospora* leads to reduce trap formation (Chen et al. 2013). During the early stage of trap formation, the expression of genes encoding enzymes involved in amino acid biosynthesis and the general regulator of amino acid biosynthesis GCN4 are induced, suggesting that nematodes induce autophagy probably by triggering intracellular amino acid starvation.

A transcriptomic analysis of *Drechslerella stenobrocha*, which mechanically traps nematodes using a constricting ring also shed some light on the signal transduction cascade that is involved: like in entomopathogenic fungi during insect infection (Zheng et al. 2011), trap formation may involve the protein kinase C (PKC) pathway, as suggested by the strong upregulation of the *pkc1* gene during trap formation. The authors proposed that one of the Gα-proteins (DRE_07451) could be the first step in this PKC pathway, which could be activated by a signal from nematodes (see above). The authors also detected several putative transcriptional regulators of the fungal-specific Zn(2)Cys(6) type that could regulate the downstream genetic responses. Finally, they found that the transcription of genes encoding a cell division protein and a cyclin peaked during the phase of trap formation, suggesting that the formation of constricting ring would involve cell division processes. In a similar approach, global patterns of gene expression in traps and mycelium of the fungus *Monacrosporium haptotylum* were compared. In this case, the trap is a unicellular spherical structure called the knob, which develops on the apex of a hyphal branch. Substantial differences in the patterns of genes expressed in the two cell types were found, with about 23 % of the putative genes preferentially expressed in knobs. Various differentially expressed genes were similar to genes known to be involved in regulating morphogenesis and cell polarity in other fungi. This set of differentially expressed genes included several putative small GTPases, such as *rho1*, *rac1*, and *ras1*, and a rho GDP dissociation inhibitor (*rdi1*). Genes involved in stress responses, protein synthesis and protein degradation, transcription, and carbon metabolism were also among this set. A number of the differentially expressed genes are also differentially regulated during infection structure formation in plant-pathogenic fungi. Interestingly, *gks1* a homologue of the *Magnaporthe grisea* (*gas1*/*mas3*) gene, which is specifically expressed in appressoria, was found (Ahrén et al. 2005).

The initial phase of penetration is believed to be associated with recognition mediated by a lectin–carbohydrate interaction (Nordbring-Hertz 1983). These lectins are located on fungal traps or adhesive conidia that can specifically bind a carbohydrate on the nematode cuticle. It is has been suggested that after the recognition event, the fungus immobilizes the nematode and secretes extracellular enzymes at the point of contact that allow the posterior parasitism (Tunlid et al. 1994). The nematode cuticle con-

sists mainly of proteins, including collagens (Cox 1992), and the nematode eggshell contains chitin fibrils embedded in a protein matrix, with the chitin complex as a major barrier against fungal infections (Warton 1980). Extracellular enzymes that are capable of digesting the main chemical constituents of the nematode cuticle and eggshell (protein, chitin, and lipids) have been isolated and identified in various nematophagous fungi (e.g., Lopez-Llorca 1990; Tunlid et al. 1994; Yang et al. 2005a, b; for review see Yang et al. 2013a, b). When the hyphae from the endoparasitic fungi *Drechmeria coniospora* and *Hirsutella rhossiliensis* reach the eggshell, they form appressoria from which these extracellular enzymes are then secreted (Lopez-Llorca and Robertson 1992). This formation of appressoria depends on the recognition of the host surface, and surface hydrophobicity is considered an important recognition factor in this process (Lopez-Llorca et al. 2002).

V. Virulence Factors

Virulence factors are molecules produced by pathogens, which are essential for major contribution to their pathogenicity, by enabling them to attach to the host, escape the defense mechanisms, and finally feed on it. The previous chapter on the molecular mechanisms that take place during attack of the nematode by the fungus has already pointed to potential candidates for virulence factors. In this chapter, we will discuss those components for which detailed scientific information is already available.

A. Proteases

Being saprophytes, most Pezizomycota are known to possess a rich arsenal of proteolytic enzymes. According to the MEROPS database, the main groups found are aspartyl proteases, cysteine proteases, metalloproteases, and serine proteases, the latter two making up for the bulk of secreted proteases (cf. Lai et al. 2014). Comparative genomics indeed showed that some families of the metalloproteases (particularly those that show collagenase activity) and of the subtilisin-type of the serine proteases are strongly enriched in the nematophagous fungi (Meerupati et al. 2013; Lai et al. 2014; Fig. 13.4).

1. Collagenases

The ascarid cuticle is a three-layered, fibrous structure, which contains nematode-specific types of collagen and keratin (Bird 1971). Collagens are among the most complex of proteins and are slowly degraded in natural soils and waters (Weiss 1976). During the infection of nematodes, nematophagous fungi must penetrate the nematode cuticle, and particularly collagenases have been considered important enzymes involved in the pathogenicity of nematophagous fungi (Dackman et al. 1992; Tunlid et al. 1994). Meerupati et al. (2013) showed that peptidases belonging to the MEROPS family M10, which display strong collagenolytic activity, are strongly enriched in *A. oligospora* and *M. haptotylum*, whereas these genes are almost absent from insect pathogenic fungi. This is in agreement with earlier studies that nematode-trapping fungi produced collagenase in the growth media of all tested species (Schenck et al. 1980).

The M10 metallopeptidases belong to a group of peptidases known as the “metzincins” due to a conserved methionine C-terminal to the zinc ligands. Both subfamilies of M10 contain mosaic proteins, which contain, e.g., glycine-rich C-terminal domains that can bind calcium ions or domains homologous to hemopexin and vitronectin that aid in binding to the extracellular matrix. The gelatinases have acquired three domains homologous to type II segments of fibronectin nested within the peptidase unit. Details about the structure of the fungal M10 metalloproteases, as well as genetic proof for their action as virulence factors, are however yet lacking.

2. Subtilisins

Almost all the proteases from entomopathogenic and nematophagous fungi that were iden-

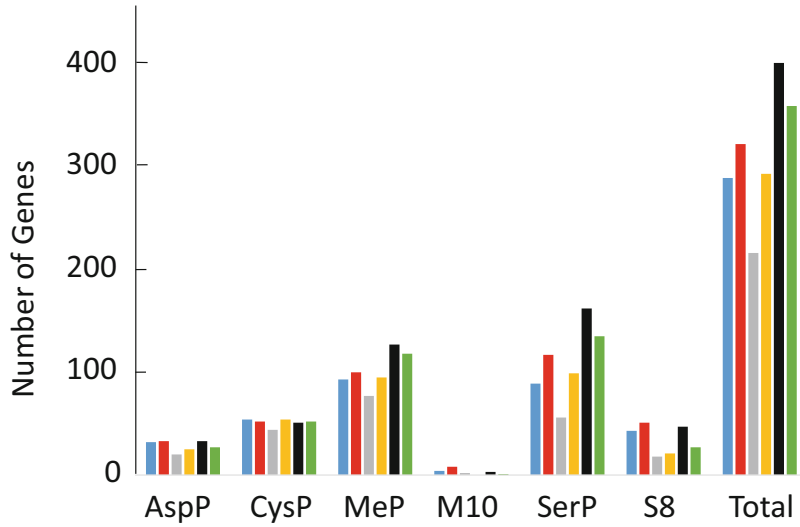

Fig. 13.4 Number of selected protease genes in the genomes of nematophagous and insect pathogenic fungi. Fungal species are indicated by color bars: *blue*: *A. oligospora*, *red*: *M. haptotylum*, *grey*: *D. stenobrocha*, *yellow*: *H. minnesotensis*, *black*: *M. anisopliae*, *green*: *B. bassiana*. Abbreviations: *AspP* aspartyl proteases, *CysP* cysteine proteases, *MeP* metalloproteases, *M10* MEROPS family M10 metalloproteases within MeP, *SerP* serine proteases, S8 MEROPS family S8 subtilisins within SerP, *Total* all protease genes encoded in the respective genome. Data taken from Yang et al. (2011a, b), Meerupati et al. (2013), Liu et al. (2014), Lai et al. (2014), Gao et al. (2011), and Xiao et al. (2012)

tified and shown to have nematicidal activity in the pre-genomic era belonged to the large subtilisin family of endopeptidases MEROPS M8 found only in fungi and bacteria. This family is enriched in nematophagous fungi, but also in insect pathogens (Meerupati et al. 2013; Lai et al. 2014). The role of serine proteases as virulence factors was first demonstrated in invertebrate pathogenesis by the entomopathogenic fungi *Metarhizium anisopliae* (St. Leger et al. 1987) and *B. bassiana* (Bidochka and Khachatourians 1987), where a 30 kDa serine protease (Pr1) was found to play an important role in the infectious process (Morton et al. 2004). Proteases that share characteristics with Pr1, such as size, sensitivity to inhibitors, and substrate pattern (thus called Pr1-like), were first purified and characterized from the opportunistic nematophagous fungi *Paecilomyces lilacinus* (Bonants et al. 1995), *P. rubescens* (Lopez-Llorca 1990), and *P. chlamydospora* (Segers et al. 1994).

