Edited by
Karl Esser

THE MYCOTA

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

Agricultural Applications

XI

Second Edition

Frank Kempken
Volume Editor

Springer

The Mycota

Edited by
K. Esser

The Mycota

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The Mycota

A Comprehensive Treatise
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XI *Agricultural Applications*
2nd Edition

Volume Editor:
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Frank Kempken
(born 1960) is professor for genetics in botany and molecular biology at the Christian-Albrechts-Universität at Kiel (Germany). A major focus of his scientific work is the different aspects of filamentous fungi including transposons and the biological function of secondary metabolites. His work covers both fundamental and applied research. He received the Bennigsen-Foerder-Preis from the State of Nordrhein-Westfalen.

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 dothey 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 off ungi, 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 thaningest, nutrients and they have rigid cellwalls. 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 amultitude 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 micro fungi 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 contributeto 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 there in 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*

Addendum to the Series Preface

During the Fourth International Mycological Congress in Regensburg (1989) while relaxing in a beer garden with Paul Lemke (USA), Dr. Czeschlik (Springer-Verlag) discussed with us the possibility to publish a series about Fungi. We both were at first somewhat reserved, but after a comprehensive discussion this idea looked promising. We decided to name this new series *The Mycota*.

Then Paul Lemke and I created a program involving seven volumes covering a wide area of Mycology. The first volume was presented in 1994 at the Fifth International Mycological Congress in Vancover (Canada). The other volumes followed step by step. After the early death of Paul Lemke (1995) I proceeded alone as Series Editor. However for Vols. X-XII I received support by Joan Bennett.

Since evidently the series was well accepted by the scientific community and since the broad area of Fungi was not completely covered, it was decided to proceed with eight more volumes. In addition, second editions of ten volumes were published and three more are in preparation.

I would like to thank Springer-Verlag, represented by Hanna G. Hensler-Fritton and Andrea Schlitzberger for their support and cooperation.

Bochum, Germany
April 2013

KARL ESSER

Volume Preface to the Second Edition

Fungi are of great relevance to human agriculture, as they may provide food, enrich food, contaminate food, be symbiotic partners of plants, or be plant pathogens. As such, fungi have a tremendous impact on humanity and of course are highly relevant for agriculture. This volume is the second edition of Mycota XI Agricultural Applications. While some of the authors of the first edition were willing or available to provide an updated version or new topic for the second edition, quite a number of new authors contributed gladly. Now fourteen excellent chapters have been put together, which belong to five different sections, i.e. ***Food and Fodder, Fungal Secondary Metabolites and Detoxification, Biology, Disease Control and Management, Symbiontic Fungi and Mycorrhiza, and Phytopathogenicity***. While the 14 chapters cover a broad area, it was not possible to include all possible aspects of agricultural applications, due to size limitations of this volume, availability of authors, and to avoid overlaps with The Mycota X Industrial Applications.

Two chapters belong to the first section, ***Food and Fodder***. Jean-Michel Savoie (INRA, France) and colleagues report about **genetics and genomics of cultivated mushrooms as well as applications to breeding of agarics**, which is believed to contribute significantly to the improvement of fruiting induction or food quality. This chapter deals mainly with the saprophytic edible mushrooms belonging to the genus *Agaricus*. Breeding strategies using molecular markers and quantitative genetics are suggested for genetic improvement. The second chapter by Jan Dijksterhuis (CBS, the Netherlands) and colleagues highlights the relevance of **fungal spoilage of crops and food**. Food spoilage is a major threat for our food stock and is responsible for enormous losses worldwide, which makes this a research area that is highly relevant with respect to the increasing demand on food during the next decennia.

The second section with three chapters is devoted to ***Fungal Secondary Metabolites and Detoxification***. Nancy p. Keller (Madison, Wisconsin, USA) and coworkers report on **genetics, biosynthesis and regulation of *Aspergillus flavus* secondary metabolites**, among these aflatoxins being the most potent natural carcinogens known. The chapter discusses recent advances in the understanding of aflatoxin and sterigmatocystin production and regulation. The second chapter by Richard D. Johnson (AgResearch, New Zealand) and colleagues deals with **fungal toxins of agricultural importance**. While it is devoted to secondary metabolites it also would fit into the ***Food and Fodder*** or ***Plant Pathology*** sections of this volume, as the authors describe phytotoxins which have defined roles in plant disease as well as mycotoxins which generally have no direct role in disease but have significant impacts on animals that feed on infected host

plants. The third chapter by Yitzhak Hadar (Hebrew University of Jerusalem, Israel) and Daniel Cullen (USDA, Madison, Wisconsin, USA) covers **organopollutant degradation by wood decay basidiomycetes**. Ligninolytic 'white-rot fungi' degrade a wide range of organopollutants including polycyclic aromatic hydrocarbons, pharmaceuticals, pentachlorophenol or pesticides. The biochemistry of enzymes like lignin peroxidase, manganese peroxidase and laccase is described in great detail by the authors.

The third section ***Biology, Disease Control and Management*** is comprised of four chapters. Harry C. Evans (CAB International, United Kingdom) writes about **biological control of weeds with fungi**, involving the use of classical biological control and inundative biological control. The later is based on the development of a product or mycoherbicide, incorporating an indigenous necrotrophic fungal pathogen that can be produced with high yield and formulated, and applied in the same manner as a chemical herbicide. Johannes Wöstemeyer (Friedrich-Schiller-University of Jena, Germany) provides data regarding **disease management of *Phoma* infections**. Fungi belonging to the genus *Phoma* form a phylogenetically heterogeneous group with a broad range of possible plant hosts. *Phoma lingam* is the causative agent of devastating field losses in rapeseed cultivation. Efficient disease management requires profound fundamental knowledge on biology and genetics of these organisms for disease control in the field. Stefan G.R. Wirsel (Martin-Luther-University, Halle, Germany) and colleagues report about the current knowledge on **biology, diversity and management of *Fusarium* species in small-grain cereals**. Fusarium Head Blight is an important cereal ear disease with potential for provoking high economic losses. In addition, the fungi accumulate a variety of mycotoxins in the grain, providing another link to the ***Food and Fodder*** section. The chapter presents achievements in the taxonomy and population biology of Fusarium Head Blight. Kerstin Voigt (Friedrich-Schiller-University of Jena, Germany) and colleagues present a comprehensive view on the **ecological and economical importance of parasitic zoosporic true fungi**. Most of the described species are saprotrophs or mutualists, but there are also examples of parasites of higher plants or animals, which play significant ecological roles or cause economically important diseases.

The fourth section ***Symbiontic Fungi and Mycorrhiza*** is new and was not part of the first edition. Symbiontic interactions and in particular mycorrhizal associations are highly relevant for plant growth and as such provide an important addition to agricultural applications. Claire Veneault-Fourrey and Francis Martin (Université de Lorraine-INRA, France) provide us with **new insights into ectomycorrhizal symbiosis evolution and function.** Understanding of the biology of ectomycorrhizal fungi is important for sustainable forest management and to improve the productivity of tree plantations in marginal soils. This chapter includes eco-biotechnological applications in forestry and bioremediation. Likewise Erika Kothe (Friedrich-Schiller-University of Jena, Germany) and coworkers provide a view into signaling processes in the mutually beneficial symbiosis on the basis of transcription analyses. This is summarized in the section genome-wide expression profiling. The authors provide examples of genetic modifications as tools for functional gene analysis.

In the fifth and final section, ***Phytopathogenicity***, three chapters look into different aspects of plant-pathogenic interactions. Diana Fernandez (Institut de

Recherche pour le Développement, France) and colleagues investigate **rust fungi: achievements and future challenges on genomics and host-parasite interactions**. The authors focus on the most recent progresses in molecular research of the rust fungal genomes and biology, but also the interaction with the plant host and the establishment of a successful infection. Importantly, the latest antifungal strategies available, including novel drug targets discovery and the use of RNA interference to engineer plants resistance to rust fungi, are covered as well. This provides a link to the section on ***Disease Management***. Ralf Horbach and Holger B. Deising (Martin-Luther-University, Halle) focus on **the biotrophy–necrotrophy switch in fungal pathogenesis**. Increasing numbers of plant pathogenic fungi have been identified as hemibiotrophs, i.e. pathogens sequentially differentiating biotrophic and necrotrophic hyphae in the host tissue, allowing to analyse biotrophic and necrotrophic lifestyles at the molecular level in the same genetic background. The chapter provides a great overview on the recent development and understanding of fungal hemibiotrophy. Last but not least, Lisha Zhang and Jan A.L. van Kan (Wageningen University The Netherlands) report in their chapter about the role of **pectin as a barrier and nutrient source for fungal plant pathogens**. They show that plant cell walls are both, a barrier for penetration and a food source for fungi.

The fourteen chapters provide a comprehensive view on agricultural applications, which I hope will prove to be useful for scientists working in the field, but also as important insight for people from other areas of research. I thank all authors for their contributions, and also the Springer staff for their help.

Frank Kempken

Volume Preface to the First Edition

The development of agriculture was an essential prerequisite to the establishment of permanent settlements and eventually complex human society. At all times, and possibly even more so now, humanity depended on the annual crop yield, which may be influenced by weeds, pathogens, or poor weather conditions. Fungi are important plant pathogens and can reduce yield significantly. In fact, many examples can be cited where fungal pathogens have actually made history, e.g., the infections by the ergot fungus *Claviceps purpurea* causing ergotism in the Middle Ages or the disastrous Phytophthora infections of potato in nineteenth century Ireland, leading to the emigration of millions of Irish people to the United States of America. In addition, the occurrence of mildew can severally spoil food and fodder, and mycotoxins produced by these fungi may cause illness or even death. Clearly, fungi had and still have a tremendous impact on humanity. However, while only a minority of fungi are pathogens, many others can be quite useful, e.g., to nutritionally enrich straw or to ferment food and drink.

In this volume, the relevance of fungi for agriculture is discussed in 18 chapters, which are divided into four sections: (1) food and fodder production, (2) mycotoxins and detoxification, (3) disease control, diagnosis, and management, and finally (4) update on host–parasite interactions.

Chapters "Genetics and Genomics of Cultivated Mushrooms, Application to Breeding of Agarics, Fungal Spoilage of Crops and Food, and Genetics, Biosynthesis, and Regulation of Aflatoxins and other *Aspergillus flavus* Secondary Metabolites" discuss various aspects of food and fodder production, featuring the application and potential of mushrooms, straw enrichment, and food or crop spoilage. The first article by Paul Horgan and Alan Castle provides insight into the use and genetics of mushrooms, especially *Agaricus*. Dusan Jalc contributes a chapter about straw enrichment by fungi, giving many details about this field of research. Jan Dijksterhuis and Rob Samson present the current knowledge about the important problem of food and crop spoilage. I should add that other aspects of food production have already been reviewed in Vol. X *(Industrial Applications)* of *The Mycota*, and are therefore not covered again here.

The second section contains two chapters which are devoted to knowledge about the biosynthesis of mycotoxins and the use of fungi in organopollutant degradation. Contamination with mycotoxins is of course a problem, particularly in humid climates, and may increase when anti-fungal agents are not available or are rejected, as is the case in organic farming. Naney Keller and colleagues provide a detailed insight into the synthesis of some mycotoxins.

Organopollutant degradation, the second chapter in this section, has a high potential for future pollution management, and was written by Daniel Cullen.

As fungal phytopathogens are of great concern in agriculture, a large section of this volume deals with various aspects of biological control (three chapters), diagnostics (two chapters) and disease management (three chapters). Fungal biological control is covered by Yigal Elad and Stanley Freeman, insect control by Tariq Butt, and weed control by Harry Evans.

Disease control is another focus, with emphasis on the example of *Magnaporthe grisea* given by Nicholas Talbot. Another chapter, by Diana Fernandez and Thierry Langin, deals with the use of repeated DNA and transposons as diagnostic tools, a rather recent development. Finally, disease management is covered in three chapters dealing with important fungal pathogens such as *Phoma,* by Kerstin Voigt and Johannes Wostemeyer, *Fusarium,* by Kerstin Voigt, and rusts and powdery mildew, by Holger Deising and collaborators.

In the fourth section, host–parasite interactions are the main topic. This section has been named "update on ..." to acknowledge work presented in a previous volume of *The Mycota* (Vol. V). In this volume, five chapters present the current knowledge in this important field, discussing relevant issues such as signal transduction, by Michael Bolker, avirulence determinants, by Wolfgang Knogge, phytotoxins, by Dan Panacchione and colleagues, and cell wall degradation, by Jan van Kan and collaborators. The final chapter, written by Jacques Mugnier, gives insight into the co-evolution of pathogenic fungi and grass hosts.

As this volume is restricted in size, certainly not all aspects of fungal applications in agriculture are covered. However, the 18 chapters provide an important insight into this area of research, and I sincerely hope that it will serve as a guide for readers from outside the field and as a valuable reference for those unfamiliar with this type of research.

Finally, I wish to express my gratitude to all contributors to this volume.

Kiel, Germany, April 2002

Frank Kempken
Volume Editor

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Food and Fodder

1 Genetics and Genomics of Cultivated Mushrooms, Application to Breeding of Agarics

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CONTENTS

I. Introduction

Cultivation of edible mushrooms is the premier agricultural application of mycology. Humanity's use of mushrooms extends back to Palaeolithic times. According to Boa (2004), the archaeological record reveals edible species associated with people living 13,000 years ago in Chile, but it is in China where the eating of wild fungi is first reliably noted, several hundred years before the birth of Christ. Edible fungi were collected from forests in ancient Greek and Roman times and highly valued, though more by high-ranking people than by peasants. At the beginning of the twenty-first century, **gourmet mushrooms** may contribute to the development of a **new agriculture** by addressing the consumer demand for healthy and sustainable products and some of the non-nutritional use of agricultural productions in developed countries, as well as making substantial contributions to the diets of poor people in developing countries. They may either be commercially collected in forests or cultivated, and present an interesting biodiversity.

By compiling more than 200 different sources from 110 countries, but excluding detailed review of species from developed countries, over 2,300 **wild useful species of mushrooms** were identified by Boa (2004). Because of a decline in forest-based industries in some countries, wild mushrooms are now considered as new sources of income even in northern countries (Román and Boa 2006). A mushroom is defined as a macrofungus with a distinctive fruiting body that is large enough to be seen with the naked eye and to be picked up by hand, and can be either a Basidiomycete or an Ascomycete, aerial or underground (Chang and Miles 1992). Mushrooms can be roughly divided into various categories depending on their ecology. **Saprophytic mushrooms** play an important role in the cycling of carbon and other elements through the breakdown

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of lignocellulosic plant residues and animal dung, whilst **ectomycorrhizal mushrooms** are involved in symbiotic associations with plant roots. Despite the important ecological and economical role of mushrooms, forest planning and management has paid little or no attention to the harvesting of wild edible fungi for a long time. A new challenge is the development of a science-based production of mushrooms in forests, sustaining the development of a **mycosilviculture**. For a review on the trends in this topic, see Savoie and Largeteau (2011).

Today, most of the mushrooms recognized as cultivated are saprophytic species. Some of them can be produced in forests on inoculated wood logs or other substrates, but this outdoor culture is dependent on local climatic conditions, and hence they are generally cultivated indoors. FAOSTAT (2011) indicates that the world mushroom production in 2010 was about 6.0 million tons, with significant progress in the past 20 years (2.1 million tons in 1991, 4.2 million tons in 2000) which shows the increasing interest for edible and medicinal mushrooms as an agricultural crop. Marshall and Nair (2009) reported 12 species that are commonly grown for food and/or medicinal purposes, across tropical and temperate zones, including the common mushroom (***Agaricus bisporus***), shiitake (*Lentinula edodes*), oyster (*Pleurotus* sp.), straw mushroom (*Volvariella volvacea*), lion's head or pom pom (*Hericium erinaceus*), ear (*Auricularia auricula*), reishi (*Ganoderma lucidum*), maitake (*Grifola frondosa*), winter (*Flammulina velutipes*), white jelly (*Tremella fuciformis*), nameko (*Pholiota nameko*), and shaggy mane mushrooms (*Coprinus comatus*). One can add at least three other species, the pavement mushroom (***Agaricus bitorquis***), the almond mushroom (***Agaricus subrufescens***), and blewit (*Lepista nuda*). Commercial markets are dominated by *A. bisporus*, *L. edodes* and *Pleurotus* spp., which represent three quarters of mushrooms cultivated globally.

Understanding the ecology of mushrooms in their natural environment is the main requirement for efficient development of a cultivation process.

- Cultivated mushrooms have two kinds of saprophytic lifestyles. Most of them are **primary decomposers** that can be cultivated on pasteurized or sterilized lignocellulosic substrates. The others are leaf-litter **secondary decomposers** cultivated on composts prepared from various agricultural wastes including manures. The cultivation substrates are both physical supports and nutrient sources for the mushrooms, which need to be able to degrade them with appropriate sets of enzymes. The choice of the **agricultural by-products** and their processing before cultivation is directed by local availabilities, and there are many projects attempting to optimize the bioconversion of these wastes by mushroom cultivation. The reader is directed to the proceedings of the International Conferences on Mushroom Biology and Mushroom Products (Savoie et al. 2011) for examples of experiments on various cultivation substrates.
- The fruiting-bodies are reproductive differentiated parts of macroscopic fungi, and the bottlenecks to large development of mushroom technologies are due to a lack of knowledge with regard to two major parts of the biology of most of the potentially interesting species: (i) **life cycle** and genetics, and (ii) factors and mechanisms responsible for **fruiting induction** and fruiting body development.
- As plant crops are, cultivated mushrooms are susceptible to a variety of viral, bacterial and fungal **diseases** as well as various **pests**. Studies concerning the mechanisms involved in **host–pathogen interactions** are intended to improve the control of pests and microbially induced diseases of mushrooms (Gaze and Fletcher 2007; Largeteau and Savoie 2010).
- The quality of the final products is the final challenge for the mushroom growers. The mushrooms should have a good quality and a long storage shelf life. **Food quality** is defined by the degree to which it meets consumers' expectations, which are mainly taste and nutritional/healthful profiles for mushrooms.

Growers and other participants in the mushroom industry are faced with these different challenges. The use of natural biodiversity and molecular genetic approaches for breeding

might contribute significantly to achieving a successful outcome of these challenges. The information discussed in the rest of this chapter deals mainly with saprophytic edible mushrooms belonging to the genus *Agaricus*. *Agaricus bisporus* (Lange) Imbach, the button mushroom, is arguably the most studied mushroom species.

II. Wild Germplasms for Mushroom Breeding

Crop wild relatives and local varieties are the elements of agricultural biodiversity most likely to contain the necessary novel, unique, and high level of genetic diversity needed to sustain innovations in breeding programs. This assertion developed for plants is also true for mushrooms but it implies a robust taxonomic and phylogenetic knowledge and a preservation of the **wild resources**.

A. Phylogeny for Identifying New Interesting Species or Varieties

Agarics are popular fungi, picked and consumed in many countries. However, their determination remains difficult even for expert mycologists in Europe. Tropical or subtropical species are less well-known than temperate ones. A review of the twentieth-century literature on *Agaricus* L.: Fr. emend Karst finds a diversity of opinion on the circumscription of natural infrageneric groups and on the relationships of species within and among the proposed groups. The group of species most closely related to the economically important, cultivated species *A. bisporus* (Lange) Imbach is no exception. This situation is changing because of recent progress in the classification facilitated by molecular characterization and phylogeny. The **genus *Agaricus*** has been shown to be **monophyletic** (Vellinga et al. 2011). Eight sections are recognized in the subgenus *Agaricus*: *Agaricus*, *Arvenses*, *Bivelares*, *Chitonioides*, *Minores*, *Sanguinolenti*, *Spissicaules*, and *Xanthodermatei* (Parra 2008; Zhao et al. 2011). The sections *Bivelares* (Kauffman) L.A. Parra and *Xanthodermatei* Singer have been phylogenetically reconstructed by analyzing DNA sequences from the ITS1+2 region of the nuclear rDNA (Challen et al. 2003; Kerrigan et al. 2006, 2008), and other sections are under investigation. Such a taxonomic and phylogenetic project is valuable for the development of the cultivation of new *Agaricus* species or varieties.

It is noteworthy that some important traits are shared by all or almost all the species of certain sections, and not by the others. For example, the **toxic species** mostly belong to *Agaricus* section *Xanthodermatei* (Kerrigan et al. 2006), and the more popular **edible species** belong to four sections, but only those of two sections (*Bivelares* with *A. bisporus* and *A. bitorquis*; *Arvenses* with *A. subrufescens*) can be easily cultivated on compost. Similar data are noted for odors, **volatile components**, and for certain macro-chemical reactions. *Agaricus* section *Xanthodermatei* comprises a group of species allied to *A. xanthodermus* and generally characterized by sporophores having phenolic odors, transiently yellowing discolorations in some parts of the sporophore, and Schaeffer's reaction negative (Kerrigan et al. 2006). Certain odors appear to be **synapomorphic characters** and are crucial for **taxonomy** (Parra 2008). It can be hypothesized that these traits and their underlying secondary metabolism have been conserved over dozens of million years because they were implicated in crucial biological processes such as spore dissemination or sporophore defence (Callac et al. 2005). We therefore predict that phylogeny will be very helpful to detect species of nutritional, biochemical or medicinal interest. Work is in progress to derive supported hypotheses about phylogenetic relationships and trends in character evolution within the genus *Agaricus*, by sequencing nuclear genes and comparing synapomorphic characters linked to the production of secondary metabolites implicated in mushroom adaptation and/or having potential interest for human industry. A new approach in mycology tends to resolve phylogenetic relationships at the infra-genus level, and to use phylogenies as tools for interpreting adaptive evolution and predicting the potentialities of this phylum to contain individuals with valuable properties. This concept of **useful phylogenetic systematics** has to be developed, and *Agaricus* are good models.

The **geographic origins** of the species and their dominant climatic conditions could be another interesting way to find specific adaptations to climate. A recent study attempting to compare temperate and tropical *Agaricus* species (Zhao et al. 2011) showed that classifying the species into climatic groups is not so easy. Among the cultivated species, the geographical range of some temperate species such as *A. bisporus* and *A. bitorquis* extends into tropical areas and, reciprocally, the tropical species *A. subrufescens* exists also in Europe (Zhao et al. 2011). This **intraspecific diversity** might also be a source of interesting traits to be selected for scientific studies of biodiversity or the production of new cultivars of edible mushrooms.

B. Lack of Diversity in the Cultivated Strains

Despite the economic importance of *A. bisporus* and its long history of cultivation since the eighteenth century, few efforts have been made in terms of breeding and strain improvement. As reported in the previous edition of this volume (Horgen and Castle 2002) the first cross-bred *A. bisporus* strains (Horst-U1 and U3) were developed in The Netherlands in the 1980s, and no new **hybrids** with a different genetic background have been developed since then. As a result, all currently grown cultivars are assumed to be related to a limited number of traditional genotypes, and *A. bisporus* appears to be nearly a **monolineage crop**. This has been observed during the past 30 years on sets of about 20 strains per study by using different markers: isoenzymes (Royse and May 1982a), RFLP (Loftus et al. 1988), RAPD (Khush et al. 1992; Moore et al. 2001; Staniaszek et al. 2002), and ISSR (Guan et al. 2008). In a recent study with 75 cultivated genotypes provided by European spawn makers from 1990 to 2005 and maintained under liquid nitrogen, the combination of the allelic patterns obtained with 14 **SSR loci** made it possible to identify 13 distinct genotypes (Foulongne-Oriol et al. 2011c). Six groups were identified which corresponded to the five **ancestral lineages** and the hybrids Horst-U1 or Horst-U3. The ancestral lineages had been previously defined by phenotypes as "off-white", "small white", "white", "brown", "small brown", and "golden white" (Royse and May 1982b; Foulongne-Oriol et al. 2009). Thirty-three cultivars showed the same genetic profile as U1/U3 hybrid strains. Using a mitochondrial marker, it was possible to separate them into two subgroups that correspond to either U1 or U3 as expected (Sonnenberg et al. 1991). In a parallel project, Sonnenberg et al. (2011) generated single nucleotide polymorphic markers (SNP's) from analysis of the genomes of the two parental homokaryons of the hybrid Horst U1, and selected 600 markers evenly distributed over the whole genome. In two traditional commercial white varieties cultivated before the release of HU1, 46 % and 50 % of all 600 SNP markers showed both alleles present in the hybrid that had been obtained by crossing these two varieties. All of the nine present-day white commercial strains show a striking similarity to Horst U1. Taking into account an error of 1 % in **SNP marker** scoring, the authors consider these varieties as identical to Horst U1 and identical to each other. All these studies show **the narrow genetic variability among the cultivars** of *A. bisporus*. An exception is a hybrid developed during the 1980s in China (Wang et al. 1995). Despite the striking genetic similarity of the present-day commercial varieties to the first released hybrid Horst U1, **phenotypic differences** can be seen in the scaling, pinning, or the size of mushrooms, as observed by growers.

An epistatic effect (Sonnenberg et al. 2011), linked to random distributions of the centromeres at the first meiotic division (see III.A.2), or epigenetic mechanisms, might cause phenotypic differences between strains with identical alleles, but these phenomena have to be studied in *A. bisporus*.

The **lack of diversity in the cultivated strains of the button mushroom is considered an important risk** for this culture, and efforts have been made during the past 30 years to overcome this problem.

Agaricus subrufescens Peck (syn. *A. blazei* Murrill sensu Heinemann, *A. rufotegulis* Nauta or *A. brasiliensis* Wasser, M. Didukh, Amazonas & Stamets), is a cultivated mushroom whose cultivation is developing in various countries

since 25 years. For taxonomy and synonymy of this taxon we followed Kerrigan (2005); Arrillaga and Parra (2006); Ludwig (2007); and Cappelli (2011). Kerrigan (2005) and Wasser et al. (2002) agreed that the name *A. blazei* Murrill sensu Heinemann was inappropriate. The homonyme *A. subrufescens* Ellis & Everh is posterior as this has been corrected in Index Fungorum. Because of its particular fragrance and taste, this basidiomycete popularly known as "**the almond mushroom**" is now considered as one of the most important culinary–medicinal biotechnological species, with rising demand in consumption and production worldwide (for reviews see Largeteau et al. 2011b; Wisitrassameewong et al. 2012a). The cultivation of the almond mushroom started on a commercial scale in the 1980s in Japan, after the isolation and study of one Brazilian isolate, from the region of Piedade, São Paulo State. The majority of the strains spread over the world most probably came from the culture originally sent from Piedade to Japan, as no further discovery in nature was reported until January 2001, when the species was found growing naturally on a heap of mown grass at Embrapa Florestas, Colombo, State of Paraná, Brazil (Amazonas 2005). The mushroom was for a long time considered as endemic. Consequently, **few commercial cultivars are currently available**. Brazilian and Japanese authors have investigated the genetic polymorphism among cultivated strains, mainly by using RAPD markers, and they showed a high genetic homogeneity (see Largeteau et al. 2011b). In each country, the strains currently cultivated probably derived from a single or very few sporophores, because the growers select the best strains. However, with the increasing interest in this mushroom, a new hybrid was recently patented in USA (Kerrigan and Wach 2008) and work is in progress to improve the genetic diversity of *A. subrufescens* for the development of its **cultivation under various conditions.**

As in plants, the progressive loss of genetic diversity in cultivated lines (genetic erosion) resulting from man's selection of the best *Agaricus* strains, or the absence of initial diversity due to a specific history of the cultivated species, raises the issue of the **sanitary and economic risks related to a mono-crop.** Wild types are important sources of breeding material to restore genetic variability as well as to improve the characteristics of commercially cultivated varieties.

C. *Agaricus* Collections, a Source of Diversity

1. Collections of Genetic Resources for *Agaricus* spp.

A prerequisite for breeding is the availability of genetically diverse source materials. In the 1980s, there appear to have been fewer than 20 independent lines of *A. bisporus* in mainstream culture collections worldwide, including those of commercial laboratories (Kerrigan 1996). There was concern about losing genetic diversity forever, and at the end of the 1980s, a few researchers decided to **support collection and conservation of the germplasm of *A. bisporus***. Two major collections, the ARP (*Agaricus* Resource Program, Kerrigan 1996) in the USA, and the CGAB (Collection du Germoplasme des Agarics à Bordeaux, Callac et al. 2002) at INRA-Bordeaux have been developed. They now contain hundreds of wild isolates originating from various habitats and numerous geographical origins, representing a source of genetic diversity.

The aim of the *Agaricus* Resource Program (**ARP**) was to encourage the discovery, acquisition, preservation, characterization, and distribution of novel germplasm of *A. bisporus* and other closely related species of *Agaricus*. Meanwhile, with the contribution of some European mycological societies, mycologists, and collaborations with scientists from North America, Greece, Mexico, and more recently Thailand and China, French mushroom scientists had gradually constituted another collection, **CGAB** (Callac et al. 2002). Some specimens are both in ARP and CGAB, but the sum of the two collections represents more than 800 wild specimens, mostly collected either on cypress or spruce litter, or on manure, but also in sandy semi-arid habitats. Their distribution mainly covers Europe, the Mediterranean region, and North America. Some wild specimens have also been isolated in other areas such as Asia and are in laboratory collections of universities

and research institutes. For instance, wild *A. bisporus* strains were collected from the Tibetan Plateau, and are in the collection of the Sichuan Academy of Agricultural Sciences (Wang et al. 2008a). The known **geographic range of *A. bisporus*** extends from the boreal region of Alaska (Geml et al. 2008) to the equatorial climate of Congo (Heinemann 1956), and from coastal dunes to mountains of more than 3,000 m elevation (Largeteau et al. 2011a), but few living specimens from extreme habitats are available in culture collections. The **geographic and ecological diversity of the strains** in collections is a positive point for the objective of biodiversity preservation and valorization. An efficient germplasm resource base is available for the commercial *Agaricus* strain development industry, while enabling scientific study of this natural resource.

For the other cultivated and potentially cultivable *Agaricus* species, germplasms are scarce. The *Agaricus subrufescens* germplasm suffered from the controversy concerning its taxonomy, and probably from the fact that it is a relatively rare species in Northern countries. In addition to the cultivated strains with their low genetic diversity presented above, the collection of genetic resources for this organism is becoming enriched with a small number of North American, European (Kerrigan 2005), and more recently Asian isolates (Wisitrassameewong et al. 2012b). Because of the new interest in this mushroom, work is in progress to increase the number of specimens in collections to study their genetic diversity.

2. Genetic Diversity in Collections

The diversity of *Agaricus* species is to be discovered with investigation in new areas. A recent project showed that approximately 50 distinct species were harvested from a small area of northern Thailand in a few days, most of them probably being novel species. Only about one-third of tropical species belong to the classical sections of the *Agaricus* based on temperate species (Zhao et al. 2011). This diversity indicates that ***Agaricus* is a species-rich genus** in the tropics as well as in temperate regions, with potentialities for identification of new cultivable species with culinary or medicinal interests. An interspecific genetic diversity is expected to be revealed in the next few years.

At the intraspecific level, due to the wild germplasms of the other *Agaricus* species being less developed than for *A. bisporus*, there is little information on their intraspecific genetic diversity.

Some projects on ITS and other taxonomic markers have used several specimens of the same species. That is the case, for instance, with *A. bitorquis* or *A. cupressicola*, for which three genotypes based on ITS sequences were identified, with five isolates of each species analyzed (Kerrigan et al. 2008). It is noteworthy that ITS1+2 sequences have been shown to be informative for the intraspecific diversity in *Agaricus* spp. With regard to *A. subrufescens*, Kerrigan (2005) did not distinguish geographical populations (with the exception of the Hawaiian samples) by sequence characters within the strains from North America, South America, or Europe which he studied.

Enrichment of the germplasm of these species is promising for an increasing availability of genetic diversity.

There have been several works using different markers showing the genetic diversity in collections of *A. bisporus* (see Horgen and Castle 2002). Recently, microsatellite markers and SNPs used to demonstrate the close relationship between the cultivated strains of *A. bisporus* (see II-B) were also useful for evaluating the available genetic diversity in collections. In 19 wild accessions on average for 29 % of all 600 SNP markers, both alleles of the cultivar strain used as reference were found by Sonnenberg et al. (2011) whilst the percentage was about 50 % for non-hybrid cultivars. Using 33 SSR markers, Foulongne-Oriol et al. (2009) observed a significant higher polymorphism among 20 wild isolates than among seven cultivars representing the six morphotype lineages assumed to represent all or almost all the genetic variability available among the traditional cultivars and the hybrid Horst U1. This clearly shows that **wild accessions are distantly related to commercial varieties.** But in clustering analysis, relatedness of cultivars with wild strains originating from France was observed (Foulongne-Oriol et al. 2009), in agreement with the hypothesis that most of the cultivars

are probably derived from a native European ancestral population (Xu et al. 1997).

Germplasm collections are often a result of historical events and arbitrary decisions, collecting missions, and specific research programmes, resulting in over-representation of certain materials, whereas other types of material can be under-represented. The *A. bisporus* germplasm is no exception. As a consequence, five known **major populations** appearing reproductively isolated from each other had been defined by Kerrigan (2004): three in North America (western Canada, coastal California, Sonoran Desert), one in the Middle East, and one in Europe. Inside the European population, the subpopulation in Greece and Crete is genotypically distinctive whilst retaining European characteristics (Callac et al. 2002). This subpopulation, as well as four different French local populations, is probably over-represented in the European germplasm. However, the **genetic diversity inside these different levels of populations is interestingly high**.

In a **fine-scale genetic analysis of diversity**, Xu et al. (2002) monitored French samples from one field (50×70 m) containing horse manure as substrate and having frequent human disturbance, and from another site (20×30 m) associated with Monterey cypress trees and without human disturbance, over a 2-year period. There were high levels of genetic variation, and their results demonstrated **limited evidence for vegetative clonality of *A. bisporus* in nature.** The largest potential genet was found in about 1 m^2. Genetic drift within a population and gene flow among neighboring populations could contribute to gene and genotype changes over years. However, the significant differentiation between the two sites located about 450 km apart suggests that long-distance gene flow was relatively limited and that **a high biodiversity might be preserved in situ in local populations** for their further use.

Besides, **hybridizations between cultivar-like strains and elements of other populations** have been truly demonstrated on California Coast samples (Kerrigan et al. 1998) whereas other populations such as that of Alberta were proven to be poorly contaminated by cultivar-like genotypes (Xu et al. 1997). When new sources of breeding material are needed to restore genetic variability in cultivars, one can collect new strains in the wild. Because of the risk of introgression in wild populations and to the dependence on climatic conditions for the collection in natural populations, **the preservation of the genetic diversity in perennial germplasm is a challenge**, both for mycologists and the commercial *Agaricus* strain development industry.

3. Phenotypic Diversity in Collections

Studies on wild germplasm have produced data on **phenotypic diversity for morphological traits and behavioral traits**. Trait diversity in wild *A. bisporus* was reviewed by Kerrigan (2004). The main characteristics that have relevance to economic development of the *A. bisporus* cultivation are cap color, post-harvest quality reaching consumer expectation, temperature tolerance, disease resistance, and differences in cultivation characteristics such as time of fruiting or number and weight of fruiting-bodies. Some recent data on this diversity are presented here.

The cap in *A. bisporus* is variably fibriollo-squamulose and **color varies from white to dark brown**, with a diversity that could be used in breeding programs.

The majority of the current button mushroom sales throughout the world are white mushrooms, while the wild specimens are mainly brown with many gradations. Pileus color indicated by brightness (L parameter measured by a chromameter) in a sample of 418 isolates studied in our research group ranged from 52 % to 93 % of the reference white color (see Kerrigan 2004). The percentage of isolates exhibiting white cap ($L > 88$) in this sample was lower than 4 %, and it may vary from 0 % in a French population to about 10 % in a Greek population (Callac et al. 2002). Using samples from an open site and from a site under cypresses (the same two sites studied for fine-scale genetic analysis of diversity described in paragraph C-2 above), it was observed that all the 21 isolates found under cypress had a brown cap color ($L < 60$), whilst 2 out of 16 isolates from the open site had a cream cap color ($80 < L < 88$), and 9 had light brown pilei ($80 < L < 88$). The colors were measured after cultivation in climatic rooms protected from daylight (Callac et al. 2005), showing the genetic origin of this trait.

Both the **available diversity and knowledge concerning genetic control** (see III-B) **meet the requirements for breeding programs** on this easily measurable trait. In breeding programs of research institutes or spawn companies, white mushrooms derived from commercial strains are crossed with brown wild strains. The objectives are either to obtain white hybrid of *A. bisporus* with given characteristics of the wild strains, or to introgress brown color and other wild traits in a white commercial strain, as recently described for instance in a United States Patent (Robles and Lodder 2009).

Cap color diversity in other cultivated *Agaricus* species is less well-documented. *Agaricus bitorquis* is one of the three species of the section *Bivelares* reported by Callac et al. (2005) which should have definitely lost putative ancestral alleles determining the brown color, whereas cap color of *A. subrufescens* isolates has been reported to vary from cream to brownish-gold in the few studied specimens (Kerrigan 2005; Llarena Hernández et al. 2011). Hybrids we obtained between brown and cream strains exhibited various colors (unpublished data). Further work is necessary for a better evaluation of the cap color diversity in *A. subrufescens*, and to know whether genetic determinants of the color are homologous to those of *A. bisporus*.

Shelf life performance and susceptibility to discoloration after harvest are other quality traits to be taken into account in addition to cap color, because they affect the commercial value of mushrooms. Mushroom discoloration is a post-harvest stress disorder caused by senescence processes and by mechanical damage as a consequence of the enzyme-catalyzed oxidation of phenols into quinones. By comparing 2-day post-harvest mushrooms with freshly harvested mushrooms, 20 genes with increased expression levels have been identified (Eastwood et al. 2001), showing a probable genetic determinism of post-harvest stress disorders in addition to effects of culture and storage conditions (Burton 2004). A collection of *A. bisporus* strains was screened for their bruising sensitivity in order to analyze the phenotypic variation in susceptibility to discoloration after mechanical damages among strains (Weijn et al. 2011). The results indicated that some brown wild strains showed less bruising sensitivity than white commercial lines. Breeding programs for improving insensitivity are in progress based on the use of this diversity (Gao et al. 2011).

Disease resistance in *A. bisporus* has recently been reviewed (Berendsen et al. 2010; Largeteau and Savoie 2010), with significant reports of work on susceptibility diversity in many cultivars and wild strains. We will not deal here in detail with the different diseases. For instance, information is available on the wide range of sensitivity to *Lecanicillium fungicola* in the wild lines of *A. bisporus*, and work published recently states the genetic bases of this trait and its interest for breeding programs (Kerrigan 2004; Largeteau et al. 2004, 2005; Sonnenberg et al. 2005; Foulongne-Oriol et al. 2011d). We tested 450 strains of *A. bisporus* from CGAB for their susceptibility to an isolate of *L. fungicola* in experiments with controlled inoculation of the pathogen, as in Juarez del Carmen et al. (2002). Between 20 % and 35 % of diseased mushrooms were recorded for commercial hybrids used as controls, and Fig. 1.1 shows the large diversity and the interesting potential of some strains on the left bottom part of the figure producing high yields with low rates of affected mushrooms. The exploitation of strains which are less affected or show fewer or milder symptoms than the commercial strains when exposed to pathogens is an objective shared by breeding companies and research institutes or universities.

There are numerous published and unpublished pieces of work in which **diversity in the yield parameters** of *Agaricus* mushrooms has been recorded. Examples of diversity in the germplasm of *A. subrufescens* have recently been reported, with yield of the better wild strains from temperate countries being 500 % that of some presently cultivated strains originating from Brazil (Llarena Hernández et al. 2011; Zied et al. 2011). This opens the possibility of improvements in hybrids that are already exploited (Kerrigan and Wach 2008). For *A. bisporus*, the standards of yield obtained with the current hybrids when cultivated under controlled conditions are

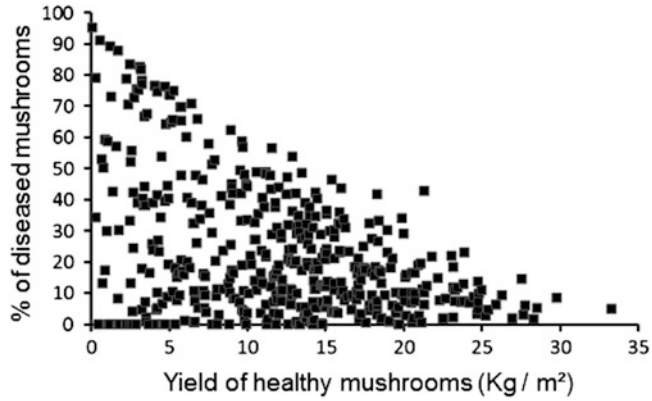

Fig. 1.1. Diversity in susceptibility of 450 strains of *Agaricus bisporus* to the dry-bubble disease caused by *Lecanicillium fungicola*

close to the maximum level, and some secondary components of the complex determinants of the yield have to be selected.

Ecological and physiological adaptation to specific environmental conditions is an important trait for the ability of the strains to colonize the substrate and to produce fruiting bodies during cultivation under various conditions. **Adaptation to the composts used as cultivation substrate** is a behavioral trait with economic interest for the diversification of production areas. Using the same two sites studied for the fine-scale genetic analysis of diversity (Xu et al. 2002) and for the correlation of cap color with the habitat (Callac et al. 2005), Savoie et al. (1996) observed a significant difference between the two populations in their ability to colonize and degrade mushroom compost. The population from the cypress litter was less efficient than that from the open area exposed to horse manure on a field. This is a second illustration, after the cap color, of the fact that **knowledge of the population may help to select samples of strains** where the chance to find a given trait is increased.

Temperature tolerance is a useful commercial trait related to the yield parameters, for which geographical variation should be expected. However, in a project considering high temperature tolerance for both mycelial growth rate and fruiting ability in *A. bisporus*, Largeteau et al. (2011a) observed that the phenotypes correlated neither with climate/microclimate nor with habitat. **Strains from the same sub-populations had contrasted phenotypes.** This illustrates the limit of the prediction of the frequency of an interesting trait based on geographical origin. However, an intercontinental difference was observed. The ability to produce mature fruiting bodies at 25 °C taken as a whole appeared higher in North American populations than in European ones. This difference could result from the different history of the two continental populations.

III. Genetic Improvement of *Agaricus* Strains

A. Various Reproducing Systems Limiting or Facilitating Breeding Strategies

As in other fungi, there are **different processes of reproduction** in *A. bisporus* that can be classified into three groups: vegetative reproduction (which produces through mitosis a mycelium theoretically genetically identical to the original), sexual reproduction through meiosis, and other processes sometimes grouped under the name of parasexual reproduction. An understanding of reproduction gives basic information for other research, for the management of natural resources, and for breeders. *A. bisporus* is an interesting model for

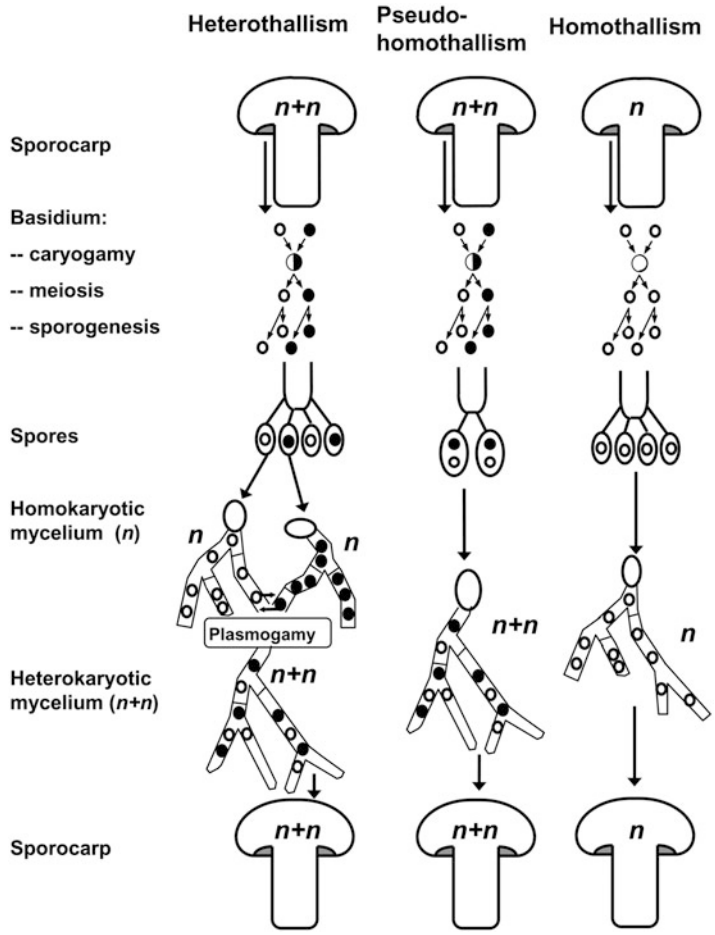

Fig. 1.2. The three life cycles of *Agaricus bisporus*. *A. bisporus* is an amphitallic species with a homothallic or heterothallic cycle depending on the ploidy level of the spores, which can be heterokaryotic ($n+n$) or homokaryotic (n) respectively. Each dominant life cycle is characteristic of a variety. *A. bisporus* var. *bisporus* is predominantly pseudohomothallic, *A. bisporus* var. *burnettii* is heterothallic, *A. bisporus* var. *eurotetrasporus*, is homothallic

addressing these questions, because different sexual life cycles can occur in this species.