In *A. oligospora*, subtilisins appear to play a key role in the early stages of infection, including immobilization of the captured nematode (Tunlid and Jansson 1991; Åhman et al. 2002; Yang et al. 2011a, b). The *A. oligospora* subtilisin PII immobilizes the active stages of *Panagrellus redivivus* and hydrolyzes its cuticle (Tunlid et al. 1994). The enzyme is expressed under starvation conditions (Åhman et al. 1996). Another subtilisin from *A. oligospora* (Aoz1), whose amino acid sequence is 97 % similar to that of PII, also produced dramatic structural changes in the nematode cuticle (Minglian et al. 2004). Deletion of the *PII* gene had only a limited effect on pathogenicity (decreased adhesion and immobilization of nematodes and formation of less traps), probably due to the presence of *aoz1*. Overexpression of the PII-encoding gene, however, resulted in a higher capacity to kill nematodes and formation of more traps (Åhman et al. 2002).

More recently, a genome-wide transcriptional analysis of *A. oligospora* revealed that in fact the gene encoding yet another subtilisin (P186) was more than 40-fold upregulated, while that encoding PII was even downregulated, suggesting that P186 may be the main protease required for penetration of the nematode cuticle. In fact, a protease belonging to the same subfamily in S8 as P186 was also found upregulated in the knob proteome of *M. haptotylum* (Andersson et al. 2013).

To better understand the cellular functions of adhesive traps, the proteome and transcriptome of trap cells versus mycelia of the fungus *Monacrosporium haptotylum* were assessed. The comparison of protein expression between mycelia and knobs revealed that 54 out of 336 detected proteins were highly expressed in the knobs compared with mycelia. Secreted proteins were overrepresented: secretion signals were predicted in 26 sequences (48 % of de proteins identified), including Small secreted cysteine-rich proteins (SSCRPs), peptidases and carbohydrate-binding proteins containing WSC and GLEYA domains, and proteins involved in stress response. WSC is a cysteine-rich domain with eight conserved cysteine residues that are required for its function (Heinisch et al. 2010; Dupres et al. 2011). All the upregulated WSC domain proteins belong to a large expanded cluster of paralogs in *M. haptotylum*. Various peptidases and homologs of experimentally verified proteins in other pathogenic fungi were also upregulated in the knob proteome. The expression of only six of the upregulated knob genes was reflected in increased protein levels. These proteins included a putative surface protein of the PA14_2/GLEYA family, a glutathion S-transferase, an alcohol dehydrogenase, and two hypothetical proteins with predicted secretion signals. In agreement with the upregulated proteome, the upregulated transcriptome was also enriched in sequences predicted to have a signal peptide (20 %). Therefore, the traps of *M. haptotylum* seem to have the necessary proteins for the early stages of infection (Andersson et al. 2013).

Subtilisins also appear to play dominant roles in the infection by non-trap forming nematophagous fungi: infection of nematode eggs by *D. coniospora* was blocked by the addition of the serine protease inhibitor, chymostatin, indicating the possible role of serine proteases in the infection process (Jansson and Friman 1999). Addition of serine protease inhibitors reduced egg penetration by the fungi *Lecanicillium lecanni* and *P. chlamydosporia*, further supporting the relevance of proteases at the early stages of the infection process (Lopez-Llorca et al. 2002). A subtilisin named P32 was immunolocalizated in appressoria of the fungus *P. rubescens* that infects eggs of the beet cyst nematode *Hetrodera schachtii* (Lopez-Llorca and Robertson 1992).

The opportunistic fungus *P. chlamydosporia* produces an alkaline subtilisin (VCP1) during the infection of nematode eggs. The incubation of nematodes eggs with purified VCP1 resulted in the removal of the outer vitellin membrane from eggs of *Meloidogyne incognita* (Segers et al. 1994). Subsequent infections of these eggs by *P. chlamydosporia* degraded extensively the eggshell to the degree of generating large holes in the structure, with no evident formation of appressoria while this was not the case when eggs of *G. pallida* were treated with VCP1, which points to the importance of the different composition of nematode eggshells (Morton et al. 2004).

Sequence analysis of the *vcp1* upstream region from 30 different isolates of *Pochonia chlamydospora* revealed that this region is highly conserved, ranging from 91 to 97 % identity, and contains putative regulatory motifs for carbon (CREA and CREB) and nitrogen repression (GATA), and pH regulation (PacC). Indeed, the presence of glucose, ammonium, and changes in pH affected the expression of this gene. For instance, addition of glucose to the growth medium significantly repressed VCP1 enzyme and mRNA levels, whereas the presence of *M. incognita* eggs did not downregulate neither the VCP1 enzyme nor the mRNA. Furthermore, the presence of ammonium chloride significantly reduced the VCP1 mRNA and proteins levels; however, at longer times (24 h), the enzyme and the mRNA levels were considerably upregulated (Ward et al. 2012). Cryo-scanning electron microscopy revealed that VCP1 production occurred only when the fungus and *P. chlamydospora* eggs are in close contact. These results indicated that the presence of preferable carbon sources and unfavorable pH in the rhizosphere/egg-mass environment might negatively affect the nematode parasitism by *P. chlamydosporia*. On the contrary, the presence of ammonium nitrate may favor the biocontrol of this nematode by the fungus at longer times (Ward et al. 2012).

The regulation of the expression of subtilisins has also been studied in the case of *prC* in *Clonostachys rosea*, whose encoded protein immobilized nematodes and hydrolysed proteins of the nematode cuticle (Li et al. 2006): the presence of putative transcription control sites in the promoter for nitrogen regulation (5′-GATA), carbon regulation (5′-SYGGRG), pH regulation (5′-GCCARG), and stress response element (STRE) (5′-AGGGG) suggested that the expression of *prC* may be regulated by nitrogen sources, environmental pH, and/or other stress conditions (Zou et al. 2010a, b). To study the effect of pH, the *C. rosea* orthologue of the pH transcriptional regulator PacC was deleted. The expression of *prC* was downregulated in Δ*pacC* mutants, and the *prC* transcript levels were significantly higher under alkaline growth conditions than under acidic growth conditions. Induction of *prC* expression by nematode cuticles was significantly suppressed by glutamine, ammonia, and serine protease inhibitors (Zou et al. 2010c).

Aside from pH and nematode cuticle-induced changes of gene expression, the expression of *prC* was also upregulated by oxidants (H_2O_2 or menadione) and heat shock, probably through a stress response pathway. Interestingly, the addition of nematode cuticle significantly attenuated the production of reactive oxygen species induced by oxidants and heat shock in the wild-type strain but not in the Δ*prC* mutants (Zou et al. 2010b). This suggests that PrC is not only involved in the degradation of nematode cuticles but also plays a role in the adaptation to environmental stresses.

The serine protease Ver112 from the nematophagous fungus *Lecanicillium psalliotae* is capable of degrading the nematode cuticle and killing nematodes effectively (Yang et al. 2005a, b). The Ver112 gene was used to genetically transform *P. lilacinus*. Protease activity of the transformants was higher than in the wild-type and correlated with a stronger ability to immobilize, infect, and degrade the nematode *Panagrellus redivivus* and *Caenorhabditis elegans* than the wild-type. The crude protein extract of the transformants showed enhanced nematicidal activity compared to the wild-type (Yang et al. 2011a, b).

The evolution of subtilisin-like serine proteases in Pezizomycotina has been analyzed (Li et al. 2010): molecular phylogeny divided the serine proteases from nematophagous fungi into two clades with neutral proteases from nematode-trapping fungi clustering in clade A and the alkaline ones from nematode-parasitic and entomopathogenic fungi clustering in clade B. Both share a high degree of sequence identity, have very similar molecular structure, and play a similar role in degrading host cuticle during fungal infection of nematodes. However, their structure reveals interesting differences in the 3D structure of the substrate-binding regions and some neighboring loops and turns (Liang et al. 2010; Yang et al. 2010a, b): disulfide bridges, which contribute to the stabilization of the local/global structures and enhance the structural flexibility of two of the substrate sites, were only present in the alkaline, but not in the neutral protease. This may explain why the alkaline proteases have higher substrate affinity and catalytic activity than neutral proteases. Since nematode-parasitic fungi, which contain only alkaline proteases, do not produce trapping devices, they likely rely mainly on the extracellular enzymes as virulence factors to help them penetrate and digest nematode cuticles (Huang et al. 2004; Yang et al. 2007a). It is likely that their subtilisins evolved towards increased activity and broad substrate specificity. In contrast, the nematode-trapping fungi degrade the trap-captured nematode without time constraints because it is already paralyzed by the trap. This interpretation is also supported by the fact that the subtilisin-like serine proteases of the nematode-trapping fungi were found to be under positive selection, suggesting co-evolution of trapping structures and proteolytic enzymes (Li et al. 2010).

B. Chitinases

Nematophagous fungi that parasitize nematode eggs must penetrate the eggshell during the infection (Lopez-Llorca and Duncan 1988; Lýsek and Krajcí 1987). As mentioned above, the structure of the eggshell is formed by several layers, including one formed by chitin (Warton 1980), which is the thickest and probably the major barrier for infection (Bird and Bird 1991).

Most fungi are able to degrade chitin, and two main enzyme classes cooperate in its degradation: chitinases (belonging to the glycoside hydrolase family GH18) and *N*-acetyl-β-D-glucosaminidases (belonging to GH20). The action of the former leads to soluble chitooligosaccharides with a chain length of at least two amino sugar units, which are subsequently further hydrolysed to NAcGln by the *N*-acetyl-β-D-glucosaminidases. Furthermore, chitin can be deacetylated by chitin deacetylases (EC 3.5.1.31) found in carbohydrate esterase family 4 (CE 4) in the Carbohydrate Active Enzymes database (CAZy) classification (Hartl et al. 2012).

Fungal chitinases can be further divided into three different subgroups, A, B, and C, based on the amino acid sequences of their GH18 modules. These subgroups differ in the architectures of their substrate-binding cleft and thus their enzymatic activities (exo vs. endo), and those from subgroups B and C contain different carbohydrate-binding modules (CBM 18 and

CBM50; see also www.cazy.org; Gruber and Seidl-Seiboth 2011; Seidl 2008). Class A chitinases comprise some of the most frequently described fungal chitinases. They typically have a Mr (Molecular weight range) between 40 and 60 kDa, their active center is located in a deep cleft, and they are exo-acting. Class B enzymes are usually somewhat smaller (30–50 kDa), endo-acting, their active center is located close to the proteins surface, and they typically contain a carbohydrate-binding domain at their C-terminus (frequently of the cellulose-binding CBM1 type). Class C chitinases, in contrast, are large proteins (120–200 kDa) that act in an exo-type with an active center in a deep and narrow cleft. Most typical for them is the presence of a CBM18 chitin-binding or CBM50 peptidoglycan-binding (LysM) domain. The presence of CBMs in class B or C chitinases enables them to bind more tightly to insoluble substrates (Eijsink et al. 2008). Bacterial proteins with LysM domains have been reported to be involved in specific recognition events between nitrogen fixing bacteria and their plant hosts (Knogge and Scheel 2006). Besides nutritional purposes, subgroup B chitinases appear also to be involved in mycoparasitic and entomopathogenic functions.

The first report for chitinase activity in nematophagous fungi was in *Verticillium* spp., isolated from infected nematode eggs, both in screening on solid media with colloidal chitin and in liquid media. Several chitinases have also been identified from egg-parasitic fungi, which were found to serve as a nematicidal factor in infecting nematode eggs (Tikhonov et al. 2002; Khan et al. 2004; Gan et al. 2007). On the basis of their properties, most of them seem to belong to the A subgroup.

The *Arthrobotrys oligospora* genome contains 16 Open Reading Frames encoding putative chitinases that belong to the glycoside hydrolases (GH) family 18. These chitinases vary considerably in their functional domains, size, and pI. Based on the phylogenetic relationship, these were grouped into four clades: I, II, III, and IV, that include an *A. oligospora*-specific subclade (Clade IV-B), which includes chitinases $\geq$100 kDa. Most of the *A. oligospora* chitinases genes are downregulated in absence of carbon; conversely nitrogen starvation upregulates all chitinase-encoding genes. Nonetheless, chitinase AO-190 was upregulated in both, carbon and/or nitrogen starvation. Furthermore, chitinases AO-59, AO-190, and AO-801 increased their transcription in the presence of colloidal chitin or *R. solani* cell wall. This suggests a role of *A. oligospora* chitinases in biocontrol. The expression patterns of *A. oligospora* chitinases suggest that they play different roles in growth, differentiation, and infection (Yang et al. 2013a, b).

When five *Trichoderma* species (*T. harzianum*, *T. virens*, *T. atroviride*, *T. rossicum*, and *T. tomentosum*) were tested against *C. elegans*, *T. harzianum* was more successful at parasitizing eggs. During egg parasitism, the expression levels of chi18-12 and chi18-5 were significantly higher than controls, which suggest a role of these endochitinases in the infection process (Szabó et al. 2012).

C. Lectins

It has for a long time been assumed that the adhesion of nematophagous fungi to their host might be mediated by the interaction between lectins on the surface of trapping devices or adhesive spores and carbohydrate ligands on the nematode cuticle (Nordbringhertz and Mattiasson 1979). Lectins are carbohydrate-binding proteins that are present in all organisms. A comparison of the genomic inventory of trap-forming fungi and nematode pathogenic or insect pathogenic fungi revealed that the trap-forming species indeed had a much higher number of lectin-encoding genes than other fungi. A detailed analysis showed that the most abundant lectin family—like in other fungi—are the concanavalin A-like lectins that bind α-D-glucose and α-D-mannose (Fig. 13.5). However, the ricin B-type lectins (PF14200), the H-type lectin (binding to *N*-acetyl-β-D-galactosamine), the fucose-specific lectin, and the bulb-type lectin were all significantly more abundant in the trap-forming species *A. oligospora*, *D. stenobrocha*, and *M. haptotylum* (Lai et al. 2014).

During trap formation and infection, all trap-forming fungi expressed transcripts encoding RicinB_lectins, which are ribosome-inactivating proteins (RIPs) consisting of a catalytic A-chain and a sugar-binding B-chain (Michiels et al. 2010). The effects of these lectins from trap-forming fungi on the nematode have not yet been studied, but a RicinB_lectin_2 domain-containing protein (MOA) of the basidiomycete *Marasmius*

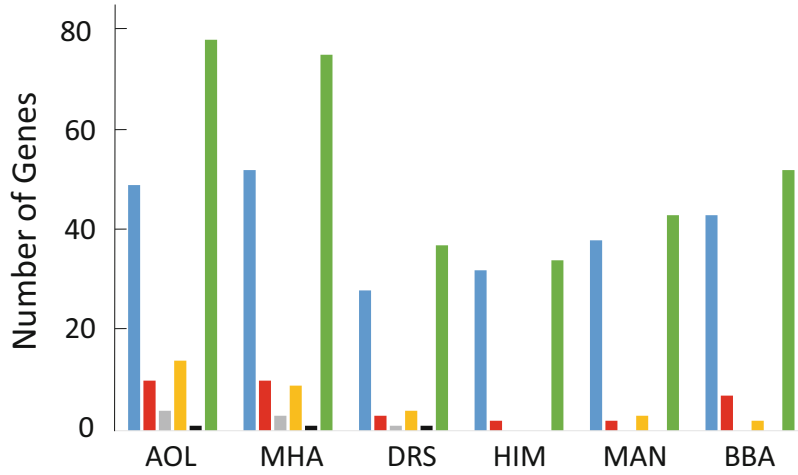

Fig. 13.5 Number of selected lectin-encoding genes in the genomes of nematophagous and insect pathogenic fungi. *Blue* Concanavalin A lectin; *red* ricin B-like lectin; *grey* H-type lectin; *yellow* fucose-specific lectin; *black* bulb-type lectin; *black* total number of lectins. Abbreviations: AOL *A. oligospora*, MHA *M. haptotylum*, DST *D. stenobrocha*, HMI *H. minnesotensis*, MAN *M. anisopliae*, BBA *B. bassiana*. Data taken from Yang et al. (2011a, b), Meerupati et al. (2013), Liu et al. (2014), Lai et al. (2014), Gao et al. (2011) and Xiao et al. (2012)

oreades displayed nematotoxic activity against *C. elegans* (Wohlschlager et al. 2011). This nematotoxicity was dependent on the cysteine protease activity of MOA and the binding of its lectin domain to glycosphingolipids in the worm intestine. A *Sclerotinia sclerotiorum* agglutinin (SSA) also contains a RicinB-lectin_2 domain and shows insecticidal properties when fed to the pea aphid *Acyrthosiphon pisum* (Hamshou et al. 2010). Most recently, a ricin B-like single-domain lectin (MpL) has been isolated and characterized from the parasol mushroom *Macrolepiota procera* (Żurga et al. 2014). MpL exhibits highest specificity for terminal *N*-acetyllactosamine and related β-D-galactosides and contains a second putative carbohydrate-binding site with a low affinity for D-galactose. MpL was shown to be toxic to *C. elegans*. Summarizing, there is now accumulating evidence that the RicinB-lectins exhibit toxicity to nematodes, and they could well be the agent that paralyzes the host after forming the trap. This is a challenging topic of further work with the trap-forming fungi.