1. The Three Life Cycles of *A. bisporus*

Agaricus bisporus* var. *bisporus has a multiallelic unifactorial system of sexual intercompatibility (Miller and Kananen 1972), the locus *MAT* (Xu et al. 1993) having 14 alleles (Kerrigan et al. 1994; Imbernon et al. 1995), **and its life cycle is amphithallic**, i.e., pseudohomothallic (= secondary homothallic) or heterothallic (Fig. 1.2), according to the ploidy level of the spores, which can be respectively heterokaryotic ($n+n$) or homokaryotic (n) (Lange 1952; Kuhner 1977). In this variety, most of the **basidia are bisporic and produce heterokaryotic spores** which confer upon it a predominant pseudohomothallic life cycle (Raper et al. 1972).

More precisely, for 215 wild French isolates examined in cultivation, Callac et al. (1996) found that the percentages of bi-, tri- and tetrasporic basidia were on average 81 %, 18 %, and 1 %. The percentage of homokaryotic offspring, possibly varying between 1 % and 10 %, cannot be easily estimated for several reasons.

The proportion of n-spored basidia depends not only on genetic factors but also on environmental conditions (Kerrigan and Ross 1987). The ploidy status of the spores of the three-spored basidia is unknown, and the germination rate of the homokaryotic spores and/or their viability can be lower than those of the heterokaryotic spores, because of the presence of lethal or deleterious recessive alleles. However, even with a low rate of spore germination, this is not negligible in absolute, if we consider that a single sporophore produces about one billion spores. These haploid spores give rise to unfertile homokaryons.

In the heterothallic life cycle, **plasmogamy** between two sexually compatible homokaryons restores a fertile heterokaryon. This is used in conventional breeding schemes. In contrast, in **the pseudohomothallic life** cycle, heterokaryotic spores give rise to fertile heterokarons. **Most of the wild populations and all the traditional cultivated strains belong to *A. bisporus* var. *bisporus*.** Consequently, the low percentage of homokaryotic offspring is a significant drawback, slowing down the breeding work (Kerrigan et al. 1992). With the enrichment of the germplasm during the past two decades, and the examination of hundreds of wild specimens, the species concept of *A. bisporus* (Lange) Imbach has been refined (Callac et al. 2002; Kerrigan 2007). Based on morphological, biological, and genetic studies, two new varieties of *A. bisporus* have been described.

A. bisporus* var. *burnettii Kerrigan & Callac has been described on the basis of specimens found in the Sonoran Desert of California (Callac et al. 1993). In the sporophores of this variety, most of the basidia are tetrasporic and, correlatively, its **amphithallic life cycle is predominantly heterothallic** (Kerrigan et al. 1994). More precisely, for 58 wild Californian isolates examined in cultivation, Callac et al. (1996) found that the percentages of bi-, tri- and tetrasporic basidia were on average 1 %, 14 %, and 85 %. This variety differs from the two other varieties by traits reflecting adaptation to dryness: smaller mean spore size and faster sporophore development. The average spore numbers per basidium, and correlatively the predominating type of life cycle, are primarily determined by the *BSN* locus (basidial spore number) which is linked to *MAT* on chromosome I (Imbernon et al. 1995, 1996; Callac et al. 1997). *A. bisporus* var. *burnettii* is known only in the population of the Sonoran Desert of California, and is completely inter-fertile with the var. *bisporus*. **Inter-varietal hybrids (var. *bisporus* x var. *burnettii*) have a predominantly heterothallic life cycle**, because of the dominance (sometimes incomplete) of the tetrasporic allele (*Bsn-t*) at the *BSN* locus. Such hybrids **make it possible to obtain large recombined homokaryotic progeny** useful for breeding work, performing genetic maps, and studying the inheritance of traits of interest (see III-B)

A. bisporus* var. *eurotetrasporus Callac & Guinberteau was described on the basis of rare tetrasporic specimens found in France and in Greece, and belonging to the same genet (Callac et al. 2003). The **life cycle of this variety is homothallic**: homokaryotic sporophores produce homokaryotic spores, giving rise to fertile homokaryons (Fig. 1.2). The basidia are mainly tetrasporic as in var. *burnettii*, but spores have the same mean size as those of var. *bisporus*. This variety is interfertile with both var. *bisporus* and var. *burnettii*, and most of the basidia of such hybrids are tetrasporic. Moreover, a natural hybrid between var. *bisporus* and var. *eurotetrasporus* has been found. Tests for allelism at *BSN* showed that both var. *eurotetrasporus* and var. *burnettii* bear similar **dominant *Bsn-t* alleles**, but we do not know whether they are ancestral or even whether they have a common origin (Callac et al. 1998).

Segregation for the haploid fruiting ability that characterizes the var. *eurotetrasporus* has been studied among the homokaryotic offspring of a hybrid between var. *eurotetrasporus* and var. *bisporus*. Genetic determinants of this trait could not be detected because numerous false positive (hybridization by unexpected inoculum of *A. bisporus*) or negative mushrooms (contamination by a competitor due to the too slow growth rate of the homokaryon) occurred in fruiting tests. The trait was inherited by at least 24 % of the homokaryotic offspring, but the haploid sporophores were generally smaller and less vigorous than those of the parent of the var. *eurotetrasporus* (Couture et al. 2004). Haploid sporophores have been also obtained exceptionally in

var. *bisporus* (Dickhardt 1985). In var. *burnettii*, haploid sporophores have been obtained experimentally, but they are weak.

Cytological studies have shown that **karyogamy and meiosis** with synaptonemal complex occur in var. *eurotetrasporus* as in the two other varieties (Kamzolkina et al. 2006). This homomictic process indicates that **the life cycle of *A. bisporus* var. *eurotetrasporus* is homothallic in the strict sense**. The persistence of sexual spores would maintain a better fitness than an asexual process (apomixis) via a more stringent screening for deleterious mutations (Bruggeman et al. 2003). Tetrasporic basidia probably also confer a better fitness than bisporic basidia, which seems to be without interest for a haploid homothallic fungus. *A. bisporus* var. *eurotetrasporus* is probably in a sympatric speciation process. Specimens of this variety were collected under cypress, and on one occasion in the company of *A. bisporus* var. *bisporus* and *A. agrinferus* (Kerrigan & Callac). This latter and *A. subfloccosus* (J.E. Lange) J. are the species most closely related to *A. bisporus*. These both edible and cultivable species are homothallic, and constituted of multiple non-recombining genets (Kerrigan et al. 1999, 2008). *A. bisporus* var. *eurotetrasporus* represents a **source of *Bsn-t* alleles useful for breeding work** as does the var. *burnettii*, but also its **haploid fruiting ability is a tool for studying development of the sporophores and the genetics of traits of interest**.

2. Special Features of Basidia

The pseudohomothallism in *A. bisporus* var. *bisporus* has three important characteristics that are not independent from each other: (1) the spores of the bisporic basidia receive two postmeiotic nuclei, which complement each other at the mating type locus (Sass 1929; Evans 1959; Royse and May 1982b; Summerbell et al. 1989; Kerrigan et al. 1993), (2) the parental heterozygosity is highly conserved in the heterokaryotic descendants, and (3) crossover is not frequent. What are the consequences in terms of **variability recovered among heterokaryotic and homokaryotic offspring?**

In the basidia, karyogamy, meiosis, and sporogenesis successively occur. In the bisporic basidia of the var. *bisporus*, the migration of the four haploid postmeiotic nuclei is not random: each spore receives two non-sister postmeiotic nuclei, one nucleus from each second division of meiosis. This model agrees with all studied offspring except an atypical one in which inclusion of sister nuclei has occurred (Spear et al. 1983). Evans (1959) proposed that the non-random distribution results from the spatial position of the two second divisions, whilst Kamzolkina et al. (2006) suggested it could result from their asynchronous divisions. Whatever the explanation, this process implies that **the heterokaryotic spores receive the two homologous centromeres of the parental heterokaryon** for each pair of chromosomes. In the absence of crossover, the heterokaryotic offspring would have the same global genotype as the parent, and 100 % of the parental heterozygosity would be conserved. However, due to the **random distribution of the centromeres at the first meiotic division**, the centromeres, like the alleles of any heterozygous loci located on different chromosomes, can be differently distributed among the two nuclei of the heterokaryotic descendants. Such heterokaryons can exhibit different phenotypes via epistatic effects that partly explain phenotypic variability among heterokaryotic offspring.

This has been shown experimentally in *Neurospora crassa* (Burton and Metzenberg 1972). If crossovers occur, alleles segregate at the second meiotic division, and as a result heterokarotic spores can be homoallelic at a locus that was heteroallelic in the parent. The probability of losing the parental heteroallelism at a given locus theoretically increases with the distance between this locus and the centromere. The fact that the heterokaryotic offspring remains heterokaryotic at *MAT* suggests that *MAT* is close to the centromere. The loss of parental heterozygosity is low in heterokaryotic offspring, suggesting that the rate of crossovers is low, but that it can also result from selection due to deleterious or lethal recessive alleles making unviable homoallelic recombined heterokaryons.

Finally, the observed variability among a heterokaryotic offspring can result from different processes: the redistribution of the centromeres in the two nuclei, the moderate loss of parental heterozygosity, heterokaryotic selection,

and other possible processes (see Moquet et al. 1998). This is the **genetic variability resulting from the intramictic process of the pseudohomothallic life cycle** which breeders try to exploit by selecting inside **monospore cultures**. Heterokaryons isolated from **multispore** cultures could additionally result from **self-cross either between homokaryon and heterokaryon** or between two compatible homokaryons (plasmogamy in the heterothallic life cycle). In the latter case, half of the parental heterozygosity is theoretically lost, and inbreeding depression occurs as has been observed by Xu (1995), but in a back-cross generation.

Today**, using the *Bsn-t* alleles of var. *burnetii* or var. *eurotetrasporus*, large homokaryotic offspring are available** to develop **methods based on controlled hybridization** (see below III-B). The variability among the homokaryotic offspring highly depends on the rate of crossovers. In a segment of chromosome I, the rates of recombination observed among the progeny of hybrids between the different varieties have suggested that an incompletely dominant allele(s), possibly *Bsn-t*, could determine a high recombination rate (Kerrigan et al. 1993; Callac et al. 1997, 1998). This is consistent with the hypothesis that high and low rates of recombination are adaptive for heterothallic and pseudohomothallic isolates respectively. In *A. bisporus* var. *bisporus*, successive generations of heterokaryotic spores may permit deleterious alleles to accumulate. A low rate of recombination maintains a high level of heterozygosity and, by complementation, high viability and fitness among most offspring.

3. Outcrosses: Which Way?

Outcrosses and recombination generate variability which is required for genetic selection in breeding programs. This variability is also crucial for adaptation in nature. From the predominantly pseudohomothallic life cycle described above for *A. bisporus* var. *bisporus*, it seems difficult to perform outcrosses; as one might expect they are infrequent in the wild populations since only a small proportion of the offspring are homokaryotic. Population studies indicate that **outcrossing occurs in the wild** (Xu et al. 1997), but although Kerrigan found in North America some evidence of pseudoclonal lineages resulting of successive pseudohomothallic generations (Kerrigan 1990), this was less evident in European studied populations, in which outcrossing could be more frequent (Xu et al. 2002). The two main ways of outcrossing are **crosses between compatible homokaryons** (heterothallic life cycle) and **crosses between homokaryons and heterokaryons.** The latter process, also called the "**Buller phenomenon**" (Buller 1931) was first reported by Raper et al. (1972) in *A. bisporus*.

In different experiments, Callac et al. (2003, 2006, 2008, and unpublished) inoculated a standard substrate for *A. bisporus* cultivation simultaneously with homokaryotic mycelium from one parent and spores from a second parent (Fig. 1.3). Culture trays have consistently produced numerous sporophores that could theoretically have resulted from five different reproductive modes (pseudohomothallism, selfing or outcrossing via heterothallism, and selfing or outcrossing via the Buller phenomenon). However, genotype analysis showed that all or almost all the sporophores consistently resulted from outcrossing between the inoculated homokaryon and the inoculated heterokaryotic spores (or mycelia that grew from them), i.e., via the Buller phenomenon. Control trays inoculated with spores only or mycelium only did not produce any sporophores. The exceptions were due to contaminations by unexpected inoculum in first experiments because the air entering in the culture room was not sterilized. This method represents **an easy way to get numerous hybrids in a single experiment.**

Such hybrids can receive recessive deleterious alleles, since there is no haploid step (no gametic/haploid selection) for the material coming from the parent of the spores, but this is not necessarily a disadvantage. For instance, it has been shown that such hybrids, which received recessive lethal alleles at loci tightly linked to *MAT* from the spores of one of the parents, were on average less susceptible to dry-bubble disease than those that did not receive these alleles (Callac et al. 2008). Such resistance

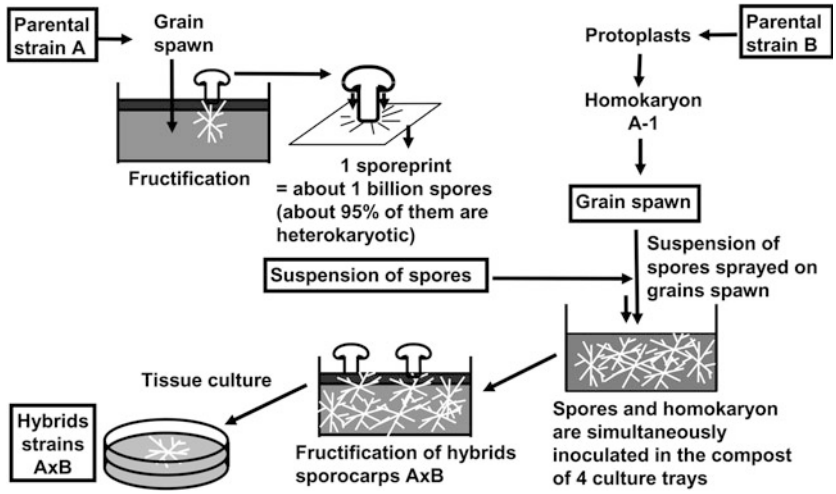

Fig. 1.3. Method of hybridization in *A. bisporus* var. *bisporus* using the Buller phenomenom. A homokaryon from a first parent and spores from a second parent are simultaneously inoculated in standard compost trays. Numerous hybrid sporophores are produced, each of them resulting from a cross between a heterokaryotic spore and the homokaryon

linked to *MAT* and to a locus bearing a lethal recessive allele could not be exploited using conventional crosses between homokaryons. A QTL of disease resistance was identified in the vicinity of *MAT* (Foulongne-Oriol et al. 2012b). It was also shown that these hybrids systematically had the mitochondrion of the homokaryon, even though in conventional crosses between homokaryons of the two parents this mitochondrion was never inherited. This method can be used to **control the effect of mitochondrial inheritance in the crosses**. In these experiments, the complete absence of sporophores directly issued from the heterokaryotic spores was unexpected, and the success of this method with different parental strains suggests that the Buller phenomenon could play a role in nature.

4. Mycelium and Anastomosis

The mycelium of *A. bisporus* can be homokaryotic (n) or heterokaryotic ($n+n$), but in both cases articles of the hyphae are without clamp connection, and multinucleate with a variable number of nuclei (Saksena et al. 1976; Hou and Elliott 1978; Kamzolkina et al. 2006). The mycelium can highly resist cold or dryness. Although vegetative spores or conidia are not detected in *A. bisporus*, **pieces of mycelium could have a role of dissemination** in the field but could also contaminate another mycelium by transmitting a virus (MVX dsRNAs; Grogan et al. 2005) or by crossing with it (Callac et al. 2003). Such contaminations occur via anastomosis. **Anastomosis** between two mycelia permits exchange not only of nuclei but also mitochondria, cytoplasm, and any other intracellular components.

Anastomosis occurs between heterokaryons that can be genetically different and allow trophic and other exchanges, as in the following cases: (1) in experimental transplantation of sporophores on a recipient mycelium (Sinden et al. 1962), (2) in experimental cocultivation of a homokaryotic mycelium and spores that produce many genetically different hybrid sporophores on the same compost tray (Callac et al. 2006), and (3) in certain processes of cultivation (CACing) in which mushroom spawn is added to the casing soil. Using a transgenic mycelium running in the compost while the spawn added in the casing layer was not transgenic, it was observed that the produced sporophores did not bear the transgene but exhibited the transgenic phenotype (Romaine et al. 2011; Woolston et al. 2011). In contrast, to avoid viral transmission, anastomosis is not

desirable, as in the hybrid strain J10165 which exhibits cultural incompatibility with the most frequently cultivated hybrid strains (Kerrigan and Wach 2010). However, until now, **genetics of the vegetative incompatibility remains unknown in *A. bisporus***.

Anastomosis plays a major role in the life history: it can occur between sexually intercompatible homokaryons, and thus restores a heterokaryon (plasmogamy in the heterothallic life cycle, see Fig. 1.2), between a homokaryon and a heterokaryon, in which case the resulting novel heterokaryon bears the nucleus of the homokaryon and a sexually compatible nucleus coming from the heterokaryon, and also between two heterokaryons. In the latter case, formation of a novel genetically different heterokaryon is generally neither reported nor detected, but cannot be excluded since (Xu et al. 1996) detected somatic recombinants in subcultures from both heterokaryon x heterokaryon and heterokaryon x homokaryon pairings. In *A. bisporus*, some processes observed in certain other agaricales (see Kues 2000) do not occur or have not been detected: for example there is no clear evidence of nuclear migration through the homokaryotic mycelium, following the Buller phenomenon. On the other hand, diploid nuclei have never been detected, except in basidia, although they could be expected to be detected through a mechanism of somatic recombination.

The heterokaryotic mycelium is homologous to a diploid organism for its genetic expression, but because it possesses individual haploid nuclei, it can play a role of gamete and crosses with a homokaryon naturally via the Buller phenomenon, or after having artificially recovered haploid status (deheterokaryotisation) in vitro, either mechanically by fragmentation of the heterokaryon (Dickhardt 1985) or biochemically by using glucanases (protoplast method; Anderson et al. 1984; Kerrigan et al. 1994). These techniques are used by breeders for recovering homokaryons.

Whatever the origin of a mycelium, germination of spores, regeneration of protoplasts, or even tissue culture from a wild sporophore that can be haploid, multilocus genotype tests using codominant markers (Kerrigan et al. 1993) are needed to know its ploidy status (n vs. $n+n$), because cytology cannot easily help, and other tests such as mycelium growth rate test, mating test or fruiting tests are not reliable enough (Kerrigan et al. 1994).

The ability to stimulate spore germination via volatile agents (Lösel 1964) such as isovaleric acid which would remove the $C0_2$-self-inhibitor in the spores by participation of β-methylcrotonyl-CoA carboxylase (Rast and Stauble 1970) is another property of the mycelium that is crucial for reproduction. In vitro, but also in semi-controlled condition (in the culture compost tray), the rate of spore germination can increase greatly when a mycelium is present in the neighborhood.

Isozymes (Royse and May 1982a), restriction fragment length polymorphisms (RFLP) markers (Castle et al. 1987), and sequence characterized amplified region (SCAR) markers or cleaved amplified polymorphic sequences (CAPS) markers (Callac et al. 1997) were successfully used.

In conclusion, **the mycelium of *A. bisporus* is far from a simple vegetative organ**. It is treated so as to maintain isolated strains, but in the wild numerous events can occur between strains and modify the genotypes without meiosis. In fact, we found poor evidence of clonality among the studied populations in Europe, even within each site (Xu et al. 2002), with the exception of a site in Portugal where several hundreds of sporophores had the same genotype (unpublished data). Mycelium in the wild generally does not extend in an area larger than 1 m diameter. But how long this particularly resistant mycelium can be maintained in place, or how far away it can be disseminated and form new colonies, remain open questions, which we are investigating. It was illustrated above how the progress in knowledge concerning mushroom reproduction is a source of innovation for developing new breeding strategies, in addition to the conventional ones that benefit also from molecular and genomic tools.

B. Molecular Breeding

Many economically important production traits, such as yield, quality, or resistance to diseases in edible mushroom cultures are under **polygenic inheritance**. Selecting for such complex traits with the classical breeding method appears quite challenging. The **dissection of these quantitative traits in individualized loci through QTL mapping** greatly facilitates their effective manipulation in a subsequent breeding program. Therefore, the development of molecular markers and linkage maps provides efficient tools to investigate genetics of desirable traits, and offers new opportunities for breeding. Although such approaches have been extensively proven to be successful in plant or animal, the use of molecular markers in mushroom breeding is a relatively new applied science that is developing mainly with *A. bisporus* as a model.

1. Quantitative Genetics

The **construction of a comprehensive linkage map** is the first step towards understanding the genetic basis of complex traits. The genetic linkage map developed for *A. bisporus* by Kerrigan et al. (1993) was the first molecular-marker-based map for an edible mushroom species. This map was based on the analysis of RAPD and RFLP segregating markers in an *A. bisporus* var. *bisporus* intravarietal offspring. It was unsaturated, with fewer linkage groups (11) than the number of chromosomes ($n=13$) and several unlinked markers. Another *A. bisporus* linkage map, based on an intervarietal var. *bisporus* x var. *burnettii* offspring, was initiated (Callac et al. 1997; Moquet et al. 1999). With only 26 markers (RAPD, CAPS) or genes spread on five linkage groups, this latter map was also far from saturation. Afterward, advances in molecular marker techniques, such as AFLP or SSR genotyping, have made it possible to enhance the level of saturation of this map. Thus, based on the same mapping progeny, the addition of hundreds of new loci (AFLP, CAPS and SSR) permitted the construction of the first comprehensive linkage map for *A. bisporus* (Foulongne-Oriol et al. 2010). This map was built with 324 markers, evenly spread over 13 linkage groups, each one assigned to the corresponding chromosome of *A. bisporus*. The map covered 1,156 cM, with an average marker spacing of 3.9 cM, and encompassed nearly the whole genome (Fig. 1.4). This reference map is a useful and adequate tool for genetic studies in *A. bisporus*.

In parallel, a **phenotypic database**, comprising numerous traits of interest assessed on the intervarietal derived materials, has been established (Fig. 1.5). **The combination of phenotypic data along with genotypic data has permitted the genomic location of either genes or QTL.** Indeed, Mendelian traits related to the reproductive mode in *A. bisporus* have been mapped as phenotypic markers. ***BSN*, the primary determinant of basidial spore number, and *MAT* which controls the mating ability** both mapped to chromosome I, but were far apart. The *MAT* locus was assumed to be in a centromeric position, while *BSN* was located in the distal portion of the chromosome (Foulongne-Oriol et al. 2010).

Cap color was first investigated as a simply inherited trait, and the *PPC1* locus has been mapped on chromosome VIII (Callac et al. 1998). QTL analysis has made it possible to refine the inheritance of this trait. In addition to the major determinant *PPC1* which explained 86 % of the phenotypic variability, two minor loci were found on two other chromosomes, confirming the oligogenic control of this trait. These two additional loci contributed to the variability observed for the color gradation of the cap within the brown genotypes (Foulongne-Oriol et al. 2012a). This could be interesting for further breeding purposes, with the renewed attraction of brown mushrooms for the consumer (Robles and Lodder 2009). The genetics of other quality traits related to **shelf life performance** and **susceptibility to discoloration** after harvest, such as bruising, stipe brittleness, cap scaling, or long-term storage behavior, remains poorly documented. This could be explained by the difficulty in establishing a reliable and reproducible assessment for these traits at a large population scale. Recently, thanks to progress in bruising sensibility assessment, Gao et al.

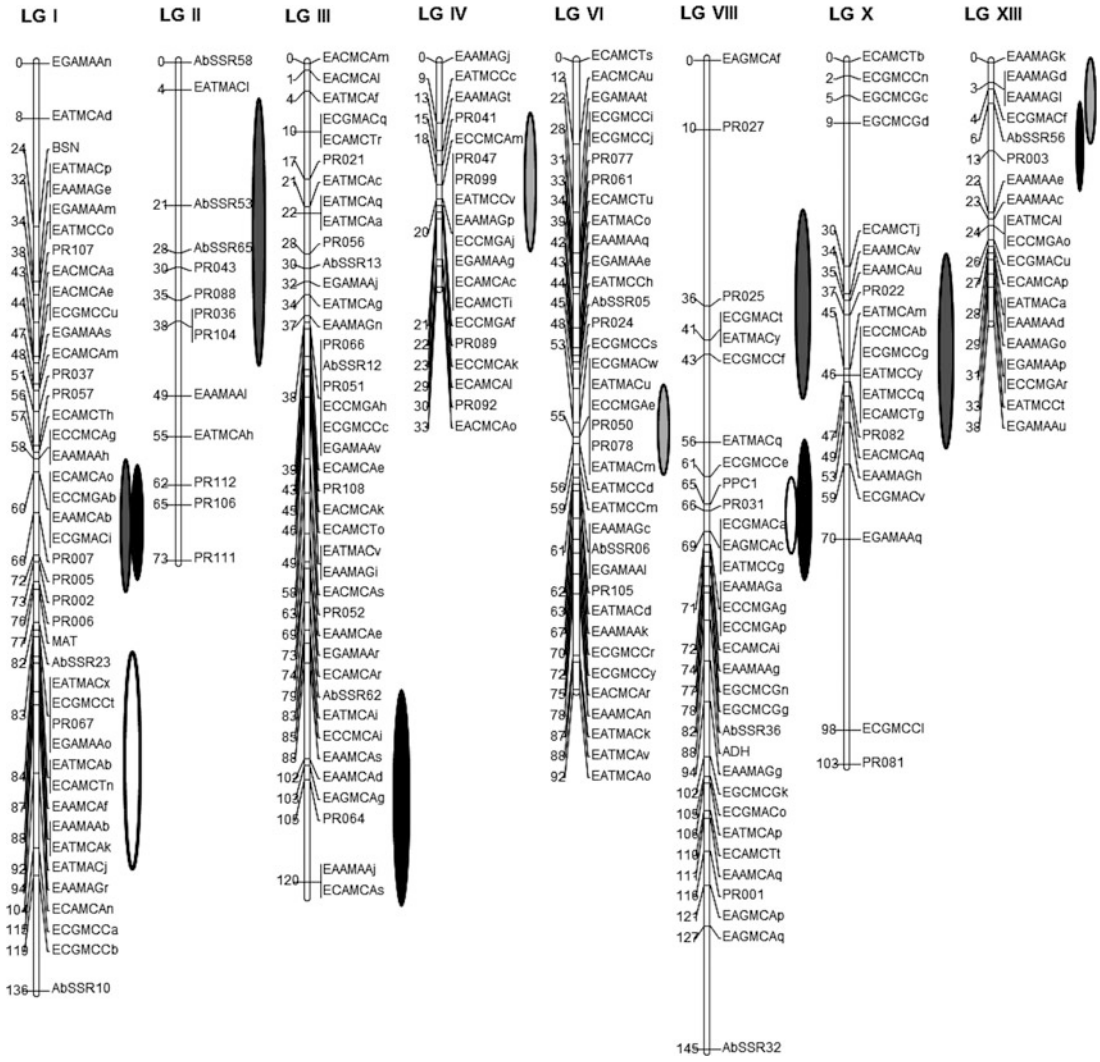

Fig. 1.4. Comparative mapping of quantitative trait loci involved in resistance to the three major diseases affecting *Agaricus bisporus*. Only the linkage groups with QTLs are represented. ○ QTL resistance to the bacterial blotch, *Pseudomonas tolaasii* (Moquet et al. 1999). ○ QTL resistance to *Trichoderma* metabolites (Foulongne-Oriol et al. 2011c). ● QTL resistance to *Lecanicillium fungicola*, bubble symptom (Foulongne-Oriol et al. 2012b). ● QTL resistance to *L. fungicola*, spotted cap symptom (Foulongne-Oriol et al. 2012b)

(2011) identified at least three inheritance patterns, and they are mapping QTLs for this trait. With QTL mapping, various quality traits are becoming available for marker-assisted selection and studies on their biological bases.

The development of resistant cultivars is the most effective, economical, and environmentally friendly approach to the management of disease control. Bacterial brown blotch (caused by *Pseudomonas tolaasii*), dry bubble (caused by *Lecanicillium fungicola*), and green mould (caused by the fungal competitor *Trichoderma aggressivum*) are the most detrimental disorders affecting yield and quality of the button mushroom throughout the world. Independent QTL mapping studies investigating the genetics of the resistance to these diseases have been performed.

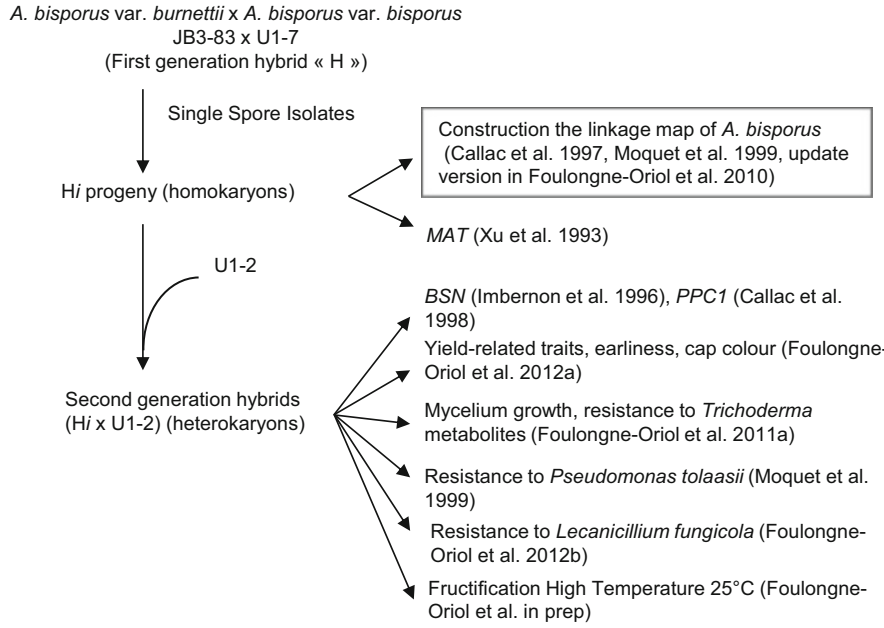

Fig. 1.5. Overview of genetic studies performed on the fungal material derived from an intervarietal *Agaricus bisporus* var. *burnettii* x *A. bisporus* var. *bisporus* cross

In one of the first QTL mapping studies in fungi, Moquet et al. (1999) described a major QTL that underlay the **resistance to *P. tolaasii***. This QTL explained about 30 % of the phenotypic variation, and was closely linked to cap color locus *PPC1* (Moquet et al. 1999). An update analysis using the saturated linkage map data made it possible to find one additional minor locus on LG I involved in *P. tolaasii* resistance (LOD = 2.85). These two loci together explained 38 % of the phenotypic variation (Foulongne-Oriol, unpublished data). Contrary to the major QTL, the second locus was not confirmed when using bacterial toxin to mime the infection. However, this was not surprising since toxin-induced symptoms partially reproduce living bacteria-induced symptoms (Moquet et al. 1999). The different mechanisms and traits that combine for the resistance to a disease might be linked to different QTLs, and be identified thanks to QTL analysis.

In contrast, one can decompose different traits that might be responsible for the final decreases of symptoms in a resistant cultivar. A part of the **resistance to *T. aggressivum*** in *A. bisporus* was indirectly assessed by the ability to counteract the growth-limiting effect of lytic enzymes and metabolites produced by *Trichoderma* sp. (Foulongne-Oriol et al. 2011a). The level of tolerance and the capacity of adaptation to these compounds were quantitatively inherited and under oligogenic control, with two QTL detected per trait. The QTL on LGIV was involved in the control of the two traits, suggesting a key role. **The colocation** with QTL related to mycelium growth in control condition makes it possible to presume that the ability to resist or adapt to *Trichoderma* metabolites are tightly linked to the fitness of *A. bisporus* strains. Even if validation in natural conditions with living *T. aggressivum* is needed, we show here that colocation of QTLs help us in the understanding of the host–pathogen interaction.

Complex **resistance to *L. fungicola*** was also dissected through QTL mapping (Foulongne-Oriol et al. 2012b). Bubble and spotted mushroom, which are the two symptoms typifying dry-bubble disease during the successive steps of the infection, were analyzed separately. The QTLs involved in the expression of these two

symptoms were detected as expected in distinct genomic regions, except on LGI. Colocations between QTLs governing *L. fungicola* resistance and production traits highlighted some **unfavorable linkage drag**, particularly on LGI, LGII, and LGX (Foulongne-Oriol et al. 2012b). The most resistant hybrids tended to produce numerous small mushrooms early. These results emphasized the difficulty **of introgressing desirable traits from wild strains while maintaining an acceptable agronomic level.**

A complex picture of the resistance mechanisms in *A. bisporus* is provided by the **comparative mapping of the QTLs controlling the resistance to three major diseases** (Fig. 1.4). A majority of the QTLs was found specific to one disease, suggesting that distinct mechanisms are involved. Nevertheless, some QTLs related to multiple resistances were highlighted on LGI, LGVIII, and LG XIII. Interestingly, on LGVIII the region in the vicinity of the *PPC1* locus was found to be significantly involved in the resistance against *L. fungicola* (during secondary infection) and *P. tolaasii*, two pathogens that produce spotted cap symptoms. In each case, the resistance allele was associated with the brown allele at *PPC1*. A shared mechanism of resistance based on melanin biosynthesis could explain such reactions. The colocation of QTL observed on LGI and LGXIII may reflect linkage or pleiotropic effects. The QTL controlling the two dry-bubble symptoms on LGI mapped in the same genomic interval as earliness (see below), and thus may be related to fitness. On LGXIII, a common genomic region was involved both in dry-bubble spotted cap symptom and adaptation to *Trichoderma* metabolites, but the parental origin of the resistance allele was not the same. These results suggested that **selecting for multi-resistance** in *A. bisporus* could be quite arduous. A multi-way breeding scheme could be conceived to combine all the resistance in one genotype.

As for disease resistance, inheritance and QTL analyses **of parameters composing the final yield of mushrooms** could be informative both for the understanding of the mushroom biology and for modifying the characteristics of the harvest (i.e., earliness, distribution of the yield between the flushes ...). The complex genetic architecture of yield-related traits has been disentangled through QTL mapping (Foulongne-Oriol et al. 2012a).

As an example, earliness was also analyzed, and was found to be linked to production traits. The earliest hybrids tended to produce the highest number of smaller mushrooms. The development of new strains that cover a large range of earliness could be interesting to diversify mushroom industry outlets (Foulongne-Oriol et al. 2012a).

Work is in progress also for the analysis of the inheritance of adaptive traits such as temperature tolerance (Fructification at High Temperature 25 °C in Fig. 1.5) or ability to growth and fruit on compost. New information is expected.

2. Marker-Assisted Selection

The basic concept of **marker-assisted selection (MAS)** is to **select on the genotype of the marker(s) tightly linked to the trait rather than to select on the phenotype** (Collard and Mackill 2008; Hospital 2009). Since most of the interesting traits in edible mushrooms are only displayed at fruiting stage, early selection with molecular markers allows an accurate screening of desirable offspring without the cultivation step. Molecular markers in *A. bisporus* have been already used for homokaryotic spore isolation (Kerrigan 1992), cap color selection (Loftus et al. 2000; Foulongne-Oriol et al. 2011b), or mating-type compatibility design (Sonnenberg et al. 2005). A first marker-assisted backcrossing program for a polygenic trait introgression was mentioned in Sonnenberg et al. (2005). The authors used genetic markers to select not only for the trait of interest but also for the genetic background, in order to limit unfavorable linkage drag. They highlighted the difficulty of having all favourable loci in a unique genotype in the advanced generation of the breeding scheme in the case of a polygenic trait (Sonnenberg et al. 2005). To counterbalance this drawback, a promising QTL pyramiding strategy was proposed. The *a posteriori* assessment of the reliability and the

effectiveness of multitrait marker-assisted selection in an *A. bisporus* breeding scheme has also been undertaken in our laboratory.

It's noteworthy that the **successful use of molecular markers for selection is tightly linked to the recombination ability of the species** (Collard and Mackill 2008). In this way, the availability of linkage maps offers also new insights into genome organization and recombination frequencies. It has been demonstrated that a higher rate of recombination occurred in the intervarietal linkage map (var. *bisporus* x var. *burnettii*) compared to the intravarietal one (Kerrigan et al. 1993; Foulongne-Oriol et al. 2010). As mentioned above, this phenomenon had been supposed to be adaptive and related to the different life cycles which typify the two *A. bisporus* varieties. Furthermore, the recombination ability in *A. bisporus* seems to be strongly impacted by the genetic background, as illustrated by a comparative linkage mapping study (Foulongne-Oriol et al. 2011b). Such **variability in recombination behavior could be exploited judiciously in mushroom breeding programs**. For example, a high recombination rate would greatly facilitate the introgression of a desirable trait, while limiting linkage drag. However, subsequent drawbacks would be the spreading of small unfavorable donor segments into the recipient genome, and the loss of linkage between markers used in selection and the selected trait. In this way, an acceptable compromise may be achieved.

C. Genetic Improvement of Mushrooms in the Era of Genomics

The recent sequencing of whole genomes of edible mushrooms is going to contribute hugely to our understanding of their biology, life cycles, and ecological behavior, and will contribute in the near future to crop improvements.

1. Genomics of *A. bisporus*

In 2007, the Joint Genome Institute, from the US Department of Energy (DOE), agreed to sequence the whole genome of *A. bisporus* due to the position of *Agaricus* spp. in forest ecosystems as a humicolous species, able to deploy a specific enzymatic pattern in comparison to other detritophilic fungi (Kerrigan 2011). Fungi which play key roles in the ecosystem process, particularly in the carbon cycle, are preferential targets supported by the Fungal Genomics Program (Grigoriev et al. 2011). Two homokaryons (haploid genome) of *A. bisporus* have been proposed. H97, obtained from the historically-cultivated stock Horst U1, was sequenced using the Sanger method with a depth of 8.29x. The H97 genome sequence consists of 30 Mb assembled in 29 scaffolds. A hundred of the sequenced markers located on the *A. bisporus* linkage maps (Foulongne-Oriol et al. 2010, 2011b) have made possible the assignment of the scaffolds to the 13 chromosomes. A few small gaps remain, but comprise less than 0.7 % of the nuclear genome. Ten thousand four hundred and thirty-eight gene models have been identified. Thanks to the genome coverage and the quality of the finished assembly, the H97 genome sequence constitutes the 'reference' for the species (Morin et al. 2012). The other homokaryon, JB137-s8, belonging to the tetrasporic var. *burnettii*, was sequenced with 454 pyrosequencing and Illumina HiSeq methods, and could be aligned on the H97 sequence. **Both genomes are now in the public domain** on the JGI's portal web site (http://genome.jgi-psf.org).

Therefore, the whole-genome sequence of *A. bisporus* is the first to be available from the large family of *Agaricaceae,* and provides a solid foundation for **understanding its particular nutrition mode and ecological adaptation** (Morin et al. 2012). It will become a highly valuable resource for performing genomic and metabolic comparisons among fungi. Beyond its interest for fundamental knowledge, **the release of the *A. bisporus* genome sequence opens a new era for breeding applications**. First, the whole-genome sequence is a resource for new target DNA markers, especially SSRs (Labbe et al. 2011; Murat et al. 2011). The density of SSRs in the *A. bisporus* genome (approx. 60 SSRs per Mb, considering di, tri, and tetra motifs with at least five repeats; *personal data*)

will provide a considerable molecular toolbox for map construction and further genetic applications. High-throughput sequencing also allows the development of single nucleotide polymorphism (SNP) markers. In *A. bisporus*, the sequencing of the two nuclei that constitute the Horst U1 strain has allowed the identification of more than 280,000 SNP, which corresponds to a frequency of about 0.93 %. However, less than 0.5 % of these SNP has been used for the development of 600 operational molecular markers (Sonnenberg et al. 2011). Their routine use implies high-throughput genotyping methods such as SNP microarrays. To date, these approaches have been rarely described in fungi, but are likely to increase. For example, in *M. graminicola,* the Diversity Arrays Technology (DArT) permitted the mapping of 1,793 markers (Wittenberg et al. 2009).

The tight relationship between linkage map and genome sequence will also make possible **custom-made molecular markers tightly linked to target loci** for further marker-assisted selection. It is premature to already expect concrete examples of applications in *Agaricus* breeding, but we can speculate that it will greatly facilitate cropping. This makes it possible to imagine that one would be able to perform some genomic selection in the next few years. It also offers a milestone towards the understanding of the molecular mechanisms that underlie traits of interest, through map-based cloning approach or candidate gene identification. Furthermore, other biotechnological tools such as transformation, reporter-gene expression, or gene-silencing are well established for *A. bisporus* (Burns et al. 2005, 2006; Eastwood et al. 2008), and will support subsequent functional validation of the gene(s) of interest. In combination with the genetic resources available, the whole genome sequence also offers the opportunity of performing genome-wide association studies to disentangle complex traits.

The challenge now for scientists and mushroom breeders will be to take advantage of the best of all the genome-based tools. It appears that the major limitation will be the way of implementing these new tools in research pipelines with, for example, the accessibility of a high-throughput genotyping platform, the ability to develop and use new statistical models and tools for bioinformatics, or the capacity of large-scale phenotypic screens.

2. Molecular Organization, Evolution, and Transmission of Mitochondrial Genes and Genomes in Mushrooms

Horgen and Castle (2002) have previously illustrated that the **mitochondrion is a genetic component of mushrooms susceptible of being manipulated** and resulting in unique and different genetic combinations involving nuclear genomes mixed with specifically chosen mitochondrion haplotypes and having potential consequences on strain performance. Improvements in the knowledge of the mitochondrial genes and genome organization, expression, and inheritance in Agaricomycetes will undoubtedly give a major contribution to the development of fungal biology and to the improvement of cultivated mushrooms.

a) Mitochondrial Genomics of Mushrooms

To date, only four complete mitochondrial genome (mtDNA) sequences have been reported and correctly annotated for the whole Agaricomycetes class: for *Schizophyllum commune* (49,704 bp) (GenBank Accession No: AF402141), *Moniliophtora perniciosa* (109,103 bp) (Formighieri et al. 2008), *Pleurotus ostreatus* (73,242 bp) (Wang et al. 2008b), and *Trametes cingulate* (91,500 bp) (Haridas and Gantt 2010). It is to be noted that the complete sequence of the mtDNA of *A. bisporus* (135,005 bp) is now available, in parallel with the achievement of its complete nuclear genome. The Agaricomycete mitochondrial genomes show a great variation in size from 36 kbp in *Suillus cavipes* (Bruns et al. 1988) to 176 kbp in *Agaricus bitorquis* (Hintz et al. 1985), and several Agaricomycete mtDNA possess larger size than those reported in the Ascomycota phylum (higher than 100,000 bp).