D. Small Secreted Cysteine-rich Proteins

One of the largest groups of proteins secreted by mycoparasitic fungi like *Trichoderma* spp. are the so-called "small secreted cysteine-rich proteins (SSCPs)." They were identified by the criteria that their M_r should be $\leq$300 amino acids long and containing four or more cysteine residues. Among them, hydrophobins, hydrophobin-like proteins, and elicitor-like proteins make up for a major part, but many others are found for which no function has been predicted. In *Trichoderma* spp., some members of this cluster contain CFEM domains or consensus sequences for glycosylphosphatidylinositol (GPI anchors), suggesting that they could be cell surface proteins with important roles in the interaction with other organisms, as in *C. albicans* (Druzhinina et al. 2012). Meerupati et al. (2013) detected that "SSPs" make up for a large amplified group of orphan genes of the "knob-forming" fungus *M. haptotylum*—but not in the "net-forming" *A. oligospora*. 27.6 % of them were actually clustered in the genome, of which 34 genes that were >10-fold upregulated in *M. haptotylum* during early infection were located in clusters. This suggests that the SSPs could play an important role in knob formation in this species.

Proof for a function of SSPs has been obtained in the case of cerato-platanins, a group of small, secreted, cysteine-rich proteins that have been implicated in virulence of certain plant pathogenic fungi and also shown to

stimulate plant defense against pathogenic fungi (Djonovic et al. 2007; Salas-Marina et al. 2015; Pazzagli et al. 2014).

The nematophagous fungus *Dactylellina cionopaga*, which is a known parasite of the nematode plant pathogens *Meloidogyne javanica* and *Heterodera schachtii*, develops adhesive columns and two-dimensional networks (Khan et al. 2006; Jaffee and Muldoon 1995). The cerato-platanin family of proteins plays roles in parasitism, recognition, adhesion, cell-wall morphogenesis, fungal growth and development, and induction of the systemic resistance to pathogens in plants (Djonovic et al. 2007; Salas-Marina et al. 2015). The transcription levels of *D. cionopaga snodprot* increased in the presence of nematodes and was induced during the development of traps and conidia. The recombinant protein changed the chemotaxis and increased the body-bend frequency of *C. elegans*, but did not induce immunity in plants. In agreement with the parasitism mechanisms of nematophagous fungi, chemotaxis, and locomotion mechanism of *C. elegans*, the possible targets of snodprot could be ASE and ASI neurons, which are involved in the process of chemotaxis to NaCl, the response to serotonin, and the locomotion of *C. elegans* (Yu et al. 2012). Together these results indicated that snodprot is a novel parasitism-related protein of nematophagous fungi with a non-described activity.

E. Secondary Metabolites

In order to antagonize or kill their competitors, many microorganisms produce toxic metabolites, such as antibiotics. Toxins are particularly important for parasitic microorganisms, because they facilitate infection by debilitating the host (Morton et al. 2004). In addition, *P. lilacinus* produces acetic acid to paralyze juvenile nematodes (Djian et al. 1991).

So far, the majority of nematicidal secondary metabolites characterized are those produced by opportunistic fungi. *Fusarium equiseti* produces compounds that reduce hatch of root-knot nematode eggs and immobilize infective juveniles (Nitao et al. 1999); a metabolite with nematicidal activity against infective juvenile, phomalactone, was isolated from *P. chlamydosporia* (Hellwig et al. 2003); and culture filtrates from several fungi grown in malt extract broth were toxic to infective juveniles and eggs (Chen et al. 2000). Several secondary metabolites have been isolated from the nematode egg-parasite *P. chlamydosporia*, including radicicol (=monorden; a resorcylic acid lactone), tetrahydromonorden, pseurotin A, pochonins A to J (Hellwig et al. 2003 and Shinonaga et al. 2009; Zhou et al. 2010), and various aurovertin-type metabolites (Niu et al. 2010). Radicicol biosynthesis has been studied in detail, as usually found in fungal genomes, the genes encoding the corresponding biosynthetic pathways are clustered (*rdc1–rdc5*; Zhou et al. 2010) and encode two fungal iterative polyketide synthases (PKS). Rdc5, the highly reducing IPKS, and Rdc1, the nonreducing IPKS, are required for the biosynthesis of radicicol. The biochemical pathway, by which Rdc1 and Rdc5 catalyze the biosynthesis of radicicol, and how the remaining genes of the cluster contribute, has been elucidated (Zhou et al. 2010). During endophytic root colonization, *P. chlamydosporia* expressed 56 % of the secondary metabolism pathway genes found, including seven of the radicicol cluster.

So far, 179 nematicidal compounds belonging to diverse chemical groups have been identified from nematophagous fungi, of which only three (oligosporon, 4′,5′-dihydro-oligosporon, and linoleic acid) were from *A. oligospora* (Li et al. 2007). A genomic comparison of PKS, nonribosomal peptide synthases, and terpene synthases (TPS) revealed that the trap-forming fungi contained the lowest number of these genes (Fig. 13.6), whereas the nematode endoparasite *H. minnesotensis* exhibited the highest number of secondary metabolite synthases, even in comparison with insect pathogens, particularly of the PKS-1, NRPS, and TPS class (Lai et al. 2014). Six NRPS and two TPS genes were unique to the nematode endoparasitic fungus, suggesting lineage-specific expansion of these families in the *H. minnesotensis* genome. Thus, while trap-forming fungi do not seem to make strong use of secondary metabolites (possibly because the lectins already paralyzed the nematodes; vide supra), the endoparasites heavily rely on these metabolites to kill the host.

Trichoderma spp. are biocontrol agents widely used in plant protection due to their capacity to antagonize phytopathogenic fungi. Nevertheless, it is known that some *Trichoderma* spp. produce secondary metabolites with nematicidal activity, including trichoder-

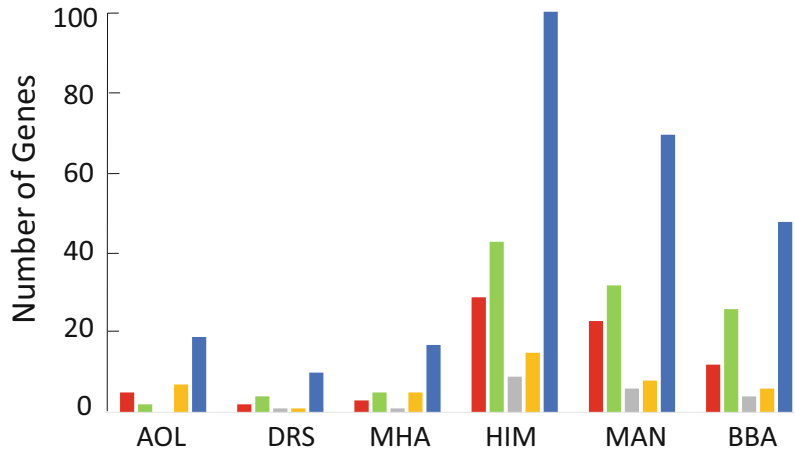

Fig. 13.6 Number of secondary metabolite synthases in the genomes of nematophagous and insect pathogenic fungi. *Red*: polyketide synthases (PKS), *green*: non-ribosomal peptide synthases (NRPS), *grey*: hybrid PKS-NRPS, *yellow*: terpene synthases, *blue*: total number of synthases lectins. Abbreviations: AOL *A. oligospora*, MHA *M. haptotylum*, DST *D. stenobrocha*, HMI *H. minnesotensis*, MAN *M. anisopliae*, BBA *B. bassiana*. Data taken from Yang et al. (2011a, b), Meerupati et al. (2013), Liu et al. (2014), Lai et al. (2014), Gao et al. (2011), and Xiao et al. (2012)

min (Yang et al. 2010a, b), acetic acid (Djian et al. 1991), gliotoxin (Watanabe et al. 2004; Anitha and Murugesan 2005), and the peptide cyclosporin A. Recently, the volatile organic compound 6-pentyl-2H-pyran-2-one from *Trichoderma* spp. was shown to kill >85 % of *Panagrellus redivivus*, *Bursaphelenchus xylophilus*, and *C. elegans* in 48 h at 200 mg/l (Yang et al. 2012).

VI. Plant Endophytism by Nematophagous Fungi

Several nematophagous fungi may be found as endophytes of plant roots. A plant endophyte is a plant microbial endosymbiont, which lives part of its live cycle in the plant, without provoking negative effects to its host. On the contrary, the presence of endophytes frequently results in positive effects in plant growth and development. Nematophagous fungi such as *P. chlamydosporia* and *A. oligospora* can present endophytic life style for both monocots (Lopez-Llorca et al. 2002) and dicots (Bordallo et al. 2002). Both fungi were capable of growth inter- and intracellularly and form appresoria when penetrating the cell wall of epidermis and cortex cells, from tomato and barley. However, these fungi never penetrate the vascular tissue (Lopez-Llorca et al. 2002; Bordallo et al. 2002). Plants endophytically colonized by nematophagous fungi show enhanced defense responses and biomass gaining (Maciá-Vicente et al. 2009a, b). LopezLlorca and coworkers detected the production of serine protease P32, VCP1, and SCP1 from a nematophagous fungus in roots colonized endophytically by this microorganism. As mentioned above, these proteases are produce by nematophagous fungi on their nematode host. Thus, the expression of these proteins in the absence of their host would imply that plants colonized by these fungi could be protected from nematode attack before contact (Lopez-Llorca et al. 2010).