Plasmid-derived sequences are involved in the large size of the Agaricomycete mtDNA. In *P. ostreatus* mtDNA, Wang et al. (2008b) have

described the presence of a DNA polymerase gene and a RNA polymerase gene, nearly identical to the genes harboured by a mitochondrial linear plasmid described in another *P. ostreatus* strain, confirming that this **selfish genetic element is able to integrate and to be maintained in the mitochondrial genome.** The *Agrocybe aegerita* mitochondrial genome has also been shown to possess two copies of a DNA polymerase gene (*polB*) with linear plasmid origin: one copy (Aa-polB) was putatively intact and consequently functional (Bois et al. 1999), and the other (Aa-polB P1) appeared eroded (Barroso et al. 2001). Interestingly, the regions flanking this eroded copy region (5834 nt) carried two large inverted repeats (higher than 2,421 nt), and contained identical copies of the *nad4* gene (Ferandon et al. 2008). In the *Agaricus* genus, a linear mitochondrial plasmid, pEM, was described in isolates of *A. bitorquis*. The mitochondrial genome of *A. bitorquis* and *A. bisporus* were shown to possess a fragmented and potentially non-functional copy of the plasmid RNA polymerase (Robison et al. 1991), which had probably integrated the *Agaricus* mitochondrial genome before the divergence of *A. bisporus* and *A. bitorquis* (Robison and Horgen 1996). In addition to this remnant, RNA *pol* gene, the *A. bisporus* mitochondrial genome contains a second site of plasmid integration occupied by an intact DNA *polB* gene (G. Barroso, personal communication), and located near a copy of a previously reported (Jin and Horgen 1993) large inverted repeat (IR: >4,000 nt). The large (>38,000 nt) sequence separating both IR contains several typical mitochondrial genes (*rps3*, seven tRNAs, the LSU-rDNA, the *nad3* and *nad2* genes). This constitutes the second report, after *A. aegerita* (Ferandon et al. 2008), of the **presence of a large inverted repeat located near a plasmid integration site.**

Hence, **mitochondrial linear plasmids appear as mobile genetic elements frequently found in mushroom mitochondria** and able to integrate the mtDNA. The integrational event can be accompanied by molecular rearrangements such as large duplications of the flanking region, and is followed or not by an erosion of the integrated plasmid sequences. Finally, the Agaricomycete mitochondrial genomes seem frequently to integrate linear plasmid sequences able to promote large inverted repeats whose significance and consequences are still unknown. In this context, it will be noted that in *A. bisporus* as well as in *A. aegerita,* the duplication leads to a significant increase in the amount of mitochondrial genes such as several tRNAs in *A. bisporus* (G. Barroso, personal communication) or the *nad4* gene in *A. aegerita* (Ferandon et al. 2008).

The size variations in mitochondrial genomes of agarics are mainly due to **the presence of numerous large group I introns in most of the mitochondrial genes** (Ferandon et al. 2010). The *cox1* gene is the mitochondrial gene showing the highest number of introns. The complete sequence of the mitochondrial *cox1* gene of *A. bisporus* has been recently achieved, and has shown that this longest *cox1* gene (29,902 bp) is the largest group I intron reservoir (18 group I introns) reported to date in a eukaryote. It contains 18 out of 24 of the fungal introns described in all the fungal *cox1* genes from Dikaria (Ascomycota and Basidiomycota). These long mitochondrial genes pose an appealing and still unresolved question about why and how some fungal species organize their mitochondrial genes and genomes in such an expensive manner, and what are the consequences on their biology.

mtDNA sequences are used to develop markers to follow mitochondrial genetic material during breeding experiments and strain improvement. Moreover, mitochondrial genomes, because of their rapid rate of sequence divergence, are also appealing molecules to use in the study of fungal wild populations or evolutionary biology. Two major types of mitochondrial sequences appear interesting for adding molecular markers suitable as taxonomic and/or phylogenetic tools at the species level and/or for strain fingerprinting, to the well-studied nuclear ribosomal unit. The first one is the compiled sequences of two variable domains (V6 and V9) of the SSU-rDNA, encoding the 16S RNA of the small-subunit of the mito-ribosome. Indeed, these domains mainly evolve by length mutations involving indel (insertion/deletion) sequences and,

consequently, can easily lead to CAPS markers for species determination (Barroso et al. 2003). The variable domains V4, V6, and V9 of the mitochondrial SSU-rDNA have been successfully used to discriminate between closely related species in the *Pleurotus* and *Agrocybe* genera (Gonzalez and Labarère 1998, 2000). The second kind of sequence is constituted by the orthologs of the *iAbi7* intron of the *A. bisporus cox1* gene (Ferandon et al. 2010). Indeed, this group I intron appears widely distributed in the eukaryote kingdom, but also in the *Agaricus* genus (data not shown). Moreover, this mobile genetic element carries a structural gene, encoding a homing endonuclease (HE). It appears to be maintained in a functional state during the evolution course. Consequently, it can constitute a performing phylogenetic marker replacing the "barcoding region" of the *cox1* gene, which is split by several large group I introns in the fungi, and this especially in the *Agaricus* genus.

b) Mitochondrial Effects on Mushroom Development

Influence of mitochondrial haplotype on mycelial growth of *A. bisporus* was studied by De La Bastide et al. (1997). Pairs of heterokaryon strains, each pair having the same nuclear genomes but a different mitochondrial genome, were produced by controlled crosses of homokaryons with wild or commercial origins. Seven genetically distinct mitochondrial DNA (mtDNA) haplotypes were evaluated in different nuclear backgrounds. The growth of heterokaryon pairs differing only in their mtDNA haplotype was compared at three temperatures similar to those utilized in commercial production facilities (18 °C, 22 °C, and 26 °C). Statistically significant differences were detected in most of the heterokaryon pairs evaluated. Some heterokaryon pairs showed differences of growth at all three temperatures of incubation, suggesting a temperature-independent difference. Others showed differences at only a single temperature, suggesting a temperature-dependent difference. **The influence of some mtDNA haplotypes on growth was dependent on the nuclear genetic background,** showing that mtDNA haplotype can influence growth of *A. bisporus* heterokaryons in some nuclear backgrounds. Combinations of mitochondrial and nuclear genomes may also affect other traits involved in agronomical performances and ageing during storage.

Ageing of biological systems is a fundamental process controlled by a complex network of molecular pathways, and mitochondria have been shown to play a crucial role in programmed cell death and ageing.

Hence, in the yeast *S. Cerevisiae* (for a review see Braun and Westermann 2011), different stimuli have been shown to activate distinct mitochondrion-dependent cell death pathways, and it has been demonstrated that ageing is associated with a progressive increase in mitochondrial damage, culminating in oxidative stress and cellular dysfunction. In the same way, in the filamentous fungus *Podospora anserine* (for review see Osiewacz 2011), a wide range of pathways have been identified that contribute to the maintenance of a population of functional mitochondria. These pathways act in a hierarchical manner, but all are limited in capacity. At the end of the life cycle, when the pathways are overwhelmed and damage has reached certain thresholds, programmed cell death brings the life of individual *P. anserina* to an end.

In *A. bisporus* sporophores, the gene of a superoxide dismutase (SOD) of the iron/manganese family usually located in mitochondria is up-regulated following harvest (Henderson et al. 2005). This renders them capable of responding rapidly to a potentially lethal level of superoxide radicals. These **ageing processes linked to mitochondria might probably affect the shelf life of mushrooms**, and are worth studying to produce improved strains.

Some cultivars of *A. bisporus* subcultured for a long time have shown phenotypic variability or vegetative decline with variation in growth which might be related to changes in mitochondrial genome organization (Jin et al. 1992). Such reports suggest that mitochondrial genetic material could be correlated with still unknown physiologically important functions of fungi and, consequently, give an important interest to the investigations on mitochondrial genomics and heredity. Identification of mitochondrial genotypes and evaluation of different nuclear–mitochondrial combinations in strain

improvement programs are promising directions for the button mushroom industry.

c) Mitochondrial Inheritance in Agaricomycetes

In fungi of the Agaricomycete class, sexual mating does not rely on organs or cells specialized for sexual reproduction, but relies on a fusion between two vegetative homokaryotic hyphae (somatogamy). After cell fusion, both hyphae exchange nuclei, leading to a dikaryon with both parental nuclei formed in the existing thallus of the homokaryon. In most of the studied species, **reciprocal (bidirectional) nuclear migration** occurs from the junction line of the two mating colonies, since donor nuclei extensively migrate through the resident cells of each recipient homokaryon, **resulting in two discrete dikaryons with the same nuclei but different cytoplasms**. *Agrocybe aegerita* (Barroso and Labarère 1995) and *L. edodes* are examples (Fukuda et al. 1995). In *Coprinopsis cinerea* (May and Taylor 1988) and *A. bitorquis* (Hintz et al. 1988), **mating asymmetry caused by nonreciprocal nuclear migration** was described, and in *A. bisporus*, no nuclear migration was observed (Jin et al. 1992; Jin and Horgen 1994). In any case, it is obvious that one of the most important consequences of plasmogamy is the mixing of cytoplasmic genetic elements from different individuals. It is generally accepted that a competition between different cytoplasmic elements can lead to conflict that impairs the fitness of the cell (Billiard et al. 2010). For instance, mitochondrial mutants with an impaired contribution to the cell's performance but possessing a more rapid replication will be able to invade. The idea that zygotes formed by the fusion of gametes with uniparental inheritance of organelles are the fittest leads to the hypothesis that processes involved in **the uniparental inheritance of the cytoplasm should have been selected during the course of evolution.** In accordance with this, uniparental mitochondrial inheritance, occurring at plasmogamy, has been reported in most of the studied mushrooms. However, biparental mitochondrial inheritance at plasmogamy has been observed in *C. cinerea* (May and Taylor 1988) or *L. edodes* (Fukuda et al. 1995).

Mitochondrial inheritance in *A. bisporus* analyzed with RFLP markers on 16 crosses obtained by combinations of 13 homokaryotic strains supported the occurrence of **uniparental mitochondrial inheritance in *A. bisporus*,** with one mtDNA haplotype usually favored in the new heterokaryon (De la Bastide and Horgen 2003). Non-parental mtDNA haplotypes were seen in heterokaryons produced from seven to 16 crosses. Evidence for the occurrence of two mtDNA haplotypes in one heterokaryotic mycelium was observed in eight out of 16 crosses, suggesting the maintenance of true heteroplasmons after three successive subculturing steps. The mating protocol described by the authors could be utilized to generate novel mtDNA haplotypes for strain improvement as an alternative to the use of the Buller phenomenon proposed above.

Mitochondrial transmission in *A. aegerita* was studied not only at plasmogamy, but also during vegetative growth of the resulting dikaryons, and also during sporophore differentiation (Barroso and Labarère 1995). It was demonstrated that plasmogamy between homokaryons from progeny of three wild-type strains resulted in bidirectional nuclear migration, whereas little mitochondrial migration accompanied the nuclear migration. A total of 75 % of the dikaryons from the fusion lines had both parental mitochondrial haplotypes (mixed dikaryons), and 25 % had only a single haplotype (homoplasmic dikaryons). Moreover, **in some matings, there was a strong bias in favour of one parental mitochondrial haplotype.**

The heteroplasmic nature of mixed dikaryons was demonstrated by isolating and subculturing apical cells in micromanipulation experiments, and also by identifying recombinant mitochondrial genomes. Conversion of heteroplasmons into homoplasmons was shown to occur (i) during long-term storage, (ii) in mycelia regenerated from isolated apical cells, and (iii) during sporophore differentiation. Homokaryons that readily accepted foreign nuclei were the most efficient homokaryons in maintaining their mitochondrial haplotype during plasmogamy, long-term storage, and sporophore differentiation.

This strongly argues for the presence of still-unknown mechanisms responsible for a non-random sorting out of mitochondrial genomes, acting at different times in the life cycle of the heteroplasmic dikaryon.

Both models, *A. bisporus* and *A. aegerita*, suggest that the mechanism responsible for the **non-random retention or elimination of a given haplotype** *may be* related to the nuclear genotype or the mitochondrial haplotype, or both in interaction. This has consequences with regard to the diffusion of mitochondrial genotypes in natural populations, and to the possibilities of strain improvements using nuclear–mitochondrial combinations.

IV. Conclusion

In the previous edition of this book, Horgen and Castle (2002) concluded their chapter with a sentence on the necessity for the mushroom industry to invest in genetic manipulation through intensive breeding programs, utilizing and adapting new technologies as they evolve to mushroom research. It "will position the industry for success in the 21st century", they wrote. Ten years later, several results of mushroom research have been confirmed by the industry, with the development of new *A. bisporus* hybrids based on the utilization of both genetic diversity in germplasm and molecular approaches. The problem of monocultural practice is going to be solved with a new interest from growers in the production of more diversified strains of the button mushroom adapted to different local conditions and to the consumer demand for safe and healthy food. The recent developments in genetics and the whole genome sequence open new ways to understand fundamental biological mechanisms in the button mushroom, and also to foresee new breeding strategies. To our knowledge, the sum of the studies performed on the *A. bisporus* species makes up the most accomplished and integrative analysis of trait genetics in an edible mushroom. Diversification of the species is a second way for a new development of the industry. It will be reached thanks to progress in knowledge concerning the biodiversity of *Agaricus* species and the possibility of optimizing the development of efficient strains and culture conditions by using the findings acquired on *A. bisporus*.

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References

Amazonas MAL de A (2005) Champignon do Brasil (*Agaricus brasiliensis*): nutrition, health, market demands and regulatory concerns. In: Tan Q, Chen ZM, Cao H, Buswell JA (eds) Proceedings of the 5th international conference on mushroom biology and mushroom products. Acta Edulis 12 sup, pp 111–119

Anderson JB, Petsche DM, Herr FB, Horgen PA (1984) Breeding relationships among several species of *Agaricus*. Can J Bot 62:1884–1889

Arrillaga P, Parra LA (2006) El género *Agaricus* L. en España. XI *Agaricus subrufescens*, primera cita para España. Bol Soc Micol Madrid 30:201–207

Barroso G, Labarère J (1995) Genetic evidence for non-random sorting of mitochondria in the basidiomycete *Agrocybe aegerita*. Appl Environ Microbiol 63:4686–4691

Barroso G, Bois F, Labarère J (2001) Duplication of a truncated paralog of the family B DNA polymerase gene Aa-polB in the *Agrocybe aegerita* mitochondrial genome. Appl Environ Microbiol 67:1739–1743

Barroso G, Sirand-Pugnet P, Mouhamadou B, Labarère J (2003) Secondary structure and molecular evolution of the mitochondrial small subunit ribosomal RNA in Agaricales (Euagarics clade, Homobasidiomycota). J Mol Evol 57:383–396

Berendsen RL, Baars JJP, Kalkhove SIC, Lugones LG, Wosten HAB, Bakker PAHM (2010) *Lecanicillium fungicola*: causal agent of dry bubble disease in white-button mushroom. Mol Plant Pathol 11:585–595

Billiard S, López-Villavicencio M, Devier B, Hood ME, Fairhead C, Giraud T (2010) Having sex, yes, but

with whom? Inferences from fungi on the evolution of anisogamy and mating types. Biol Rev Camb Philos Soc 86:421–442

Boa E (2004) Non-wood forest products 17. Wild edible fungi a global overview of their use and importance to people. FAO, Rome, ISBN 92-5-105157-7 http://www.fao.org/docrep/007/y5489e/y5489e00.htm#TopOfPage

Bois F, Barroso G, Gonzalez P, Labarère J (1999) Molecular cloning, sequence and expression of Aa-polB, a mitochondrial gene encoding a family B DNA polymerase from the edible basidiomycete *Agrocybe aegerita*. Mol Gen Genet 261:508–513

Braun RJ, Westermann B (2011) Mitochondrial dynamics in yeast cell death and aging. Biochem Soc Trans 39:1520–1526

Bruggeman J, Debets AJM, Wijngaarden PJ, deVisser JAGM, Hoekstra RF (2003) Sex slows down the accumulation of deleterious mutations in the homothallic fungus *Aspergillus nidulans*. Genetics 164:479–485

Bruns TD, Palmer JD, Shumard DS, Grossman LI, Hudspeth ME (1988) Mitochondrial DNAs of *Suillus:* three fold size change in molecules that share a common gene order. Curr Genet 13:49–56

Buller AHR (1931) Researches on fungi, vol IV. Longmans/Green and Co, London

Burns C, Gregory KE, Kirby M, Cheung MK, Riquelme M, Elliott TJ, Challen MP, Bailey A, Foster GD (2005) Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. Fungal Genet Biol 42:191–199

Burns C, Leach KM, Elliott TJ, Challen MP, Foster GD, Bailey A (2006) Evaluation of agrobacterium-mediated transformation of *Agaricus bisporus* using a range of promoters linked to hygromycin resistance. Mol Biotechnol 32:129–138

Burton KS (2004) Cultural factors affecting mushroom quality- cause and control of bruising. In: Romaine CP, Keil CB, Rinker DL, Royse DJ (eds) Mushroom science XVI, science and cultivation of edible and medicinal mushrooms. The Pennsylvania State University, Penn State, ISBN-1-883956-01-13, pp 397–404

Burton EG, Metzenberg RL (1972) Novel mutation causing derepression of several enzymes of sulfur metabolism in *Neurospora crassa*. J Bacteriol 109:140–151

Callac P, Billette C, Imbernon M, Kerrigan RW (1993) Morphological, genetic, and interfertility analyses reveal a novel, tetrasporic variety of *Agaricus bisporus* from the Sonoran Desert of California. Mycologia 85:335–351

Callac P, Imbernon M, Kerrigan RW, Olivier JM (1996) The two life cycles of *Agaricus bisporus*. In: Royse J (ed) Mushroom biology and mushroom product, proceeding of the second international conference. University Park, Pennsylvania, pp 57–66

Callac P, Desmerger C, Kerrigan RW, Imbernon M (1997) Conservation of genetic linkage with map expansion in distantly related crosses of *Agaricus bisporus*. FEMS Microbiol Lett 146:235–240

Callac P, Moquet F, Imbernon M, Ramos Guedes-Lafargue M, Mamoun M, Olivier JM (1998) Evidence for *PPC1* a determinant of the pilei-pellis color of *Agaricus bisporus* fruiting bodies. Fungal Genet Biol 23:181–188

Callac P, Theochari I, Kerrigan RW (2002) The germplasm of *Agaricus bisporus*: main results after ten years of collection in France, in Greece, and in North America. Acta Hortic 579:49–55

Callac P, Gaubert J, Imbernon M, Guinberteau J, Desmerger C, Olivier JM (2003) Ressources génétiques chez les agarics: résultats récents et premiers essais expérimentaux d'interfécondation libre chez le champignon de Paris. Les Actes du BRG 4:331–346

Callac P, Guinberteau J, Rapior S (2005) New hypotheses from integration of morphological traits, biochemical data and molecular phylogeny in *Agaricus* spp. In: Tan Q, Chen ZM, Cao H, Buswell JA (eds) Proceedings of the 5th international conference on mushroom biology and mushroom products, Shanghai. Acta Edulis Fungi 12 sup, pp 37–44

Callac P, Spataro C, Caillé A, Imbernon M (2006) Evidence for outcrossing via Buller phenomenon in substrate simultaneously inoculated with spores and mycelium of *Agaricus bisporus*. Appl Environ Microbiol 72:2366–2372

Callac P, Imbernon, Savoie JM (2008) Outcrossing via the Buller phenomenon in a substrate simultaneously inoculated with spores and mycelium of *Agaricus bisporus* creates variability for agronomic traits. In: Lelley JI, Buswell JA (eds) Proceedings of the 6th international conference on mushroom biology and mushroom products, GAMU, Krefeld, pp 113–119

Cappelli A (2011) Approccio al Genere *Agaricus*-IV. Rivista di Micologia 54:3–27

Castle AJ, Horgen PA, Anderson JB (1987) Restriction fragment length polymorphisms in the mushrooms *Agaricus brunnescens* and *Agaricus bitorquis*. Appl Environ Microbiol 53:816–822

Challen MP, Kerrigan RW, Callac P (2003) A phylogenetic reconstruction and emendation of *Agaricus* section *Duploannulatae*. Mycologia 95:61–73

Chang ST, Miles PG (1992) Mushroom biology, a new discipline. Mycologist 6:64–65

Collard BC, Mackill DJ (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philos Trans R Soc Lond B Biol Sci 363:557–572

Couture C, Michel A, Imbernon M, Callac P (2004) Inheritance of the haploid fruiting ability in *Agaricus bisporus*. In: Romaine CP, Keil CB, Rinker DL, Royse DJ (eds) Mushroom science

XVI, science and cultivation of edible and medicinal mushrooms. The Pennsylvania State University, Penn State, pp 45–52

De la Bastide PY, Horgen PA (2003) Mitochondrial inheritance and the detection of non-parental mitochondrial DNA haplotypes in crosses of *Agaricus bisporus* homokaryons. Fungal Genet Biol 38:333–342

De La Bastide PY, Sonnenberg A, Van Griensven L, Anderson JB, Horgen PA (1997) Mitochondrial haplotype influences mycelial growth of *Agaricus bisporus* heterokaryons. Appl Environ Microbiol 63:3426–3431

Dickhardt R (1985) Homokaryotisation of *Agaricus bitorquis* (Quel.) Sacc. and *Agaricus bisporus* (Lange) Imb. Theor Appl Genet 70:52–56

Eastwood DC, Kingsnorth CS, Jones HJ, Burton KS (2001) Genes with increased transcript levels following harvest of the sporophore of *Agaricus bisporus* have multiple physiologic roles. Mycol Res 105:1223–1230

Eastwood DC, Challen MP, Zhang C, Jenkins H, Henderson J, Burton KS (2008) Hairpin-mediated down-regulation of the urea cycle enzyme argininosuccinate lyase in *Agaricus bisporus*. Mycol Res 112:708–716

Evans HJ (1959) Nuclear behaviour in the cultivated mushroom. Chromosoma 10:115–135

FAOSTAT (2011) Food and Agriculture Organization of the United Nations. https://www.Faostat.fao.org. Cited 10 Jan 2012

Ferandon C, ChatelSel K, Castandet B, Castroviejo M, Barroso G (2008) The *Agrocybe aegerita* mitochondrial genome contains two inverted repeats of the *nad4* gene arisen by duplication on both sides of a linear plasmid integration site. Fungal Genet Biol 45:292–301

Ferandon C, Moukha S, Callac P, Benedetto JP, Castroviejo M, Barroso G (2010) The *Agaricus bisporus cox1* gene: the longest mitochondrial gene and the largest reservoir of mitochondrial group i introns. PLoS One 5:e14048

Formighieri EF, Tiburcio RA, Armas ED, Medrano FJ, Shimo H, Carels N, Góes-Neto A, Cotomacci C, Carazzolle MF, Sardinha-Pinto N, Thomazella DP, Rincones J, Digiampietri L, Carraro DM, Azeredo-Espin AM, Reis SF, Deckmann AC, Gramacho K, Gonçalves MS, MouraNeto JP, Barbosa LV, Meinhardt LW, Cascardo JC, Pereira GA (2008) The mitochondrial genome of the phytopathogenicbasidiomycete*Moniliophthora perniciosa* is 109 kb in size and contains a stable integrated plasmid. Mycol Res 112:1136–1152

Foulongne-Oriol M, Spataro C, Savoie JM (2009) Novel microsatellite markers suitable for genetic studies in the white button mushroom *Agaricus bisporus*. Appl Microbiol Biot 1184:1125–1135

Foulongne-Oriol M, Spataro C, Cathalot V, Monllor S, Savoie JM (2010) An expanded genetic linkage map of an intervarietal *Agaricus bisporus* var. *bisporus* x *A. bisporus* var. *burnettii* hybrid based on AFLP, SSR and CAPS markers sheds light on the recombination behaviour of the species. Fungal Genet Biol 47:226–236

Foulongne-Oriol M, Minvielle N, Savoie JM (2011a) QTL for resistance to *Trichoderma* lytic enzymes and metabolites in *Agaricus bisporus*. In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 2. pp 17–25. http://wsmbmp.org/Previous_Conference_7.html. Cited 27 Feb 2012

Foulongne-Oriol M, Dufourcq R, Spataro C, Devesse C, Rodier A, Savoie JM (2011b) Comparative linkage mapping in the white button mushroom *Agaricus bisporus* provides foundation for breeding management. Curr Genet 57:39–50

Foulongne-Oriol M, Rodier A, Caumont P, Spataro C, Savoie JM (2011c) *Agaricus bisporus* cultivars: hidden diversity beyond apparent uniformity? In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 2. pp 9–16. http://wsmbmp.org/Previous_Conference_7.html. Cited 10 Feb 2012

Foulongne-Oriol M, Rodier A, Rousseau T, Largeteau M, Savoie JM (2011d) Quantitative genetics to dissect the fungal-fungal interaction between *Lecanicillium verticillium* and the white button mushroom *Agaricus bisporus*. Fungal Biol 115:421–431

Foulongne-Oriol M, Rodier A, Rousseau T, Savoie JM (2012a) QTL mapping of yield-related components and oligogenic control of the cap color in the button mushroom *Agaricus bisporus*. Appl Environ Microbiol 78:2422–2434

Foulongne-Oriol M, Rodier A, Savoie JM (2012b) Relationship between yield components and partial resistance to Lecanicillium fungicola in *Agaricus bisporus* assessed by QTL mapping. Appl Environ Microbiol 78:2435–2442

Fukuda M, Harada Y, Imahori S, Fukumasa-Nakai Y, Hayashi Y (1995) Inheritance of mitochondrial DNA in sexual crosses and protoplast cell fusions in *Lentinula edodes*. Curr Genet 27:550–554

Gao W, Baars JJP, Sonnenberg ASM, Richard Visser R (2011) Inheritance pattern of bruising sensitivity trait in *Agaricus bisporus*. In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 1. pp 43–51. http://www.wsmbmp.org/Previous_Conference_7.html. Cited 10 Feb 2012

Gaze R, Fletcher J (2007) Mushroom pest and disease control. Academic/Elsevier, Waltham

Geml J, Laursen GA, Taylor DL (2008) Molecular diversity assessment of artic and boreal *Agaricus* taxa. Mycologia 100:577–589

Gonzalez P, Labarère J (1998) Sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6, and V9 domains reveal highly species-specific variations within the genus *Agrocybe*. Appl Environ Microbiol 64:4149–4160

Gonzalez P, Labarère J (2000) Phylogenetic relationships of *Pleurotus* species according to the sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6 and V9 domains. Microbiology 146:209–221

Grigoriev IV, Cullen D, Goodwin SB, Hibbett D, Jeffries TW, Kubicek CP, Kuske C, Magnuson JK, Martin F, Spatafora JW, Tsang A, Baker SE (2011) Fueling the future with fungal genomics. Mycology 2:192–209

Grogan H, Gaze R, Holcroft S (2005) Viral dsRNAs in *Agaricus bisporus*: transmisson, symptom expression and control. In: Tan Q, Chen ZM, Cao H, Buswell JA (eds) Proceedings of the 5th international conference on mushroom biology and mushroom products. Acta Edulis Fungi 12 sup, pp 363–367

Guan XJ, Xu L, Shao YC, Wang ZR, Chen FS (2008) Differentiation of commercial strains of *Agaricus* species in China with inter-simple sequence repeat marker. World J Microbiol Biot 24:1617–1622

Haridas S, Gantt JS (2010) The mitochondrial genome of the wood-degrading basidiomycete*Trametes cingulata*. FEMS Microbiol Lett 308:29–34

Heinemann P (1956) Champignons récoltés au Congo Belge par Madame M. Goosens-Fontana. II *Agaricus* Fries s.s. Bulletin du Jardin Botanique de l'Etat à Bruxelles 26:1–127

Henderson J, Eastwood D, Bains N, Burton K (2005) Superoxide dismutase – mushroom under stress. In: Tan Q, Chen ZM, Cao H, Buswell JA (eds) Proceedings of the 5th international conference on mushroom biology and mushroom products. Acta Edulis Fungi 12sup, pp 61–65

Hintz W, Mohan M, Anderson JB, Horgen PA (1985) The mitochondrial DNAs of *Agaricus*: heterogeneity in *A. bitorquis* and homogeneity in *A. brunnescens*. Curr Genet 9:127–132

Hintz W, Anderson JB, Horgen PA (1988) Nuclear migration and mitochondrial inheritance in the mushroom *Agaricus bitorquis*. Genetics 119:35–41

Horgen PA, Castle AJ (2002) The application and potential of molecular approaches to mushrooms. In: Kempken F (ed) The Mycota XI agricultural applications. Springer, Berlin/Heidelberg, pp 3–17

Hospital F (2009) Challenges for effective marker-assisted selection in plants. Genetica 136:303–310

Hou HH, Elliott TJ (1978) Comparative cytology in the genus *Agaricus*. Mushroom Sci 10(1):51–62

Imbernon M, Callac P, Granit S, Pirobe L (1995) Allelic polymorphism at the mating type locus in *Agaricus bisporus* var. *burnettii*, and confirmation of the dominance of its tetrasporic trait. Mushroom Sci 14(1):11–19

Imbernon M, Callac P, Gasqui P, Kerrigan RW, Velcko AJ Jr (1996) BSN, the primary determinant of basidial spore number and reproductive mode in *Agaricus bisporus*, maps to chromosome I. Mycologia 88:749–761

Jin T, Horgen PA (1993) Further characterization of a large inverted repeat in the mitochondrial genomes of *Agaricus bisporus* (= *A. brunnescens*) and related species. Curr Genet 23:228–233

Jin T, Horgen PA (1994) Uniparental mitochondrial transmission in the cultivated button mushroom, *Agaricusbisporus*. Appl Environ Microbiol 60:4456–4460

Jin T, Sonnenberg AS, Van Griensven LJ, Horgen PA (1992) Investigation of mitochondrial transmission in selected matings between homokaryons from commercial and wild-collected isolates of *Agaricus bisporus* (= *Agaricus brunnescens*). Appl Environ Microbiol 58:3553–3560

Juarez del Carmen S, Largeteau-Mamoun ML, Rousseau T, Regnault-Roger C, Savoie JM (2002) Genetic and physiological variation in isolates of *Verticillium fungicola* causing dry bubble disease of the cultivated button mushroom, *Agaricus bisporus*. Mycol Res 106:1163–1170

Kamzolkina O, Volkova V, Kozlova M, Pancheva E, Dyakov Y, Callac P (2006) Karyological evidence for meiosis in the three different types of life cycles existing in *Agaricus bisporus*. Mycologia 98:763–770

Kerrigan RW (1990) Evidence of genetic divergence in two populations of *Agaricus bisporus*. Mycol Res 94:721–733

Kerrigan RW (1992) Strategies for the efficient recovery of *Agaricus bisporus* homokaryons. Mycologia 84:575–579

Kerrigan RW (1996) Characteristics of a large collection of wild edible mushroom germ plasm: the *Agaricus* resource program. Centraalbureau voor Schimmelcultures and the World Federation for Culture Collections, Veldhoven, pp 302–308

Kerrigan RW (2004) Trait diversity in wild *Agaricus bisporus*. In: Romaine CP, Keil CB, Rinker DL, Royse DJ (eds) Mushroom science XVI, science and cultivation of edible and medicinal mushrooms, The Pennsylvania State University, Penn State, ISBN-1-883956-01-13, pp 31–38

Kerrigan RW (2005) *Agaricus subrufescens*, a cultivated edible and medicinal mushroom, and its synonyms. Mycologia 97:12–24

Kerrigan RWK (2007) Lectotypification of *Agaricus brunnescens*. Mycologia 99:906–915

Kerrigan RW (2011) Whole-genome sequencing of the cultivated button mushroom *Agaricus bisporus*: history, status and applications. In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 1. pp 1–6. http://www.wsmbmp.org/Previous_Conference_7.html. Cited 01 Feb 2012

Kerrigan RWK, Ross K (1987) Basidiospore number variation in *Agaricus*. In: Wuest PJ, Royse DJ, Beelman RB (eds) Cultivating edible fungi. Elsevier Science Publishers, Amsterdam, pp 155–162

Kerrigan RW, Wach MP (2008) *Agaricus subsrufescens* mushroom plant named 'H1X1'. US Patent USPP19, 313P3

Kerrigan RWK, Wach MP (2010) *Agaricus bisporus* mushroom plant named J10165 US Patent Application: 20100218294

Kerrigan RW, Baller LM, Horgen PA, Anderson JB (1992) Strategies for the efficient recovery of *Agaricus bisporus* homokaryons. Mycologia 84:575–579

Kerrigan RW, Royer JC, Baller LM, Kohli Y, Horgen PA, Anderson JB (1993) Meiotic behavior and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. Genetics 133:225–236

Kerrigan RW, Imbernon M, Callac P, Billette C, Olivier JM (1994) The heterothallic life cycle of *Agaricus bisporus* var. *burnettii*, and the inheritance of its tetrasporic trait. Exp Mycol 18:193–210

Kerrigan RW, Carvalho DB, Horgen PA, Anderson JB (1998) The indigenous coastal Californian population of the mushroom *Agaricus bisporus*, a cultivated species, may be at risk of extinction. Mol Ecol 7:35–45

Kerrigan RW, Callac P, Xu J, Noble R (1999) Population and phylogenetic structure within the *Agaricus subfloccosus* complex. Mycol Res 103:1515–1523

Kerrigan RW, Callac P, Challen M, Guinberteau J, Parra LA (2006 [2005]) *Agaricus* section *Xanthodermatei*: a phylogenetic reconstruction with commentary on taxa. Mycologia 97:1292–1315

Kerrigan RW, Callac P, Para LA (2008) New and rare taxa in *Agaricus* section *Bivelares* (*Duploannulati*). Mycologia 100:876–892

Khush R, Becker E, Wach M (1992) DNA amplification polymorphisms of the cultivated mushroom *Agaricus bisporus*. Appl Environ Microbiol 58:2971–2977

Kue U (2000) Life history and developmental processes in the Basidiomycete *Coprinus cinereus*. Microbiol Mol Biol Rev 64:316–353

Kuhner R (1977) Variation of nuclear behaviour in the Homobasidiomycetes. Trans Brit Mycol Soc 68 (Kuhner R):1–16

Labbe J, Murat C, Morin E, Le Tacon F, Martin F (2011) Survey and analysis of simple sequence repeats in the *Laccaria bicolor* genome, with development of microsatellite markers. Curr Genet 57:75–88

Lange M (1952) Species concepts in the genus *Coprinus*. Dansk Bot Ark 14:1–140

Largeteau ML, Savoie JM (2010) Microbially-induced diseases of *Agaricus bisporus*: biochemical mechanisms and impact on commercial mushroom production. MiniReview. Appl Microbiol Biot 86:63–73

Largeteau ML, Rodier A, Rousseau T, Juarez del Carmen S, Védie R, Savoie JM (2004) *Agaricus* susceptibility to *Verticillium fungicola*. In: Romaine CP, Keil CB, Rinker DL, Royse DJ (eds) Mushroom science XVI, science and cultivation of edible and medicinal mushrooms. The Pennsylvania State University, Penn State, ISBN-1-883956-01-13, pp 515–522

Largeteau ML, Baars JPP, Juarez del Carmen S, Regnault-Roger C, Savoie JM (2005) Wild strains of *Agaricus bisporus*: a source of tolerance to dry bubble disease. In: Pisabarro AG, Ramirez L (eds) IV congress genetics and cellular biology of basidiomycetes. Universidad Pública de Navarra, Pamplona, pp 77–87

Largeteau ML, Callac P, Navarro-Rodriguez AM, Savoie JM (2011a) Diversity in the ability of *Agaricus bisporus* wild isolates to fruit at high temperature (25 °C). Fungal Biol 115:1186–1195

Largeteau ML, Llarena-Hernandez RC, Regnault-Roger C, Savoie JM (2011b) The medicinal *Agaricus* mushroom cultivated in Brazil: biology, cultivation and non-medicinal valorization. Appl Microbiol Biot 92:897–907

Llarena Hernández RC, Largeteau M, Farnet AM, Minvielle N, Regnault-Roger C, Savoie JM (2011) Phenotypic variability in cultivars and wild strains of *Agaricus brasiliensis* and *Agaricus subrufescens*. In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 2. pp 38–49. http://www.wsmbmp.org/Previous_Conference_7.html. Cited 25 Jan 2012

Loftus MG, Moore D, Elliot TJ (1988) DNA polymorphisms in commercial and wild strains of the cultivated mushroom, *Agaricus bisporus*. Theor Appl Genet 76:712–718

Loftus M, Bouchti KL, Robles C, Van Griensven LJLD (2000) Use of SCAR marker for cap color in *Agaricus bisporus* breeding programs. Mushroom Sci 15:201–205

Lösel DM (1964) The stimulation of spore germination in *Agaricus bisporus* by living mycelium. Ann Bot 28:54I–554I

Ludwig E (2007) Pilzkompendium 2: 61. Fungicon-Verlag, Berlin

Marshall E, (Tan) Nair NG (2009) FAO Diversification booklet number 7: make money by growing mushrooms, 53 p. Food and Agriculture Organization of the United Nations. ftp://ftp.fao.org/docrep/fao/011/i0522e/i0522e00.pdf. Cited 25 Jan 2012

May G, Taylor JW (1988) Patterns of mating and mitochondrial DNA inheritance in the agaric Basidiomycete *Coprinus cinereus*. Genetics 118:213–220

Miller RE, Kananen DL (1972) Bipolar sexuality in the mushroom. Mushroom Sci 13:713–718

Moore AJ, Challen MP, Warner PJ, Elliott TJ (2001) RAPD discrimination of *Agaricus bisporus* mushroom cultivars. Appl Microbiol Biot 55:742–749

Moquet F, Guedes-Lafargue MR, Mamoun M, Olivier JM (1998) Selfreproduction induced variability in agronomic traits for a wild *Agaricus bisporus*. Mycologia 90(1998):806–812

Moquet F, Desmerger C, Mamoun M, Ramos-Guedes-Lafargue M, Olivier JM (1999) A quantitative trait locus of *Agaricus bisporus* resistance to *Pseudomonas tolaasii* is closely linked to natural cap color. Fungal Genet Biol 28:34–42

Morin E, Kohler A, Baker A, Foulongne-Oriol M, Lombard V, Ohm RA, Patyshakuliyeva A, Brun A, Aerts AL, Bailey AM, Billette C, Coutinho PM, Doddapaneni H, Grimwood J, Hildén K, Kües U, LaButti KM, Lapidus A, Lindquist EA, Lucas SM, Lundell T, Murat C, Riley RW, Salamov AA, Schmutz J, Subramanian V, Wösten HAB, Xu JP, Eastwood DC, Foster GD, Sonnenberg ASM, Cullen D, de Vries RP, Henrissat B, Burton KS, Kerrigan RW, Challen MP, Grigoriev IV, Martin F (2012) The genome sequence of the button mushroom *Agaricus bisporus* reveals mechanisms governing adaptation to a humic-rich ecological niche. PNAS 109(43):17501–17506

Murat C, Riccioni C, Belfiori B, Cichocki N, Labbé J, Morin E, Tisserant E, Paolocci F, Rubini A, Martin F (2011) Distribution and localization of microsatellites in the Perigord black truffle genome and identification of new molecular markers. Fungal Genet Biol 48:592–601

Osiewacz HD (2011) Mitochondrial quality control in aging and lifespan control of the fungal aging model *Podospora anserina*. Biochem Soc Trans 39:1488–1492

Parra LA (2008) *Agaricus* L. Allopsalliota Nauta & Bas. Pars 1Edizioni Candusso Alassio, Italy

Raper CA, Raper JR, Miller RE (1972) Genetic analysis of the life cycle of *Agaricus bisporus*. Mycologia 64:1088–1117

Rast D, Stauble EJ (1970) On the mode of action of isovaleric acid in stimulating the germination of *Agaricus bisporus*. New Phytol 69:557–566

Robison MM, Horgen PA (1996) Plasmid RNA polymerase-like mitochondrial sequences in *Agaricus bitorquis*. Curr Genet 29:370–376

Robison MM, Royer JC, Horgen PA (1991) Homology between mitochondrial DNA of *Agaricus bisporus* and an internal portion of a linear mitochondrial plasmid of *Agaricus bitorquis*. Curr Genet 19:495–502

Robles CW, Lodder SC (2009) Brown mushrooms for commercial production. US Patent 7,608,760 B2

Romaine CP, Schlagnhaufer CD, Woolston BM (2011) Strategies for the transgenic manipulation of filamentous fungi. Patent WO/2011/130247 International Application PCT/US2011/032087

Román M, Boa E (2006) The marketing of *Lactarius deliciosus* in northern Spain. Econ Bot 60:284–290

Royse DJ, May B (1982a) Use of isozyme variation to identify genetic classes of *Agaricus brunnescens*. Mycologia 74:93–102

Royse DJ, May B (1982b) Genetic relatedness and its application in selective breeding of *Agaricus brunnescens*. Mycologia 74:569–575

Saksena KN, Marino R, Haller MN, Lemke PA (1976) Study on development of *Agaricus bisporus* by fluorescent microscopy and scanning electron microcopy. J Bacteriol 126:417–428

Sass JE (1929) The cytological basis for homothallism and heterothallism in the *Agaricaceae*. Am J Bot 16:663–701

Savoie JM, Largeteau ML (2011) Production of edible mushrooms in forests: trends in development of a mycosilviculture. MiniReview. Appl Microbiol Biot 89:971–979

Savoie JM, Bruneau D, Mamoun M (1996) Resource allocation ability of wild isolates of *Agaricus bisporus* on conventional mushroom compost. FEMS Microbiol Ecol 21:285–292

Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (2011) Proceedings of the 7th international conference on mushroom biology and mushroom products. http://www.wsmbmp.org/Previous_Conference_7.html

Sinden JW, Tschierpe HJ, Hauser E (1962) Transplantation of sporophores as a new method for studying growth and nutritional factors of mushrooms. Mushroom Sci 5:250–266

Sonnenberg AS, Van Loon PC, Van Griensven LJ (1991) The occurrence of mitochondrial genotypes and inheritance of mitochondria in the cultivated mushroom *Agaricus bisporus*. Mushroom Sci 13:85–92

Sonnenberg, ASM, Baars JP, Hendrickx PM, Kerrigan KW (2005) Breeding mushrooms: state of the art. In: Tan Q, Chen ZM, Cao H, Buswell JA (eds) Proceedings of the 5th international conference on mushroom biology and mushroom products, Acta Edulis Fungi 12sup, 163–173

Sonnenberg, ASM, Baars JP, Hendrickx PM, Lavrijssen B, Gao W, Weijn A, Mes JJ (2011) Breeding and strain protection in the button mushroom *Agaricus bisporus*. In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 1. pp 7–15. http://wsmbmp.org/Previous_Conference_7.html. Cited 30 Jan 2012

Spear MC, Royse DJ, May B (1983) Atypical meiosis and joint segregation of biochemical loci in *Agaricus brunnescens*. J Hered 74:417–420

Staniaszek M, Marczewski W, Szudyga K, Maszkiewicz J, Czaplicki A, Qian G (2002) Genetic relationship between Polish and Chinese strains of the mushroom *Agaricus bisporus* (Lange) Sing., determined by the RAPD method. J Appl Genet 43:43–47

Summerbell RC, Castle ZJ, Horgen PA, Anderson JB (1989) Inheritance of restriction fragment length polymorphisms in *Agaricus brunnescens*. Genetics 123:293–300

Vellinga EC, Sysouphanthong P, Hyde KD (2011) The family of *Agaricaceae*: phylogenies and two new white-spored genera. Mycologia 103:494–509

Wang ZS, Liao JH, Li FG (1995) Studies on breeding hybrid strain As2796 of *Agaricus bisporus* for canning in China. Mushroom Sci 14:71–72

Wang ZS, Liao J, Li H, Wang B, Chen M, Lu Z, Guo Z (2008a) Study on the biological characteristics of wild *Agaricus bisporus* strains from China. In: van Gruening M (ed) Science and cultivation of edible and medicinal fungi: Mushroom Science XVII, Proceedings of the 17th congress of the international society for mushroom science. South African Mushroom Farmers Association, Pretoria, South Africa (CD-ROM). ISBN 978-0-620-40808-0

Wang Y, Zeng F, Hon CC, Zhang Y, Leung FC (2008b) The mitochondrial genome of the Basidiomycete fungus *Pleurotus ostreatus* (oyster mushroom). FEMS Microbiol Lett 280:34–41

Wasser SP, Didukh MY, Amazonas MAL, Nevo E, Stamets P, Eira AF (2002) Is a widely cultivated culinary-medicinal royal sun *Agaricus* (the Himematsutake mushroom) indeed *Agaricus blazei* Murill? Int J Med Mushrooms 4:267–290

Weijn A, Tomassen MMM, Bastiaan-Net S, Hendrix EAHJ, Baars JJP, Sonnenberg ASM, Wichers HJ, Mes JJ (2011) Browning sensitivity of button mushrooms. In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 1. pp 203–211. http://www.wsmbmp.org/Previous_Conference_7.html. Cited 10 Feb 2012

Wisitrassameewong K, Karunarathna SC, Thongklang N, Zhao R, Callac P, Moukha S, Férandon C, Chukeatirote E, Hyde KD (2012a) *Agaricus subrufescens*: a review. Saudi J Biol Sci 19:131–146

Wisitrassameewong K, Karunarathna SC, Thongklang N, Zhao RL, Callac P, Chukeatirote E, Bahkali AH, Hyde KD (2012b) *Agaricus subrufescens*: new to Thailand. Chiang Mai J Sci 39:281–291

Wittenberg AH, van der Lee TA, Ben M'Barek S, Ware SB, Goodwin SB, Kilian A, Visser RG, Kema GH, Schouten HJ (2009) Meiosis drives extraordinary genome plasticity in the haploid fungal plant pathogen *Mycosphaerella graminicola*. PLoS One 4: e5863

Woolston BM, Schlagnhaufer C, Wilkinson J, Larsen J, Shi Z, Mayer K, Walters DS, Curtis WR, Romaine CP (2011) Long-distance translocation of protein during morphogenesis of the fruiting body in the filamentous fungus, *Agaricus bisporus*. PLoS ONE 6:e28412

Xu J (1995) Analysis of inbreeding depression in *Agaricus bisporus*. Genetics 141:137–145

Xu J, Kerrigan RW, Horgen PA, Anderson JB (1993) Localization of the mating-type gene in *Agaricus bisporus*. Appl Environ Microbiol 59:3044–3049

Xu J, Horgen PA, Anderson JB (1996) Somatic recombination in the cultivated mushroom *Agaricus bisporus*. Mycol Res 100:188–192

Xu J, Kerrigan RW, Callac P, Horgen PA, Anderson JB (1997) Genetic structure of natural populations of *Agaricus bisporus*, the commercial button mushroom. J Hered 88:482–488

Xu J, Desmerger C, Callac P (2002) Fine-scale genetic analyses reveal unexpected spatial-temporal heterogeneity in two natural populations of the commercial mushroom *Agaricus bisporus*. Microbiology 148:1253–1262

Zhao R, Karunarathna S, Raspé O, Parra LA, Guinberteau J, Moinard M, De Kesel A, Barroso G, Courtecuisse R, Hyde KD, Guelly AK, Desjardin D, Callac P (2011) Major clades in tropical *Agaricus*. Fungal Divers 51:279–296

Zied DC, Pardo-Gimenez A, Savoie JM, Pardo-Gonzalez JR, Callac P (2011) "Indoor" method of composting and genetic breeding of the strains to improve yield and quality of the almond mushroom *Agaricus subrufescens*. In: Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G (eds) Proceedings of the 7th international conference on mushroom biology and mushroom products, vol 1. pp 424–432. http://www.wsmbmp.org/Previous_Conference_7.html. Cited 25 Jan 2012

2 Fungal Spoilage of Crops and Food

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CONTENTS

I. Introduction: Food Is an Ecological Resource

Food products are a rich nutrient source that will attract both bacterial and fungal colonizers. As such, the food product can be regarded as an **ecological resource**. After successful colonization of the product, its nutritional properties are altered. When the nutritional value, structure, and taste of the product are negatively influenced, this colonization is called food spoilage. It can be accompanied by the production of toxic secondary metabolites which may result in grave medical problems, and is an issue that needs our continual awareness with respect to food safety. This will be the topic of another chapter in this book.

In other cases, colonization with a number of food-borne microorganisms is beneficial with respect to nutritional value and prolonged storage of the food product, which is dubbed as food fermentation. These two aspects of food colonization are two sides of the same coin.

Food spoilage is a major threat for our food stock and is responsible for **enormous losses worldwide**, which makes it a research area that is very relevant with respect to the **increasing demand on food** during the next decennia. Knowledge concerning the specific mechanisms that occur during food spoilage might generate novel insights that result in increased net amounts of food without an increase of land use.

This chapter highlights fungal spoilage, including the fact that it deals mostly with **plant-based food products**. Fungi are the main degraders of the sturdy plant cell wall components that otherwise would accumulate within the ecosystems of the world. Prior to spoilage, the fungi can be present on or inside the crop in low numbers, or as survival structures. Spoilage fungi can also be introduced to an empty habitat if the food is previously treated by pasteurization treatments.

Food products include two main groups, namely **living crops and processed food**. Colonization of food products is hence very diverse. This chapter evaluates different fungal–food relationships. At first, the relationship between the living crop and fungi is illustrated. Then the association of fungi with different types of processed food is described. Different preservation techniques make the food product a difficult environment to colonize, although it is also a rich medium. Only fungi

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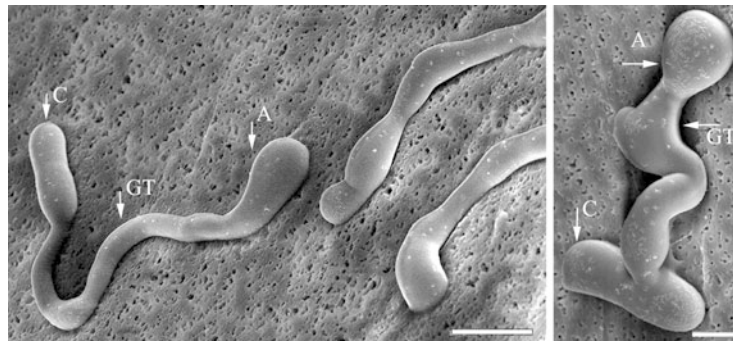

Fig. 2.1. Conidia (*C*) of a *Colletotrichum* species germinate on an avocado surface. The germ tube (*GT*) differentiates into an appressorium (*A*) that is firmly attached to the plant cell wall (Micrograph made by Jan Dijksterhuis, CBS-KNAW Fungal Biodiversity Centre, The Netherlands). Bars are 10 and 5 μm respectively

that can survive certain adverse conditions including high osmolarity and heat can successfully spoil processed food. Different aspects of stress resistance are addressed in this chapter, including osmotolerance, protective compounds inside cells, and heat-resistant structures.

Several books on food spoilage fungi summarize many different aspects of fungi and food. Pitt and Hocking (2009) and Samson et al. (2004) provide overviews on the taxonomic description and specificity of food spoilage fungi, and Dijksterhuis and Samson (2007) highlight numerous aspects of the relation between food and fungi including spoilage and fermentation.

II. Infection of Living Crops: Post-Harvest Diseases

The **relation between fungi and living agricultural crops can be regarded as plant-pathogenic** in nature, which includes a **complex communication** between parasite and host. Some of these fungi enter intact crop cells without direct killing the host. They initially **establish a fungus–host interface** as a biotrophic fungus that can exhibit prolonged survival in a quiescent state, which can be **followed by a necrotrophic** infection stage in which plant tissue is killed and lesions develop. The **true necrotrophic fungi** start to kill plant tissue directly upon entering the host. The so-called **opportunistic fungi** cause infections of fruit, vegetables, or flower bulbs by entering cracks, wounds or natural orifices on the surface of crops. The total number of fungal species involved in post-harvest diseases is much larger than ever can be covered in this short overview; we would like to illustrate some principles of infection and the fungi involved.

A. Anthracnose as an Example of Complex Crop–Fungus Interaction

Members of the fungal genus ***Colletotrichum*** (teleomorph; *Glomerella*) cause **anthracnose** disease in a wide range of fruits and vegetables. For instance, the species *C. gloeosporioides* (*Glomerella cingulata*) infects **over 100 different host species**. These fungi produce highly specialized structures called **appressoria** that provide fungal entry into healthy plant tissue (Fig. 2.1). Appressoria generate enormous turgor pressure, which enables the penetration peg formed on it to breach the sturdy plant cell walls with pure mechanical force (Bechinger et al. 1999). In the case of infection of the climacteric fruit of the tomato with *C. gloeosporioides*, the fungus actually waits for the right moment to infect. The **plant hormone ethylene** that is produced by the tomato during senescence is an important trigger for proper infection (Flaishman and Kolattukudy 1994).

On avocado, appressoria are formed on unripe fruit, and penetrate the plant cell wall. The **subcuticular hyphae then become**

quiescent until ripening of the fruit has occurred (Prusky and Lichter 2007). Quiescence is correlated with the presence of an **antifungal compound**, **AFD,** which is degraded by the enzyme lipoxygenase. Indirectly, AFD levels are controlled by the antioxidant epicatechin which is present in the avocado peel and acts as a lipoxygenase inhibitor (Ardi et al. 1998).

Synthesis of the antifungal compound is correlated with the expression of a Δ12-fatty acid desaturase (Wang et al. 2004). Interestingly, a cold treatment that resulted in increase of unsaturated fatty acid also gave rise to increased antifungal diene (AFD). *C. gloeosporioides* also reacts to **other chemical triggers**, namely the host surface wax in the case of anthracnose of avocado fruit (Podila et al. 1993). Analysis of wax fractions showed that differentiation of appressoria was maximal in the presence of certain long-chain fatty acid alcohols. That this aspect of communication is also very specific is illustrated by a strongly lowered appressorium formation by wax from other fruit (jade wax). Another basic requirement for appressorium formation is **the presence of a surface** (Kim et al. 1998). Thus, different thigmotropic (sense-reactive) and chemical signaling pathways cooperate during differentiation and infection. Wax- and ethylene-dependent signaling pathways are not identical, but share two proteins that must be phosphorylated (Kolattukudy et al. 1995). Unripe avocado tissue is also able to react on the presence of fungal elicitor with **the formation of ROS**; during ripening this ability is almost absent (Beno-Moualem and Prusky 2000).

Following the initial stage of infection, the fungus resumes growth and develops from a biotrophic parasite characterized by fungal cells that are compatible with living plant cells towards a necrotrophic parasite that actively kills the host cells. This is characteristic for a **hemibiotrophic lifestyle** (as reviewed in Prusky and Lichter 2007; Prusky and Kolattukudy 2007; Münch et al. 2008). Necrotrophic hyphae are thinner than biotrophic hyphae, and produce a variety of plant-cell-wall-degrading enzymes, and also produce other factors that lead to cell death such as reactive oxygen species or secondary metabolites.

A typical phenomenon which is correlated with the onset of the necrotrophic stage is the accumulation of ammonium at the leading edge of the developing lesion (Alkan et al. 2008, 2009), as is observed with *C. coccodes* (on tomato), *C. gloeosporioides* (on avocado), and *Alternaria alternata* (on persimmon fruit). This **alkalinization** is induced by the usually low pH of the host fruit, and is a prerequisite for the activation of a host NADPH oxidase, a ROS-producing enzyme. The presence of ROS increases local cell death, a hallmark of necrotrophic growth.

Tissue alkalinization in *C. gloeosporioides* also results in the secretion of a pectate lyase (encoded by PELB) via increased expression of the transcription factor PacC. Loss of function mutants of the latter factor have shown strongly reduced pathogenity and pectate lyase secretion (Miyara et al. 2008). In addition, *C. gloeosporioides* forms a laccase in the avocado peel that is able to degrade epicatechin and thus shortens the period of quiescence in case of some active isolates of the fungus (Guetsky et al. 2005). Thus, hemibiotrophic pathogens are able to establish complex interactions with the host.

B. True Necrotrophs

Necrotrophs directly start to kill plant tissue upon entering the host and some of them develop into **broad spectrum pathogens** that destroy many different and large amounts of vegetables and fruits upon harvesting. ***Botrytis cinerea*** is fungus that causes widespread infection of grapes, strawberries, and other fruits, as well as vegetables. The fungus enters the host by means of appressorium-like structures or via wounds.

The appressoria can breach intact plant tissues, and require the presence of tetraspanins (the gene *BcPls1*) for successful penetration (Gourgues et al. 2004). Homologues of these specialized membrane proteins are also found in the plant parasite *Magnaporthe grisea* that forms similar appressoria as *Colletotrichum*.

After entrance into the plant tissue, the fungus starts to kill host cells with the help of toxic secondary metabolites such as botrydial. There is evidence that the fungus uses the host hypersensitivity response for further infection (as reviewed by Choquer et al. 2007). The fungal genome of *B. cinerea* contains families of plant-cell-wall-degrading enzymes, and up to 12 different lipases have been identified (van Kan 2006). In particular, the enzymes involved in

pectin degradation, including endopolygalacturonases, are important for *B. cinerea*, and hosts with high pectin contents are an excellent target for the fungus. In tomato fruit, the activity of expansins and polygalacturonases produced by the host loosens the plant cell wall during ripening. Transgenic tomato strains that did not have these activities showed clearly reduced and delayed infection development after inoculation with *B. cinerea* (Cantu et al. 2008). These findings show that the **interaction between necrotroph and the host is considerably more complex** than thought before (Amselem et al. 2011), and that there exists a balance between host and pathogen. *B. cinerea* is an avid producer of **oxalic acid** inside the lesion. This organic acid stimulates cell-wall-degrading enzymes, and also has a strong calcium-chelating activity that helps to **destabilize the pectin network** in which calcium ions are embedded (van Kan 2006; Prusky and Lichter 2007). Oxalic acid production is even more a hallmark of infection by another widespread necrotrophic pathogen ***Sclerotinia sclerotiorum*** (Kim et al. 2007; Hegedus and Rimmer 2005) that is related to *B. cinerea* (Amselem et al. 2011). This fungus is notorious as a post-harvest pathogen of carrot, sunflower seeds, and bean pods among 400 plant species, most of them dicots.

Here, oxalic acid also modulates the hypersensitivity response, including programmed cell death around the pathogen, in delaying the oxidative burst and prevention of callose deposition at the leading edges of the lesions. (Williams et al. 2011)

C. Opportunistic Fungi

Opportunistic fungi can grow well without plant hosts as saprotrophs on decaying plant material or in soil. They also infect crops mostly without the help of specialized infection structures, and need a natural opening or a wound in the outer layer of the crop. Despite their dual growth mode, they can develop into **true pests of harvested crops.** Opportunists can also enter via the **dying leaves of the flower** before the fruit is fully grown (Snowdon 1990). For tomatoes, fungi often develop first on the remnants of the leaves present on the fruit (the so-called calix), and then colonize the tissues of the fruit (Smid et al. 1996). **Careful handling of crops** directly after harvesting is vital for the quality of the product. The more small wounds that are introduced by, for instance, rough treatment of the crop, the more damage occurs as a result of post-harvest diseases.

For example, the fungus ***Alternaria alternata***, a post–harvest pathogen of many vegetables and fruits (Thomma 2003), can attack apples via the calycine tube and causes core rot in susceptible cultivars (Niem et al. 2007). *A. alternata* also causes rot of persimmon fruit, melon, and tomato, and is a fungus that alkalinizes the host tissue.

An important factor in the disease is an endo-1,4-β-glucanase that is more highly expressed in the presence of cell-wall polymers and a higher pH (above 6, Eshel et al. 2002). The fungus can survive in a quiescent state in plant material, and enters plant tissues that are weakened as a result of senescence or wounding, but the formation of small appressoria is not ruled out.

Like all other fungi involved in post-harvest rot, growing hyphae of the opportunistic fungi release enzymes that degrade the plant cell wall, which results in **dry or wet rot** of the food crop. This depends on the selection of enzymes formed by the pathogen. **Cellulolytic enzymes** do not disrupt the pectin middle lamella, and therefore do not dissociate plant cells, which results in a more preserved structure of the tissue after infection known as dry rot (as reviewed in Prusky and Kolattukudy 2007). **Pectin-degrading enzymes** destroy the connection between the cells, resulting in maceration and wet rot. The variety of the secreted polysaccharide-degrading enzymes is large. The variability of these enzymes have been reviewed (De Vries 2000; De Vries and Visser 2001; Pel et al. 2007) in the case of the fungus ***Aspergillus niger***. The genome of this fungus contains ORFs of 131 secreted carbohydrate active enzymes, which illustrates the versatility of the tool box to degrade plant cell walls.

A. niger is a cosmopolitan fungus, and causes serious opportunistic infections in onions and hyacinth bulbs.

In citrus fruit, the fungi *Penicillium italicum* and *P. digitatum* (blue and green rot of citrus respectively) cause the most serious and widespread rots of these crops. Other opportunistic fungi on these fruits are *Alternaria alternata*, *A. niger*, *Fusarium spp.*, *Geotrichum candidum*, and *Trichoderma viride* (Snowdon 1990). **In apple, *P. expansum*** is a post-harvest problem of similar magnitude. *P. italicum*, *P. digitatum*, and *P. expansum* are all able to **acidify** the host tissue and form citric acid in liquid culture (Prusky et al. 2004). In citrus and apple fruit, citric acid and **gluconic acid** accumulate, and expression of an endopolygalacturonase (*pepg1*) was highest at pH 4.0.

The fungi have a preference for ammonium as the nitrogen source. Ammonium levels had dropped sharply in decaying tissue because of uptake by the fungal cells, which excrete H^+ and lower the pH inside the lesion. The production of gluconic acid was accompanied with the expression of a glucose oxidase gene (*gox2*), and virulent isolates showed more of both (Hadas et al. 2007). Interestingly, GOX activity, gluconic acid accumulation, and decay dropped significantly when oxygen levels dropped to 10 % or lower. This indicates that gluconic acid and not citric acid is an important factor for disease.

That opportunistic fungi are markedly adapted to infection of harvested crops is illustrated by conidia of *P. digitatum* that germinate quicker and in higher numbers in **the presence of volatiles** that surround wounded oranges (Eckert and Ratnayake 1994). Interestingly, the strongest stimulation was observed when the "authentic" volatile mixture was applied, and was invariably lower in preparations of single compounds or mixtures with concentrations above and below that of the wounded oranges.

Microconidia of *Fusarium oxysporum* f. sp. *tulipae* cause a devastating dry rot in tulip bulbs, and only germinate in wounds on the surface of the bulbs and are not able to grow on undamaged epidermis, even when it is very close to a wound (Fig. 2.2).

Hyphae then grow out underneath the epidermis and a lesion is formed. At later stages, numerous microconidia are formed on the surface of the bulbs. Dependent on environmental factors such as humidity and temperature, fungal cells stop developing but remain alive and can enter a quiescent stage. When tulip bulbs are planted out upon storage, fungal development resumes after many months, and during outgrowth of the bulbs leads to infection of plant tissues and subsequent death (Dijksterhuis, van der Lee and de Boer, unpublished results). **The quiescent stage is called latency**, and is an important aspect of post-harvest diseases; and the mechanisms of prolonged survival of fungal cells during this stage are a **potential important research topic** in order to develop novel strategies to prevent damage to food products.

III. Processed Foods: Spoilage as Colonization of a Medium

Many processed foods contain vegetables, fruits, and other plant material that are treated to **make the nutrients more available** for the human digestive system. In a way, these foods are comparable to plant-based media that are used in microbiological laboratoria, and as such can be colonized and spoiled by fungi. Spoilage fungi can enter the food via the **basic components** of the product. For example, some spoilage fungi enter the product via added spices (small pieces of plant material). In other cases, they are introduced **during the food production chain** or subsequent storage. In particular, **airborne spores** can enter food products that are not effectively shielded. Airborne contamination is characterized by the simultaneous outgrowth of more species of fungi in a product. The density of fungal spores in the (indoor) air varies greatly, and is correlated with the ability of certain fungal species to form large numbers of them. The propagules then enter food and crop, and can cause damage.

In the case of *Penicillium expansum* infecting apple, high spore densities in the air are probably caused by growth of the mould in high concentration in rotten organic material in orchards (Borner 1963). Fungi also develop **inside buildings** (where storage occurs), and their proliferation is then often related to leakage,

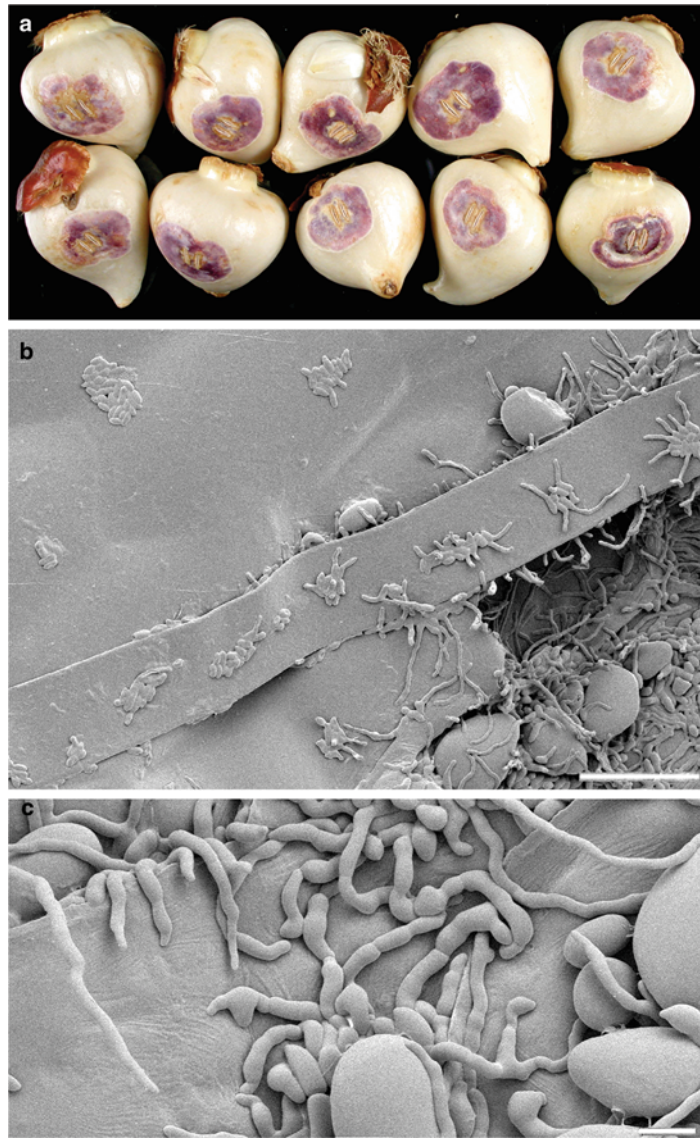

Fig. 2.2. Post-harvest infection of tulip bulbs. (**a**) Tulip bulbs are infested with conidia of *Fusarium oxysporum* f. sp. *tulipae*, and develop purple stained lesions after 120 h following inoculation. (**b**) Microconidia do not germinate outside a wound in the epidermis of the bulb. (**c**) Germination of conidia on plant cell wall and starch granules (Photographs made by Jan Dijksterhuis, CBS-KNAW Biodiversity Centre, The Netherlands). Bars are 20 and 5 μm respectively

flooding, condensation, and humidity. Occupants inside homes also contribute to mould growth as a result of activities generating humidity (cooking, breathing) in combination with the obstruction of venting of the building caused by, for instance, the insulation of buildings. Therefore, the composition of the **fungal indoor mycobiota is very dynamic and correlated with and depending on human activity** (Flannigan et al. 2011; Adan and Samson 2011). Previous indoor food spoilage may grossly enhance the inoculum pressure on newly introduced food products. For example, in Dutch cheese warehouses, *Penicillium*

discolor commonly occurs, and can cause serious spoilage when poor hygienic conditions increase the sporulation of this fungus.

Massive production of conidia can be regarded as a vital strategy for dispersion of a number of important food-borne fungi. The order *Eurotiales* includes many relevant food-spoilage fungi (Samson et al. 2004; Pitt and Hocking 2009), with an emphasis on the genera *Paecilomyces, Penicillium* and *Aspergillus*. With respect to food spoilage, *Aspergillus* seems to be more suitable for tropical areas than *Penicillium*, which is observed more in temperate areas.

Fungal spoilage organisms can **build up considerable biomass** in certain areas of the food production chain, and when not sufficiently cleaned act as a **recurrent source** of contamination. In this way a "house flora" can develop inside certain factories; e.g., *Penicillium roqueforti* which causes spoilage in rye bread factories, and *Fusarium oxysporum* in dairy products. *Geotrichum candidum* is known as the "machinery mould" or "dairy mould", and is responsible for slime building in processing equipment and off-smells in finished products (Wildman and Clark 1947).

In time, **different preservation techniques** are developed with the aim of discouraging fungal development in the food product. These include fermentation, addition of salts or high concentrations of sugars, pickling, drying, cooling, the addition of preservatives or a heating treatment before packaging. More recent techniques include modified atmosphere packaging and the application of high-pressure treatment (Barbosa-Cànovas 1998; Smelt 1998) of the food product, but heat-resistant ascospores clearly show survival of treatment (Butz et al. 1996; Palou et al. 1998). In addition, high-pulse fields are applied to food products in order to evaluate if these are able to kill spoiling organisms.

Novel food-preserving techniques include the application of the preservatives sorbate and benzoate on the surface of fruits and vegetables, and maybe also to processed food by the use of edible coatings (Valencia-Chamorro et al. 2010; Mehyar et al. 2011). In addition, biocontrol agents such as the yeast *Pichia anomala* are an interesting option that counteracts spoilage fungi in case of high-moisture feed grain under airtight conditions (Petersson et al. 1999). The latter is in fact similar to the use of fermentation as stated at the beginning of this chapter.

In certain aspects, the ecological niche of processed food products therefore can be regarded as an **extreme environment with rich nutrients**. This is of evolutionary interest; the fungi that are able to overcome these stresses are heavily rewarded.

A. Association of Fungal Species with Food Products

It was already recognized by Johanna Westerdijk in 1949 that there might be an **association between specific fungal species with certain food products or crops**. For instance, *P. expansum* is specific for pomaceous and stone fruits, while the species *P. italicum* and *digitatum* cause damage to citrus fruit. The adaptability of the fungal species to overcome the restrictions of the crop or the limitations introduced by preservation techniques determines the dominance of the species in relation to the relevant food product.

Food parameters are **surprisingly restrictive to the spectrum of species which are able to grow and thus spoil the individual food types**. Normally, less than ten and often one to three species are responsible for spoilage (Frisvad and Filtenborg 1988, 1993; Frisvad et al. 2007b). Table 2.1 shows a survey of different classes of food products and associated fungi (see also Frisvad et al. 2007b). Frisvad et al. (2007a) have described the **importance of accurate identification of spoilage fungi**. The wrong identification will blur the development of a conclusive scheme of food spoilage and disturb research on the (cellular) mechanisms responsible for this specificity as well as the specificity of mycotoxin production. Knowledge of these parameters will lead to novel tailor-made preservation strategies.

Fungal culture collections may play an important role in this development. As an

Table 2.1. Most common associated fungal species (From Frisvad et al. 2007, with courtesy of Taylor and Francis, CRC Press)

Crop	Product	Fungal species
Beans & peas	Black beans, cowpeas	*Alternaria alternata, Aspergillus flavus, Asp. ochraceus, Asp. parasiticus, Fusarium proliferatum, Penicillium citrinum*
Cereal	Maize	*Asp. flavus, Asp. niger, Asp. ochraceus, F. graminearum, F. proliferatum, F. verticillioides, P. citrinum*
	Rice	*Asp. flavus, Asp. niger, P. citrinum*
	Rye bread	*Eurotium repens, Eur. rubrum, P. carneum, P. paneum, P. roqueforti*
	Sorghum	*Alt. alternata, Asp. flavus, F. verticillioides, F. semitectum, P. citrinum*
	Wheat bread	*Asp. flavus, Eur. repens, Eur. rubrum*
	Wheat, rye, barley, oat	*Alt. tenuissima* and *infectoria* sp.-grps., *Asp. flavus, Asp. parasiticus, F. avenaceum, F. culmorum, F. graminearum, P. aurantiogriseum, P. cyclopium, P. freii, P. melanoconidium, P. polonicum, P. verrucosum*
Cheeses	Hard cheese	*Asp. versicolor, P. commune, P. discolor, P. nalgiovense, P. solitum*
Coffee	Coffee—monsoon	*Asp. candidus, Asp. niger, Asp. tamarii*
	Coffee—traditional	*Asp. carbonarius, Asp. steynii, Asp. westerdijkiae, P. citrinum*
Fruit	Citrus	*Alt. tangelonis, Alt. tenuissima* sp.-grp., *Alt. turkisafria, P. digitatum, P. italicum*
	Dried fruits	*Asp. carbonarius, Asp. flavus, Asp. niger, Asp. ochraceus, Xeromyces bisporus, Wallemia sebi*
	Fruit juice	*Byssochlamys nivea, B. spectabilis (= Paecilomyces variotii), Eupenicillium* spp., *Neosartorya* spp., *Talaromyces* spp.
	Grapes	*Asp. carbonarius, Asp. niger, Asp. tubingensis, P. expansum*
	Pomaceous & stone	*Alt. arborescens* sp.-grp., *Alt. tenuissima* sp.-grp., *F. lateritium, P. crustosum, P. expansum, P. solitum*
Meat	Sausages	*P. nalgiovense, P. nordicum, P.olsonii, P. chrysogenum, Eurotium* spp.
Nuts	Almonds, hazelnuts, pistachio, walnuts	*Alt. arborescens* sp.-grp., *Asp. flavus, Asp. niger, Asp. tamarii, F. acuminatum, F. avenaceum, F. semitectum, P. crustosum, P. discolor*
Oil crop	Olives	*Alt. alternata, Asp. versicolor, P. citrinum, P. expansum*
	Peanuts	*Asp. flavus, Asp. niger*
	Sunflower	*Alt. alternata, Asp. flavus, Asp. niger, Asp. parasiticus, F.verticilloides, F. semitectum*
Vegetables	Ginger	*P. brevicompactum*
	Onion & garlic	*P. allii, P. glabrum, Petromyces alliaceus*
	Pepper—bell	*Alt. alternata*
	Pepper—black	*Asp. flavus, Asp. parasiticus, Asp. tamarii*
	Potatoes	*Alt. alternata, Alt. solani, F. coeruleum., F. sambucinum*
	Tomatoes	*Alt. alternata, Alt. subtropica, Alt. tenuissima* sp.-grp., *P. expansum, P. olsonii, P. tularense, Stemphylium eturmiunum, Stemphylium solani*
	Yams	*Botryosphaeria rhodina, F. verticillioides, P. sclerotigenum*
	Yam chips	*Asp. flavus, Asp. niger*

example, Samson and Frisvad (2004) developed a taxonomy of *Penicillium* subgenus *Penicillium* based on a polyphasic approach that includes macro- and microscopic morphology, growth characteristics, DNA sequences, and the excretion of secondary metabolites. More recently, similar approaches have been applied to the genera *Aspergillus* (Samson and Varga 2007) and other genera within *Penicillium* (Samson and Houbraken 2011). *Penicillium* and *Aspergillus* belong to the most abundant fungi in air, and are dominant fungi with respect to food spoilage. **The correct classification and deposition of the identified strains** in a culture collection may become an important element in our struggle to prevent food spoilage.

B. Food-Spoiling Fungi

One of the main preservation strategies is the **increase of the osmolarity** caused by the addition of sugar or salt. Several yeasts (***Debaryomyces hansenii, Zygosaccharomyces rouxii* and *Z. bailii***) are very osmotolerant, and can grow in environments with extremely high concentrations of sugar and salt.

Ecologically, *D. hansenii* is a marine fungus (Clipson and Jennings 1992), but it is also isolated from penguin droppings, anthills, and soils. It is observed in hypersaline habitats and subglacial ice (Gunde-Cimerman 2009). It spoils **brine foods and contaminates fruit powders**, but is also the dominant yeast species isolated from dry cured meat products, and plays a role in taste development by the production of specific volatile compounds (Andrade et al. 2010).

The *Zygosaccharomyces* species, including the extremely osmotolerant species *Z. rouxii* and *Z. bailii*, cause **major food spoilage** in many different products including **fruit juices, sauces, carbonated soft drinks, and ketchup** (Pitt and Hocking 2009). These yeasts have a reputation of being able to grow on a very high concentration of sugars (e.g., 5 M glucose for *Z. rouxii*, Martorell et al. 2007) and form high amounts of carbon dioxide, but also degrade food preservatives as sorbic acid. They are halotolerant, but to a lesser extent than *D. hansenii* (Lages and Silva-Graça 1999).

Other filamentous fungi can also grow **at very low water activities (*Eurotium amstelodami, Wallemia sebi, Aspergillus penicilloides* and *Xeromyces bisporus*).** *E. amstelodami* is known for spoilage in **corn silos**, where it can develop at 15–16 % moisture levels, and *A. penicilloides* is probably the pioneer species for fungal spoilage of **stored grains** (Pitt and Hocking 2009). *W sebi* grows on **dried figs, dates, chocolate, and fruit bars**; *X. bisporus* is the most xerophilic fungus known to date, and develops on pure marzipan (Williams and Hallsworth 2009; Vinnere-Pettersson et al. 2011; Leong et al. 2011).

Fungi that are present on food products or are introduced via the food production chain are inactivated by heat treatments. Spoilage can occur **after pasteurisation treatments** when heat-resistant fungi survive high temperatures. Fungi that cause damage worth millions of dollars in the fruit-juice industry are, among others, ***Byssochlamys nivea* (*fulva*), *Talaromyces flavus* (*macrosporus*),** and ***Neosartorya fischeri*** (as reviewed by Tournas 1994). These are soil-borne fungi, and fruits that develop in contact with soil (such as strawberries) are more prone to contamination. These fungi can survive temperatures of 85 °C for time intervals that are markedly longer than those used for pasteurization treatments. The heat resistance is conveyed by **sexual ascospores** that are candidates to be the most stress-resistant eukaryotic described to date (Dijksterhuis 2007). **The dormant state of these spores is broken** by temperatures used for pasteurization.

Storage of food products in refrigerators is often accompanied by the presence and growth of the so-called **psychrotolerant fungi** (cold-tolerant fungi). These fungi often belong to the genera *Alternaria*, *Fusarium*, *Penicillium*, and *Cladosporium*. In addition, *Botritys cinerea* is also a fungus that develops well at a surprisingly low temperature (Hoogerwerf et al. 2002).

Several fungi (including filamentous species and yeasts) are able to **degrade the important food preservative sorbate** by the action of enzymic activity. In particular, the species ***Penicillium roqueforti*** and ***Paecilomyces variotii*** are notorious for the spoilage of rye bread, drinks, and margarine that contain sorbic acid, benzoic acid, and propionic acid.

Aspergillus niger is also capable of degrading sorbic acid, but is not a major spoiler of these products. This illustrates the subtle interplay of different parameters during food spoilage.

Low-oxygen packaging is a relatively novel method of keeping products free of spoilers. However, some fungi have traits that make them suitable for **development under very low oxygen and/or high carbon dioxide.** These include ***Saccharomycopsis fubiliger*** and ***Hyphopichia burtonii,*** the “chalk molds”, well-known from products such as pre-backed bread.

In addition, the fungi *P. roqueforti* and *Fusarium oxysporum* are able to grow at very low levels of oxygen. The main question is: are these fungi able to grow anaerobically, or are they microaerophilic?

The following paragraph elaborates on some cellular traits which these organisms have developed as an answer to adverse conditions that are similar to the typical difficulties of growing in food.

IV. Coping with Adverse Conditions

A. Osmotolerance

The number of **yeast species** that are involved in food spoilage is small compared to their total number, and include **extreme osmotolerant organisms**. Lages and Silva-Graça (1999) summarize 33 yeast species that have a maximum tolerance above 2 M NaCl. The yeast *D. hansenii* grows at sodium chloride concentrations up to 2.5 M, and growth is stimulated in 0.5 M (as reviewed in Prista et al. 2005). During mid-exponential growth, ***D. hansenii* accumulates high concentrations of sodium** (approx. 750 mM) and potassium (300 mM, Prista et al. 1997). For this reason, the yeast species can be regarded as a **"sodium includer"**, in contrast to "sodium excluders" that have a strategy of keeping the internal concentration of the sodium ion low (Prista et al. 2005). While higher internal concentrations of sodium are beneficial for the biological performance of the organism, one can state that *D. hansenii*, is not only halotolerant, but is also a halophilic organism.

During salt stress, different **transporter proteins located in the plasma and vacuolar membrane** (Prista et al. 2005) are active in both efflux and influx of protons, sodium, and potassium ions.

D. hansenii expresses two P-type ATPases, DhENA1 and DhENA2, one specific for sodium efflux at higher pH. When expressed in *S. cerevisiae* without any Na^+ efflux activity, these proteins were able to recover growth of the yeast in NaCl containing media (Almagro et al. 2001). Alternatively, the DhHAK1 and DhTRK1 transporters enable the cell to take up monovalent cations, especially potassium (Martínez et al. 2011).

These observations indicate that *D. hansenii* realizes halotolerance as a result of **the interplay of different transport processes.**

In addition, another major process is important for the salt-tolerance of these cells, namely the **accumulation of compatible solutes** (Jennings and Burke 1990). The term compatible means that high intracellular concentrations of certain solutes are compatible with enzyme functioning. For fungi, the most common solutes are the polyols **glycerol,** erithreitol, arabinitol, and **mannitol**, as well as the disaccharide **trehalose.**

Remarkably, **the type of solute may change with the growth phase**; arabinitol (arabitol) is the major solute present in the cells of *D. hansenii* in the stationary growth phase (Adler and Gustafsson 1980). During mid-exponential growth, this yeast accumulates glycerol up to approx. 35 % dry weight of the cells in 16 % (w/v) NaCl (Adler et al. 1985). At the beginning of the stationary phase, a major portion of the glycerol leaks out of the cells. Simultaneously, glycerol is actively taken up by the cell and metabolized. This could be the result of the activity of **a proton/glycerol symporter** or even a still-putative sodium/glycerol symporter (Lucas et al. 1990; Prista et al. 2005). The precise functioning of the compatible solutes in *D. hansenii* clearly has its enigmas, as the yeast preferentially accumulates trehalose at low salt levels and glycerol at high salt (2.0 M or higher, Gonzlez-Hernndez et al. 2005).

Z. rouxii also **accumulates high levels of glycerol, but is better able to retain it** inside the cell (Hosono 2000). *Z. rouxii* can still grow in 875 g sugar/l and at pH 2.5 (Membre et al. 1999) or in 3.1 M NaCI (Hosono 2000). In addition, *Z. bailii* is also **extremely tolerant to organic acids** such as sorbic and benzoic acid (Steels et al. 2000; Martorell et al. 2007).

Z. rouxii expresses a proton-ATPase in combination with a Na^+/H^+ antiporter to remove sodium ions from the cell (Watanabe et al. 1991, 1995). A *S. cerevisiae* strain that was very sensitive to salt stress was made more osmotolerant with the antiporter genes (ZrSod2 and ZrSod22) from *Z. rouxii* (Iwaki et al. 1998). When the antiporter was deleted from *Z. rouxii*, the organism could not grow on medium with high salt concentration, but was still able to develop on very high concentrations of sugar (Watanabe et al. 1995). Recently,

ZrNha1 was identified, an antiporter that is thought to be indispensible for maintaining potassium homeostasis (Pribylova et al. 2008).

However, how do these yeasts realize growth even at sugar concentrations **above 5 M (90 % w/v)?** Is it their ability to ferment sugars at **high rates**, even in the presence of oxygen, a factor in their survival (Leyva et al. 1999)? Under anaerobic conditions, the yeast can also grow exponentially, with vigorous fermentation given that the medium is complex (as food and beverages are; Rodriguez et al. 2001). *Z rouxii* employs two unique uptake systems for fructose molecules (Leandro et al. 2011), and is called **fructophilic** as it can transport these molecules with a higher capacity. Alternatively, the structure of the cell wall is very responsive to growth conditions, and is found to be variable with salt tolerance (Přibylova et al. 2007). For example, it might be that a more elastic cell enables the cell to deal with these straining conditions.

The moderate osmotolerant fungus ***Geotrichum candidum*** is an important spoilage organism (**dairy, vegetables and fruit**) and can heavily contaminate food production chains. It forms numerous one-celled arthrospores, and cultures show some resemblance with yeasts in development and morphology. Arabitol, a sugar alcohol, accumulates in *Geotrichum* species in 1 M NaCl. It reaches amounts above 30–40 % dry weight, which decreases slightly at the stationary phase of culturing. One species of *Geotrichum* (out of five), however, accumulated mannitol as a compatible solute in similar amounts. Mannitol is also formed during the stationary growth phase without salt (Luxo et al. 1993).

B. Xerophilic Fungi

We discussed osmotolerance in relation to high salt and sugar concentrations in growing media. Strictly speaking, all halophiles and osmophiles are **xerophiles**, fungi that develop at low water activity. For living organisms, the availability of water molecules is a prerequisite for development. Water availability is restricted as a result of **high concentrations of solutes** in the growth medium, and also when the **relative humidity in air** is low, when fungi grow on inert surfaces, a situation common in indoor conditions. The latter are conditions that may also prevail during large-scale storage of cereals. A number of filamentous fungi are able to grow in the presence of low amounts of water, including *Wallemia sebi, Eurotium amstelodami*, and related species, *Aspergillus penicilloides* and the most xerophilic organism known to date, *Xeromyces bisporus*. The genome of *W. sebi* has recently been sequenced (Padamsee et al. 2012), and revealed adaptations to osmotic stress.

Recently, a number of studies have appeared (Williams and Hallsworth 2009; Chin et al. 2010) that reflect on the role of intra- and extracellular solutes on survival and growth at low water activities. Williams and Hallsworth (2009) studied growth of xerophilic fungi on different media containing solutes with varying degrees of **chaotropic activity**. These solutes weaken macromolecular interactions and disorder cellular structures, while **kosmotropic solutes** stabilize these interactions. Chaotropic solutes include **glycerol**, magnesium chloride and **fructose**, and kosmotropic solutes are ammonium sulphate and **sucrose**.

The authors observed that growth media with very low water activity and relatively low chaotropic activity showed relatively better growth of xerophilic fungi. The authors asked the question whether the chaotropicity of glycerol-supplemented media at very low water activity limited hyphal growth, and whether a more kosmotropic environment might result in growth at even lower water activities. Indeed, the authors realized growth of *A. penicilloides* at a water activity of 0.647 on a neutral medium, compared to 0.653 for *X. bisporus* in 7.6 M glycerol. Chin et al. (2010) evaluate the influence of chaotropy on growth at low temperatures, and observe that *Eurotium herbariorum* grows much better in fructose (chaotropic) at 1.7 °C, and best on sucrose at 30 °C. The extreme halotolerant yeast *Mrakia frigida* even can grow slowly at −5 °C in 1.1 M glycerol, which is not possible in 0.73 M sucrose (kosmotropic). Conidia (spores) of *X. bisporus* from glycerol containing cultures survive freezing treatments better than conidia from sucrose-containing medium, which are better survivors after heat and high-pressure treatments.

The rationale behind this is that **chaotropic solutes counteract the rigidity of macromolecular interactions at low temperatures** (and drying?), and kosmotropic solutes stabilize these interactions at high temperatures. This **link** between xerophilic and psychrophilic growth habit is nicely illustrated in the isolation of fungi from high-altitude Nepalese soil (Petrovič et al. 2000). Biophysical relationships between water, macromolecules, and solutes reveal novel aspects of cellular survival under adverse conditions.

C. Fungal Survival Structures

This brings us to fungal structures that are used **for dispersion in time and space;** fungal spores. Fungal spores are extremely variable; the way they are formed and their shape are still very important for the determination of fungal genera and species. They bear different names such as sporangiospores in the case of *Zygomycetes* (reviewed by Dijksterhuis and Samson 2006), ascospores when they are sexual and conidia when they are asexual (see for reviews Chitarra and Dijksterhuis 2007; Magan et al. 2012). Some fungal ascospores belong to the strongest eukaryotic cells described to date (Dijksterhuis 2007). **Fungal genera that are important for food spoilage such as *Aspergillus*, *Paecilomyces* and *Penicillium* are also avid sporeformers** (Berbee et al. 1995).