Lopez-Llorca and coworkers showed that fungi other than *A. oligospora* and *P. chlamydosporia* grow endophytically in roots (Lopez-Llorca et al. 2006). For instance, the endoparasitic basidiomycete *Nematocnus robustus*, which infects nematodes through adhesive conidia, penetrated and colonized barley roots and formed clamp connections, whereas *N. pachysporus* did not colonize the root system, but colonized the root surface (Lopez-Llorca et al.

2006). The nematode endoparasitic fungus *Hirsutella rhossiliensis* has a similar behavior since it only colonizes the root surface. Furthermore, fungi belonging to the basidiomycota, such as *Pleurotus djamor* that immobilizes nematodes with a toxin (Kwok et al. 1992) prior to infection and digestion of its prey, also colonizes and penetrates barley roots (Lopez-Llorca et al. 2006). The nematode-trapping fungus *Arthrobotrys dactyloides* is also a root colonizer that penetrates the epidermal cells and forms coiling structures in barley root cells (Lopez-Llorca et al. 2006).

To better understand the endophytic process, the *P. chlamydospora* genome was recently sequenced and consists of 41.2 Mb, from which 12,122 gene models were predicted. Under the endophytic relationship with barley roots, 63 % (7586 genes) of the genome was expressed. From the 1432 predicted secreted proteins, 57 % were expressed under this condition. 663 predicted genes did not exhibit any homologue in the NCBI database and almost a half of them (277) were expressed during *P. chlamydospora* endophytic lifestyle. Phylogenetic analysis of genome-encoded orthologous showed that *P. chlamydospora* is most closely related to *Metarhizium anisopliae* and *M. acridium*. A search for pathogenesis-related genes in the Pathogen–Host Interaction (PHI) database that collects pathogenesis-related genes of fungi, bacteria, and oomycetes (Winnenburg et al. 2008) showed that 1981 genes (16 %) shared homology with genes included in the PHI database, 24 % (468 genes) of which encoded putatively secreted proteins. The majority of genes putatively associated with pathogenesis and endophytism encode hydrolytic enzymes and signal transduction proteins. The hydrolases found in PHI included metalloproteases and chitinases, whilst those expressed under endophytism include serine and rhomboid protease families and a protein phosphatase. The *P. chlamydospora* genome contains a wide set of genes encoding hydrolytic enzymes, from which almost a half were expressed during endophytism. In addition it contains 15 PKS and 12 putative non-ribosomal peptide synthases (PRPS), together with a number of PKS- and NRPS-like proteins, and 4 NRPS–PKS hybrid genes. A radicicol gene cluster was also found. *P. chlamydospora* expressed 56 of the secondary metabolism pathway genes identified. From these seven genes belonging to the radicicol cluster were expressed. The *P. chlamydospora* genome contains 290 transporters of the major facilitator superfamily (MFS), 58 ATP-binding cassette (ABC) transporters, and 113 general transporters, most of which exhibited homologs included in PHI database. *P. chlamydospora* expressed drug resistance, sugar/inositol, oligopeptide, and amino acid transporters during endophytism. Furthermore, a number of genes encoding oxidoreductases related to detoxification were also detected. Genes involved in cell wall biosynthesis and modification, including chitin synthesis activators, chitin synthases, lipopolysaccharide modifying proteins, and hydrophobins, were also found expressed under endophytism. Additionally, the *P. chlamydospora* genome encodes 409 putative transcription factors (TFs), grouped in six families, of which the Zn2Cys6 fungal-specific type TF contains the highest number of genes expressed under endophytism. In addition, the *P. chlamydospora* genome encodes G-protein subunits (8), putatively involved in vegetative growth, conidiation conidium attachment, appresorium formation, mating, and pathogenicity, from which six were expressed in endophytism. The genome of *P. chlamydospora* also contains 54 homologues to Pth11-like G protein-coupled receptors (GPCR), 27 small GTPase regulators, and 12 Rab GTPase activators, all of which presumably regulate its endophytic behavior. Similarly to entomopathogens or plant pathogens, the *P. chlamydospora* genome contains 153 genes for protein kinases involved in the regulation of cell and metabolic processes, from which 75 had matches in the PHI database. Together with PKs, genes coding for histidine kinases (HKs) were identified, nearly all which had homologous in the PHI database. From these 14 were expressed during endophytism (Larriba et al. 2014). All together these results provide information for understanding the molecular mechanism involved in the multitrophic lifestyle of *P. chlamydospora*.

Recently, a fusion PCR-based deletion method was developed for *P. chlamydosporia*, using the split marker strategy and PEG-mediated protoplast transformation. The authors were capable of generating three stable deletion mutants resistant to neomycin (G418 sulfate) of genes induced during infection of nematode eggs by *P. chlamydosporia*: one chitinase (VFPPC_01099) and two protease genes (VFPPC_10088 and VFPPC_06535) (Shen et al. 2014). After screening ~100 mutant candidates by PCR, the average rate of gene knockout was 13 %. This method resulted in an efficient homologous gene knockout strategy for *P. chlamydosporia*, which together with the availability of the genome sequences opens the opportunity for high-throughput genetic analysis in this fungus (Shen et al. 2014).

VII. Concluding Remarks

The last decade has resulted in a burst of scientific research on nematophagous fungi, fueled

by the advance in techniques for genome sequencing and transcriptome analysis. Thus, the knowledge that has previously accumulated in relation to the structural characteristics of the specialized structures produced by nematode-trapping fungi during the interaction with their hosts and even in the life cycle and ecology of this organisms, and the now obtained complement by "-omics" data on several species, renders the nematophagous fungi an attractive subject to investigate their molecular physiology in detail. Despite all the abovementioned success, clear-cut evidence for the involvement of the described virulence factors is still lacking. In this regard, it will be of crucial importance to develop a toolbox for the genetic manipulation of nematophagous fungi, as is now the standard for other filamentous fungi such as the model systems *Neurospora crassa* and *Aspergillus nidulans*, but not for many plant pathogenic and industrially used fungal species. The availability of techniques for high throughput preparation of genetically manipulated strains would stimulate the analysis of whole gene groups, and the results to be obtained thereby could be used to generate improved biological control fungi against nematodes by genetic engineering. In addition, the analysis of different isolated structures such as knobs, trapping nets, appressoria, and other structures will help us better understand their role in the parasitic process as infection structures.

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14 Beetles versus Fungi: Trophic Interactions in Boreal Forests

Dmitry Schigel[1]

CONTENTS

I. Introduction

Nutrient cycling of ecosystems depends on fungi, which exit the communities by local extinctions or reproduction. The vast amount of woody organic matter generated by trees is isolated from potential animal consumers by the chemically challenging macromolecules that comprise wood, such as lignin, cellulose and hemicellulose. Fungi are able to transform living and dead plant organics into a more palatable—for insects—fungal organics. Fruit bodies of macrofungi produce spores but are also consumed by a variety of insects from several orders. This chapter aims to illustrate trophic interactions between fungivorous Coleoptera and their fungal hosts in relatively well-studied European boreal forests.

Descriptive terminology varies from study to study, but generally process of organisms consuming mycelia, fruit bodies or spores of fungi is called **fungivory** (mycophagy: Lawrence 1989). Many dead-wood and coarse woody debris-dependent (saproxylic) insects utilise enzymatic abilities of fungi, feed on mycelia and inhabit wood altered by fungi. The unique adaptations and dispersal abilities of both fungi and insects result in a diversity of interaction types, which are highly dynamic, and can be direct or indirect (Boddy and Jones 2008). One of the major guilds is comprised by fungivorous beetles. Fungivory, most commonly in combination with saprophagy and xylophagy, is a characteristic of many families of Coleoptera. Hundreds of beetle species are known from boreal environments as associated with dead wood, but apparently very few, if any, of those can be declared pure xylotrophs. The resource-exploiting fungal mycelia are most frequently consumed by beetles together with the woody substrata. Shaw (1992) argues that only organisms that selectively feed on fungi should be called fungivorous, while others separate obligate or facultative fungivory to demarcate these two types of fungal diet. Some researchers only call species fungivorous if their larvae are feeding on fungi.