Some conidia are **hyaline** and are dispersed by **water splashes**, others are **airborne** and have to survive conditions of **drought** during transport through the air and are moderately stress-resistant. Interestingly, the method of dispersion of the conidia is correlated to **membrane composition** (ergosterol level) or **cytoplasmic parameters** (viscosity of the cytoplasm). Van Leeuwen et al. (2010) showed that hyaline, water dispersed conidia of *F. oxysporum*, and *Verticillium fungicola* showed less resistance to the antifungal natamycin, lower cytoplasmic viscosity and higher staining of ergosterol (with filipin, van Leeuwen et al. 2008) compared to airborne conidia of *A. niger* and *P. discolor*. Extremely heat-resistant ascospores of *Talaromyces macrosporus* and *Neosartorya fischeri* exhibit the **highest cytoplasmic viscosity** (Dijksterhuis et al. 2007, Wyatt et al., unpublished results).

In fungal spores, often a combination of mannitol, a polyol, and trehalose, a disaccharide is observed, e.g., in the species *Aspergillus oryzae*, *A. nidulans*, *A. niger*, and *P. rubens* (Horikoshi and Ikeda 1966; D' Enfert and Fontaine 1997; van Leeuwen et al. 2013b; Bekker et al. 2012). Trehalose accumulation is an important factor in yeast **heat tolerance** and protection of cell components (Wiemken 1990). Both **membranes** (Crowe et al. 1984) **and proteins** (Hottiger et al. 1994) **are stabilised** by trehalose. Hallsworth and Magan (1994, 1996) showed that different **environmental conditions** during cultivation **influence the solute composition** inside conidia in the case of insect pathogenic fungi.

Optimal conditions might result in stronger spores, which have higher **shelf lives** if they are used for biological control of insect pests. The principles found in the entomopathogenic species may also apply for fungal species occurring on food, with the difference that now conidia maybe have to be eradicated as effectively as possible. Primary models for the inactivation of fungal spores are reviewed (Dijksterhuis et al. 2012), and can be used for different sporocidal conditions including heat, drying, or vapour treatments (Dao and Dantigny 2009; Dao et al. 2010).

Fungal colonization of food is often initiated by the deposition of conidia on the product, with the prerequisite that the **dormant state is effectively broken** and germination can occur. The **transition** from a dormant conidium towards a vegetative growing fungal hyphae includes changes of the cell wall (Tiedt 1993; Fontaine et al. 2010), breakdown of compatible sugars (including trehalose and mannitol) (D' Enfert et al. 1999; Fillinger et al. 2001; van Leeuwen et al. 2013b), reorganization of the transcriptome including major mRNA breakdown, and strong upregulation of specific gene categories (van Leeuwen et al. 2013a). Water is a basic compound needed for cellular functioning, and when added to spores directly influences the formation of polyribosomes (Bonnen and Brambl 1983). **Isotropic growth** of conidia of *A. niger* occurs after incubation in distilled water (Morozova et al. 2002), and conidia of *A. oryzae* germinate on water agar (Sakamoto et al. 2009). Nutrients such as phosphate,

amino acids, glucose, and their combinations reactivated dried sporangiospores of the tempe fungus *Rhizopus oligosporus* to a different extent, but rich media were far more effective in this (Thanh and Nout 2004; Thanh et al. 2005). Small amounts of glucose or ammonium sulphate in water resulted in **germ tubes** on conidia of *A. niger* and in branched mycelium when combined. Other conidia (*A. nidulans)* specifically need carbon sources such as glucose for germination (d'Enfert 1997; Osherov and May 2000).

A micro-array analysis of *A. niger* conidia clearly indicates that the expression of genes involved in protein synthesis changes most during the first 2 h of germination, which can be seen as the major strategy for early germination (van Leeuwen et al. 2013a). Osherov and May (2000) already treated conidia of *A. nidulans* with various inhibitors, and only the protein synthesis inhibitor cycloheximide prevented isotropic growth, in contrast to inhibitors of other cell processes.

Germination of conidia is also affected by volatile compounds such as, for instance, the volatile 1-octen-3-ol. This compound is produced by fungi during crowding of conidia of *Penicillium paneum* and *A. nidulans* (Chitarra et al. 2004; Herrero-Garcia et al. 2011), and in the absence of germination when spores are present in high densities. 1-Octen-3-ol is also produced by *A. niger* (Karlshøy et al. 2007). It is hypothesized that 1-octen-3-ol acts as **a fungal self-inhibitor** that prevents premature germination of conidia on conidiophores.

The compound had a profound influence on protein expression patterns (Chitarra et al. 2005), blocked isotropic growth, but had only mild physiological effects on germinating conidia in solution. **Volatiles also activate germination of conidia** in the case of *Penicillium digitatum*, which causes post-harvest citrus rot. Conidia, when provoked with volatiles from damaged oranges, showed enhanced germination (Eckert and Ratnayake 1994).

D. Heat Resistance

Fungal survival structures can be regarded as more or less heat-resistant compared to vegetative cells, and conidia, sclerotia, chlamydospores, and ascospores survive heat treatments between 55 °C and 95 °C. Yeast ascospores isolated from soft drinks and fruit products (mainly *S. cerevisiae, Z. bailii* and *chevalieri* strains) had D_{60} values that were 25–350 times higher than those of the corresponding vegetative cells (Put and De Jong 1980). Recent measurements in our laboratory show that **heat resistance of ascospores above 70 °C** (see Fig. 2.3) is a common trait that occurs in all fungal genera of the *Eurotiales*: *Eupenicillium*, *Neosartorya*, *Eurotium*, *Hamigera*, *Xeromyces*, *Byssochlamys*, *Thermoascus*, and *Talaromyces*, and most probably occurs in more fungal clades (e.g., *Neurospora* and *Daldinia* species). Ascospores of the fungus *Talaromyces macrosporus* survive at **85 °C for 100 min** (Dijksterhuis and Teunissen 2004), which is similar to some bacterial spores (e.g., *Bacillus subtilis*). Ascospores of *Neosartorya spinosa* and *Byssochlamys spectabilis* shows similar heat resistance (Houbraken et al. 2008; Wyatt and van Leeuwen, unpublished results).

The **heat resistance** of ascospores in food products generally **increases with the sugar concentration** of the surrounding medium (Splittstoesser and Splittstoesser 1977; Beuchat 1988a; King and Whitehand 1990) Additional factors are **pH** and the presence of **organic acids** such as those present in fruits or used for preservation, which counteract heat resistance of ascospores, but only at low values of pH (lower than 4).

Benzoic and sorbic acid had effects on *T. flavus* and *N. fischeri* ascospore heat resistance (Beuchat 1988b; Rajashekhara et al. 1998). Combination of different factors may lead to some unpredictable variations in heat resistance. For instance, *N. fischeri* exhibited a far higher heat resistance in 0.1 M phosphate buffer (pH 7.0) than in grape jellies with large amounts of cane sugar (pH 3.1−3.3, Beuchat and Kuhn 1997), and *B. nivea*, *B. fulva*, and *N. fischeri* were approximately twice as heat resistant in tomato juice (pH of 4.2) as in phosphate buffer (pH 7.0, Kotzekidou 1997).

Factors inside the ascospore are important for heat resistance, for instance as a result of the **age of the culture** in case of *N. fischeri*, *T flavus*, and *B. nivea* (Conner and Beuchat 1987; Beuchat 1988a; Casella et al. 1990) or the **growth temperature** of the

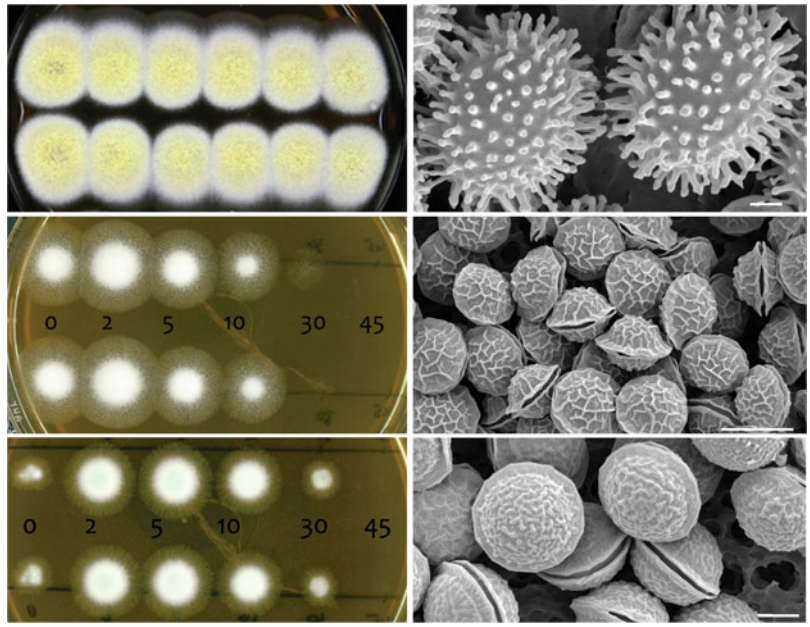

Fig. 2.3. Heat resistant fungi survive 75–85 °C for periods that are longer than conventional pasteurization times. *Left panel*; ca. 5,000 ascospores were inoculated in a small droplet on agar after a heat treatment for 0, 2, 5, 10, 30, and 45 min (from *left* to *right*). Top (*Talaromyces macrosporus* at 75 °C, middle *Neosartorya fischeri* and bottom *Neosartorya hiratsukae,* the latter at 85 °C). The *right panel* shows images of the ascospores as taken with low temperature scanning electron microscopy. Bars are 1, 5 and, 2 μm from *top* to *bottom*

spore-generating colony (Conner and Beuchat 1987; King and Whitehand 1990).

Harvested and washed ascospores also showed maturation in case of *T. macrosporus* (i.e., increase of heat resistance in time, Dijksterhuis and Teunissen 2004) when stored at 30 °C. This phenomenon did not occur at 10 °C, suggesting a temperature-dependent acquisition of resistance. Furthermore, King and Whitehand (1990) report higher heat resistance of *T macrosporus* on solid medium, and also the type of medium used is important (Beuchat 1988a). Finally, heat resistance varies with the fungal isolate used (Bayne and Michener 1979; Beuchat 1986; King and Whitehand 1990).

Conner et al. (1987) investigated the nature of heat resistance. They studied younger and older ascospores of *N. fischeri* that had increasing heat resistance. Ascospores showed **changes in the inner cell wall** region during aging. Older spores contained more mannitol and trehalose. Polyols and disaccharides may play an important role in heat protection as compatible solutes. Recent work indicates that additional compatible solutes may also exist in heat-resistant **ascospores** made by different fungal species (Wyatt et al., unpublished results). HPLC studies showed that ascospores of *T. macrosporus* contain **very high concentrations of trehalose,** up to 15–20 % of the wet cell weight (that is, 24–32 % of the dry weight, Dijksterhuis et al. 2002). The **low water content** of the spores (38 %) introduces a **very high viscosity** inside the spores as measured by means of EPR (electron paramagnetic resonance) studies (Dijksterhuis et al. 2007). When these spores are in solution at room temperature no **glassy state** occurs, but under dry conditions this may occur. A glassy state is an amorphous phase characterized by very low movement of the molecules inside the cytoplasm. A sudden lowering of the temperature or a reduction of the water content might introduce a glass transition situation inside the cell, which virtually brings all processes in the cell to a stand still.

Constitutive dormancy of ascospores includes a **metabolic block,** a **barrier to the penetration of nutrients,** or the production of

a **self-inhibitor** (as defined by Sussman and Halvorson 1966), or as a result of a **specific physical state** of the cytoplasm such as a glassy state. Ascospores often need a robust physical signal such as heat for breaking of dormancy, where the number of viable counts after treatment is increased by several log cycles (e.g., *Eurotium herbariorum* at 60 °C, Splittstoesser et al. 1989). For ascospores of *Talaromyces flavus*, activation is observed at 80 °C and, at 85 °C, activation is followed by killing (Beuchat 1986).

These extreme characteristics may also favour a very long shelf life of the ascospores; they can be still viable for up to 17 years in the case of *T. flavus* (Nagtzaam and Bollen 1994). At lower temperatures, activation fails, and only low numbers of germinated spores are observed. Remarkably, the speed of activation increases with higher temperatures with *T. macrosporus* (Kikoku 2003). Apart from heat, also a **drying treatment** can result in activation. For *N. fischeri*, the dormant state can be broken by a drying treatment of 18 h at 40 °C (Beuchat 1992), but *T. flavus* ascospores did not show a release of dormancy. Heating at 50 % r.h. (dry heat treatment) at 95 °C (for 30 or 60 min) activated *N. fischeri* ascospores, but the temperature of the wetting or recovery buffer was crucial for the viable count obtained (Gomez et al. 1989, 1993).

Further, **high pressure treatments** (6,000 Bar) that are used for non-thermal "pasteurization" of a number of food products can activate ascospores of *T. macrosporus* to germinate (Reyns et al. 2003; Dijksterhuis and Teunissen 2004), in which physical disruption of the thick outer cell wall may play a role.

Recent work at our laboratory indicated that a cell-wall protein is related to the permeability of the cell wall of ascospores and dormancy of these spores (Wyatt et al., unpublished results).

Could activated ascospores resume dormancy again when cytoplasm is forced into a glassy state by a sudden lowering of the temperature or drying to very low water levels? Heat-activated spores of *T. macrosporus* after cooling in liquid nitrogen or kept at −20 °C directly germinated upon introduction into conducive conditions (Dijksterhuis and Samson 2006). Further, ascospores remained dormant after drying, and could be effectively activated by a heat treatment after resuspension in buffer. **These findings indicate that irreversible changes occur during breaking of dormancy.**

E. Food Preservatives

Food preservatives are added to food products in order to prevent outgrowth of fungi. The food additive **sorbic acid** is widely used in the food industry as a preservative of low pH sugar-containing products (Stratford et al. 2012). The minimally inhibitory concentration of sorbic acid is dependent on the density of the inoculum of conidia in the case of *A. niger* (Plumridge et al. 2004). Sorbic acid delays conidial germination and lowers the cytoplasmic pH, but at 3 h of germination, conidia start to **degrade the preservative** (Plumridge et al. 2010) and resume development. This is the result of the activity of a **phenylacrylic acid decarboxylase** encoded by the *padA1* gene and a putative 2-hydroxybenzoic acid decarboxylase encoded by *ohbA1* (Plumridge et al. 2008). One of the characteristic degradation products of sorbic acid is **1,3-pentadiene**, which has a kerosene-like smell. Several **osmophilic yeasts** including *Z. rouxii* and *D. hanseni* are able to perform degradation of sorbate, as well as several *Penicillium* species and *Trichoderma* (Cheng et al. 1999; Casa et al. 2004; Pinches and Apps 2007). Yeast cells also strongly upregulate the expression of transporters in answer to weak acid stress, which is imposed on the cell by sorbic acid, including Pdr12 that specifically removes the anions from the cells (Piper et al. 2001).

Another preservative, **natamycin**, is used for the protection of cheese and sausages against fungal development (Stark 2007). This polyene antibiotic binds to ergosterol, which is most available at growing tips of germinating spores and vegetative hyphae (van Leeuwen et al. 2008, 2010) and blocks active fungal growth. Natamycin is active at very **low concentration** (micromolars), and affects many species of fungi. Other polyenes such as amphotericin B and nystatin form complexes that lead to leakage at the plasma membrane, but natamycin does not permeabilize cells

(Te Welscher et al. 2008), but acts directly on different aspects of cellular physiology and membrane trafficking and fusion. It inhibits endocytosis in germinating conidia of *P. discolor* (Van Leeuwen et al. 2009) and fusion of prevacuolar compartments in *S. cerevisiae* (Te Welscher et al. 2012). Very recent work shows that natamycin inhibits transport of amino acids and sugar into cells in a reversible manner, and via a hitherto unknown mechanism (Te Welscher et al. 2012). In natamycin-containing solutions, development of the spores was halted at or before isotropic growth (Van Leeuwen et al. 2013b).

A micro-array study on treated conidia of *A. niger* showed that 8-h-treated cells showed certain similarities with dormant conidia compared to the controls that had formed germ tubes. These included the elevated presence of transcripts of protective proteins (heat shock proteins, dehydrins, LEA-like proteins), genes involved in glyoxylate cycle, fermentation, glycerol, trehalose, and mannitol synthesis. Conidia of *A. niger* and *P. discolor* are able to survive 20 h in a concentration of natamycin of ten times the minimal inhibitory concentration, and germinate at high percentages after removal of the antifungal compound (Van Leeuwen et al. 2010). Mann and Beuchat (2008) suggest a combinatory use of reduced concentrations of organic acids and natamycin to control those fungi that degrade sorbic acid.

References

Adan OCG, Samson RA (2011) Fundamentals of mold growth in indoor environments and strategies for healthy living. Wageningen Academic Press, The Netherlands, 523 pp

Adler L, Gustafsson L (1980) Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. Arch Microbiol 124:123–130

Adler L, Blomberg A, Nilsson A (1985) Glycerol metabolism and osmoregulation in the salt-tolerant yeast *Debaryomyces hansenii*. J Bacteriol 162:300–306

Alkan N, Fluhr R, Sherman A, Prusky D (2008) Role of ammonia secretion and pH modulation on pathogenicity of *Colletotrichum coccodes* on tomato fruit. Mol Plant Microb Interact 21:1058–1066

Alkan N, Davydov O, Sagi M, Fluhr R, Prusky D (2009) Ammonium secretion by *Colletotrichum coccodes* activates host NADPH oxidase activity enhancing host cell death and fungal virulence in tomato fruits. Mol Plant Microbe Interact 22:1484–1491

Almagro A, Prista C, Benito B, Loureiro-Dias MC, Ramos J (2001) Cloning and expression of two genes coding for sodium pumps in the salt-tolerant yeast *Debaryomyces hansenii*. J Bacteriol 183:3251–3255

Amselem J, Cuomo CA, Van Kan J, Viaud M, Benito EP, Couloux A, Coutinho PM, De Vries RP, Dyer PS, Fillinger S, Fournier E, Gout L, Hahn M, Kohn L, Lapalu N, Plummer KM, Pradier J-M, Quévillon E, Sharon A, Simon A, Ten Have A, Tudzynski B, Wincker P, Andrew M, Anthouard V, Beever RE, Beffa R, Benoit I, Bouzid O, Brault B, Chen Z, Choquer M, Collémare J, Cotton P, Danchin EG, Da Silva C, Gautier A, Giraud C, Giraud T, Gonzalez C, Grossetete S, Güldener U, Henrissat B, Howlett B, Kodira C, Kretschmer M, Lappartient A, Leroch M, Levis C, Mauceli E, Neuvéglise C, Oeser B, Pearson M, Poulain J, Poussereau N, Quesneville H, Rascle C, Schumacher J, Ségurens B, Sexton A, Silva E, Sirven C, Soanes DM, Talbot NJ, Templeton M, Yandava C, Yarden O, Zeng Q, Rollins J, Lebrun M-H, Dickman M (2011) Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genet 7:e1002230

Andrade MJ, Córdoba JJ, Casado EM, Córdoba MG, Rodríguez M (2010) Effect of selected strains of *Debaryomyces hansenii* on the volatile compound production of dry fermented sausage "salchichón". Meat Sci 85:256–264

Ardi R, Kobiler I, Keen NT, Prusky D (1998) Involvement of epicatechin biosynthesis in the resistance of avocado fruits to postharvest decay. Physiol Mol Plant Pathol 53:269–285

Barbosa-Cànovas GV, Pothakamury U, Palou E, Swanson B (1998) High hydrostatic pressure food processing. In: Barbosa-Cànovas GV, Pothakamury U, Palou E, Swanson B (eds) Nonthermal presevation of foods. Marcel Dekker, New York, pp 9–52

Bayne HG, Michener HD (1979) Heat resistance of *Byssochlamys* ascospores. Appl Environ Microbiol 37:449–453

Bechinger C, Giebel K-F, Schnell M, Leiderer P, Deising HB, Bastneyer M (1999) Optical measurement of invasive forces exerted by appressoria of a plant pathogenic fungus. Science 285:1896–1899

Bekker M, Adan OA, Huinink H, Samson RA, Dijksterhuis J (2012) Production of an extra cellular matrix is an isotrophic growth phase in *Penicillium rubens* on gypsum. Appl Environ Microbiol 78:6930–6937

Beno-Moualem D, Prusky D (2000) Early events during quiescent infection development by *Colletotrichum gloeosporioides* in unripe avocado fruits. Pythopathol 90:553–559

Berbee ML, Yoshimura A, Sugiyama J, Taylor JW (1995) Is *Penicillium* monophyletic? An evaluation of phylogeny in the family *Trichocomaceae* from 18S, 5.8S and ITS ribosomal DNA sequence data. Mycologia 87:210–222

Beuchat LR (1986) Extraordinary heat resistance of *Talaromyces flavus* and *Neosartorya fischeri* ascospores in fruit products. J Food Sci 51:1506–1510

Beuchat LR (1988a) Thermal tolerance of *Talaromyces flavus* ascospores as affected by growth medium and temperature, age and sugar content in the inactivation medium. Trans Br Mycol Soc 90:359–374

Beuchat LR (1988b) Influence of organic acids on heat resistance characteristics of *Talaromyces flavus* ascospores. Int J Food Microbiol 6:97–105

Beuchat LR (1992) Survival of *Neosartorya fischeri* and *Talaromyces flavus* ascospores in fruit powders. Lett Appl Microbiol 14:238–240

Beuchat LR, Kuhn GD (1997) Thermal sensitivity of *Neosartorya fischeri* ascospores in regular and reduced-sugar grape jelly. J Food Prot 60:1577–1579

Bonnen A, Brambl R (1983) Germination physiology of *Neurospora crassa* conidia. Exp Mycol 7:197–207

Borner H (1963) Untersuchungen uber die Bildung antiphytotischer und antimicrobieller Substanzen durch Mikroorganism im Bodem und ihre mögliche Bedeutung für die Bodenmüdigkeit beim Apfels *Pirus malus* L. Phytopathol Z 49:1–28

Butz P, Funtenberger S, Haberditzl T, Tausher B (1996) High pressure inactivation of *Byssochlamys nivea* ascospores and other heat resistant moulds. LebensmWiss Technol 29:404–410

Cantu D, Vicente AR, Greve LC, Dewey FM, Bennett AB, Labavitch JM, Powell ALT (2008) The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*. Proc Natl Acad Sci USA 105:859–864

Casa E, De Ancos B, Valderrama MJ, Cano P, Peinado JM (2004) Pentadiene production from potassium sorbate by osmotolerant yeasts. Int J Food Microbiol 94:93–96

Casella MLA, Matasci F, Schmidt-Lorenz W (1990) Influence of age, growth medium, and temperature on heat resistance of *Byssochlamys nivea* ascospores. Lebensm Wiss Technol 23:404–411

Cheng L, Moghraby J, Piper PW (1999) Weak organic acid treatment causes a trehalose accumulation in low-pH cultures of *Saccharomyces cerevisiae*, not displayed by the more preservative-resistant *Zygosaccharomyces bailii*. FEMS Microbiol Lett 170:89–95

Chin JP, Megaw J, Magill CL, Nowotarski K, Williams JP, Bhaganna P, Linton M, Patterson MF, Underwood GJC, Mswaka AY, Hallsworth JE (2010) Solutes determine the temperature windows for microbial survival and growth. Proc Natl Acad Sci USA 107:7835–7840

Chitarra GS, Dijksterhuis J (2007) The germinating spore as a contaminating vehicle. In: Dijksterhuis J, Samson RA (eds) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, pp 83–100

Chitarra GS, Abee T, Rombouts FM, Posthumus MA, Dijksterhuis J (2004) Germination of *Penicillium paneum* conidia is regulated by a volatile self-inhibitor. Appl Environ Microbiol 70:2823–2829

Chitarra GS, Abee T, Rombouts FM, Dijksterhuis J (2005) 1-Octen-3-ol has mild effects on membrane permeability, respiration and intracellular pH, but blocks germination and changes the protein composition of *Penicillium paneum* conidia. FEMS Microbiol Ecol 54:67–75

Choquer M, Fournier E, Kunz C, Levis C, Pradier J-M, Simon A, Viaud M (2007) *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous –pathogen. FEMS Microbiol 277:1–10

Clipson NJW, Jennings DH (1992) *Dendryphiella salina* and *Debaryomyces hansenii:* models for ecophysical adaptation to salinity by fungi that grow in the sea. Can J Bot 70:2097–2105

Conner DE, Beuchat LR (1987) Efficacy of media for promoting ascospore formation by *Neosartory fischeri*, and the influence of age and culture temperature on heat resistance of ascospores. Food Microbiol 4:229–238

Conner DE, Beuchat LR, Chang CJ (1987) Age-related changes in ultrastructure and chemical composition associated with changes in heat resistance of *Neosartorya fischeri* ascospores. Trans Br Mycol Soc 89:539–550

Crowe JH, Crowe LM, Chapman D (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science 223:701–703

D' Enfert C, Fontaine T (1997) Molecular characterization of the *Aspergillus nidulans* treA gene encoding an acid trehalase required for growth on trehalose. Mol Microbiol 24:203–216

D' Enfert C, Bonini BM, Zapella PDA, Fontaine T, Da Silva AM, Terenzi HF (1999) Neutral trehalase catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. Mol Microbiol 32:471–483

Dao T, Dantigny P (2009) Preparation of fungal spores impacts their susceptibility to inactivation by ethanol vapours. Int J Food Microbiol 135:268–273

Dao T, Dejardin J, Bensoussan M, Dantigny P (2010) Use of the Weibull model to describe the inactivation of dry-harvested conidia of different *Penicillium* species by ethanol vapours. J Appl Microbiol 109:408–414

De Vries RP (2000) Accessory enzymes from *Aspergillus* involved in xylan and pectin degradation. Ph.D. thesis, Wageningen University, 192 pp

De Vries RP, Visser J (2001) Enzymes from *Aspergilli* involved in the degradation of plant cell wall polysaccharides. Microbiol Mol Biol Rev 65:497–522

d'Enfert C (1997) Fungal Spore Germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. Fungal Genet Biol 21:163–172

Dijksterhuis J (2007) Heat-resistant ascospores. In: Dijksterhuis J, Samson RA (eds) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, pp 101–117

Dijksterhuis J, Samson RA (2006a) Zygomycetes. In: de Blackburn CW (ed) Food spoilage microorganisms. Woodhead Publishing Ltd, Cambridge, pp 415–436

Dijksterhuis J, Samson RA (2006b) Activation of ascospores by novel food preservation techniques. Adv Exp Med Biol 571:247–260

Dijksterhuis J, Samson RA (2007) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, 403 pp

Dijksterhuis J, Teunissen PGM (2004) Dormant ascospores of *Talaromyces macrosporus* are activated to germinate after treatment with ultra high pressure. J Appl Microbiol 96:162–169

Dijksterhuis J, Van Driel K, Sanders M, Houbraken J, Molenaar D, Samson RA, Kets EPW (2002) Glucose efflux and cell ejection during germination of heat activated ascospores of *Talaromyces macrosporus*. Arch Microbiol 78:1–7

Dijksterhuis J, Nijsse J, Hoekstra FA, Golovina EA (2007) High viscosity and anisotropy characterize the cytoplasm of dormant stress-resistant spores. Eukaryot Cell 6:157–170

Dijksterhuis J, de Rodriquez MP, Da Silva P, Dantigny P (2012) Primary models for inactivation of fungal spores. In: Dantigny P, Panagou EZ (eds) Predictive mycology. Nova, New York

Eckert JW, Ratnayake M (1994) Role of volatile compounds from wounded oranges in induction of germination of *Penicillium digitatum* conidia. Phytopathology 84:746–750

Eshel D, Miyara I, Ailing T, Dinoor A, Prusky D (2002) pH regulates endoglucanase expression and virulence of *Alternaria alternata* in persimmon fruit. MPMI 15:774–779

Fillinger S, Chaveroce MK, Van Dijck P, De Vries RP, Ruijter G, Thevelein J, D'Enfert C (2001) Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. Microbiol 147:1851–1862

Flaishman MA, Kolattukudy PE (1994) Timing of fungal invasion using host's ripening hormones as a signal. Proc Natl Acad Sci USA 91:6579–6583

Flannigan B, Samson RA, Miller JD (2011) Microorganisms in home and indoor work environments. Diversity, health impacts, investigation and control, 2nd edn. Taylor and Francis, Boca Raton, 539 pp

Fontaine T, Beauvais A, Loussert C, Thevenard B, Fulgsang CC, Ohno N, Clavaud C, Prevost M-C, Latgé JP (2010) Cell wall α1-3glucans induce the aggregation of germinating conidia of *Aspergillus fumigatus*. Fung Genet Biol 47:707–712

Frisvad JC, Filtenborg O (1988) Specific mycotoxin producing *Penicillium* and *Aspergillus* mycoflora of different foods. Proc Jpn Assoc Mycotoxicol Suppl 1:163–166

Frisvad JC, Filtenborg O (1993) Mycotoxin production by *Penicillium* and *Aspergillus* species associated with different foods and other substrates. In: Scudamore K (ed) Occurrence and significance of mycotoxins. Central Science Laboratory, Slough, pp 138–145

Frisvad JC, Thrane U, Samson RA (2007a) Mycotoxin producers. In: Dijksterhuis J, Samson RA (eds) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, pp 135–159

Frisvad JC, Andersen B, Samson RA (2007b) Association of moulds to foods. In: Dijksterhuis J, Samson RA (eds) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, pp 199–239

Gomez MM, Busta FF, Pflug IJ (1989) Effect of the post-dry heat treatment temperature on the recovery of ascospores of *Neosartorya fischeri*. Lett Appl Microbiol 8:59–62

Gomez MM, Pflug IJ, Busta FF (1993) Factors affecting recovery of *Neosartorya fischeri* after exposure to dry heat. J Par Sci Technol 47:300–305

Gonzlez-Hernndez JC, Jiménez-Estrada M, Peña A (2005) Comparative analysis of trehalose production by *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under saline stress. Extremophiles 9:7–16

Gourgues M, Brunet-Simon A, Lebrun M-H, Levis C (2004) The tetraspanin BcPls1 is required for appressorium-mediated penetration of *Botrytis cinerea* into host plant leaves. Mol Microbiol 51:619–629

Guetsky R, Kobiler I, Wang X, Perlman N, Gollop N, Avila-Quezada G, Hadar I, Prusky D (2005) Metabolism of the flavonoid epicatechin by laccase of *Colletotrichum gloeosporioides* and its effect on pathogenicity on avocado fruits. Pythopathol 95:1341–1348

Gunde-Cimerman N, Ramos J, Plemenitas A (2009) Halotolerant and halophilic fungi. Mycol Res 13:1231–1241

Hadas Y, Goldberg I, Pines O, Prusky D (2007) Involvement of gluconic acid and glucose oxidase in the pathogenicity of *Penicillium expansum* in apples. Phytopathol 97:384–390

Hallsworth JE, Magan N (1994) Effects of KCl concentrations on accumulation of acyclic sugar alcohols and trehalose in conidia of three entomopathogenic fungi. Lett Appl Microbiol 18:8–11

Hallsworth JE, Magan N (1996) Culture age, temperature, and pH affect the polyol and trehalose contents of fungal propagules. Appl Environ Microbiol 62:2435–2442

Hegedus DD, Rimmer SR (2005) *Sclerotinia sclerotiorum*: when "to be or not to be" a pathogen? FEMS Microbiol Lett 251:177–184

Herrero-Garcia E, Garzia A, Cordobes S, Espeso EA, Ugalde U (2011) 8-Carbon oxylipins inhibit germination and growth, and stimulate aerial conidiation in *Aspergillus nidulans*. Fung Biol 115:393–400

Hoogerwerf SA, Kets EPW, Dijksterhuis J (2002) High-oxigen and high-carbon dioxide containing atmospheres inhibit growth of food-associated moulds. Let Appl Microbiol 35:419–422

Horikoshi K, Ikeda Y (1966) Trehalase in conidia of *Aspergillus oryzae*. J Bacteriol 91:1883–1887

Hosono K (2000) Effect of nystatin on the release of glycerol from salt-stressed cells of the salt-tolerant yeast *Zygosaccharomyces rouxii*. Arch Microbiol 173:284–287

Hottiger T, DeVirgilio C, Hall MN, Boller T, Wiemken A (1994) The role of trehalose for the acquisition of thermotolerance in yeast. II. Physiological concentrations of trehalose increase the stability of proteins in vivo. Eur J Biochem 219:187–193

Houbraken JAMP, Varga J, Rico-Munoz E, Johnson S, Samson RA (2008) Sexual reproduction as the cause of heat resistance in the food spoilage fungus *Byssochlamys spectabilis* (Anamorph *Paecilomyces variotii*). Appl Environ Microbiol 74:1613–1619

Iwaki T, Higashida Y, Tsuji T, Tamai Y, Watanabe Y (1998) Characterization of a second gene (ZSOD22) of Na^+/H^+ antiporter from salt-tolerant yeast *Zygosaccharomyces rouxii* and functional expression of ZSOD2 and ZSOD22 in *Saccharomyces cerevisiae*. Yeast 14:1167–1174

Jennings DH, Burke RM (1990) Compatible solutes – the mycological dimension and their role as physiological buffering agents. New Phytol 116:277–283

Karlshøj K, Nielsen PV, Larsen TO (2007) Fungal volatiles: biomarkers of good and bad food quality. In: Dijksterhuis J, Samson RA (eds) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, pp 279–302

Kikoku Y (2003) Heat activation characteristics of *Talaromyces ascospores*. J Food Sci 68:2331–2335

Kim YK, Li D, Kolattukudy PA (1998) Induction of Ca^{2+}calmodulin signalling by hard-surface contact primes *Colletotrichum gloeosporioides* conidia to germinate and form appressoria. J Bacteriol 180:5144–5150

Kim YT, Prusky D, Rollins JA (2007) An activating mutation of the *Sclerotinia sclerotiorum pac*1 gene increases oxalic acid production at low pH but decreases virulence. Mol Plant Pathol 8:611–622

King AD, Whitehand LC (1990) Alteration of *Talaromyces flavus* heat resistance by growth conditions and heating medium composition. J Food Sci 55:830–832

Kolattukudy PA, Rogers LM, Li D, Hwang CS, Flaishman MA (1995) Surface signalling in pathogenesis. Proc Natl Acad Sci USA 92:4080–4087

Kotzekidou P (1997) Heat resistance of *Byssochlamys nivea*, *Byssochlamys fulva* and *Neosartorya fischeri* isolated from canned tomato paste. J Food Sci 62:410–412

Lages F, Silva-Graça LC (1999) Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. Microbiol 145:2577–2585

Leandro MJ, Sychrová H, Prista C, Loureiro-Dias MC (2011) The osmotolerant fructophilic yeast *Zygosaccharomyces rouxii* employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters. Microbiology 157:601–608

Leong SL, Vinnere-Pettersson RT, Hocking AD, Schnürer J (2011) The extreme xerophilic mould *Xeromyces bisporus* – growth and competition at various water activities. Int J Food Microbiol 145:57–63

Leyva JS, Manrique M, Prats L, Loureiro-Dias MC, Peiñado JM (1999) Regulation of fermentative CO_2 production by the food spoilage yeast *Zygosaccharomyces bailii*. Enzyme Microbiol Technol 24:270–275

Lucas C, Da Costa M, Van Uden N (1990) Osmoregulatory active sodium-glycerol co-transport in the halotolerant yeast *Debaryomyces hansenii*. Yeast 6:187–191

Luxo C, Fernanda Nobre M, Da Costa MS (1993) Intracellular polyol accumulation by yeast-like fungi of the genera *Geotrichum* and *Endomyces* in response to water stress (NaCl). Can J Microbiol 39:868–873

Magan N, Aldred D, Dijksterhuis J (2012) The physiological state of fungal spores and survival dynamics. In: Dantigny P, Panagou EZ (eds) Predictive mycology. Nova, New York

Mann DA, Beuchat LR (2008) Combinations of antimycotics to inhibit the growth of molds capable of producing 1,3-pentadiene. Food Microbiol 25:144–153

Martínez JL, Sychrova H, Ramos J (2011) Monovalent cations regulate expression and activity of the Hak1 potassium transporter in *Debaryomyces hansenii*. Fungal Gen Biol 48:177–184

Martorell P, Stratford M, Steels H, Fernndez-Espinar MT, Querol A (2007) Physiological characterization of spoilage strains of *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* isolated from high sugar environments. Int J Food Microbiol 114:234–242

Mehyar GF, Al-Qadiri HM, Abu-Blan HA, Swanson BG (2011) Antifungal effectiveness of potassium sorbate incorporated in edible coatings against spoilage molds of apples, cucumbers, and tomatoes during refrigerated storage. J Food Sci 76:210–217

Membre JM, Kubaczka M, Chene C (1999) Combined effects of pH and sugar on growth rate of *Zygosaccharomyces rouxii*, a bakery product spoilage yeast. Appl Environ Microbiol 65:4921–4925

Miyara I, Shafran H, Kramer Haimovich H, Rollings J, Sherman A, Prusky D (2008) Multi-factor regulation of pectate lyase secretion by *Colletotrichum gloeosporioides* pathogenic on avocado fruit. Mol Pathol 9:281–291

Morozova EV, Kozlov VP, Tereshina VM, Memorskaya AS, Feofilova EP (2002) Changes in lipid composition and carbohydrate composition of *Aspergillus niger* conidia during germination. Prikl Biokhim Mikrobiol 38:149–154

Münch S, Lingner U, Floss DS, Ludwig N, Sauer N, Deising H (2008) The hemibiotrophic lifestyle of *Colletotrichum* species. J Plant Physiol 165:41–51

Nagtzaam MPM, Bollen GJ (1994) Long shelf life of *Talaromyces flavus* in coating material of pelleted seed. Eur J Plant Pathol 100:279–282

Niem J, Miyara I, Ettedgui Y, Reuveni M, Flaishman M, Prusky D (2007) Core rot development in red delicious apples is affected by susceptibility of the seed locule to *Alternaria alternata* colonization. Phytopathol 97:1415–1421

Osherov N, May G (2000) Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. Genetics 155:647–656

Padamsee M, Arun Kumar TK, Riley R, Binder M, Boyd A, Calvo AM et al (2012) The genome of the xerotolerant mould *Wallemia sebi* reveals adaptations to osmotic stress and suggests cryptic sexual reproduction. Fungal Gen Biol 49:217–226

Palou E, Lopez-Malo A, Barbosa-Canovas GV, Welti-Chanes J, Davidson PM, Swanson BG (1998) Effect of oscillatory high hydrostatic pressure treatments on *Byssochlamys nivea* ascospores suspended in fruit juice concentrates. Lett Appl Microbiol 27:375–378

Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van Dijk A, Dijkhuizen L, Driessen AJ, D'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CAMJJ, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pl K, van Peij NNME, Ram AFJ, Rinas U, Roubos JA, Sagt CMJ, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wösten HAB, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nature Biotechnol 25:221–231

Petersson S, Jonsson N, Schnürer J (1999) *Pichia anomala* as a biocontrol agent during storage of high-moisture feed grain under airtight conditions. Postharvest Biol Technol 15:175–184

Petrovič U, Gunde-Zimmerman N, Zalar P (2000) Xerotolerant mycobiota from high altitude Annapurna soil, Nepal. FEMS Microbiol Let 182:339–342

Pinches SE, Apps P (2007) Production in food of 1,3-pentadiene and styrene by *Trichoderma* species. Int J Food Sci 116:182–185

Piper P, Ortiz Calderon C, Hatzixanthis K, Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiol 147:2635–2642

Pitt JI, Hocking AD (2009) Fungi and food spoilage, 3rd edn. Springer, Dordrecht/Heidelberg, 519 pp

Plumridge A, Hesse SJA, Watson AJ, Lowe KC, Stratford M, Archer DB (2004) The weak-acid preservative, sorbic acid, inhibits conidial germination and mycelial growth of *Aspergillus niger* through intracellular acidification. Appl Environ Microbiol 70:3506–3511

Plumridge A, Stratford M, Lowe KC, Archer DB (2008) The weak-acid preservative, sorbic acid, is decarboxylated and detoxified by a phenylacrylic acid decarboxylase, PadA1, in the spoilage mould *Aspergillus niger*. Appl Environ Microbiol 74:550–552

Plumridge A, Melin P, Stratford M, Novodvorska M, Shunburne L, Dyer PS, Roubos JA, Menke H, Stark J, Stam H, Archer DB (2010) The decarboxylation of the weak-acid preservative, sorbic acid, is encoded by linked genes in *Aspergillus* spp. Fung Genet Biol 47:683–692

Podila GK, Rogers LM, Kolattukudy PA (1993) Chemical signals from avocado wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. Plant Physiol 103:267–272

Přibylova L, Farkas V, Slaninova I, De Montigny J, Sychrova H (2007) Differences in osmotolerant and cell-wall properties of two *Zygosaccharomyces rouxii* strains. Folia Microbiol 52:241–245

Pribylova L, Papouskova K, Sychrova H (2008) The salt tolerant yeast *Zygosaccharomyces rouxii* possesses two plasma-membrane Na^+/H^+ -antiporters (*Zr*Nha1p and *Zr*Sod2-22p) playing different roles in cation homeostasis and cell physiology. Fung Genet Biol 45:1439–1447

Prista C, Almagro A, Loureiro-Dias MC, Ramos J (1997) Physiological basis for the high salt tolerance of *Debaryomyces hansenii*. Appl Environ Microbiol 63:4005–4009

Prista C, Loureiro-Dias C, Montiel V, García R, Ramos J (2005) Mechanisms underlying the halotolerant way of *Debaryomyces hansenii*. FEMS Yeast Res 5:693–701

Prusky D, Kolattukudy PE (2007) Cross-talk between host and fungus in postharvest situations and its effect on symptom development. In: Dijksterhuis J, Samson RA (eds) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, pp 3–25

Prusky D, Lichter A (2007) Activation of quiescent infections by postharvest pathogens during transition from the biotrophic to the necrotrophic stage. FEMS Microbiol Lett 268:1–8

Prusky D, McEvoy JL, Saftner R, Conway WS, Jones R (2004) Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. Phytopathol 94:44–51

Put HMC, De Jong J (1980) The heat resistance of selected yeasts causing spoilage of canned soft drinks and fruit products. In: Skinner FA, Passmore SM, Davenport RR (eds) Biology and activities of yeasts, vol 9, Society for applied bacteriology symposium series. Academic, London

Rajashekhara E, Suresh ER, Ethiraj S (1998) Thermal death rate of ascospores of *Neosartorya fischeri* ATCC 200957 in the presence of organic acids

and preservatives in fruit juices. J Food Prot 61:1358–1362

Reyns KMFA, Verbeke EAV, Michiels CEW (2003) Activation and inactivation of *Talaromyces macrosporus* ascospores by high hydrostatic pressure. J Food Prot 66:1035–1042

Rodriguez F, Côrte-Real M, Leão C, Van Dijken JP, Pronk JT (2001) Oxygen requirements of the food spoilage yeast *Zygosaccharomyces bailii* in synthetic and complex media. Appl Environ Microbiol 67:2123–2128

Sakamoto K, Iwashita K, Yamada O, Kobayashi K, Mizuno A, Akita O, Mikami S, Shimoi H, Gomi K (2009) *Aspergillus oryzae* atfA controls conidial germination and stress tolerance. Fung Genet Biol 46:887–897

Samson RA, Frisvad JC (2004) Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud Mycol 49:1–174

Samson RA, Houbraken JAMP (eds) (2011) Phylogenetic and taxonomic studies on the genera *Penicillium* and *Talaromyces*. Stud Mycol 70, 183 pp

Samson RA, Varga J (2007) *Aspergillus* systematic in the genomic era. Stud Mycol 59:1–206

Samson RA, Hoekstra ES, Frisvad JC, Filtenborg O (2004) Introduction to food- and airborne fungi, 7th edn. Centraalbureau voor Schimmelcultures, Utrecht, 389 pp

Smelt JPPM (1998) Recent advances in the microbiology of high pressure processing. Trends Food Sci Technol 9:152–158

Smid EJ, Hendriks L, Boerrigter HAM, Gorris LGM (1996) Surface disinfection of tomatoes using the natural plant compound trans-cinnamaldehyde. Postharvest BioI Technol 9:343–350

Snowdon AL (1990) A colour atlas of post-harvest diseases and disorders of fruits and vegetables, vol 1, General introduction and fruits. Wolfe Scientific Ltd, London

Splittstoesser DF, Splittstoesser CM (1977) Ascospores of *Byssochlamys fulva* compared with those of a heat resistant *Aspergillus*. J Food Sci 4:685–688