Polypores, wood-rotting agarics and corticioid fungi compete for resources and succeed each other, co-occurring with other saproxylic organisms in dead wood. Trees, fungi and beetles form a **fundamental ecological triangle** (Crowson 1981), with all elements and ages of trees subjected to fungal colonisation, and all stages of fungal life cycle, from spore to mycelium to fruit body targeted by grazing beetles. Species interactions peak during the tree and fruit body afterlives. Interactions between fungi and beetles are among the most complex, diverse and species rich in terrestrial landscapes.

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Fungus–insect interactions can be compared with plant–herbivore systems (Shaw 1992). Herbivore invertebrates often specialise on genera or genus groups, but certain guilds are monophagous. Noteworthy that higher diversity of herbivores in tropics is mostly explained by higher diversity of plants, while there is no clear latitudinal trend in numbers of consumers per host, consumer specialisation and beta diversity (Lewinsohn and Roslin 2008). There are much fewer studies on tropical (Yamashita et al. 2015), subtropical or Mediterranean (Quinto et al. 2015) saproxylic and fungivorous food webs than on boreal and temperate systems, and a meta-analysis or a gradient study would be needed to explore the **parallels between plant–herbivore and fungus–insect host use systems**. As in plant–herbivore systems, interactions between fungi and insects include grazing, defence (Rohlfs et al. 2007) and dispersal interactions.

II. Species Diversity

European temperate and especially boreal forests harbour relatively low numbers of native tree species compared to subtropical and tropical forests. Only recently we came closer to realise the huge number of species supported by wood (Stokland et al. 2012). Of around 7000 of **saproxylic** (dead-wood dependent) species in North Europe, 2000 species are fungi (Stokland et al. 2006). At least 1000 species of lignicolous fungi in boreal Europe are voluminous enough to host fungivorous insects in the fruit body interiors or at their surfaces. In combination with soil saprotrophs and mycorrhiza species, as well as with macroscopic non-lichenized Ascomycetes, boreal forests harbour **several thousand fungal species** that are potentially colonised or visited by insects. Through physical and chemical barriers, only a fraction of this fungal diversity is utilised by insects, and various temporal and spatial factors contribute to population equilibrium of fungi and fungivores.

Coleoptera is one of the most important, in numbers of species and the diversity of associations, orders of fungivorous insects (Hammond and Lawrence 1989). **Other insect orders** that share compact but structured fruit bodies with beetles are Diptera (Wertheim et al. 2000; Yamashita and Hijii 2007b), Hymenoptera, Hemiptera, Lepidoptera and Thysanoptera. Ambrosia bark beetles, attine ants and termites are well known for their abilities to cultivate fungal gardens. Noninsect invertebrate groups (Takahashi et al. 2005), such as mites (Makarova 2004), millipedes, molluscs, oligochaetes, nematodes, woodlice and springtails, are also tied into the complex network of ecological interactions.

The **number of insect** species associated with fungi in the UK was estimated by Paviour-Smith (1960) as 600 species. There are estimated 300–400 **fungivorous beetle species** in Finland (Schigel 2011b) and potentially 500–1000 species from at least 20 families in Europe. Adults of truly fungivorous beetles are attracted by the fungal odours to the fruit bodies, they meet and mate there, and their larvae exploit fruit bodies until pupation, which takes place inside the fruit body or in the soil. Certain species of Ciidae are able to exploit the fungal fruit body for several generations, until nothing is left. Similarly to colonisation of trees by fungi, the visible traces of beetle colonisation of fungal fruit bodies are mostly apparent when fruit bodies are dead. There are certain exceptions of this general rule—numerous beetles breed in the living fruit bodies of *Polyporus*, *Laetiporus* and *Inonotus*, and fewer beetle species and individuals are found in the dead remnants of their fruit bodies.

The smallest beetles, Ptiliidae, especially Nanosellinae have fungivorous forms (Fogel and Peck 1975; Polilov 2008), including the smallest beetle in Europe *Baranowskiella ehnstromi*. Fungivory of Staphylinidae (Lawrence 1989) is one of the least studied trophic specialisations in this family. Saprophagous beetles such as Scarabaeidae, Silphidae and Hydrophilidae can be found in decaying fungi. Clambidae, Cryptophagidae and Corylophidae frequently visit sporulating fruit bodies and anamorphic fungi on decaying plant debris. In European fauna, key polypore destructors are found among Anobiidae, Trogossitidae, Erotylidae, Endomychidae, Tenebrionidae, Mycetophagidae, Tetratomidae, Ciidae and Melandryidae (Schigel 2009).

III. Functional and Life-Form Diversity

Fungal fruit bodies vary in size, **shape**, architecture and consistency, from flat, soft and ephemeral annual, through various intermediate forms, to massive, hard and perennial fruit bodies which may last sporulating for decades (Niemelä 2005). For beetles, fungal fruit bodies offer the rich feeding and breeding substrate.

Fungi transform chemically challenging organic molecules of wood. Fungal fruit bodies, unlike mycelium, form the compact, protected and relatively stable habitat and food source exploited by invertebrates. The kaleidoscope of differences in **longevity and seasonality** and chemistry and structure of fungal fruit bodies support a variety of associated insect species, in particularly of beetles. Perennial polypores have more persistent fruit bodies than annual polypores, which are longer living than those of boletes and of agarics, with the diversity – stability hypothesis is a likely explanation of species richness patterns among fungivores (Hanski 1989).

Different stages of fungal life cycle attract various beetle colonisers and consumers: for instance, in European taiga larvae of Melandryidae occur mostly in living fruit bodies of polypores, Ciidae—in dead ones, Latridiidae on sporulating fruit bodies. The roles of fungivorous beetles as spore vectors are rarely documented (but see Shaw 1992). The direct negative (destructive) impact of the fungivores on the fungal community by, for example, reducing spore productivity has been shown in a number of cases (Shaw 1992; Guevara et al. 2000a). Shaw (1992) observes that few studies investigate mycelial **grazing**, most focusing on dipteran pests of commercial mushroom cultivation or explore laboratory microcosms. Boddy and Jones (2008) review in detail direct positive and negative effects of *Basidiomycota* on invertebrates, as well as role of invertebrates in dispersal, colonisation, modification of environment and metabolism of fungi.

McGonigle (2007) reviews impact that fungivore grazing on fungi in soil and litter, suggesting that it should be high in natural condition based on estimates that up to 75 % of the soil fauna biomass are fungivores (McGonigle 1995), while fruit bodies are less than 1 % of fungal biomass (Frankland 1982). Most of reviewed studies concern the laboratory experiments in controlled environments. Grazing is reported to affect species richness by elimination or of fungal species, relative abundances and community diversity, comminution of substrate and fungal dispersal of fungi. In mycelial fungivory, Annelida, Nematoda and Arthropoda are identified as key taxa; within the latter phylum, Acari and Collembola are the best studied groups through direct observation, examination of gut contents and monitoring enumeration of hyphae. Grazing causes a variety of responses of single species of grazed fungi and of community effects: reducing roles of dominant and not dominant fungi, species replacements, selectivity and intensity of grazing, enhanced diversity or polarisation of fungal communities. McGonigle (2007) concludes that three factors control impact of grazing: selectivity, intensity and fungal responses.

IV. Ecology of Fungus – Beetle Relationships and Interaction Types

Ecology of beetle fungivory is based on fungal fruit bodies, mycelia and their spores providing beetles with patchy and unpredictable habitats and food sources. The variability of these habitats is influenced by the speed of hyphal growth and decomposition, yearly fluctuations in fruit body production, architecture of fruit bodies (Ryvarden 1991) and sporulation patterns.

Fungi and beetles are involved in a variety of **interaction types**, including commensal, mutualistic and combative interactions with fungi killing insects and insects grazing on fungi, insects vectoring fungal propagules and fungal symbionts of insects. The *Biodiversity in Dead Wood* book (Stokland et al. 2012) challenges the classic pyramidal schemas of trophic saproxylic food webs and suggests understanding the complexity through interaction web where various wood-dependent taxa are interconnected through a dense network of relationships, which include tropic, nesting and habitat uses.

Six types of fungivory can be identified since two life stages of beetles, larvae and adults can be interacting with fungal spores, mycelia or fruit bodies. Moreover, fungivorous insects can be divided into consumers of living and of

dead fungi (Yakovlev 1995), though this border is fuzzy. Lawrence (1989) separates micro- and macrophagy of fungivorous beetles based on mouthpart morphology and the type of feeding and illustrates dramatic differences between the mouthpart adaptations of larvae feeding on loose food particles, such as spores and surface mycelia, and those adapted for dealing with dense hyphal masses.