Splittstoesser DF, Lammers JM, Downing DL, Churey JJ (1989) Heat resistance of *Eurotium herbariorum,* a xerophilic mold. J Food Sci 54:683–685

Stark J (2007) Cheese and fermented sausages. In: Dijksterhuis J, Samson RA (eds) Food mycology: a multifaceted approach to fungi and food. Taylor and Francis, Boca Raton, pp 319–331

Steels H, James SA, Roberts IN, Stratford M (2000) Sorbic acid resistance: the inoculum effect. Yeast 16:1173–1183

Stratford M, Plumridge A, Pleasants MW, Novodvorska M, Baker-Glenn CAG, Pattenden G, Archer DB (2012) Mapping the structural requirements of inducers and substrates for decarboxylation of weak acid preservatives by the food spoilage mould *Aspergillus niger*. Int J Food Microbiol 157:375–383

Sussman S, Halvorson HO (1966) Spores, their dormancy and germination. Harper and Row, New York

Te Welscher YM, ten Napel HH, Balagué MM, Souza CM, Riezman H, de Kruijff B, Breukink E (2008) Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. J Biol Chem 283:6393–6401

Te Welscher YM, Jones L, Van Leeuwen MR, Dijksterhuis J, De Kruijff B, Eitzen G, Breukink EJ (2010) Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. Antimicrob Agents Chemother 54:2618–2625

Te Welscher YM, Van Leeuwen MR, De Kruijff B, Dijksterhuis J, Breukink EJ (2012) Polyene antibiotic that inhibits membrane transport proteins. Proc Natl Acad Sci USA 109:11156–11159

Thanh NV, Nout MJ (2004) Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores. Int J Food Microbiol 92:171–179

Thanh NV, Rombouts FM, Nout MJ (2005) Effect of individual amino acids and glucose on activation and germination of *Rhizopus oligosporus* sporangiospores in tempe starter. J Appl Microbiol 99:1204–1214

Thomma BPHJ (2003) *Alternaria* spp.: from general saprophyte to specific parasite. Mol Plant Pathol 4:225–236

Tiedt LR (1993) An electron microscope study of conidiogenesis and wall formation of conidia of *Aspergillus niger*. Mycolog Res 12:1459–1462

Tournas V (1994) Heat-resistant fungi of importance to the food and beverage industry. Crit Rev Microbiol 20:243–263

Valencia-Chamorro SA, Pérez-Gago MB, Del Río MA, Palou L (2010) Effect of antifungal hydroxypropyl methylcellulose-lipid edible composite coatings on *Penicillium* decay development and postharvest quality of cold-stored "Ortanique" mandarins. J Food Sci 75:418–426

Van Kan JAL (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci 11:247–253

Van Leeuwen MR, De Boer W, Smant W, Dijksterhuis J (2008) Filipin is a reliable in situ marker of ergosterol in the plasma membrane of germinating conidia (spores) of *Penicillium discolor* and stains intensively at the site of germ tube formation. J Microbiol Methods 74:64–73

Van Leeuwen MR, Golovina EA, Dijksterhuis J (2009) The polyene antimycotics nystatin and filipin disrupt the plasma membrane, whereas natamycin inhibits endocytosis in germinating conidia of *Penicillium discolor*. J Appl Microbiol 106:1908–1918

Van Leeuwen MR, Van Doorn T, Golovina EA, Stark J, Dijksterhuis J (2010) Water- and air-distributed conidia exhibit differences in sterol content and

cytoplasmic microviscosity. Appl Environ Microbiol 67:366–369

Van Leeuwen MR, Krijgsheld P, Bleichrodt RJ, Menke H, Stam H, Stark J, Wösten HAB, Dijksterhuis J (2013a) Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. Stud Mycol 74:59–70

Van Leeuwen MR, Wyatt TT, Golovina EA, Stam H, Menke H, Dekker A, Stark J, Wösten HAB, Dijksterhuis J (2013b) Germinating conidia, transcriptomes and antifungals. Stud Mycol 74: 71–85

Vinnere-Pettersson O, Leong SL, Lantz H, Rice T, Dijksterhuis J, Houbraken J, Samson RA, Schnürer J (2011) Phylogeny and intraspecific variation of the extreme xerophile, *Xeromyces bisporus*. Fungal Biol 115:1100–1111

Wang X, Beno-Moualem D, Kobiler I, Leikin-Frenkel A, Lichter A, Prusky D (2004) Expression of Δ^{12} fatty acid desaturase during the induced accumulation of the antifungal dienen in avocado fruits. Mol Plant Pathol 5:575–585

Watanabe Y, Yamaguchi M, Sanemitsu Y, Tarnai Y (1991) Characterisation of plasma membrane protonATPase from salt-tolerant yeast *Zygosaccharomyces rouxii* cells. Yeast 7:599–606

Watanabe Y, Miwa S, Tarnai Y (1995) Characterization of Na^+/H(+)-antiporter gene closely related salt-tolerance of yeast *Zygosaccharomyces rouxii*. Yeast 11:829–838

Wiemken A (1990) Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie van Leeuwenhoek 68:209–217

Wildman JD, Clark PB (1947) Some examples of the occurrence of machinery slime in caning factories. J Ass Off Anal Chem 30:582–585

Williams JP, Hallsworth JE (2009) Limits of life in hostile environments: no barriers to biosphere function? Environ Microbiol 11:3292–3308

Williams B, Kabbage M, Kim H-J, Britt R, Dickman MB (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defences by manipulating the host redox environment. PLoS Pathog 7:e1002107

Fungal Secondary Metabolites and Detoxification

3 Genetics, Biosynthesis, and Regulation of Aflatoxins and other *Aspergillus flavus* Secondary Metabolites

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CONTENTS

I. Introduction

In England in the 1960s, thousands of poultry were killed by a mysterious disease called Turkey X disease. After an investigation, peanut feed contaminated with aflatoxins (AFs) was deemed the causal agent (Forgacs and Carll 1962). Since then, AF B1 has been reported as the most potent natural carcinogen known (Squire 1981). **AFs and their penultimate precursor sterigmatocystin (ST), which has biological activities similar to AF B1, are produced by filamentous ascomycete fungi, primarily species in the genus *Aspergillus*.** At least twelve *Aspergillus* species, including the two plant pathogens *A. flavus* and *A. parasiticus,* have been reported as AF producers, while a number of Aspergilli produce ST (Perrone et al. 2009; Cole and Cox 1981; Rank et al. 2011). Other genera capable of producing AFs, ST, and related compounds include *Chaetomium, Botryotrichum, Podospora* and *Humicola* (Rank et al. 2011; Slot and Rokas 2011). These AF-producing fungi are capable of producing at least 14 different chemical types of AF. The most prevalent AFs are AFs B1 and B2, produced by both *A. flavus* and *A. parasiticus*. *A. parasiticus* is also able to produce AFs G1 and G2. B and G AFs were designated as such because of their fluorescent colors, blue (B) and green (G), under UV light (Sweeney and Dobson 1999). Other AFs, M1 and M2, were first isolated from milk of cows that had eaten contaminated feed, and are hydroxylated derivatives of AF B1 and B2 respectively (Yu et al. 2004a).

AFs are the causal agents of a group of disease symptoms collectively called aflatoxicoses, which result from inhaling or ingesting high levels of AF-contaminated food or feed. Acute toxicity is associated with both animal and human deaths, as has been seen among Kenyan populations in the last few years (Lewis et al. 2005; Yu et al. 2007). AF B1 is also a potent hepatocarcinogen, and induces tumors in human and animals. Hepatocellular carcinoma (HCC), or liver cancer, is associated with AF B1 consumption in Asian and African countries. Geographical regions of high AF exposure often overlap with high incidences of chronic hepatitis virus B (HBV) infections, and the two factors associate synergistically to increase HCC (Cardwell and Henry 2004; Liu and Wu 2010; Wang and Tang 2005). A tumor

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suppressor protein, p53, has been implicated in HCC. AF B1 epoxides and AF B1-exo-epoxides intercalate between the bases of DNA and bind at codon 249 in the p53 gene, resulting in a mutation (AGG to AGT, R249S) often found in HCC patients (Ozturk 1991). This mutation is considered the signature of AF exposure, and is present in up to 75 % of HCC patients in Asia and Africa (Gouas et al. 2009).

The problems of AF contamination have been reported not only in developing countries, but also in the United States. The AF producer *A. flavus* has been isolated from a wide range of climate zones, especially between latitudes 16° and 35° in warm climate zones, and it is commonly found in the southeastern parts of the United States (Klich 2007). AF-contaminated food and feed cause annual yield losses in the million-dollar range in the United States (Rubens and Cardwell 2005). Contaminated maize in commercial dog foods in the United States caused lethal aflatoxicosis outbreaks among dogs in 2005–2006 (Dereszynski et al. 2008), and recalls are frequent (http://efoodalert.net/2011/12/28/fda-aflatoxin-and-pet-food-recalls/). Allowable AF levels—typically set for AF B1—according to federal regulations vary worldwide, which makes it difficult to trade and regulate AF-contaminated food and feed. The Food and Drug Administration (FDA) of the United States sets the amount of allowable AF contamination at 20 ppb in crops, and 0.5 ppb in milk for humans. The AF concentrations allowed for animal consumption are slightly higher (Klich 2007; Payne and Yu 2010). However, peanuts containing more than 2 ppb of AF B1 or 4 ppb of total AFs are rejected in Europe (Van Egmond and Jonker 2005).

Because of the dangers that AFs pose to human and animal health worldwide, methods of controlling AF contamination and AF-producing fungi are greatly needed. **Elucidating how AF/ST are synthesized and regulated is considered a key goal for development of control measures.** In the half-century since its initial discovery, AF biosynthesis has become one of the most well-studied fungal secondary metabolite pathways. AF intermediate metabolites and the biosynthetic pathway have been identified over the last couple of decades. Moreover, recent molecular techniques and genome sequencing projects have revealed new insights into AF/ST production and their regulation in the Aspergilli since our previous Mycota chapter (Hicks et al. 2002). Here we will discuss these new insights, based on recent genetic and biochemical findings.

II. Aflatoxin and Sterigmatocystin Genetics and Biosynthesis

Genome sequence, EST, and bioinformatic data show that the genome size of the Aspergilli ranges from 30 to 35 Mb, with approximately 10,000–12,000 functional genes (Cleveland et al 2009; Galagan et al. 2005; Nierman et al. 2005; Pel et al. 2007; Yu et al. 2004b). One main focus of the *Aspergillus* genome-sequencing projects is to identify and characterize the genes necessary for fungal physiology/development, secondary metabolism, and/or pathogenesis. **Secondary metabolites, such as AF and ST, are produced by clusters of biosynthetic and regulatory genes, including backbone genes encoding hallmark enzymatic functions (e.g., polyketide synthase, PKS, for AF and ST) or transcriptional factors, such as AflR, which regulate the expression of the enzymatic genes** (Hoffmeister and Keller 2007, Fig. 3.1 and Table 3.1). The names of the AF genes have been changed several times, with not all authors picking up the newer preferred '*afl*' acronym, and the reader is cautioned to keep this in mind when reading AF literature and to refer to Table 3.1 for clarity.

The AF and ST biosynthetic gene clusters span about 70 kb and contain ca. 25–30 genes. AF/ST biosynthesis initially begins with two fatty acid synthase (FAS) genes, *aflA* (*fas2*, *stcJ*) and *aflB* (*fas1*, *stcK*), which encode the alpha and beta subunits of FAS respectively. The PKS is encoded by *aflC* (*pksA*, *stcA*). Together, the PKS and two FAS form a complex called norsolorinic acid synthase that results in the production of norsolorinic acid (NOR) (Watanabe and Townsend 2002). NOR is the first stable intermediate of AF/ST biosynthesis (Bennett et al. 1997). NOR is further metabolized by up to 23 enzymatic genes, resulting in

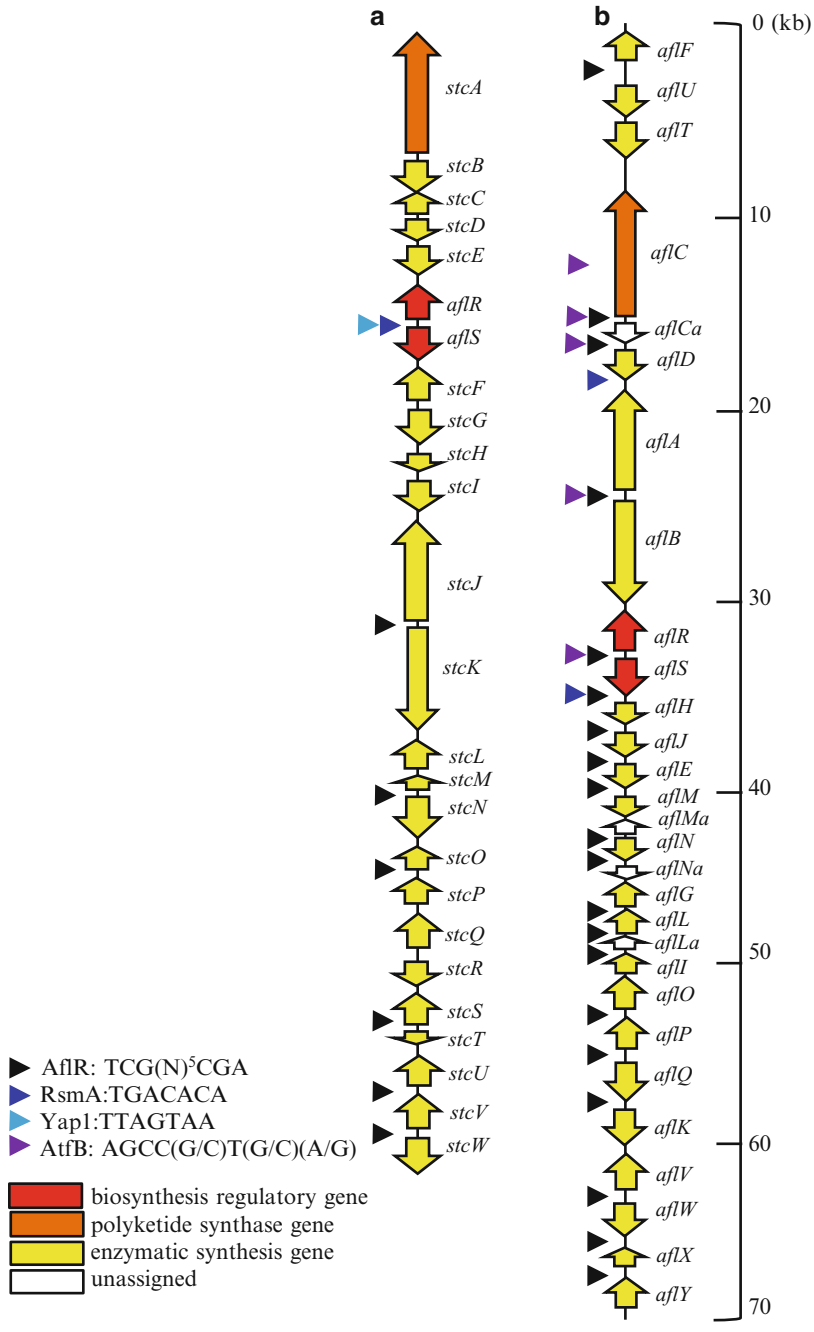

Fig. 3.1. AF and ST Cluster Gene Localization and Transcription Factor Binding Sites. Localization of 29 genes of the aflatoxin cluster gene, categorized into four different groups: polyketide synthase (PKS) gene, regulatory genes, enzymatic synthetic genes, and unassigned genes, which are described as newly assigned genes in the text. Data are based on Aspergillus flavus JCVI-afl1-v.2.0 Jan. 2009 data, available at www.aspergillusflavus.org. Arrows indicate the binding sites of transcription factors AflR, RsmA (RsmA and Yap1), AtfB, and MeaB (Yap1)

Table 3.1. AF and ST Cluster Genes and Biosynthetic Roles. The aflatoxin cluster consists of 29 different genes, located in secondary metabolite cluster #54 in the Aspergillus flavus genome. Data are based on Aspergillus flavus JCVI-afl1-v.2.0 Jan. 2009 data, available at www.aspergillusflavus.org.

AF			
New	Old	ST	Biosynthetic roles
aflF	norB	Not present	Dehydrogenase
aflU	cypA	AN9313/AN5360	P450 monooxygenase
aflT	aflT	Not present	Transmembrane protein/potential toxin transporter
aflC	pksA, pksL1	stcA	Polyketide synthase (PKS)
aflCa	hypC	stcM	Monooxygenase
aflD	nor-1	stcE	Norsolorinic acid (NOR) reductase
aflA	fas-2, hexA	stcJ	Fatty acid synthase (FAS) alpha-subunit
aflB	fas-1	stcK	Fatty acid synthase (FAS) beta-subunit
aflR	aflR, apa-2, afl-2	aflR	Transcriptional activator
aflS	aflJ	aflJ/aflS	Transcriptional enhancer/pathway regulator
aflH	adhA	stcG	Short-chain alcohol dehydrogenase
aflJ	estA	stcI	Esterase
aflE	norA, aad, adh-2	stcV	NOR reductase/dehydrogenase
aflM	ver-1	stcU	Dehydrogenase/ketoreductase
aflMa	hypE	Not present	Hypothetical protein
aflN	verA	stcS	Monooxygenase
aflNa	hypD	AN7822	Hypothetical protein
aflG	avnA, ord-1	stcF	Cytochrome P450 monooxygenase
aflL	verB	stcL	Desaturase/P450 monooxygnase
afLa	hypB	stcM	Hypothetical protein
aflI	avfA	stcO	Oxidase/cytochtome P450 monooxygenase
aflO	omtB, dmtA	stcP	O-methyl transferase B
aflP	omtA, omt-1	Not present	O-methyl transferase A
aflQ	ordA	AN1601	Oxidoreductase/cytochrome P450 monooxygenase
aflK	vbs	stcN	Versicolorin B synthase
aflV	cypX	stcB	Cytochtome P450 monooxygenase
aflW	moxY	stcW	Monooxygenase
aflX	ordB	stcQ	Monooxygenase/oxidase
aflY	hypA, hypP	stcR	Hypothetical protein

the synthesis of at least 15 different AF intermediate products, including ST. These steps have been described in great detail (Hicks et al. 2002; McDonald et al. 2005; Yu et al. 2004b, 2007) (Table 3.1), and for this review we will just focus on newly discovered enzymatic or putative enzymatic steps in this complex pathway.

A. Newly Assigned Genes and Their Biosynthetic Roles

The original AF and ST gene clusters were manually sequenced, and genes assigned with the technologies of that time frame (Brown et al. 1996; Trail et al. 1995; Yu et al. 1995). **In the nearly 20 years since, re-sequencing, bioinformatic examinations, EST, and microarray analyses have resulted in the identification and reassignment of new genes associated with either the AF or ST clusters.** In the ST cluster, the assigned putative *stcX* gene (Brown et al. 1996) has been discarded as a pseudogene and *aflJ* (*aflS*, Table 3.1), originally not reported in the ST cluster, has now been acknowledged (Yin et al. 2012).

Genome sequence analysis revealed an additional gene in the AF cluster, *aflY* (*hypA, stcR*) (Yu et al. 2004a). Disruption of *aflY* in *A. parasiticus* resulted in the accumulation of the ST precursor, versicolorin A (VA), and this was

also true for *A. nidulans* (Maggio Hall and Keller, unpublished). VA is converted to ST in a multi-step process, and the study suggested that *aflY* encodes an enzyme that catalyzes the Baeyer–Villiger oxidation of a dienone intermediate. This forms a xanthone ring that is required for dimethyl-ST production (Ehrlich et al. 2005). *aflX* has also been found to be involved in the VA-to-ST conversion. It encodes an oxidoreductase, though its exact mechanism is unknown (Ehrlich et al. 2005).

In addition to the *A. flavus* genome sequencing project, **sequencing of *A. flavus* expressed sequence tags (EST) revealed three small gene transcripts, intergenically located in the AF cluster, that had not been previously identified.** These genes were originally called *hypB*, *hypC*, and *hypD*, but preferably should be referred to as *aflLa*, *aflCa*, and *aflNa* respectively based on location and nomenclature; however, the *hyp* names persist so we will use them concurrently (Yu et al. 2004b; Cleveland et al. 2009).

hypB/aflLa and *hypC/aflCa* share high identity and are putative orthologs of the ST cluster gene, *stcM*. Enzymatic analyses have revealed that *hypC/aflCa* encodes a monooxygenase that can convert norsolorinic acid anthrone to norsolorinic acid (NOR). HypB/AflLa, on the other hand, is unable to catalyze this reaction. This may be due to the absence of a key tryptophan residue that is present in the HypC/AflCa predicted catalytic motif. Interestingly, an *A. parasiticus* Δ*hypC/aflCa* mutant still produces NOR and AF, suggesting that non-enzymatic oxidation is sufficient to continue AF biosynthesis (Ehrlich et al. 2010). Deletion of *stcM* in *A. nidulans* lowered the amount of ST produced (Maggio-Hall and Keller, unpublished).

Conversion of O-methylsterigmatocystin (OMST) to AF requires at least three oxidative steps, and currently the only enzyme shown to be involved is AflQ (OrdA, Udwary et al. 2002). Ehrlich (2009) modified a scheme for how this may be occurring, to include several other enzymes. HypB/AflLa was predicted to catalyze the second oxidation, following oxidation by AflQ. HypE (AflMa) and AflE were also predicted to catalyze steps in this process (Ehrlich 2009). Similar to the other *hyp* genes, *hypE* (*aflMa*) was found in the intergenic region of the AF cluster in *A. flavus* (Holmes 2008). Disruption of *hypE/aflMa* led to a decrease in AF production and the accumulation of an unknown metabolite. This metabolite was absent in a strain overexpressing *hypE* (Holmes 2008). Based on mass spectrometry data, this metabolite is predicted to be a 328 Da intermediate that could be converted to AF B1 by the combined action of HypE and any of the AF cluster's five cytochrome P450 monooxygenases (Ehrlich 2009).

The role of *hypD/aflNa* in AF and ST biosynthetic pathways remains unclear. *hypD* is predicted to encode an integral membrane protein containing a DUF6 domain that is highly conserved in other fungi. Because no aflatoxin transporter has been identified, and transporters are integral membrane proteins, it has been suggested that HypD/AflNa might be involved in AF efflux. Disruption of *hypD* in *A. parasiticus* decreased AF production and increased conidia production, relative to the wildtype. This may be due to regulatory feedback that shuts off AF production and increases conidiation in the absence of *hypD/aflNa*, which is possible given the tight relationship between development and secondary metabolism (Ehrlich 2009; Bayrum et al. 2008; Brown et al. 2009). BLAST analysis now indicates that all *hyp* genes except for *hypE/aflMa* are also present in the ST cluster of *A. nidulans* (Table 3.1) and analogous clusters in other fungi.

B. Other Secondary Metabolites

In contrast to the extensive research on AF, relatively little is known about the biosynthesis of other secondary metabolites in *A. flavus*, with a few exceptions. To date, *A. flavus* has been reported to produce at least fourteen different secondary metabolites, ten of which are made by the AF biosynthesis pathway. However, **the *A. flavus* genome is predicted to contain at least 55 secondary metabolite clusters** (Khaldi et al. 2010). Unlike AF and ST, many secondary metabolite clusters are not expressed in typical lab conditions, which can make the identification of additional compounds

challenging. Of the known metabolites, cluster assignments have been made for AFs (cluster #54), aflatrem (clusters #15 and 32, Nicholson et al. 2009), and cyclopiazonic acid (CPA, cluster #55, Chang et al. 2009; Georgianna et al. 2010). Recently, the kojic acid gene cluster has also been characterized, raising the cluster numbers to 56 (Marui et al. 2011), and clusters #35 and 48 are both associated with the production of novel piperazines (Forseth et al. 2013).

As described before, the genes that are involved in secondary metabolite production are often clustered together, and these clusters contain synthetic genes ("backbone" synthase genes, "decorating" enzymatic genes, etc.), transporter genes, and regulatory genes (transcription factors). Two methods to identify secondary metabolite clusters in the genome are via software tools (e.g., *S*econdary *M*etabolite *U*nknown *R*egions *F*inder, SMURF, Khaldi et al. 2010 and *anti*biotics and *S*econdary *M*etabolite *A*nalysis *SH*ell, antiSMASH, Medema et al. 2011) or via microarray/RNA-seq analysis coupled with genome sequence (Yu et al. 2011).

Both SMURF and antiSMASH work by identifying synthase genes by their domains and predicting gene clusters based on their genomic context. The synthase genes, encoding functional enzymes that generate the secondary metabolite core structure, include non-ribosomal peptide synthases (NRPS), polyketide synthases (PKS), their hybrids (NRPS-PKS), indole alkaloids, and terpenoids (Hoffmeister and Keller 2007; Keller et al. 2005). These synthase genes have multiple domains that build upon a starter molecule, such as malonyl-CoA or acetyl-CoA for PKS, or amino acids for NRPS, to synthesize the secondary metabolite precursor.

For example, the AF/ST PKS gene, *pksA*, is classified as a non-reducing (NR)-iterative PKS (IPKS) and has several domains: ketosynthase (KS), malonyl-CoA: ACP transacylase (MAT), acyl–carrier protein (ACP), and thioesterase-cyclase (TE/CLC) domains, as well as the recently identified starter unit–acyl carrier protein transacylase (SAT) and product template (PT) domains. These domains assemble a hexanoyl starter unit and seven malonyl-CoA extender units to synthesize the precursor of AF and ST, norsolorinic acid anthrone (Crawford et al. 2008). SMURF and antiSMASH are designed to identify synthase genes on the basis of their various domains, and then assess whether neighboring genes are cluster members based on their predicted annotations (such as decorating or regulatory genes).

SMURF was applied to 27 fungal genomes, and the resulting clusters were compared with the genetically characterized clusters of six fungal species. The SMURF data contained all previously discovered clusters, as well as additional ones (Khaldi et al. 2010). Thus, SMURF is a useful and reliable tool for identifying secondary metabolite clusters. antiSMASH works in a similar fashion. The data also highlighted some of the evolutionary aspects of secondary metabolism, and suggested adaptation to environmental effect in organisms (Khaldi et al. 2010).

Another way to identify secondary metabolite clusters is through microarray analysis of fungal mutants lacking or overexpressing secondary metabolism global regulatory genes. For example, LaeA (found in a screen for genes regulating ST synthesis and discussed in greater depth in the following section) is a global regulator of secondary metabolite clusters first found in the genetic model *A. nidulans* (Bok and Keller 2004). Later, LaeA function was found to be conserved in other Aspergilli as well as other filamentous fungi, such as *Fusarium fujikuroi* (Wiemann et al. 2010), *Penicillium chrysogenum* (Kosalková et al. 2009), and *Cochliobolus heterostrophus* (Wu et al. 2012). Mutants lacking *laeA* are unable to produce ST and penicillin (PN) in *A. nidulans* and gliotoxin in *A. fumigatus,* while over-expression of *laeA* increases ST and lovastatin production in *A. nidulans* and *A. terreus* (Bok and Keller 2004). Moreover, aflatoxin, cyclopiazonic acid, aflatrem, paspaline, kojic acid and aflavinine biosynthesis are regulated by *laeA* in *A. flavus* (Kale et al. 2008; Georgianna et al. 2010; Oda et al. 2011). Because of its global role in secondary metabolism regulation, *laeA* has been used as a molecular tool to characterize secondary metabolite clusters. For example, Bok et al. identified a novel *laeA*-regulated secondary metabolite produced by *A. nidulans*, terrequinone A, by using microarray data (2006).

The terrequinone A cluster contains five genes, including *tdiA*, a putative NRPS-encoding gene, and all five cluster genes were regulated by *laeA* (Bok et al. 2006).

Microarray analyses and SMURF data can be used together to identify ideal culture conditions for expression of secondary metabolite clusters. Georgianna et al. (2010) performed microarrays of *A. flavus* grown under 28 different conditions, including growth on plant hosts, various media, and with *laeA* mutants (Georgianna et al. 2010). They monitored the expression of the backbone genes in all 55 of *A. flavus*' SMURF-predicted clusters. Eight gene clusters were regulated similarly to the AF cluster, including cluster #55, which was later found to produce cyclopiazonic acid. Comparison of wildtype, Δ*laeA* and *OE::laeA* strains' expression patterns revealed that up to 39 of the clusters are positively regulated by *laeA* (Georgianna et al. 2010; Amaike and Keller 2011). In fact, two clusters—which appear to have arisen from a duplication event—regulated by *laeA* and predicted to encode non-ribosomal peptides have been recently characterized as both contributing to the production of the same piperazine-like compound. (Forseth et al. 2013)

While Georgianna et al. (2010) utilized these data to characterize the CPA cluster in *A. flavus*, the aflatrem cluster was identified by *A. flavus* genome analysis. Aflatrem is an indole-diterpene, and this class of molecules is synthesized by geranylgeranyl diphosphate (GGPP) synthases. Using consensus primers for the GGPP synthase sequence, *atmG* was amplified from *A. flavus* genomic DNA, and *atmC* and *atmM* were found adjacent to *atmG* (Zhang et al. 2004). This cluster is referred to as *ATM1,* and it is located near the telomere of chromosome 5. *Penicillium paxilli* synthesizes the indole–diterpene paxilline via seven clustered genes, three of which are orthologous to *ATM1* genes. Putative orthologs for the other four genes, as well as for an additional gene in the paxilline cluster, were found at another locus, called *ATM2* and located at chromosome 7. These genes include *atmD, atmQ, atmB, atmA*, and *atmP*. Expression of *ATM1* and *ATM2* genes increased with the onset of aflatrem production. The aflatrem clusters are also present in *A. oryzae*, though it does not produce aflatrem. Interestingly, the putative cytochrome P450 monooxygenase *atmQ* in *A. oryzae* has a frame-shift mutation that leads to a nonfunctional protein (Nicholson et al. 2009). The kojic acid cluster was found through examination of highly induced genes during kojic acid production conditions in *A. orzyae* (Terabayashi et al. 2010), a species now considered as a non-aflatoxigenic version of *A. flavus.*

III. Regulation of Aflatoxin and Sterigmatocystin Production

Regulation of secondary metabolite synthesis is composed of complex hierarchical cross-feeding pathways. Our knowledge of AF and ST regulatory factors has greatly expanded in the last 10 years, as considered below (Yin and Keller 2011).

A. AflR

There are several $Zn(II)_2Cys_6$ transcription factors involved in secondary metabolite regulation in *Aspergillus* and other fungi (Yin and Keller 2011). The genes for these types of transcription factors are typically found embedded in secondary metabolite clusters, where the encoded protein acts to activate the other genes in the cluster. The AF/ST $Zn(II)_2Cys_6$ factor, AflR, has been studied for about 20 years, and was instrumental in elucidating the mechanism of ST/AF regulation and production. AflR (formerly called Afl-2 or Apa-2) binds the motif 5′-$TCG(N)_5CGA$ that is located within most promoters of ST/AF cluster genes (Chang et al. 1993; Payne et al. 1993; Brown et al. 1996; Fernandes et al. 1998, Fig. 3.1). AflR also weakly binds a non-consensus site in its own promoter in *A. parasiticus* and *A. flavus* (Ehrlich et al. 1999). Microarray data of wild-type *A. parasiticus* and a Δ*aflR* mutant showed that AF cluster genes were downregulated in the Δ*aflR* strain compared to wildtype (Price et al. 2006). AflR is both transcriptionally and post-transcriptionally regulated by protein kinase A (Shimizu and Keller 2001; Shimizu et al. 2003).

Sharing a promoter with *aflR* is *aflS* (*aflJ*). The function of AflS is unclear, but it could act as an enhancer for AflR regulation of ST/AF cluster genes (Chang 2003). Disruption of *aflS* in *A. flavus* results in a loss of AF production, as

well as an inability to convert exogenous precursors to AF, suggesting that *aflS* is required for AF biosynthesis (Meyers et al. 1998). More recently, RNA-Seq data indicated that AF cluster genes, including *aflR* and *aflS*, were expressed much higher at 30 °C than at 37 °C, suggesting that temperature affects AF production via these regulators (Yu et al. 2011).

B. bZIP Transcriptional Factors

Another type of transcription factor recently associated with secondary metabolite regulation, including AF and ST, is the basic leucine zipper domain (bZIP) protein associated with stress responses, development, and metabolite biosynthesis in many fungi (Rodrigues-Pousada et al. 2010). These transcription factors contain two major motifs: a basic region, which facilitates sequence-specific DNA binding, and a leucine zipper region, which allows dimerization of bZIP proteins (Fernandes et al. 1997). bZIPs can be both positive and negative regulators.

atfB encodes a bZIP transcription factor belonging to the cAMP response element (CRE) binding protein family. Microarray data in *A. oryzae* revealed that several stress response genes, including a catalase gene and trehalose biosynthesis genes, were downregulated in a Δ*atfB* mutant compared to the wild-type. The Δ*atfB* strain germinated similarly to wildtype in stress-free conditions, but Δ*atfB* conidia were much more susceptible to heat-shock and H_2O_2 stresses (Sakamoto et al. 2008). Recently, chromatin immunoprecipitation (ChIP) in *A. parasiticus* revealed that AtfB binds to the promoters of seven genes in the AF cluster, all of which contain CRE sites, under AF-inducing, but not AF-repressing conditions, suggesting AtfB to be a positive regulator of AF gene expression (Roze et al. 2011b). Interestingly, binding at these sites was nearly absent in a strain lacking *veA*, a global regulator of secondary metabolism and fungal development, and a member of the Velvet Complex (see discussion below). Electrophoretic mobility shift analysis (EMSA) confirmed that AtfB is part of a protein complex that binds to the *aflD* (formerly called *nor-1*) promoter in the AF cluster, and that both a CRE1 and an AP-1 site are needed for binding. AP-1 is another conidial stress tolerance bZIP transcription factor (Reverberi et al. 2008), and this work suggests that AtfB may form a heterodimer with AP-1. Together, these findings revealed a link between the oxidative stress response in conidia to production of secondary metabolites (Roze et al. 2011a, b).

RsmA (*r*emediation of *s*econdary *m*etabolism) is another bZIP transcription factor that was identified in a multicopy-suppressor screen for restoration of secondary metabolism in an *A. nidulans* Δ*laeA* mutant. Overexpression of *rsmA* greatly increases ST synthesis (Shaaban et al. 2010). Microarray analysis of an *rsmA* overexpression strain showed that the entire ST cluster was upregulated in this strain (Yin et al. 2012). Two putative RsmA-binding sites were identified by bioinformatic analysis, and both were found in the *aflR-aflS* bidirectional promoter. EMSA revealed that RsmA binds to both of these motifs, and this activates *aflR* to regulate ST production. One of these motifs is similar to the canonical binding site (TTAGTAA) of a subclass of *S. cerevisiae* bZIP proteins known as YAP proteins, and the other is an RsmA-specific binding site, TGACACA. Not only are these sites required for RsmA binding in vitro, they are also required for *aflR* expression and ST production in vivo (Yin et al. 2012).

Another positively acting bZIP protein is *meaB* (*me*thyl*a*mmonium-resistant), first described in *A. nidulans*, where it was shown to be involved in nitrogen metabolite repression. Deletion of *meaB* increased colony diameter on the ammonium analog methylammonium, and sensitivity to chlorate and nitrite with ammonium sources (Polley and Caddick 1996). MeaB activates expression of *nmrA* in *A. nidulans*, which represses nitrogen metabolism, by binding to a motif, TTGCACCAT, found in the *nmrA* promoter (Wong et al. 2007). Later work, however, showed that while MeaB may play a role in *nmrA* regulation, it is not its sole activator (Wagner et al. 2010). In addition, MeaB binds the same Yap-like binding site as RsmA, and positively regulates ST production in *A. nidulans* (Amaike et al.,

unpublished). MeaB also regulates the NRPS-derived pigment metabolite, bikaverin, and the plant hormone, gibberellin in *Fusarium fujikuroi* (Wagner et al. 2010), which suggests that MeaB might bind the promoter regions of synthases or transcription factors of these clusters. MeaB is also an important plant pathogenicity factor in the vascular wilt pathogen, *Fusarium oxysporum* (López-Berges et al. 2010). When *meaB* is overexpressed in *A. flavus,* it decreases conidiation on peanut seed (Amaike et al. 2013 submitted). *meaB* is also involved in fungal development. In *A. nidulans,* overexpression of *meaB* decreased colony diameter and conidiation, while disruption of *meaB* increased conidiation and decreased cleistothecia formation, suggesting that MeaB may be binding to the promoter region of asexual and sexual development regulators, and inducing feedback mechanisms (S. Amaike and N.P. Keller, unpublished data). These data indicate that MeaB is a regulator of secondary metabolism, nitrate utilization, plant pathogenicity, and fungal development through the binding-specific sites in the fungal genome.

A. parasiticus ApyapA and *A. ochraceus* Aoyap1 are orthologous bZIPs associated with negative regulation of AF and the mycotoxin ochratoxin respectively (Reverberi et al. 2007, 2008, 2012). Both proteins are orthologs of *A. nidulans* NapA, characterized for its role in protecting the fungus from oxidative stress (Asano et al. 2007). Deletion of *ApyapA* and *Aoyap1* increases oxidative stress and AF/ochratoxin levels in the two fungi. Here, the authors suggest that these bZIPs are required for proper redox balance in the cell, and loss of this balance stimulates AF/ochratoxin levels.

C. Velvet Complex

As mentioned, ***laeA* (loss of *aflR* expression) regulates many secondary metabolites. LaeA is a member of a heterotrimeric nuclear complex called the Velvet Complex, along with two other proteins, the aforementioned VeA and VelB** (Bayrum et al. 2008). The Velvet Complex is a conserved fungal-specific transcriptional regulator of several fungal processes including secondary metabolism, spore development, and stress responses to the environment (Bayrum et al. 2008; Baba et al. 2012; Wiemann et al. 2010; Wu et al. 2012). Little is known about the role of VelB in this complex, but VeA is required for production of cleistothecia, or sexual fruiting bodies, in *A. nidulans* (Kim et al. 2002), as well as sclerotia, overwintering structures in both *A. parasiticus* (Calvo et al. 2004) and *A. flavus* (Duran et al. 2007; Amaike and Keller 2009). Moreover, VeA regulates the same set of secondary metabolites as LaeA in *A. nidulans* and *A. flavus* (Bok and Keller 2004; Kale et al. 2008; Amaike and Keller 2009). Deletion of either VeA or LaeA eliminated AF/ST production, whereas overexpression increases AF production (ST not assessed, Bok and Keller 2004; Kale et al. 2008; Amaike and Keller 2009). **VeA and LaeA orthologs have been found in various fungi, such as *Fusarium verticillioides, Magnaporthe grisea,* and *Cochliobolus heterostrophus*** (Li et al. 2006; Calvo 2008; Wu et al. 2012). Both LaeA and VeA also play a key role in pathogenicity in both plant and animal pathogens (Amaike and Keller 2009; Bok et al. 2005; Myung et al. 2012; Wiemann et al. 2010; Wu et al. 2012).

VeA interacts with at least one other protein in addition to LaeA and VelB, FphA. FphA is a phytochrome that acts as a red-light receptor that represses sexual development in *A. nidulans* in red-light conditions (Blumenstein et al. 2005). It forms a protein complex with light response proteins LreA and LreB. LreA and LreB are orthologous to white collar proteins WC-1 and WC-2, which are key for sensing blue light in *Neurospora crassa* (Purschwitz et al. 2008). Thus, the Velvet Complex appears to be the link between light sensing, fungal development, and secondary metabolism. (Calvo 2008)

Although *laeA*-mediated regulation of secondary metabolism has been shown to occur at the transcriptional level, the mechanism of LaeA has not yet been elucidated (Bok and Keller 2004; Kale et al. 2008; Amaike and Keller

2009). LaeA is a nuclear protein and a putative methyltransferase containing an S-adenosylmethionine (SAM) binding site required for function (Bok et al. 2006). LaeA activity is associated with epigenetic mechanisms, where LaeA-regulated regions of the genome display heterochromatin marks when LaeA is absent but euchromatin marks when LaeA is activated (Reyes-Dominguez et al. 2010). Loss of LaeA can be partially remediated by deletion/inactivation of heterochromatin gatekeepers (Shwab et al. 2007; Lee et al. 2009).

D. Chromatin

The first inkling that chromatin regulation may be part of the hierarchical pathways regulating AF and ST synthesis came from a study in *A. parasiticus* where placement of an AF cluster gene in another part of the genome resulted in abberant regulation of this gene (Chiou et al. 2002). Later, the identification of LaeA as a methyltransferase with similarities to histone methytransferases, and the fact that removal of AflR from the ST cluster released it from LaeA regulation (Bok et al. 2006), coupled with similar observations of AflR rescue of AF cluster silencing by ectopic placement of AflR in *A. flavus* (Smith et al. 2007), strengthened the notion that AF and ST clusters were regulated in part through chromatin activation. Roze et al. (2007) also found that the spread of histone H4 acetylation paralleled the order of transcriptional activation of genes in the AF cluster. Since this time, a series of studies aimed at activating chromatin through either genetically manipulating genes encoding enzymes modifying histone charge through acetylation and methylation, or by growing fungal cultures with epigenetic modifiers, has **clearly supported a role for heterochromatin/euchromatin control of fungal secondary metabolism** (Shwab et al. 2007 and reviewed in Palmer and Keller 2010; Strauss and Reyes-Dominguez 2011). A role for chromatin regulation of gene expression has also been described for the trichothecene family toxin, dioxynivalenol (DON) in *Fusarium graminearum* (Reyes-Dominguez et al. 2012).

E. Host-Microbe Interactions

Several plant metabolites have been implicated in AF gene regulation. The compounds receiving the most study are oxygenated fatty acids called oxylipins. Oxylipins are produced in all organisms. In fungi, they mediate development and production of secondary metabolites, and in plants, they are key to development and environmental adaptation. Moreover, they are critical signaling molecules in fungal/host interactions (reviewed in Christensen and Kolomiets 2011). Fungal oxylipins are produced by oxygenases including Ppo and Lox enzymes, and oxygenase mutants in *A. flavus* and *A. nidulans* affect the fungus' ability to sporulate, produce AF and ST, and colonize seed. For example, an *A. nidulans* Δ*ppoABC* mutant was unable to produce ST in growth medium and *in planta*. The Δ*ppoABC* mutant was also extremely impaired in its ability to colonize peanuts, showing a drastic reduction of asexual and sexual sporulation compared to the wildtype (Tsitsigiannis and Keller 2006). In a reciprocal fashion, maize lipoxygenase *Zmlox3* null mutant corn kernels are more resistant to several fungal pathogens (Gao et al. 2007), but are more susceptible to both *A. nidulans* and *A. flavus*, seen by an increase in conidiation and AF/ST production (Gao et al. 2009).

Plant oxylipins appear to mimic endogenous oxylipin signals to affect development of *A. flavus*, with various exogenously applied plant oxylipins altering sporulation, mostly to increase conidiation (Calvo et al. 1999). The plant lipoxygenase (13-Lox) product 13*(S)*-HPODE decreases ST in *A. nidulans* and AF in *Aspergillus parasiticus* but 9*(S)*-HPODE production by plant 9-Lox stimulate AF/ST synthesis (Burow et al. 1997). Furthermore, maize-derived *Zmlox3* (a 9*(S)*-HPODE producer) expressed in *A. nidulans* causes a dramatic increase in conidiation, ST production, and cleistothecia size (Brodhagen et al. 2008). Several studies show that infection of plant seeds with *Aspergillus* species impacts host lipoxygenase expression (Burow et al. 2000; Wilson et al. 2001; Tsitsigiannis et al. 2005). This induction is, in part, mediated by

fungal oxylipins, as *A. nidulans ppo*-deletion mutants were no longer able to induce plant Lox expression, demonstrating the crosstalk between fungal and plant oxylipins (Brodhagen et al. 2008). This impact of oxylipins on AF and ST synthesis has been associated with oxidative stress levels in the fungus, in part regulated by the bZIP protein Apyap discussed above.