The **life cycles** of fungivorous beetles are synchronised with those of fungi. Beetles emerging from pupae seek mating and disperse to colonise new fungal habitats or continue using the parental fruit body. Most species of fungivorous beetles consume recently dead fungal fruit bodies soon after final (or, in case of annuals, the only) sporulation period. Spatial complexity of perennial fruit bodies allows accommodation and niche isolation of beetle larvae of different species. Fungivorous beetles, visitors and colonisers come in waves during the warm season, but succession also takes place during the whole life of a fruit body. Structural similarity, decomposition stage and phylogenetic relations serve as a basis for attraction of certain fungivorous guilds. Many beetles feed on fungi only as adults, while those most closely associated have larvae with fungal diet. In some cases (e.g. many Ciidae) the entire life cycle of a beetle takes place within a fruit body. Most of fungal fruit bodies can be called ephemeral substrates, but perennial fruit bodies of some polypores, such as *Phellinus* spp., may stay on trees for decades (Niemelä 2005).

Significant species diversity of fungivorous beetles from distantly related phylogenetic clades indicates an important role of fungi in the **evolution** of this insect order. Saprophagy and fungivory are ancestral types of feeding in Coleoptera (Lawrence 1989; Leschen 2000), and fungivory has evolved several times independently in this order of insects (Crowson 1981). Palaeontology shed little light on history of insect fungivory; however, massive fruit bodies of *Basidiomycetes* are known since Early Cretaceous, and there are evidences that fungi have been consumed by insects for at least 100 million years (Schmidt et al. 2010).

A gradient of **trophic specialisation types** is demonstrated by fungivorous beetles, from mono- and oligophagy to the most common polyphagy. At one extreme, *Cis bilamellatus* is an example of a very broad host range in northern Europe (Orledge et al. 2010); at another extreme of trophic specialisation some polypores "escape" by producing fruit bodies unpalatable for insect consumers (Schigel 2011a, 2012a).

Trophic interactions between fungi and their beetles could be visualised using **food web** diagrams (Schigel 2011a). Frequencies of fungal fruit bodies in forest compartments were used to measure host abundance and frequencies of occurrence of beetle larvae in these fungi to measure consumer abundances in seven protected boreal forests in northern, eastern and southern Finland. Twenty-four out of 37 commonest polypore species proved to be colonised by 32 beetle species, resulting in 87 fungus–beetle links (Fig. 14.1).

Fungivorous beetles demonstrate at least **three types of host–consumer interactions** and exploitation of host polypores: wide generalists, specialist (strict and not) and beetles forming species-rich communities which jointly share a host fungus. With more links to discover and more species of fungi to explore, both the number of fungivorous species developing in polypores and the assumed level of beetle generality are likely to increase in accordance with Martinez (1992).

In boreal forests, and most likely in other wooded landscapes, diversity of fungivorous insects seems to positively correlate with **volume** of fully grown fungal fruit body, with gigantic *Polyporus squamosus* (Klimaszewski and Peck 1987), *Laetiporus sulphureus* (Benick 1952), large *Piptoporus betulinus* and *Fomes fomentarius* (Thunes 1994), *Fomitopsis pinicola* and *Fomes fomentarius* (Hågvar 1999; Økland 2002) hosting tens of beetle species. Soft and voluminous polypores, such as *Polyporus squamosus* and *Grifola frondosa*, as well as pleurotoid fungi, are also likely to host more species and individuals of parasitoids than the dry and tough polypores with perennial or annual hibernating fruit bodies. Commonness, architectural complexity, seasonal and age changes and longevity of fungal fruit body also increase the number of niches and number of associated species, though fewer species at a time can be recorded or reared from a sample from a perennial than from a soft large annual fruit body.

Seasonality plays an important role in fungus–insect interactions. While perennial fruit bodies are available all year round, certain fungi produce ephemeral fruit bodies for only a few weeks. Adult beetles hatch from pupae in advance, feed on various sources and travel to

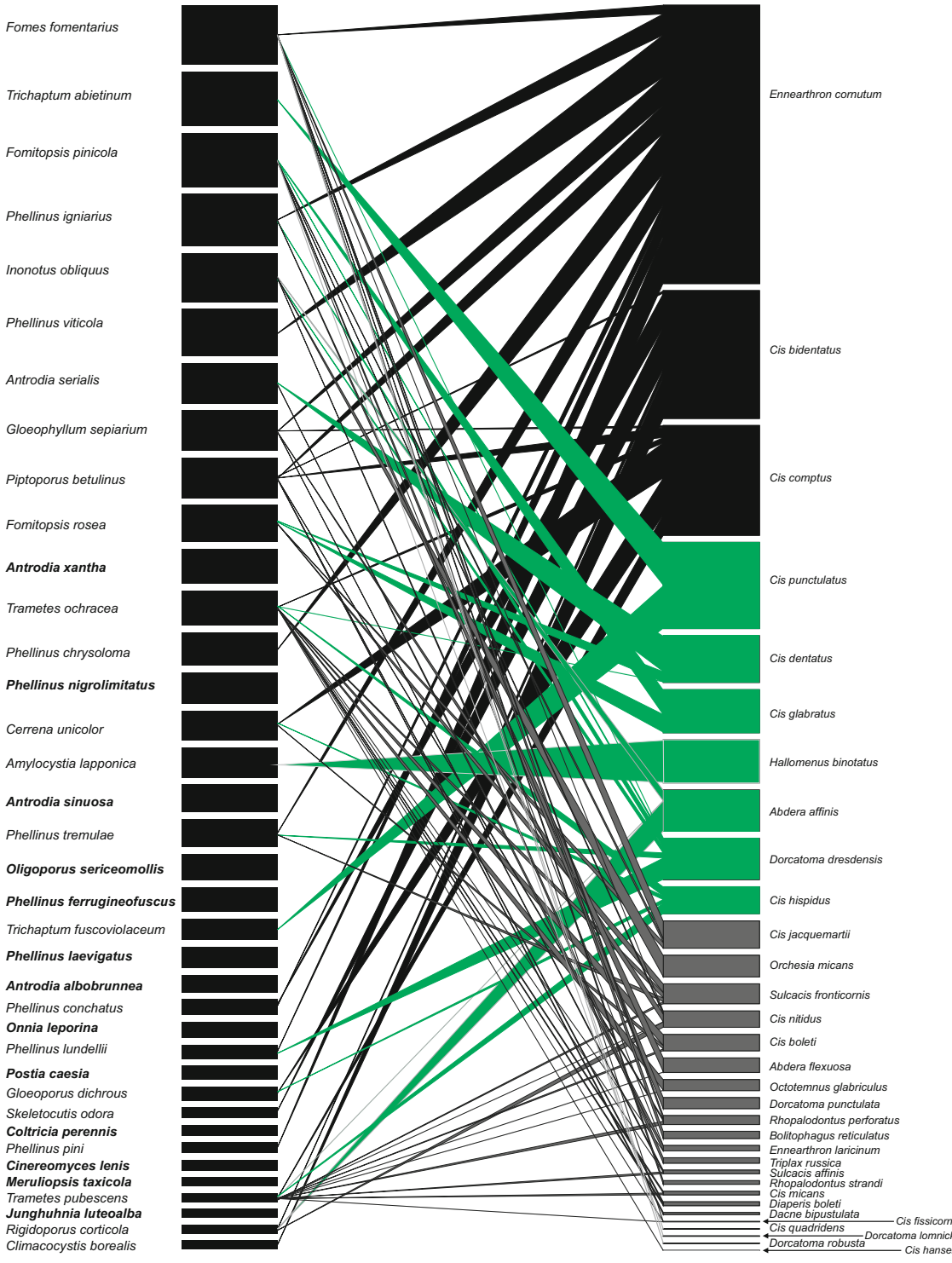

Fig. 14.1 Fungus–beetle food web in Finnish boreal forests (Schigel 2011a). *Bars* on the *left* represent frequencies of fruit body occurrence of the host polypores from the commonest (*top left*) to least frequent in seven sites in Finland (*bottom left*). Only species with more than 10 % fruit body frequency scores in forest compartments were included. Names of fungi which hosted no beetle larvae are set in *bold face*. *Bars* on the *right* represent the gradient of sums of beetle larvae frequencies in the selected fungi, from the most frequent in all

breeding sites, when those become available. Various sporulating fungi are regularly visited by adults of beetles breeding elsewhere. For instance, sporulating fruit bodies of *Fomes fomentarius* attract tens of beetle species during such period in spring, while fruit bodies appear nearly deserted for the rest of the year. Among fungal spore feeders, Leiodidae, Latridiidae and Sphingidae are frequently present in North Europe, whose larvae require even more unpredictable substrate—the myxomycetes. Adult visitors feeding on the polypore spores, or on the fungal fruit bodies, are often different species from those breeding in the fungus as larvae, or adult visitors and coloniser may co-occur. After being exposed to spore masses, these visitors ramble at various feeding and breeding sites and very likely contribute to directional transport of fungal spores. Closer to the end of decomposition of fungal fruit body and of the succession of beetles species, secondary anamorphic fungi may grow over the wet remnants of the fungus. These "moulds" are many and diverse, though poorly studied, their species diversity may become less mysterious with availability of DNA methods and they attract yet another guild of fungal feeders, such as Cryptophagidae.