Oxylipin production has also been implicated in quorum sensing in *A. flavus*. Quorum sensing, originally described in bacteria, is now known to exist in fungi as well. Horowitz Brown et al. (2008) demonstrated an *A. flavus* conidia–sclerotia morphology shift dependent on cell density, with increasing conidial production at high cell densities. In addition to morphological alterations, the profile of secondary metabolites changed as a consequence of cell densities, with decreased AF synthesis at high cell densities (Brown et al. 2009). A critical property associated with quorum-sensing was demonstrated in *A. flavus*, when it was shown that high cell density extracts induced high cell density development, and vice versa. As deletions in *A. flavus ppo* and *lox* genes block the developmental shift (as does exogenous addition of various oxylipins), it has been proposed that oxylipins may serve as quorum-sensing signals in *Aspergillus* spp. adding yet another layer of complexity in cross-kingdom communications. (Brown et al. 2009)

IV. Conclusions

The specter of disease and economic costs caused by AF contamination of food and feed crops continues to drive studies in *Aspergillus* research. **Great advances have been made in understanding the complex genetic regulation of AF/ST in the last 10 years, enhanced by the sequence completion of several *Aspergillus* genomes including those of *A. flavus* and *A. nidulans*.** Insights into chromatin regulation and plant signals, which are important in activating and repressing AF synthesis, provide platforms for new methodologies to control production of this potent carcinogen.

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References

Amaike S, Keller NP (2009) Distinct roles for VeA and LaeA in development and pathogenesis of *Aspergillus flavus*. Eukaryot Cell 8:1051–1060

Amaike S, Keller NP (2011) *Aspergillus flavus*. Ann Rev Phytopathol 49:107–133

Amaike S, Affeldt KJ, Yin WB, Franke S, Choithani A, Keller NP (2013) The bZIP protein MeaB mediates virulence attributes in *Aspergillus flavus* (submitted)

Asano Y, Hagiwara D, Yamashino T, Mizuno T (2007) Characterization of the bZip-type transcription factor NapA with reference to oxidative stress response in *Aspergillus nidulans*. Biosci Biotechnol Biochem 71:1800–1803

Baba S, Kinoshita H, Nihira T (2012) Identification and characterization of *Penicillium citrinum* VeA and LaeA as global regulators for ML-236B production. Curr Genet 58:1–11

Bayrum O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stromeyer S, Kwon NJ, Keller NP, Yu JH, Braus GH (2008) The VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320:1504–1506

Bennett JW, Chang PK, Bhatnagar D (1997) One gene to whole pathway: the role of norsolorinic acid in aflatoxin research. Adv Appl Microbiol 45:1–15

Blumenstein A, Vienken K, Tasler R, Purschwitz J, Veith D, Frankenberg-Dinkel N, Fischer R (2005) The *Aspergillus nidulans* phytochrome FphA represses sexual development in red light. Curr Biol 15:1833–1838

Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in *Aspergillus* spp. Eukaryot Cell 3:527–535

Bok JW, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Keller NP (2005) LaeA, a regulator of morphogenetic fungal virulence factors. Eukaryot Cell 4:1574–1582

Bok JW, Hoffmeister D, Maggio-Hall LA, Murillo R, Glasner JD, Keller NP (2006) Genomic mining for *Aspergillus* natural products. Chem Biol 13:31–37

Brodhagen M, Tsitsigiannis DI, Hornung E, Göbel C, Feussner I, Keller NP (2008) Reciprocal oxylipin-mediated cross-talk in the *Aspergillus*–seed pathosystem. Mol Microbiol 67:378–391

Brown DW, Yu JH, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH, Leonard TJ (1996) Twenty-five co-regulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc Natl Acad Sci USA 93:1418–1422

Brown SH, Scott JB, Bhaheetharan J, Sharpee WC, Milde L, Wilson RA, Keller NP (2009) Oxygenase coordination is required for morphological transition and the host-fungus interaction of *Aspergillus flavus*. Mol Plant Microbe Interact 22:882–894

Burow GB, Nesbitt TC, Dunlap J, Keller NP (1997) Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis. Mol Plant Microbe Interact 10:380–387

Burow GB, Gardner HW, Keller NP (2000) A peanut seed lipoxygenase responsive to *Aspergillus* colonization. Plant Mol Biol 42:689–701

Calvo AM (2008) The VeA regulatory system and its role in morphological and chemical development in fungi. Fungal Genet Biol 45:1053–1061

Calvo AM, Hinze LL, Gardner HW, Keller NP (1999) Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. Appl Environ Microbiol 65:3668–3673

Calvo AM, Bok JW, Brooks W, Keller NP (2004) *veA* is required for toxin and sclerotial production in Aspergillus parasiticus. Appl Environ Microbiol 70:4722–4739

Cardwell KF, Henry SH (2004) Risk of exposure to mitigation of effects of aflatoxin on human health: a west African example. J Toxicol 23:217–247

Chang PK (2003) The Aspergillus parasiticus protein AflJ interacts with the aflatoxin pathway-specific regulator AflR. Mol Genet Genomics 268:711–719

Chang PK, Cary JW, Bhatnagar D, Cleveland TE, Bennett JW, Linz JE, Woloshuk CP (1993) Cloning of the Aspergillus parasiticus apa-2 gene associated with the regulation of aflatoxin biosynthesis. Appl Environ Microbiol 59:3273–3279

Chang PK, Horn BW, Dorner JW (2009) Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis cluster in Aspergillus flavus. Fungal Genet Biol 46:176–182

Chiou CH, Miller M, Wilson DL, Trail F, Linz JE (2002) Chromosomal location plays a role in regulation of aflatoxin gene expression in Aspergillus parasiticus. Appl Environ Microbiol 68:306–315

Christensen SA, Kolomiets MV (2011) The lipid language of plant-fungal interactions. Fungal Genet Biol 48:4–14

Cleveland TE, Yu J, Fedorova ND, Bhatnagar D, Payne GA, Nierman WC, Bennett JW (2009) Potential of Aspergillus flavus genomics for applications in biotechnology. Trends Biotechnol 3:151–157

Cole RJ, Cox RH (1981) In: Cole RJ, Cox RH (eds) Handbook of toxic fungal metabolites. Academic, New York, pp 67–93

Crawford JM, Thomas PM, Scheerer JR, Vagstad AL, Kelleher NL, Townsend CA (2008) Deconstruction of iterative multidomain polyketide synthase function. Science 320:243–246

Dereszynski DM, Center SA, Randolph JF, Brooks MB, Hadden AG, Palyad KS, McDonough SP, Messick J, Stokol T, Bischoff KL, Gluckman S, Sanders SY (2008) Clinical and clinicopathologic features of dogs that consumed foodborne hepatotoxic aflatoxins: 72 cases (2005–2006). J Am Vet Med Assoc 232:1329–1337

Duran RM, Cary JW, Calvo AM (2007) Production of cyclopiazonic acid, aflatrem, and aflatoxin by Aspergillus flavus is regulated by veA, a gene necessary for sclerotia formation. Appl Micobiol Biotechnol 73:1158–1168

Ehrlich KC (2009) Predicted roles of the uncharacterized clustered genes in aflatoxin biosynthesis. Toxins 1:37–58

Ehrlich KC, Montalbano BG, Cary JW (1999) Binding of the C6-zinc cluster protein, AflR, to the promoters of aflatoxin pathway biosynthesis genes in Aspergillus parasiticus. Gene 230:249–257

Ehrlich KC, Montalbano Boué SM, Bhatnagar D (2005) Aflatoxin biosynthesis cluster gene encodes a novel oxidase required for conversion of versicolorin A to sterigmatocystin. Appl Environ Microbiol 71:8963–8965

Ehrlich KC, Scharfenstein PLL, Chang PK (2010) HypC, the anthrone oxidase involved in aflatoxin biosynthesis. App Environ Microbiol 76:3374–3377

Fernandes L, Rodrigues-Pousada C, Struhl K (1997) Yap, a novel family of eight bZIP proteins in Saccharomyces cerevisiae with distinct biological functions. Mol Cell Biol 17:6982–6993

Fernandes M, Keller NP, Adams TH (1998) Sequence-specific binding by Aspergillus nidulans AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. Mol Microbiol 28:1355–1365

Forgacs J, Carll WT (1962) Mycotoxicoses. In: Bradly CA, Jungherr EL (eds) Advances in veterinary science. Academic, New York, pp 273–382

Forseth RR, Amaike S, Schwenk D, Affeldt KJ, Hoffmeister D, Schroeder FC, Keller NP (2013) Homologous NRPS-like gene clusters mediate redundant small-molecule biosynthesis in *Aspergillus flavus*. Angew Chem Int Ed Engl 52:1590–1594

Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, Baştürkmen M, Spevak CC, Clutterbuck J, Kapitonov V, Jurka J, Scazzocchio C, Farman M, Butler J, Purcell S, Harris S, Braus GH, Draht O, Busch S, D'Enfert C, Bouchier C, Goldman GH, Bell-Pedersen D, Griffiths-Jones S, Doonan JH, Yu J, Vienken K, Pain A, Freitag M, Selker EU, Archer DB, Peñalva MA, Oakley BR, Momany M, Tanaka T, Kumagai T, Asai K, Machida M, Nierman WC, Denning DW, Caddick M, Hynes M, Paoletti M, Fischer R, Miller B, Dyer P, Sachs MS, Osmani SA, Birren BW (2005) Sequencing of Aspergillus nidulans and comparative analysis with A. fumigatus and A. oryzae. Nature 438:1105–1115

Gao X, Shim WB, Göbel C, Kunze S, Feussner I, Meeley R, Balint-Kurti P, Kolomiets M (2007) Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. Mol Plant Microbe Interact 20:922–933

Gao X, Brodhagen M, Isakeit T, Brown SH, Göbel C, Betran J, Feussner I, Keller NP, Kolomiets MV (2009) Inactivation of the lipoxygenase ZmLOX3 increases susceptibility of maize to Aspergillus spp. Mol Plant Microbe Interact 22:222–231

Georgianna DR, Fedorova ND, Burroughs JL, Dolezal AL, Bok JW, Brown SH, Woloshuk CP, Yu J, Keller

NP, Payne GA (2010) Beyond aflatoxin: four distinct expression patterns and functional roles associated with Aspergillus flavus secondary metabolism gene clusters. Mol Plant Pathol 11:213–226

Gouas D, Shi H, Hainaut P (2009) The aflatoxin-induced TP53 mutation at codon 249 (R249S): biomarker of exposure, early detection and target for therapy. Cancer Lett 286:29–37

Hicks JK, Shimizu K, Keller NP (2002) Genetics and biosynthesis of aflatoxins and sterigmatocystin. In: Esser K, Bennett JW (eds) The mycota: a comprehensive treatise on as experimental systems for basic and applied research, vol XI. Springer, Berlin

Hoffmeister D, Keller NP (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat Prod Rep 24:393–416

Holmes RA (2008) Characterization of an aflatoxin biosynthetic gene and resistance in maize seeds to Aspergillus flavus. Ph.D. thesis, NCSU, 122 pp

Horowitz Brown S, Zarnowski R, Sharpee WC, Keller NP (2008) Morphological transitions governed by density dependence and lipoxygenase activity in Aspergillus flavus. Appl Environ Microbiol 74:5674–5685

Kale SP, Milde L, Trapp MK, Frisvad JC, Keller NP, Bok JW (2008) Requirement of LaeA for secondary metabolism and sclerotial production Aspergillus flavus. Fungal Genet Biol 45:1422–1429

Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism: from biochemistry to genomics. Nat Rev Microbiol 3:937–947

Khaldi N, Seifuddin FT, Turner G, Haft D, Nierman WC, Wolfe KH (2010) SMURF: genomic mapping of fungal secondary metabolite clusters. Fungal Genet Biol 47:736–741

Kim H, Hank K, Kim K, Han D, Jahng K, Chae K (2002) The veA gene activates sexual development in Aspergillus nidulans. Fungal Genet Biol 37:72–80

Klich MA (2007) Aspergillus flavus: the major producer of aflatoxin. Mol Plant Pathol 8:713–722

Kosalková K, García-Estrada C, Ullán RV, Godio RP, Feltrer R, Teijeira F, Mauriz E, Martín JF (2009) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in Penicillium chrysogenum. Biochimie 92:214–225

Lee I, Oh JH, Shwab EK, Dagenais TR, Andes D, Keller NP (2009) HdaA, a class 2 histone deacetylase of Aspergillus fumigatus, affects germination and secondary metabolite production. Fungal Genet Biol 46:782–790

Lewis L, Onsongo M, Njapau H, Schurz-Rogers H, Luber G, Kieszak S, Nyamongo J, Backer L, Dahiye AM, Misore A, DeCock K, Rubin C, Kenya Aflatoxicosis Investigation Group (2005) Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. Environ Health Perspect 113:1763–1767

Li S, Myung K, Guse D, Donkin B, Proctor RH, Grayburn WS, Calvo AM (2006) FvVE1 regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in Fusarium verticillioides. Mol Microbiol 62:1418–1432

Liu Y, Wu F (2010) Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. Environ Health Perspect 118:818–824

López-Berges MS, Rispall N, Prados-Rosales RC, Pietro A (2010) A nitrogen response pathway regulates virulence functions in Fusarium oxysporum via the protein kinase tor and the bZIP protein MeaB. Plant Cell 22(7):2459–2475

Marui J, Yamane N, Ohashi-Kunihiro S, Ando T, Terabayashi Y, Sano M, Ohashi S, Ohshima E, Tachibana K, Higa Y, Nishimura M, Koike H, Machida M (2011) Kojic acid biosynthesis in Aspergillus oryzae is regulated by a Zn(II)(2)Cys(6) transcriptional activator and induced by kojic acid at the transcriptional level. J Biosci Bioeng 112:40–43

McDonald T, Noordermeer D, Zhang Y-Q, Hammond T, Keller NP (2005) The ST cluster revisited: lessons from a genetic model. In: Abbas HK (ed) Aflatoxin and food safety. CRC Press, Boca Raton, pp 117–136

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R (2011) antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 39: W339–W346

Meyers DM, O'Brian G, Du W, Bhatnagar D, Payne GA (1998) Characterization of aflJ, a gene required for conversion of pathway intermediates to aflatoxin. Appl Environ Microbiol 64:3713–3717

Myung K, Zitomer NC, Duvall M, Glenn AE, Riley RT, Calvo AM (2012) The conserved global regulator VeA is necessary for symptom production and mycotoxin synthesis in maize seedlings by Fusarium verticillioides. Plant Pathol 61:152–160

Nicholson MJ, Koulman A, Monahan BJ, Pritchard BL, Payne GA, Scott B (2009) Identification of two aflatrem biosynthesis gene loci in Aspergillus flavus and metabolic engineering of Penicillium paxilli to elucidate their function. Appl Environ Microbiol 75:7469–7481

Nierman WC, Pain A, Anderson MJ, Wortman MJ, Kim SH, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, Bennett J, Bowyer P, Chen D, Collins M, Coulsen R, Davies R, Dyer PS, Farman M, Fedorova N, Fedorova N, Feldblyum TV, Fischer R, Fosker N, Fraser A, García JL, García MJ, Goble A, Goldman GH, Gomi K, Griffith-Jones S, Gwilliam R, Haas B, Haas H, Harris D, Horiuchi H, Huang J, Humphray S, Jiménez J, Keller N, Khouri H, Kitamoto K, Kobayashi T,

Konzack S, Kulkarni R, Kumagai T, Lafon A, Latgé JP, Li W, Lord A, Lu C, Majoros WH, May GS, Miller BL, Mohamoud Y, Molina M, Monod M, Mouyna I, Mulligan S, Murphy L, O'Neil S, Paulsen I, Peñalva MA, Pertea M, Price C, Pritchard BL, Quail MA, Rabbinowitsch E, Rawlins N, Rajandream MA, Reichard U, Renauld H, Robson GD, Rodriguez de Córdoba S, Rodríguez-Peña JM, Ronning CM, Rutter S, Salzberg SL, Sanchez M, Sánchez-Ferrero JC, Saunders D, Seeger K, Squares R, Squares S, Takeuchi M, Tekaia F, Turner G, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu JH, Fraser C, Galagan JE, Asai K, Machida M, Hall N, Barrell B, Denning DW (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438:1151–1156

Oda K, Kobayashi A, Ohashi S, Sano M (2011) Aspergillus oryzae laeA regulates kojic acid synthesis genes. Biosci Biotechnol Biochem 75:1832–1834

Ozturk M (1991) P53 mutation in hepatocellular carcinoma after aflatoxin exposure. Lancet 338:1356–1359

Palmer JM, Keller NP (2010) Secondary metabolism in fungi: does chromosomal location matter? Curr Opin Microbiol 13:431–436

Payne GA, Yu J (2010) Ecology, development and gene regulation in Aspergillus flavus. In: Machida M, Gomi K (eds) Aspergillus: molecular biology and genomics. Caister Academic Press, Norwich, pp 157–171

Payne GA, Nystrom GJ, Bhatnagar D, Cleveland TE, Woloshuk CP (1993) Cloning of the afl-2 gene involved in aflatoxin biosynthesis from Aspergillus flavus. Appl Environ Microbiol 59:156–162

Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van Dijk A, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wösten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nat Biotechnol 25:221–231

Perrone G, Gallo A, Susca A (2009) Aspergillus. In: Liu D (ed) Molecular detection of foodborne pathogens. CRC Press, Boca Raton, pp 529–548

Polley SD, Caddick MX (1996) Molecular characterisation of meaB, a novel gene affecting nitrogen metabolite repression in Aspergillus nidulans. FEBS Lett 388:200–205

Price MS, Yu J, Nierman WC, Kim HS, Pritchard B, Jacobus CA, Bhatnagar TE, Cleveland TE, Payne GA (2006) The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. FEMS Microbiol Lett 225:275–279

Purschwitz J, Müller S, Kastner C, Schöser M, Haas H, Espeso EA, Atoui A, Calvo AM, Fischer R (2008) Functional and physical interaction of blue and red-light sensors in Aspergillus nidulans. Curr Biol 18:1–5

Rank C, Nielsen KF, Larsen TO, Varga J, Samson RA, Frisvad JC (2011) Distribution of sterigmatocystin in filamentous fungi. Fungal Biol 115:406–420

Reverberi M, Zjalic S, Punelli F, Ricelli A, Fabbri AA, Fanelli C (2007) Apyap1 affects aflatoxin biosynthesis during Aspergillus parasiticus growth in maize seeds. Food Addit Contam 24:1070–1075

Reverberi M, Zjalic S, Ricelli A, Punelli F, Camera E, Fabbri C, Picardo M, Fanelli C, Fabbri AA (2008) Modulation of antioxidant defense in Aspergillus parasiticus is involved in aflatoxin biosynthesis: a role for the ApyapA gene. Eukaryot Cell 7:988–1000

Reverberi M, Gazzetti K, Punelli F, Scarpari M, Zjalic S, Ricelli A, Fabbri AA, Fanelli C (2012) Aoyap1 regulates OTA synthesis by controlling cell redox balance in Aspergillus ochraceus. Appl Microbiol Biotechnol. doi:10.1007/s00253-012-3985-4

Reyes-Dominguez Y, Bok JW, Berger H, Shwab EK, Basheer A, Gallmetzer A, Scazzocchio C, Keller N, Strauss J (2010) Heterochromatic marks are associated with the repression of secondary metabolism clusters in Aspergillus nidulans. Mol Microbiol 76:1376–1386

Reyes-Dominguez Y, Boedi S, Sulyok M, Wiesenberger G, Stoppacher N, Krska R, Strauss J (2012) Heterochromatin influences the secondary metabolite profile in the plant pathogen Fusarium graminearum. Fungal Genet Biol 49:39–47

Rodrigues-Pousada C, Menezes R, Pimentel C (2010) The Yap family and its role in stress response. Yeast 27:245–258

Roze LV, Arthur AE, Hong SY, Chanda A, Linz JE (2007) The initiation and pattern of spread of histone H4 acetylation parallel the order of transcriptional activation of genes in the aflatoxin cluster. Mol Microbiol 66:713–726

Roze LV, Chanda A, Linz JE (2011a) Compartmentalization and molecular traffic in secondary metabolism: a new understanding of established cellular processes. Fungal Genet Biol 48:35–48

Roze LV, Chanda A, Wee J, Awad D, Linz JE (2011b) Stress-related transcription factor AtfB integrates secondary metabolism with oxidative

stress response in Aspergilli. J Biol Chem 286:35137–35148
Rubens J, Cardwell KF (2005) The cost of mycotoxin management in the United States. In: Abbas HK (ed) Aflatoxin and food safety. CRC Press, Boca Raton, pp 1–13
Sakamoto K, Arima TH, Iwashita K, Yamada O, Gomi K, Akita O (2008) Aspergillus oryzae atfB encodes a transcription factor required for stress tolerance in conidia. Fungal Genet Biol 45:922–932
Shaaban MI, Bok JW, Lauer C, Keller NP (2010) Suppressor mutagenesis identifies a velvet complex remediator of Aspergillus nidulans secondary metabolism. Eukaryot Cell 9:1816–1824
Shimizu K, Keller NP (2001) Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. Genetics 157:592–600
Shimizu K, Hicks JK, Huang TP, Keller NP (2003) Pka, Ras and RGS protein interactions regulate activity of AflR, a Zn(II)2Cys6 transcription factor in Aspergillus nidulans. Genetics 165:1095–1104
Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S, Keller NP (2007) Histone deacetylase activity regulates chemical diversity in Aspergillus. Eukaryot Cell 6:1656–1664
Slot JC, Rokas A (2011) Horizontal transfer of a large and highly secondary metabolic gene cluster between fungi. Curr Biol 21:134–139
Smith CA, Woloshuk CP, Robertson D, Payne GA (2007) Silencing of the aflatoxin gene cluster in a diploid strain of Aspergillus flavus is suppressed by ectopic aflR expression. Genetics 176:2077–2086
Squire RA (1981) Ranking animal carcinogens, a proposed regulatory approach. Science 214:877–880
Strauss J, Reyes-Dominguez Y (2011) Regulation of secondary metabolism by chromatin structure and epigenetic codes. Fungal Genet Biol 48:62–69
Sweeney MJ, Dobson ADW (1999) Molecular biology of mycotoxin biosynthesis. FEMS Microbiol Lett 175:149–163
Terabayashi Y, Sano M, Yamane N, Marui J, Tamano K, Sagara J, Dohmoto M, Oda K, Ohshima E, Tachibana K, Higa Y, Ohashi S, Koike H, Machida M (2010) Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from Aspergillus oryzae. Fungal Genet Biol 47:953–961
Trail F, Mahanti N, Rarick M, Mehigh R, Liang SH, Zhou R, Linz JE (1995) Physical and transcriptional map of an aflatoxin gene cluster in Aspergillus parasiticus and functional disruption of a gene involved early in the aflatoxin pathway. Appl Environ Microbiol 61:2665–2673
Tsitsigiannis D, Kunze S, Willis D, Feussner I, Keller NP (2005) *Aspergillus* infection inhibites the expression of peanut 13S-HPODE-forming seed lipoxygenase. Mol Plant Microbe Interact 18:1081–1089
Tsitsigiannis DI, Keller NP (2006) Oxylipins act as determinants of natural product biosynthesis and seed colonization in Aspergillus nidulans. Mol Microbiol 59:882–892
Udwary DW, Casillas LK, Townsend CA (2002) Synthesis of 11-hydroxy-O-methylsterigmatocystin and the role of a cytochrome P-450 in the final step of aflatoxin biosynthesis. J Am Chem Soc 124:5294–5303
Van Egmond HP, Jonker MA (2005) Worldwide regulations on aflatoxins. In: Abbas HK (ed) Aflatoxin and food safety. CRC Press, Boca Raton, pp 77–93
Wagner D, Schmeinck A, Mos M, Morozov IY, Caddick MX, Tudzynski B (2010) The bZIP transcription factor MeaB mediates nitrogen metabolite repression at specific loci. Eukaryot Cell 9:1588–1601
Wang JS, Tang L (2005) Epidemiology of aflatoxin exposure and human liver cancer. In: Abbas HK (ed) Aflatoxin and food safety. CRC Press, Boca Raton, pp 195–211
Watanabe CMH, Townsend CA (2002) Initial characterization of a type I fatty acid synthase and polyketide synthase multienzyme complex NorS in the biosynthesis of aflatoxin B1. Chem Biol 9:981–988
Wiemann P, Brown DW, Kleigrewe K, Bok JW, Keller NP, Humph HU, Tudzynski B (2010) FfVel1 and FfLae1, components of a velvet-like complex in Fusarium fujikuroi, affect differentiation, secondary metabolism and virulence. Mol Microbiol 77:972–994
Wilson R, Gardner H, Keller NP (2001) Cultivar-dependent of a maize lipoxygenase responsive to seed infecting fungi. Mol Plant Microbe Interact 14:980–987
Wong KH, Hynes MJ, Todd RB, Davis MA (2007) Transcriptional control of nmrA by the bZIP transcriptional factor MeaB reveals a new level of nitrogen regulation in Aspergillus nidulans. Mol Microbiol 66:534–551
Wu D, Oide S, Zhang N, Choi MY, Turgeon BG (2012) ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen Cochliobolus heterostrophus. PLoS Pathog 8:e1002542
Yin WB, Keller NP (2011) Transcriptional regulatory elements in fungal secondary metabolism. J Microbiol 49:329–339
Yin WB, Amaike S, Wohlbach DJ, Gasch A, Chiang YM, Wang CC, Bok JW, Rohlfs M, Keller NP (2012) Aspergillus nidulans bZIP response pathway hardwired for defensive secondary metabolism operates through aflR. Mol Microbiol 83:1024–1034
Yu J, Chang PK, Cary JW, Wright M, Bhatnagar D, Cleveland TE, Payne GA, Linz JE (1995) Comparative mapping of aflatoxin pathway gene clusters in Aspergillus parasiticus and Aspergillus flavus. Appl Environ Microbiol 61:2365–2371

Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW (2004a) Clustered pathway genes in aflatoxin biosynthesis. Appl Environ Microbiol 70:1253–1262

Yu J, Whitelaw CA, Nierman WC, Bhatnagar D, Cleveland TE (2004b) Aspergillus flavus expressed sequence tags for identification of genes with putative roles in aflatoxin contamination of crops. FEMS Microbiol Lett 237:333–340

Yu J, Payne GA, Campbell BC, Guo B, Cleveland TE, Robens JF, Keller NP, Bennett JW, Nierman WC (2007) Mycotoxin production and prevention of aflatoxin contamination in food and feed. In: Osmani S, Goldman G (eds) The Aspergilli: genomics, medical aspects, biotechnology, and research methods. CRC Press, Boca Raton, pp 457–472

Yu J, Fedorova ND, Montalbano G, Bhatnaga D, Cleveland TE, Bennett JW, Nierman WC (2011) Tight control of mycotoxin biosynthesis gene expression in Aspergilus flavus by temperature as revealed by RNA-seq. FEMS Microbiol Lett 322:145–149

Zhang S, Monahan BJ, Tkacz JS, Scott B (2004) Indole-diterpene gene cluster from Aspergillus flavus. Appl Environ Microbiol 70:6875–6883

4 Fungal Toxins of Agricultural Importance

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I. Introduction

This chapter aims to broadly cover a range of **toxins that are produced only by symbiotic fungi**; that is, fungi that are either pathogenic or mutualistic on their host plants. Toxins produced by saprophytic fungi that impact feed and food spoilage are covered in the chapters entitled "Fungal Spoilage of Crops and Food" and "Genetics, Biosynthesis, and Regulation of Aflatoxins and other *Aspergillus flavus* Secondary Metabolites" in this edition.

Fungi produce a diverse array of toxins that contribute both positively and negatively in an agricultural context. In this regard they can be loosely classified into **phytotoxins** or **mycotoxins**. **Phytotoxins can have a direct effect on the host plant, whereby they rapidly**

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kill affected cells, whilst others cause disease indirectly through modulation of host defence responses, allowing pathogen colonization of the host tissue. Phytotoxins can be further characterised as **host specific toxins (HSTs)**, which include both **secondary metabolites** (e.g., HC-toxin described below) and **proteinaceous effectors** (e.g., ToxA described below), or non-specific toxins (e.g., Cercosporin described below). HSTs are only toxic to specific plant varieties or genotypes that are susceptible to the producing fungus, whereas non-specific toxins affect a wide range of plants, including in some cases non-host plants. Some of these non-specific phytotoxins include fungal secondary metabolites traditionally considered as mycotoxins, such as the fumonosins and trichothecenes discussed below. However, **the majority of mycotoxins have no direct role in host colonisation or disease but have significant impacts on both vertebrate and invertebrate animals that feed on infected host plants.** Several examples are the secondary metabolites produced in planta by **epichloae endophytes of grasses** which are described in detail below. Unlike the pathogenic fungi described in this chapter, **epichloae endophytes form mutualistic symbioses with cool-season grasses** (Schardl et al. 2004), and are included here because they have significant economic impacts on pasture-based systems. Whereas fungal pathogens of plants have evolved to produce toxins that promote disease, mutualistic epichloae endophytes have evolved, often through interspecific hybridisation, to produce chemically diverse secondary metabolites that benefit their host grasses (Schardl et al. 2012).

We have divided this chapter into sections that describe particular classes of toxins based on their core biosynthesis. Our objective is to review agriculturally important examples within these classes, and we focus on the role of each toxin in the plant–fungus interaction, including their activities against vertebrate and invertebrate pests where applicable. The biochemistry and molecular basis of toxin biosynthesis are also described.

II. Nonribosomally Synthesized Peptides

A wide range of biologically active peptides are synthesized, nonribosomally, by multifunctional enzymes called peptide synthetases (von Döhren et al. 1997; Marahiel et al. 1997; Schwarzer et al. 2003). In contrast to ribosomally synthesized polypeptides, which may contain only the 21 proteinogenic amino acids, nonribosomally synthesized peptides may contain a diverse array of residues (Schwarzer et al. 2003) that include nonproteinogenic amino acids (as found in HC-toxin, victorin, and AM-toxin, discussed below), *N*-methylated amino acids (as found in enniatins and destruxins), D-amino acids (as found in HC-toxin and destruxins), and α-hydroxy acids (as found in AM-toxin and enniatins). In addition, the peptide backbone can be linear, cyclic, or cyclic-branched, and may be further modified by glycosylation, acylation, or heterocyclic ring formation (Marahiel et al. 1997). These diverse bioactive peptides range from 2 to 48 residues (to date) and include antibiotics (e.g., penicillin), immunosuppressive agents (e.g., cyclosporin), iron-chelating siderophores and compounds active against insects (e.g., peramine, discussed below), mammals (e.g., ergot alkaloids, discussed below), and plants (e.g., HC-toxin, victorin, and AM-toxin, discussed below). More recently, nonribosomal peptide synthetase/polyketide synthase hybrid enzymes have also been identified with important roles in fungal pathogenicity towards plants. *Magnaporthe grisea* synthesizes the PKS/NRPS hybrid enzyme ACE1 (***a***virulence ***c***onferring ***e***nzyme 1), which is recognized by rice (*Oryza sativa*) cultivars carrying the Pi33 resistance gene and confers resistance to the pathogen (Böhnert et al. 2004), and a PKS/NRPS hybrid enzyme from *Trichoderma virens* appears to be involved in the synthesis of a metabolite that causes induction of host defence responses with a putative role in systemic acquired resistance (Mukherjee et al. 2012). In many cases, the genes encoding these peptide synthetases have been cloned

and characterized (e.g., Bailey et al. 1996; Diez et al. 1990; Haese et al. 1993; Johnson et al. 2000a; Scott-Craig et al. 1992; Weber et al. 1994; Böhnert et al. 2004; Tanaka et al. 2005; Fleetwood et al. 2007; Mukherjee et al. 2012), and all share a common modular structure.

Without exception, peptide synthetases are multifunctional enzymes that link amino acid (or occasionally α-hydroxy acid) residues according to the multiple-carrier thiotemplate mechanism (Kleinkauf and von Döhren 1990; Marahiel et al. 1997; Stein et al. 1994). A minimal module, of about 650 amino acids, has been defined (Marahiel et al. 1997) which is composed of an amino-acid-activating domain, a thiolation domain, and a condensation domain on a single polypeptide chain. The activation domain recognizes, specifically in most cases, a substrate amino (or α-hydroxy) acid and activates it as its acyladenylate by reaction with ATP. The activated ester then becomes covalently linked as a thioester to an enzyme-bound 4′-phosphopantetheine cofactor located within the module. Finally, the condensation domain mediates transfer to another acylamino acid intermediate on the adjacent downstream module to form a peptide bond. In some cases, modifications (such as epimerization, *N*-methylation, reduction, or cyclization) are catalyzed by additional domains or by modified domains within a module.

Many peptide synthetase enzymes were initially identified biochemically using the ATP-pyrophosphate exchange reaction (Lipmann 1971), which takes advantage of the reversible activation of amino acid substrates to acyladenylates. This technique, which requires the addition of particular amino acid substrates, is also the most commonly used method to determine the substrate specificity of the adenylate-activating domain. However, this technique is limited by the availability of amino acid substrates and, as such, is not feasible for the analysis of many peptides containing nonproteinogenic residues. A number of methods to predict the specificity of NRPS adenylation domains have been developed, based on previously characterised domains with known specificity (Challis et al. 2000; Rausch et al. 2005; Stachelhaus et al. 1999), which work reasonably well for predicting the substrates for adenylation domains from prokaryotic NRPSs but tend to fail for fungi because of the divergence of their signature sequences. Recently, the three-dimensional structure of the third adenylation domain of *SidN* (SidNA3) from the fungal endophyte *Neotyphodium lolii* has been obtained (Lee et al. 2010). This is the first structure of a eukaryotic NRPS domain to have been reported, and details the architecture of the specificity-determining pocket for eukaryotic adenylation domains.

The cloning and sequencing of peptide synthetase genes has led to the identification of highly conserved motifs (of three to eight amino acids) within the functional domains already discussed (Marahiel et al. 1997; Turgay and Marahiel 1994). Degenerate oligonucleotides, based on these core motifs, have been successfully used in PCR experiments to identify several genes encoding peptide synthetases (Johnson et al. 2000a, 2007; Nikolskaya et al. 1995; Panaccione 1996; Turgay and Marahiel 1994). These approaches have been largely superseded by whole genome sequencing, which has identified the full complement of NRPS genes in a number of sequenced fungal species. For example, *C. carbonum* has 11 functional NRPS genes (Lee et al. 2005) with diverse functions. The availability of fungal genome sequences has also provided insights into the evolution of NRPS genes. Bushley and Turgeon (2010) described nine major subfamilies of fungal NRPSs, which fall into two main groups. The biosynthesis of several important NRPS-based fungal secondary metabolites and their significance in agriculture is discussed below.

A. HC-toxin

1. Biological Significance of HC-toxin

Cochliobolus carbonum causes **northern corn leaf spot disease of maize** (*Zea mays* L.), and was first observed in the late 1930s on certain inbred lines (Ullstrup 1941). Two races of *C. carbonum*, races 1 and 2, have been well-characterized. Both races are generally weak pathogens on the majority of corn lines that contain the dominant Mendelian loci *HM1* and *HM2*, both of which confer insensitivity to HC-toxin and resistance to *C. carbonum* race 1

(Ullstrup and Brunson 1947). The leaf spot disease caused by *C. carbonum* race 1 first appeared as a result of a combination of spontaneous mutation in both genes, coupled with inbreeding for hybrid seed production that bred out both resistance genes (Multani et al. 1998). In the absence of this resistance, *C. carbonum* can colonise all parts of the maize plant, leading to complete death of the crop (Walton 2006). Durable resistance to *C. carbonum* is now provided through the use of the *HM1* gene, and its importance as a pathogen has since diminished. Nevertheless, the interaction of *C. carbonum* race 1 and maize has become a model system to understand the genetics, biochemistry, and molecular biology of this plant–fungus interaction.

HC-toxin was first demonstrated to have **host specificity** by Scheffer and Ullstrup (1965), who showed that maize lines susceptible to *C. carbonum* race 1 are sensitive to HC-toxin, and only HC-toxin-producing strains of the fungus are capable of causing disease (Scheffer et al. 1967). Further to this, the addition of HC-toxin to the infection court of a non-toxin producing race 2 isolate of *C. carbonum* on susceptible maize results in symptoms comparable to those caused by race 1 of the fungus (Comstock and Scheffer 1973). "Despite HC-toxin's demonstrated role in causing disease of maize, it is not a true phytotoxin in the strictest sense, as it does not cause cell death. Rather, HC-toxin has been shown to be stimulatory rather than inhibitory, promoting responses such as increased dark CO_2 fixation (Kuo and Scheffer 1970) and increased uptake of several ions and organic compounds (Yoder and Scheffer 1973a, b), as well as promoting the survival of non-dividing maize mesophyll cells (Wolf and Earle 1991). HC-toxin has also been shown to inhibit root growth of susceptible genotypes (Scheffer and Ullstrup 1965; Rasmussen and Scheffer 1988), and has been reported to inhibit growth of other plants (Walton 2006)."

The mode of action for HC-toxin took an interesting turn when it was discovered that the closely related compound trapoxin acts in a similar way to trichostatin, a previously characterised inhibitor of **histone deacetylase** (HDAC) (Yoshida et al. 1990; Yoshida and Sugita 1992; Kijima et al. 1993). Proof that HC-toxin also acted as an **HDAC inhibitor** was obtained in experiments that demonstrated that all three isoforms of HDAC from susceptible maize, but not from resistant maize, were indeed inhibited by HC-toxin (Brosch et al. 1995). Further to this, *C. carbonum* infection induces early hyperacteylation of histones H3 and H4, consistent with a role in pathogenesis (Ransom and Walton 1997). Walton (2006) has also proposed that HDAC is the sole site of action of HC-toxin, and specifically that HC-toxin acts on a class of nucleolus-localised HDAC, HD2, of which maize has four members (Verdin et al. 2003). Surprisingly, the mechanism of resistance to HC-toxin is uncoupled from the site of action of the toxin. Instead, the enzyme HC-toxin reductase has been shown to detoxify HC-toxin (Meeley and Walton 1991) by an NADPH-dependent reduction of the carbonyl group at position 8 in the Aeo side chain to the corresponding alcohol, a compound previously shown to be nontoxic (Kim et al. 1987). In addition, there is an absolute co-segregation of HC-toxin reductase activity with disease resistance, and this segregates, without exception, with *HM1*, providing evidence that *HM1* encodes HC-toxin reductase (Meeley et al. 1992). Further evidence has come from sequencing of the *HM1* allele which encodes an NAD(P)H-dependent reductase, the structural gene for HC-toxin reductase (Johal and Briggs 1992). Homologues of the maize resistance gene have been found in other grasses, including important crops such as rice, sorghum, and barley (Han et al. 1997; Multani et al. 1998).

2. Biosynthesis of HC-toxin

C. carbonum race 1 and race 2 isolates were shown to differ by a single genetic locus which was subsequently named ***Tox2*** (Nelson and Ullstrup 1961), and which controls the production of HC-toxin (Scheffer et al. 1967). The structure of HC-toxin (Fig. 4.1) is a cyclic tetrapeptide of D-proline, L-alanine, D-alanine, and L- Aeo (2-amino-9,10-epoxy-8-oxodecanoic acid) (Gross et al. 1982; Kawai and Rich 1983; Leisch et al. 1982; Pope et al. 1983; Walton et al. 1982). The terminal epoxide of Aeo (Ciuffetti et al. 1983; Walton and Earle 1983) and the carbonyl group at position 8 (Kim et al. 1987)

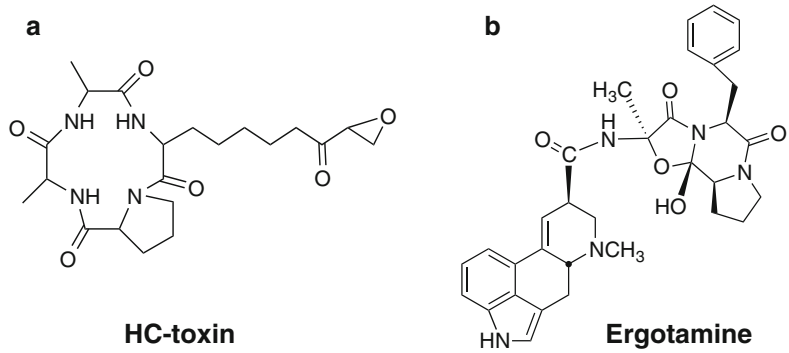

Fig. 4.1. Two non-ribosomally produced fungal secondary metabolites of agricultural importance. (**a**) HC-toxin produced by *Cochliobolus carbonum*; (**b**) ergotamine produced by *Claviceps purpurea*. Illustrations represent chemical structures but not necessarily bond angles or conformations

of Aeo are both required for toxin activity. *Tox2* is a complex of biosynthetic and regulatory genes, many of which are found in two or three copies, and all of which are absent from HC-non-toxin producing isolates of *C. carbonum* (Walton et al. 1998). Based on the peptide nature of HC-toxin, Walton (1987) hypothesized that synthesis would require the action of a nonribosomal peptide synthetase, and through characterisation of proteins catalyzing activities expected from an HC-toxin peptide synthetase (Walton 1987; Walton and Holden 1988) went on to identify ***HTS1*** (Panaccione et al. 1992; Scott-Craig et al. 1992). *HTS1* is present in all toxin-producing isolates but absent in non-toxin producing isolates, and encodes a four-module nonribosomal peptide synthetase (Scott-Craig et al. 1992) that catalyzes the assembly of the four constituent amino acids into HC-toxin (Panaccione et al. 1992). "Additional genes clustered with *HTS1* include *TOXG*, an alanine racemase (Cheng and Walton 2000), *TOXA*, a putative efflux carrier of the major facilitator super family (Pitkin et al. 1996), *TOXC*, a fatty acid synthase involved in the biosynthesis of Aeo (Ahn and Walton 1997), *TOXF*, encoding a branched-chain amino acid amino transferase (Cheng et al. 1999), and *TOXE*, a unique pathway specific transcription factor that binds to the promoters of the known *Tox2* genes (Pedley and Walton 2001). *TOXD* is found as part of the *Tox2* locus, though it is not essential for HC-toxin production (Walton et al. 1998)." The *Tox2* locus extends more than 500 kb, and many of the genes are found in two or three copies (Ahn and Walton 1996; Panaccione et al. 1992; Walton et al. 1998). Several new putative *Tox2* genes have recently been identified through high throughput sequencing of bacterial artificial chromosomes containing the *Tox2* locus (Walton 2006). One encodes a fatty acid synthase alpha subunit that might work in conjunction with TOXC and several encode P450s that may contribute to the synthesis of the epoxide group.

B. Victorin

1. Biological Significance of Victorin

The host specific peptide toxin **victorin** and its producing fungus ***Cochliobolus victoriae*** Nelson are named for the oat (*Avena sativa* L.) variety 'Victoria' and its derivatives, on which ***C. victoriae* causes the severe necrotrophic disease Victoria blight**. Oat varieties derived from 'Victoria' were widely planted in North America by the 1940s as they contained the ***Pc2* gene**, which conferred resistance to specific races of the **crown rust fungus**, ***Puccinia coronata*** Corda. Widespread dependence on this genotype made the emergence of victorin-producing *C. victoriae* particularly devastating (Meehan and Murphy 1946, 1947). The susceptibility of these varieties of oats to *C. victoriae* was due to them containing the **dominant gene *Vb*,** which confers susceptibility to victorin.

Research on the mode of action of victorin has been central to host-specific toxins being considered **necrotrophic effectors** (Wolpert et al. 2002; Friesen et al. 2008). These HSTs have **gene-for-gene relationships** with dominant disease susceptibility proteins that are analogous to **resistance protein** interactions in biotrophic pathogen–plant interactions. In the case of victorin, the susceptibility gene *Vb* and the crown rust resistance gene *Pc2* are very tightly linked, and may be the same gene (Wolpert et al. 2002). It has been proposed that the plant responses induced by ***Vb/Pc2* interactions** may lead to resistance to the biotrophic *P. coronata* but susceptibility to necrotrophic *C. victoria*. Because of the intractability of oat, *Vb/Pc2* has not been cloned, although a single quantitative trait locus for victorin sensitivity was recently identified in a resistance gene rich region of the more tractable barley genome (Lorang et al. 2010).