In reverse interactions, fungal abilities to kill insects can be exploited for human needs. Use of fungal insecticides has comparatively little impact on pest control, but is seen as a promising direction for biocontrol development, despite constraints by the environment humidity and safety concerns. Entomopathogenic fungi are found in most fungal clades except for the higher *Basidiomycota*; fungal pathogens include aggressive species such as *Metarhizium anisopliae* and opportunists like *Mucor hiemalis*. Some of those fungi demonstrate complicated life cycles changing host organisms, other cause mummification of insects. The major biological non-taxonomic division of entomopathogenic fungi is into destructively pathogenic and biotrophic fungi. Both systems display a wide range of infection and response strategies, resulting in a complexity of population biology, including epizootic, interactions (Charnley and Collins 2007).

V. Fungivory Studies

Great variety of fungus–insect interaction types and different aspects of fungus–insect interaction ecology are comprehensively covered in at least four **books** (Wheeler and Blackwell 1984; Wilding et al. 1989; Vega and Blackwell 2005; Boddy et al. 2008) and in a number of **book chapters** (Crowson 1981; Shaw 1992; Ehnström and Axelsson 2002; Stokland et al. 2012), plus at least 600 research and review articles. Since 1990s, at least 70 studies of saproxylic, including fungivorous, beetles and their fungal hosts, were carried out in North Europe. **Additional references** can be found in the bibliography reviews by Fogel (1973a, b), Jonsell (1999) and Schigel (2012b). The megadiverse order Cole-

Fig. 14.1 (continued) fungi (*top right*) to the least frequent (*bottom right*). Thickness of the interaction cline reflects the relative contribution of the fungal species to diet of the beetle larvae. **Generalist beetles**, one-to-many interactions (*upper right corner*): a few generalist species utilise a wide range of host fungi; some beetle species are narrowly dependent on one (or two closely related) host fungus, while other beetles are members of speciose communities inhabiting a certain host fungus only. Generalist beetles demonstrate ecological flexibility with larvae developing in a variety of unrelated and structurally diverse host fungi. Such flexibility is, however, not infinite: a broad preference may be identified within the diverse host species range. **Specialist beetles** (one-to-one type of host–consumer interactions) are found in the middle part of the food web diagram, e.g. *Cis punctulatus* is confined to *Trichaptum* species, pioneer decomposers of dead coniferous trees. The heterogeneous set of beetle species least frequently reared from the commonest polypores is found in the right-bottom corner of the diagram and is recognised by the up-pointed interaction clines, forming **many-to-one group**. Many of the host fungi supporting beetles in many-to-one interactions have perennial or semi-perennial (hibernating) fruit bodies. Extended durability of the fruit bodies improves chances to discover the fungus individual both for a beetle looking for habitat and for a researcher surveying the forest for polypores. These beetles tolerate competition with each other in the structured environment of relatively voluminous fruit bodies of perennial and hibernating fungi and form multispecific communities

optera is one of the key players in the saproxylic game, and fungivory, selective consumption of fungal spores, mycelia and fruit bodies, is among the most apparent interaction types.

A number of **Nordic studies** approach fungus–beetle relationships from ecological, population biology and **conservation** directions (Økland 1995; Thunes et al. 2000; Jonsell and Nordlander 2004). Habitat preference is measured by aggregation and frequency of occurrence (Jonsell and Nordlander 1995; Jonsson et al. 1997). According to Jonsell et al. (1998), presence of wood-decaying fungi determines the fauna of saproxylic beetles. Habitat loss and fragmentation pose a threat to the stability of the fungus–insect systems: Jonsson and Nordlander (2006) prove that distance from an old-growth forest reserve affects colonisation rates of fungivores.

The parallels between **trophic associations and phylogeny** and systematic position of beetle and fungal taxa were identified by Paviour-Smith (1960), Lawrence (1973), Kompantsev (1984), and Jonsell and Nordlander (2004). Orledge and Reynolds (2005), based on original and literature data, identified beetle fungal host groups among 167 species of Ciidae.

VI. Discussion and Future Prospects

A typical research project on trophic interactions would either select a model system and explore it in high detail in the controlled experiment or repeated field study or would document species diversity and ecology using heterogeneous best available data, including museum, digital, own, colleagues' and literature sources. Both **accuracy and trust** of experimental/model systems and **coverage and realism** of field-based studies require sample size of tens of fruit bodies to detect most of the beetles for each host species. The main **methods** in beetle fungivory studies are field collections and rearing beetle larvae into adults, with more than 40 publications based on each approach (Schigel 2009), followed by less widespread methods such are trapping, laboratory and field experiments (Komonen 2008; Faticov et al. 2015).

Most of beetle fungivory research has focused on associations with polypores, agaricoids and boletes, with numerous fungal and beetle **taxa left overlooked** by fungivory explorers. Links of Coleoptera with *Ascomycetes* (Lawrence 1977) and other fungi (Leschen and Carlton 1996; Stribling and Seymour 1988) and most importantly with microscopic and soil fungi are in need of further studies. Species and geographic coverage in the fungus–beetle studies needs to be expanded; more research is needed on ecological factors and processes affecting fungus–insect systems (Hanski 1989; Heard 1998; Hilszczajski et al 2005; Yorozuya 2006), including competition (Yamashita and Hijii 2007a), acoustic communication (Gilbert and Arrow 1924), subsocial behaviour (Ashe 1986; Leschen 1994) and predation.

To build **quantitative food webs**, abundances of hosts and consumers need to be measured. As a proxy to actual abundance of fungal hosts, frequencies in forest biotopes can be used. A similar compromise is available for the ranking beetle species: instead of the sampled frequency, contributions of individual beetle species into the whole spectrum of species interacting with a given fungal species are obtainable. In traditional food web analysis, abundances of hosts, consumers and interaction frequencies are measured from the same sampling. This approach uses the abundances of all interacting species and numbers of interactions per unit volume or area (Lewis et al. 2002); the **alternative food web approach** may use presence-only frequency measures, such as contributions of individual consumer species into the whole number of interactions with a host. Such methodology (Schigel 2011a) lacks higher precision (Polis 1991) and may be used in an analysis of gradually growing datasets, such as those coming from reserve surveys, citizen science initiatives, old project and museum data, and datasets on poorly known and rare species. Beetles grazing on rare fungi are slow to collect and rear in statistically analysable quantities and such studies are further complicated by identification difficulties. Building **molecular food webs** is possible using high-throughput sequencing and biomass measurements for fungi and using molecular identification and DNA barcodes for beetles; isotope studies help to clarify the nature of interspecific relationships: these are promising methodological improvements in food web research.

Chemical signals, volatile compounds, play a key role in directing adult beetles to and from certain fungi in certain conditions or life stages. A large fraction of Finnish polypore species appeared never visited nor colonised by beetles; chemical repellents are the very likely reasons for the mycelial (Shaw 1992) and fruit body (Schigel 2012a) rejection. Chemical ecology offers a range of powerful spectrometry methods to solve the mechanisms of attraction and rejection. Host odours and volatiles attract beetles to fungal fruit bodies (Guevara et al. 2000b; Thakeow et al. 2008), and volatiles may explain the broad polyphagy of fungivorous insects (Hanski 1989).

The fine details of larval trophic specialisation and mouthpart morphology remain poorly studied even in the countries with well-documented lignicolous mycotas and beetle faunas. Indirectly, presence of bacteria and fungi in the wood of living and recently dead trees is proved by isolation experiments and e-DNA studies. A more accurate disclosure of assimilation and dietary role of fungi and bacteria would require the examination of larval **gut content**, also role of beetles as fungal vectors is studied by examining spores in guts (Dodelin et al. 2005).

The growing number of **scientific publications** makes it increasingly difficult to follow the progress even in the comparatively narrow research field. Dormant dataset on fungivores often remains unpublished or difficult to access from grey literature. Occurrence and sequence records, often organised in datasets, are being deposited to the global aggregators, such as Global Biodiversity Information Facility, GenBank and Barcode of Life Data Systems. Similar attempts to improve visibility and discoverability of interaction datasets are carried out by the Global Biotic Interactions (2015) and the Interactions Web DataBase (2015). Hopefully, global trend for more transparent science and open data will transform depositing the biodiversity and interaction evidences into open repositories from a good practice of into a prerequisite for an academic publication, if not into its agile substitute.

The **complexity** of simultaneous processes in the changing seasons and the diversity of interacting species in such system are almost scary, and disclosing of its hidden logic and beauty is a never-ending research pleasure. The elements of this rich and secret world are nearby, in the parks, in the managed and in the protected forests, planted and natural. Many of saproxylic species are small, inconspicuous and hidden from eyes of an observer. Dead wood harbours saproxylic organisms which support boreal forests exist as stable habitats.

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