In *Vb*-containing oat plants, victorin induces resistance-like processes such as an **oxidative burst, phytoalexin synthesis**, and callose deposition (Wolpert et al. 2002) and **programmed cell death**-like symptoms of mitochondrial permeabilization (Curtis and Wolpert 2002, 2004), chromatin condensation (Yao et al. 2001), DNA laddering (Tada et al. 2001; Navarre and Wolpert 1999), rRNA and housekeeping mRNA degradation (Hoat et al. 2006), specific proteolysis of the Rubisco large subunit by multiple proteases (Navarre and Wolpert 1999; Vartapetian et al. 2011), and cell shrinkage and collapse (Yao et al. 2001; Curtis and Wolpert 2004). **Victorin is also a potent inhibitor of glycine decarboxylase** (GDC), and binds the P and H proteins (Wolpert et al. 1994; Navarre and Wolpert 1995). It is likely, however, that this occurs following the onset of programmed cell death (Tada et al. 2005; Curtis and Wolpert 2002), and the relative importance of GDC inhibition in victorin-induced plant disease is unclear.

Sensitivity to victorin has also been observed in the model plant *Arabidopsis thaliana* (Lorang et al. 2004), and the gene conferring sensitivity, ***LOV1***, has been cloned and characterized and shown to indeed encode a protein with the hallmarks of **resistance (R) proteins** (Lorang et al. 2007). LOV1 has coiled-coil, nucleotide-binding site and **leucine-rich repeat domains**, and defence response genes and cell death are induced upon *C. victoriae* application to LOV1-containing plants. These responses require thioredoxin *h*5, although its exact role in signalling is undetermined (Sweat and Wolpert 2007). *LOV1* has highly conserved sequence in *Arabidopsis* populations, which suggests its primary role is as a resistance gene to an *Arabidopsis* pathogen (Sweat et al. 2008), highlighting the fact that necrotrophic effectors such as victorin are likely to subvert resistance mechanisms for biotrophic pathogens.

2. Biosynthesis of Victorin

Victorin is a partially cyclic pentapeptide containing unusual, modified amino acids, some of which are chlorinated. Victorin C, the form of victorin accumulating most abundantly in cultures of *C. victoriae*, contains residues of 5,5-dichloroleucine, 3-hydroxylysine, chloroacrylic acid, 3-hydroxyleucine, and a novel amino acid named victalanine (Wolpert et al. 1985). A number of variations of victorin can be isolated from culture filtrates. These alternate forms of toxin differ in the degree of chlorination and hydroxylation of various amino acid side chains or, in one case, an amino acid substitution (Wolpert et al. 1986).

In Mendelian genetic analyses involving crosses of victorin producing isolates of *C. victoriae* with non-producing mutants of this species, or with compatible isolates of *C. carbonum* race 2, the ability to produce victorin segregated as a single genetic locus, ***Tox3*** (Scheffer et al. 1967). Based on the structural complexity of victorin, it is likely that *Tox3* is a biosynthetic gene cluster; however, to date the genes for biosynthesis of victorin remain unidentified.

C. Ergot Alkaloids

1. Biological Significance of Ergot Alkaloids

A number of ascomycetous fungi produce **ergot alkaloids**, but it is the production by the Clavicipitaceae that has had the greatest impact

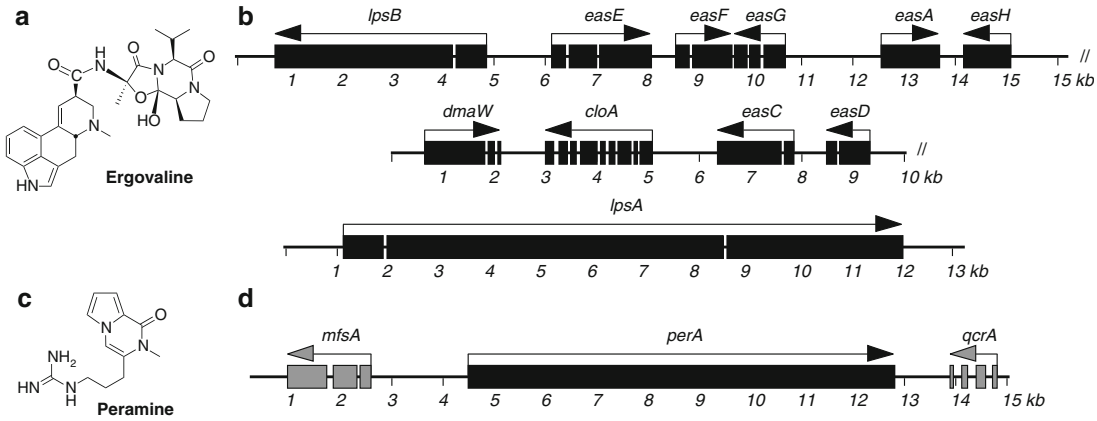

Fig. 4.2. Non-ribosomal peptides ergovaline and peramine produced by epichloid endophtyes of cool season grasses. (**a**) Structure of ergovaline; (**b**) representivive *EAS* locus from *Epichloë festucae* strain Fl1 (drawn from accession numbers JN177500, JN177501 and JN177502); (**c**) structure of peramine; (**d**) representative *PER* locus from *Epichloë festucae* strain Fl1 (drawn from accession number AB205145). A *black line* represents the gene sequence, exons of pathway-associated genes are *black boxes* on the sequence, and flanking genes are *grey boxes*. An *arrow* above the exons indicates gene orientation. The symbol // indicates repetitive sequence not included in locus map and indicates linkage between clusters. The genes encoding non-ribosomal peptide synthestases are *lpsB*, *lpsA* and *perA*

on agriculture. **Plant-associated Clavicipitaceae** are well known to produce a range of **ergot alkaloids** including the non-ribosomally synthesized peptides, **ergopeptines** (Stadler and Stütz 1975; Porter et al. 1981; Bacon 1988). Production of these alkaloids has impacted the human race for centuries because of poisoning by ingestion of contaminated grains infected with ***Claviceps purpurea***, but has also provided medicinal uses in childbirth and treatment of migraines (Merhoff and Porter 1974; De Costa 2002). In fact, studies now suggest that ergot poisoning (**ergotism**) by ingesting **ergotamine** (Fig. 4.1) was the likely culprit in the disturbed nature of people involved in the Salem witch trials in 1692 (Caporael 1976). St Anthony's fire (or the Holy Fire) described the burning sensation people experienced with ergotism that resulted with the vasoconstrictive nature of gangrenous ergotism, while hallucinations were common with the convulsive form (De Costa 2002). Cereal grains are easily infected by *Claviceps* species that produce the toxic ergopeptines, and are now managed for contamination, thus reducing outbreaks of ergotism. More recently, the ergot alkaloids have become the focus of **animal toxicity in agriculture** from production of the **epichloid endophytes** (***Epichloë* and *Neotyphodium* species**) found as a systemic symbiont in forage grasses (Porter et al. 1981). The development of a highly persistent tall fescue line known as 'Kentucky 31' resulted in **livestock toxicity** that resembled ergotism when sown as a monoculture or as a major dietary component (Bacon 1995). Ergovaline (Fig. 4.2), produced by **epichloid endophytes**, is now recognised as the causative agent of fescue toxicosis (Bacon et al. 1986). Fescue toxicosis presents as vasoconstriction, increased body temperature, immune system suppression, reduced forage intake, and low weight gains (Strickland et al. 2009). Reproductive problems can be severe in pregnant mares when grazing toxic fescue (Cross 2009).

2. Biosynthesis of Ergot Alkaloids

The **ergot alkaloids** are indole-derived compounds that start with the formation of dimethylallyltryptophan from the precursors tryptophan and dimethylallyl diphosphate. Subsequence cyclisation and enzymatic steps convert to lysergic acid, where the formation of cyclicpeptines proceeds via non-ribosomal peptide synthetases. Genes required for ergot alkaloid production have been cloned from a number of epichloid endophyte (Fig. 4.2) and *Claviceps* species, where they are found

to be present in co-regulated gene clusters (Tudzynski et al. 1999; Haarmann et al. 2005; Fleetwood et al. 2007; Wang et al. 2004; Tsai et al. 1995). Similarity is found between the gene clusters of *C. purpurea* and with those within the **epichloae** by the presence of likely orthologous genes (Fleetwood et al. 2007). However, modifications encoded by the NRPS translate into specificity differences for these enzymes and the production of diverse ergopeptines (Haarmann et al. 2005; Panaccione et al. 2001) such as ergotamine (Fig. 4.1), ergocryptine, and ergocristine produced by *Claviceps* species, and **ergovaline** (Fig. 4.2) produced by some epichloae.

D. Other Peptides

1. AM-toxin

Alternaria blotch of apple (*Malus domestica* Borkh.) is caused by the **apple pathotype of *Alternaria alternata*** (Fr.:Fr.) Keissler (previously described as a virulent form of *A. mali* Roberts). This disease is one of the most serious diseases of apple in Japan (Sawamura 1966), and has been increasing in incidence worldwide (Filajdic and Sutton 1991; Sawamura 1990), most recently in the southeastern United States (Abe et al. 2010). The causal fungus produces multiple host-specific toxins named **AM-toxin** I (**alternariolide**), AM-toxin II, and AM-toxin III, which selectively affect a narrow range of susceptible apple cultivars (Kohmoto et al. 1974). AM-toxin I is the most abundant and cytotoxic (Kohmoto et al. 1976). The chemical structure of AM-toxin I has been elucidated (Okuno et al. 1974; Ueno et al. 1975, 1977) and consists of a four-membered **cyclic depsipeptide**, consisting of one standard amino acid, L-alanine (L-Ala), and three unusual residues, L-α-amino-methoxyphenyl-valeric acid (L-amv), L-α-hydroxy-isovaleric acid, and dehydroalanine.

AM-toxin has two primary sites of action: the cell wall/plasma membrane interface (Park et al. 1977) and the chloroplast (Park et al. 1981). Dysfunction of either one of these organelles leads to suppression of the host defense reaction, fungal penetration, and induction of disease (Kohmoto and Otani 1991; Shimomura et al. 1991). Susceptible apple cultivars show tissue specificity towards photosynthetic tissues, in response to AM-toxin, and the photosynthetic activity of chloroplasts isolated from leaves is inhibited. This suggests that the chloroplast is the primary target site of AM-toxin (Tsuge et al. 2013).

Disease symptoms first appear on leaves in late spring or early summer and can result in up to 60 % defoliation on susceptible cultivars (Filajdic and Sutton 1991). Apple breeding experiments have suggested that susceptibility to Alternaria blotch is controlled by a single dominant gene (Saito and Niizeki 1988). This supports the presence of a receptor for AM-toxin on the plasma membrane and/or chloroplast of susceptible cells, but to date this receptor has not been identified.

A degenerate PCR based strategy was used to identify the AM-toxin synthetase gene, ***AMT1***, involved in AM-toxin biosynthesis (Johnson et al. 2000a). "*AMT1* is 13.1 kb in length, is present in multiple copies, only one of which appears active, and appears to be part of a large duplication (Johnson et al. 2000a) reminiscent of the HC-toxin biosynthetic locus (*Tox2*, described above). *AMT1* encodes a 479-kDa NRPS containing four catalytic domains responsible for the activation of each residue in AM-toxin." Gene knockout experiments have proven a crucial role for *AMT1* in the biosynthesis of AM-toxin (Johnson et al. 2000a), and the gene is required and present in all AM-toxin-producing strains (Johnson et al. 2000b). Apple pathotype strains also have a homolog, ***AMT2***, of *AFTS1* of the strawberry pathotype (Ito et al. 2004), which catalyses the conversion of 2-keto-isovaleric acid to 2-hydroxy-isovaleric acid. Interestingly, both *AMT1* and *AMT2* reside on **conditionally dispensable (CD) chromosomes** in apple pathotype strains (Johnson et al. 2001; Harimoto et al. 2007). Expression analysis of genes residing on these CD chromosomes identified a number of other co-regulated genes that were shown to be required for AM-toxin biosynthesis (Harimoto et al. 2007). Sequencing of CD chromosomes from AM-toxin-producing strains has identified a putative cluster of 15 *AMT* genes that are present in one to four copies (Tsuge et al. 2013).

2. Peramine

Peramine (Fig. 4.2), a pyrrolopyrazine, is a non-ribosomally synthesized peptide produced exclusively by the **epichloid endophytes** in association with their grass host (Siegel et al. 1990). Production of peramine is considered to have a positive impact on agriculture as an **insect feeding deterrent** and as such, peramine producers have been selected for and utilized in **endophyte-infected perennial ryegrass** cultivar development (Fletcher 1986, 1999).

Two amino acids, proline (or proline precursor such as pyrroline-5-carboxylate) and arginine, are thought to be precursors for peramine production catalysed via a NRPS. The ***perA* gene** (Fig. 4.2) responsible for peramine production encodes a single two-module-NRPS enzyme, containing adenylation (A1), thiolation (T1), condensation (C), adenylation (A2), methylation (M), thiolation (T2), and reductase (R) domains (Tanaka et al. 2005). Gene knockout of *perA* confirmed the role of PerA in peramine production, and confirmed the impact this metabolite had on Argentine stem weevil (ASW; *Listronotus bonariensis*) (Tanaka et al. 2005), a known destructive pest of grasses that was introduced in New Zealand in the early 1920s. "In choice assays, ASW avoided peramine-containing perennial ryegrass produced by the resident endophyte, and instead significant feeding damage could be detected on endophyte-free or endophyte-infected perennial ryegrass containing the Δ*perA* mutant. The bioprotective role of **peramine** makes it an important consideration when developing endophyte-infected cultivars of the forage grasses, perennial ryegrass (*Lolium perenne*) and tall fescue (*Lolium arundinaceum*) (Latch and Christensen 1985; Easton 2007)."

A wide distribution of epichloid endophytes symbiotic with different host tribes are known to produce peramine (Clay and Schardl 2002; Schardl et al. 2012), which suggests a strong selective pressure for maintenance of this alkaloid (Schardl 1996). However, sequence analysis of the *perA* gene from the non-peramine producer *E. festucae* E2368 revealed deletion of the reductase domain, mediated by a repetitive element (Fleetwood et al. 2007) that was common in other epichloae. (Young and Takach, unpublished)

III. Polyketide-Derived Phytotoxins

Polyketides are naturally occurring compounds that are commonly produced by plants and actinomycetes in addition to fungi. They are highly diverse in structure, ranging from simple aromatic compounds such as the tetraketide orsellinic acid to complex polycyclic compounds such as **T-toxin** (this chapter) where more carbon atoms are incorporated, and the final compound has been the subject of several reduction and dehydration reactions (Cox 2007). "Other examples of polyketides include antibiotics (e.g., tetracycline), antifungal agents (e.g., griseofulvin), immunosuppressive agents (e.g., rapamycin), mycotoxins (e.g., aflatoxin), and phytotoxins. In addition, many other metabolites (e.g., cyclosporine and HC-toxin) that are synthesized via an independent pathway also contain polyketide-derived moieties."

Although polyketides are diverse in structure, they all share a common biosynthetic mechanism involving **polyketide synthases** (PKS). **PKS** are multifunctional enzymes that synthesize the carbon backbones common to this class of molecules (reviewed in Cox 2007; Evans et al. 2011). PKSs are closely related to **fatty acid synthases** (FAS), and like them, catalyze repeated condensations of acyl CoA esters to build a linear polyketide chain. Linear fatty acids are essentially fully reduced polyketides derived exclusively from acetate and malonate precursors. **AK-toxin** (described below) from *Alternaria alternata* (Fr.:Fr.) Keissl. Japanese pear pathotype contains a fatty acid backbone, most probably synthesized by a FAS. It will therefore be considered here.

Unlike FAS, which use acetate and malonate as starter and extender units respectively, PKS can use a variety of chain starter units (e.g., acetate, propionate, benzoate, cinnamate, and amino acids), and a range of extender units (e.g., malonate, methylmalonate, or ethylmalonate), and also polyketide products from the activity of other PKS such as in the synthesis of **zearalenone** (see this chapter). "PKS synthesis is initiated by an acyl transferase (AT) domain that selects the starter and extender

units, and tethers them as thiol esters to the phosphopantetheine moiety on the ACP domain. Following initiation by ketosynthase (KS) and elongation by ACP, each condensation can be followed by a cycle of ketoreduction (KR, catalyzed by ß-ketoacyl reductase), dehydration (DH, mediated by a dehydratase), and enoyl reduction (ER, via enoyl reductase) (Wakil 1989; Hopwood and Sherman 1990; Hopwood and Khosla 1992; Cox 2007; Evans et al. 2011). The polyketide chain is typically released from the PKS by cleavage of the thiol ester by thioesterase (TE), and may be processed by further cyclization (Cyc) or c- methyl transferase (MT) domains among others. Not all PKSs have or use all of the above-mentioned catalytic domains and this, together with variation in starter and extender units, chain length, reductive modification, and stereochemistry, generates the structural diversity seen in PKS products."

There are three major classes of PKS enzymes, all of which are found to a greater or lesser extent in fungi (Evan 2011; Cox 2007). Modular **type I PKS** are large polypeptides that contain separate catalytic domains for each reaction catalyzed in the biosynthetic pathway. "The active sites for each round of processing are encoded by a single open reading frame containing DNA modules ordered as they are required biochemically. Type I PKS can be further subdivided onto non-reducing, partially reducing and highly reducing forms depending on the presence or absence of the KR, DH, and ER domains that catalyze the reducing functions (Cox 2007; Evans et al. 2011)." **Type II PKS** contain fewer active domains, which are encoded only once in the DNA open reading frame. These domains are used iteratively as required. **Type III PKS** are predominantly regarded as plant enzymes, but have also been found in bacteria and now fungi (Seshime et al. 2005). "Filamentous fungi, such as those discussed in this chapter, contain PKS from all three classes, with many structurally resembling type I enzymes. However, enzymatically they also resemble type II enzymes since they have only one domain per catalytic activity, which must be repeatedly used, for each catalytic cycle (Tkacz 2000)."

"Genome analysis of filamentous fungi indicates that the genomes of most species contain PKS genes (and clustered genes) capable of synthesizing a significant array of diverse PKS products, most of which are yet to be characterized (Kroken et al. 2003; Ma et al. 2010; Brown et al. 2012; Ahuja et al. 2012)." This chapter will cover a number of the most significant polyketide toxins in the agricultural setting, including T-toxin, **fumonisins** and **AAL toxin**, **zearalenone**, **AK-toxin**, and **cercosporin**.

A. T-toxin

1. Biological Significance of T-toxin

T-toxin (Fig. 4.3) is a host-specific polyketide toxin that played a role of central significance in the **southern corn leaf blight** (**SCLB**) epidemic of 1970 in the USA. This epidemic caused substantial losses in the corn crops of the warmer reaches of the eastern seaboard, and illustrated the critical implications of genetic uniformity in our major crops. The SCLB epidemic was caused by a previously unknown race of ***Cochliobolus heterostrophus*** (Drechs.) Drechs., **race T**, which produces T-toxin. The less virulent race (race O) of *C. heterostrophus* does not synthesise T-toxin. Whereas races T and O produce small necrotic lesions on a wide range of maize varieties, race T isolates alone are highly virulent specifically on maize varieties carrying the **Texas type male sterile cytoplasm (T-cms)**, and produce large necrotic lesions with chlorotic streaking. As described in detail below, the interaction between T-toxin and a protein (**URF13**) in T-cms mitochondria results in the increased virulence of *C. heterostrophus* race T in maize carrying this protein.

T-toxin is the general name given to a family of partially reduced linear polyketols ranging from 35 to 45 carbons in length (Kono and Daly 1979) (Fig. 4.3), all of which have similar toxicity against T-cms maize (Turgeon and Baker 2007). The distantly related maize pathogen *Didymella zeae-maydis* (Mukunya and Boothroyd), formally *Mycosphaerella zeae-maydis* (Mukunya and Boothroyd), produces a similar family of compounds called PM-toxins

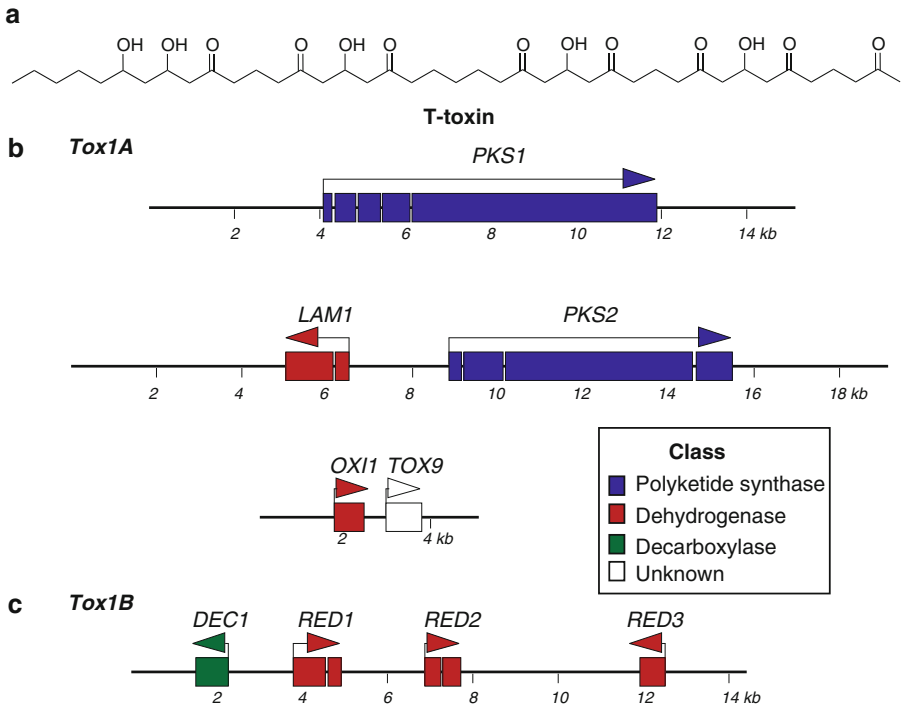

Fig. 4.3. The polyketide T-toxin (C_{41}) produced by *Cochliobolus heterostrophus* and genes found at the *Tox1A* and *Tox1B* loci that are required for T-toxin production. (**a**) T-toxin; (**b**) genes found at the *Tox1A* locus (accession numbers U68040, DQ186598 and FJ943499); (**c**) genes found at the *Tox1B* locus (accession number AF525909). A *black line* represents the gene sequence, exons of pathway-associated genes are *coloured boxes* on the sequence representing the different encoded gene classes, and an *arrow* above the exons indicates gene orientation

(chain lengths ranging from 33 to 35 carbons) that also bind to URF13 and are required specifically for virulence on T-cms maize (Danko et al. 1984; Kono et al. 1983). *D. zeae-maydis* is restricted to the cooler northern regions of the USA, and has never had the impact on maize production that the more widely distributed but predominantly southern *C. heterostrophus* race T had (Rhoads et al. 1998).

Sensitivity to T-toxin, along with resulting susceptibility to race T of *C. heterostrophus*, is due to the presence of a small chimeric protein (URF13) in the inner mitochondrial membrane of T-cms maize (Levings 1990). URF13 is the product of a novel gene (**T-*urf13***) that is proposed to have arisen through recombination of mitochondrial DNA (containing the promoter of the gene for ATPase subunit 6 fused to fragments of the 26S ribosomal RNA gene) and additional sequences with unspecified original function (Dewey et al. 1987; Wise et al. 1987). In addition to conferring sensitivity to T-toxin, URF13 also appears to be the factor responsible for Texas-type **cytoplasmic male sterility** (Levings 1990). Mutations in T-*urf13* restore male fertility and host insensitivity to the toxin (Wise et al. 1987). Because of reduced costs and labor associated with male sterility, T-cms was bred into the majority of hybrid maize germplasm, making the later appearance of *C. heterostrophus* race T exceptionally devastating.

The critical interaction between T-toxin and URF13 for disease progression has been further demonstrated by expressing T-*urf13* in organisms that are not normally sensitive to T-toxin. This was first demonstrated in *Escherichia coli,* where URF13-expressing cells responded to T-toxin in a similar manner to T-cms mitochondria, exhibiting a significant decrease in glucose-driven respiration, leakage of small molecules and spheroplast swelling (Braun et al. 1989; Dewey et al. 1988).

The untransformed control strain was not sensitive to the toxin. Likewise, in transgenic tobacco (*Nicotiana tabacum* L.), expression of URF13, regardless of its cellular localization, converted plants to T-toxin sensitivity, resulting in light-dependent bleaching on exposure to T-toxin (von Allmen et al. 1991). Conversely, yeast (*Saccharomyces cerevisiae* Hansen) cells expressing heterologous URF13 protein only become sensitive to T-toxin when URF13 is targeted to mitochondria (Huang et al. 1990). Exposure to T-toxin resulted in a greatly reduced growth rate relative to control strains (Huang et al. 1990). The ability of URF13 to confer sensitivity to T-toxin in organisms ranging from bacteria to fungi and plants indicates this protein alone is sufficient to confer the toxin-sensitive phenotype.

URF13 has been immuno-cytochemically localized in the inner mitochondrial membrane (Hack et al. 1991; Korth et al. 1991). Cross-linking studies (Kaspi and Siedow 1993; Korth et al. 1991) indicate that URF13 forms oligomers and that four molecules of URF13 associate to form an open pore upon binding of T-toxin or PM-toxin. Tritiated toxin binds directly to URF13 in a specific and saturable manner in cmsT mitochondria and in *E. coli*-expressing T-*urf13* (Braun et al. 1990). Binding of T- or PM-toxin to the URF13 oligomer results in mitochondrial membrane permeabilisation and ion leakage (Korth et al. 1991; Levings 1990; Rhoads et al. 1998). Compromised mitochondria cease ATP synthesis, and the host cell dies.

2. Biosynthesis of T-toxin

Similar to the genetics of the peptide toxin-producing *Cochliobolus* species described above, genetic studies on *C. heterostrophus* races T and O indicated that T-toxin was initially defined as a single Mendelian locus called ***Tox1*** (Leach et al. 1982; Lim and Hooker 1971). More recently it has become clear that the *Tox1* locus is located on two different chromosomes and is inseparable from a reciprocal translocation break point on those chromosomes (Chang and Bronson 1996; Kodama et al. 1999; Tzeng et al. 1992). The loci are designated ***Tox1A*** and ***Tox1B*** (Kodama et al. 1999). A detailed history of the elucidation of the complex *Tox1* locus is given in Turgeon and Baker 2007. Molecular analyses have revealed that *Tox1* contains genes that are located on two separate chromosomes, is embedded in A+T rich DNA (Turgeon and Baker 2007) and is associated with a significant insertion (1.2 Mb) in toxin-producing isolates (Kodama et al. 1999).

The first gene to be positively identified as being involved in T-toxin biosynthesis was the type I ***ChPKS1*** from *Tox1A* (Yang et al. 1996). This gene was originally tagged by the restriction enzyme mediated integration (REMI) procedure (Lu et al. 1994). Sequencing of the vector integration site revealed a 7.8-kb open reading frame that, in addition to encoding the domains required for synthesis of the primary polyketide (β-ketoacyl synthase, acyl transferase, and acyl carrier protein), also encodes domains for all the keto processing functions (Yang et al. 1996), which makes this PKS unique among fungal PKSs (Yoder 1998). This PKS gene, ***PKS1***, is present in all race T isolates and absent from all race O isolates of *C. heterostrophus*. Disruption of *ChPKS1* in a wild-type race T isolate resulted in a T-toxin minus phenotype, with virulence reduced to the level of T-non-toxin producing race O isolates (Yang et al. 1996). *D. zeae-maydis* which produces the polyketide PM-toxin contains a similar PKS (62 % identical at the nucleotide level) that is required for PM-toxin production and pathogenicity to cmsT maize (Yoder 1998; Yun et al. 1998). Later, using comparative cDNA-based methodology, a further PKS encoding gene, ***PKS2***, was isolated and also shown to map to the *Tox1A* locus of race-T (Baker et al. 2006). This gene too is essential for synthesis of T-toxin and for high virulence in T-cms corn, making it likely that both PKS are required for T-toxin biosynthesis. PKS2 shares only 32 % identity with PKS1 and is shorter in length, partly as a result of the lack of a degenerate methyltransferase domain that is present in *PKS1* but not thought to be functional (Baker et al. 2006; Turgeon and Baker 2007). Although both genes belong to a clade of fungal PKS genes encoding enzymes that synthesize linear and reduced polyketides, they are sufficiently different to eliminate gene duplication as a mechanism to explain the appearance of the second PKS. (Baker et al. 2006)

Positional cloning was also used to isolate the first two *Tox1*-associated genes from the *Tox1B* locus, ***DEC1*** and *RED1* (Rose et al. 2002). *DEC1* only was required for T-toxin biosynthesis and full virulence on cmsT maize (Yoder 1998; Rose et al. 2002). Based on DNA sequence analysis, *DEC1* is hypothesized to encode a decarboxylase that removes the terminal carboxylic acid group from the polyketide precursor, resulting in chains with odd numbers of carbons (Yoder 1998). Inactivation of *DEC1* by

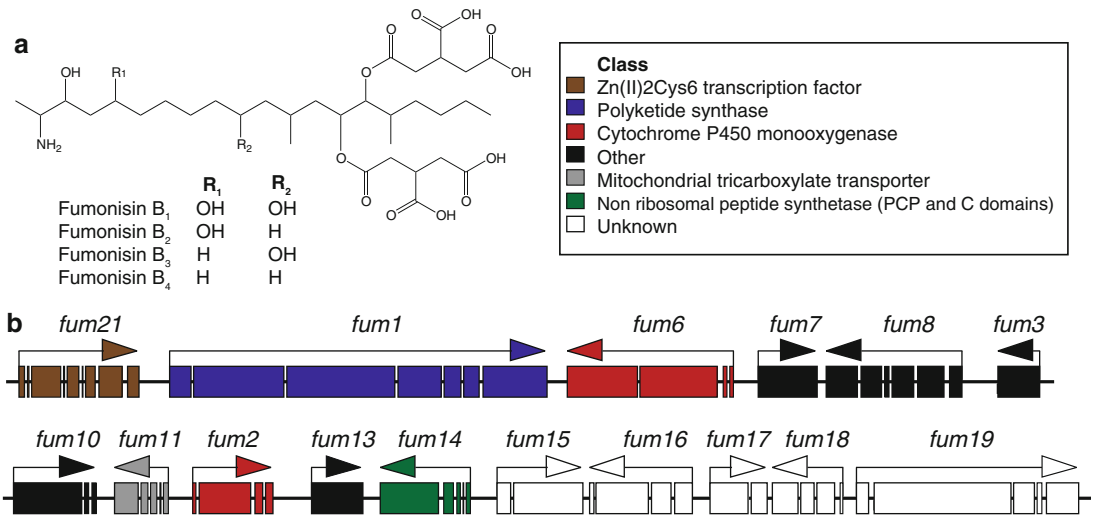

Fig. 4.4. The fumonisin B group and associated gene locus from *Gibberella moniliformis*. (**a**) Representitive core structure for fumonisin B and required substitutions; (**b**) the *FUM* locus from *G. moniliformis* (accession number AF155773) required for fumonisin production. A *black line* represents the gene sequence, exons of pathway-associated genes are *coloured boxes* on the sequence representing the different encoded gene classes, and an *arrow* above the exons indicates gene orientation

gene disruption leads to lack of toxin production and reduced virulence (Yoder 1998).

Three further genes at the *Tox1A* locus (*LAM1*, *OXI1* and *TOX9*) and two further genes at the *Tox1B* (*RED2, RED3*) have been identified through a combination of comparative cDNA expression analysis of race T vs race O strains plus the availability of the *C. heterostrophus* race T genome (Inderbitzin et al. 2010) (Fig. 4.3). A total of nine T-toxin genes have been reported at this time (Fig. 4.3). All genes, when deleted individually either eliminate toxin production (*PKS1, PKS2, DEC1, TOX9*) or reduce it to various extents (*RED1, RED2, RED3, LAM1, OXI1*). Deletions of combinations of genes in the latter category nearly completely eliminate toxin production, indicating a role for all genes (Inderbitzin et al. 2010). All nine genes are unique to race T isolates of *C. heterostrophus* and in crosses with race O, *Tox1*-associated sequences segregate together as part of a four-armed linkage group. In crosses between race T isolates, the loci can segregate independently because homologous sequences are present along the lengths of the translocated chromosomes (Kodama et al. 1999).

B. AAL-toxin and Fumonisin

1. Biological Significance of AAL-toxin and Fumonisin

In the group of highly reduced polyketides, host-specific (-selective) **AAL-toxins** and mycotoxin **fumonisins** (Fig. 4.4) are structurally related, and were originally isolated from the tomato pathotype of ***Alternaria alternata*** (synonym *A. alternata* f. sp. *lycopersici*, synonym *A. arborescens*) and from ***Fusarium verticillioides*** (synonym *F. moniliforme*, teleomorph *Gibberella moniliformis*, synonym *G. fujikuroi* mating population A) respectively (Akamatsu et al. 1997; Bottini et al. 1981; Gilchrist and Grogan 1976; Bezuidenhout et al. 1988). Both AAL-toxins and fumonisins contain a highly reduced, dimethylated, acyclic polyketide backbone chain with one nitrogen, three or four hydroxyl, two methyl and one or two tricarballylic ester functions at positions along the backbone. The main differences are the length and the modifications of the polyketide backbone. The backbone of AAL-toxins is derived from an octaketide precursor (C-2 to -17), whereas that of fumonisins is derived from a nonaketide precursor (C-3 to -20) (Caldas et al. 1995; Musser and Plattner 1997). The terminal

C-1 and amino group of AAL-toxins are derived from glycine, and the two terminal carbons, C-1 and C-2, as well as the C-2 amino group, of fumonisins are from alanine (Caldas et al. 1995; Musser and Plattner 1997). In addition, AAL-toxins and fumonisins can be divided into structurally distinct groups (AAL-toxin TA, TB, TC, TD, TE, and acetyl TA; fumonisin A, B, C, and P) (Caldas et al. 1995; Musser and Plattner 1997). Among those, AAL-toxin TA and fumonisin B1 (Fig. 4.4) are considered to be major toxins in each pathogen in terms of biological activities and productivities (Caldas et al. 1995; Musser and Plattner 1997).

Fumonisins were first isolated as mycotoxins in South Africa in 1988 from cultures of *F. verticillioides* obtained from infected maize in an area with a high esophageal cancer rate (Bezuidenhout et al. 1988; Gelderblom et al. 1988). Fumonisins have been shown to be associated with leukoencephalomalacia, pulmonary edema syndrome, acute nephrosis in animals, and were shown to have cancer-promoting activity (Gelderblom et al. 1988). In addition to the animal toxicosis, several diseases of maize, including seedling blight and ear rot, are attributed to *F. verticillioides*, and the potential role of fumonisins in plant–pathogen interactions has been investigated, with varying results. Desjardins et al. (1995) suggested that fumonisins increased the virulence of *F. verticillioides* but were not necessary or sufficient for disease development. On the other hand, a significant positive correlation between leaf lesion development on maize seedlings and the production of fumonisins by *F. verticillioides* has also been reported (Williams et al. 2006). Furthermore, transformation-mediated complementation of a fumonisin biosynthetic gene cluster in fumonisin-nonproducing strains restored both fumonisin production and pathogenicity on maize seedlings (Glenn et al. 2008). The results indicate that fumonisin production by *F. verticillioides* is important for disease development.

The tomato pathotype of *A. alternata* causes Alternaria stem canker of tomato, and the disease is characterized by the formation of dark brown cankers on the stem of tomato plants, usually in association with interveinal necrosis on the leaves (Gilchrist and Grogan 1976; Grogan et al. 1975). Production of AAL-toxins by the pathogen is responsible for those typical necrotic lesions found only on susceptible tomato cultivars. In the tomato pathotype of *A. alternata*–tomato interactions, a major factor in pathogenicity is the production of AAL-toxin that is capable of inducing cell death only in susceptible cultivars (Akamatsu et al. 1997; Brandwagt et al. 2000; Yamagishi et al. 2006). AAL-toxins can also induce infection of nonpathogenic *A. alternata* on susceptible tomato cultivars at low concentrations (Yamagishi et al. 2006). Moreover, an AAL-toxin deficient mutant was unable to cause symptoms on susceptible tomato (Akamatsu et al. 1997). These results indicate AAL-toxins play an essential role in pathogenicity of the pathogen.

AAL-toxins and fumonisins are **sphinganine-analog mycotoxins** (SAMs or SAMTs), which are toxic to some plant species and mammalian cells (Gilchrist et al. 1995). They cause apoptosis in susceptible tomato cells and mammalian cells by inhibiting **ceramide biosynthesis** (Gilchrist et al. 1995; Spassieva et al. 2002; Wang et al. 1996). AAL-toxins and fumonisins are potent and specific inhibitors of the sphinganine N-acetyltransferase (acyl CoA-dependent ceramide synthase), a key enzyme in de novo **sphingolipid** biosynthesis (Wang et al. 1996). Animal and plant diseases caused by those toxins are a consequence of disrupted sphingolipid metabolism (Brandwagt et al. 2000; Gilchrist et al. 1995; Wang et al. 1996).

In tomato and other plants, insensitivity to AAL-toxin and fumonisin is conferred by the ***Asc1*** (Alternaria stem canker resistance gene 1) gene, a homologue of the yeast longevity assurance gene *Lag1*, which mediates resistance to SAM-induced apoptosis by the production of alternative ceramide (Brandwagt et al. 2000). Yeast *Lag1* and *Lac1* (longevity assurance gene cognate 1) are thought to be encoding ceramide synthase, the target site of SAMs, and each gene can compensate for the absence of the other: deletion of either gene doesn't affect ceramide synthase activity, but deletion of both genes markedly impairs activity. *Asc1* partially compensated the growth defect in the *Lag1*/

Lac1 deleted yeast strain, indicating the common function of those plant and yeast ceramid synthase genes (Mullen et al. 2011; Spassieva et al. 2002). It has been also shown that sensitivity/insensitivity to AAL-toxin is governed by *Asc1*/*Lag1* homologues in other plants such as *Arabidopsis thaliana* and *Orobanche cumana* (de Zélicourt et al. 2009; Gechev et al. 2004).

2. Biosynthesis of AAL-toxin and Fumonisin

Fumonisins are synthesized, at least in part, through the activity of enzymes encoded by the fumonisin biosynthetic (*FUM*) gene cluster (Fig. 4.4). The cluster consists of 15 coregulated genes designated *FUM1–FUM3* (formerly *FUM5*, *FUM12* and *FUM9*, respectively Butchko et al. 2003; Proctor et al. 2003, 2006), *FUM6–FUM8*, *FUM10, FUM11,* and *FUM13–FUM19* and a transcriptional factor, *FUM21*, which are localized with 42.5 kb region located on chromosome I of *F. verticillioides* (Proctor et al. 2003) (Fig. 4.4).

The roles of some *FUM* genes have been confirmed by gene deletion (Bojja et al. 2004; Butchko et al. 2006; Ding et al. 2004; Proctor et al. 1999, 2003, 2006), and heterologous expression (Yi et al. 2005; Zaleta-Rivera et al. 2006). Deletion of *FUM1*, *FUM6*, or *FUM8,* which are predicted to encode a polyketide synthase (PKS), cytochrome P450 monooxygenase, and amino transferase, respectively, blocked accumulation of all fumonisins (Bojja et al. 2004; Proctor et al. 1999). Deletion mutants of *FUM2* (cytochrome P450 monooxygenase) produced only fumonisins B2 and B4 (Fig. 4.4), which lack the C-10 hydroxyl, suggesting that *FUM2* is involved in fumonisins C-10 hydroxylation (Proctor et al. 2006). The diruptants of *FUM3* (dioxygenase) produced only fumonisins B3 and B4, which lack the C-5 hydroxyl, indicating that *FUM3* is involved in fumonisin C-5 hydroxylation (Ding et al. 2004). The mutants of *FUM13* (ketoreductase) produced 3-keto homologues of fumonisins B3 and B4, indicating that *FUM13* is involved in the reduction of the C-3 carbonyl to a C-3 hydroxyl (Butchko et al. 2003; Yi et al. 2005). *FUM10* (fatty acyl-CoA) and *FUM14* (NRPS with only the PCP and C domains) deletion mutants produced hydrolyzed fumonisins B3 and B4, which lack tricarballylic moiety (Butchko et al. 2006), and heterologous expression of Fum14p showed that Fum14p can convert hydrolyzed fumonisins B3 and B4 to fumonisins B3 and B4 respectively (Zaleta-Rivera et al. 2006). These results suggest that Fum10p catalyzes the CoA activation of tricarballylic acid and Fum14p catalyzes the esterification of the CoA-activated tricarballylic acids to the fumonisin backbone. *FUM7* (dehydrogenase) deletion mutants gave multiple metabolites with slightly different molecular masses compared with fumonisins B1, B2 and B3 (Butchko et al. 2006). Deletion of *FUM11* (mitochondrial tricarboxylate transporter) resulted in the accumulation of the half-hydrolyzed forms of fumonisins B3 and B4 (Butchko et al. 2006). Deletion of the remaining *FUM* genes (*FUM15-19*) did not affect fumonisin production, except for a subtle change of the fumonisins B1 and B3 ratio in the *FUM19* deletion mutants (Proctor et al. 2003). The mutants of *FUM21*, which is predicted to encode a Zn(II)2Cys6 transcription factor, lost B series fumonisins production with no expression of *FUM1* and *FUM8*. Analysis of *FUM21* cDNAs identified four alternative splice forms (ASFs), and microarray analysis indicated that the ASFs were differentially expressed (Brown et al. 2007). Based on these data, a complete model for fumonisin biosynthesis by the *FUM* cluster has been proposed. (Butchko et al. 2006)

The first AAL toxin biosynthesis gene was cloned from the tomato pathotype of *A. alternata* using a degenerate PCR approach based upon conserved domains of fungal PKS genes. Sequencing revealed a type I PKS gene of 7.8 kb in length, designated ***ALT1*** (Akagi et al. 2009a, b; Akamatsu et al. 2003; Yamagishi et al. 2006). A gene deletion of *ALT1* in the tomato pathotype led to AAL-toxin-minus mutants that had lost virulence to susceptible tomatoes. The AAL-toxin biosynthetic (ALT) gene cluster was sequenced from the tomato pathotye as a BAC clone spanning 120 kb, and includes at least 13 genes (Akagi et al. 2009a, b; Akamatsu et al. 2003) which showed similarity to the genes in the *FUM* cluster from *G. moniliformis* (Proctor et al. 2003). Deletion of several genes within the cluster, including *ALT1* (type I PKS), *ALT2* (cytochrome P450 monooxygenase), *ALT4* (aminotransferase), *ALT6* (short-chain dehydrogenase/reductase), and *ALT13* (Zn(II)2Cys6 transcription factor), provided evidence for a role in AAL-toxin biosynthesis by the pathogen (Akagi et al. 2009a; Akamatsu et al. 2003; Akagi et al., unpublished).

The *ALT* gene cluster resides on a 1.0 Mb conditionally dispensable chromosome (CDC) which is present only in pathogenic and AAL-toxin-producing strains (Akagi et al. 2009a, b; Akamatsu et al. 1999, 2003; Johnson et al. 2001). The origin and evolution of CDCs has been an intriguing issue in the study of plant–microbe interactions (Covert 1998; Johnson et al. 2001; Han et al. 2001; Hatta et al. 2002; Mehrabi et al. 2011). In addition to

the tomato pathotype, other *A. alternata* pathotypes (e.g., the Apple pathotype described above) also harbour CDCs of less than 1.7 Mb, whereas nonpathogenic isolates do not (Akamatsu et al. 1999). In the tomato pathotype, at least, the genetic origin of the CDC appears to be different from other chromosomes in the genome, and through protoplast fusion CDCs could be transmitted from one strain to another and stably maintained in the new genome (Akagi et al. 2009a, b). CDCs can thus be regarded as "pathogenicity chromosomes" which may be horizontally transferred between strains, providing a possible mechanism whereby new pathogens arise in nature.

C. Zearalenone

1. Biological Significance of Zearalenone

Fusarium species are common contaminants of feed worldwide, and produce a number of notorious mycotoxins including trichothecenes and fumonisins that severely affect humans and animals (Glenn 2007, Bennett and Klich 2003). In contrast, **zearalenone** (6-[10-hydroxy-6-oxo-*trans*-1-undecenyl-]-B-resorcyclic acid lactone) (ZEA), produced by *F. graminearum*, *F. pseudograminearum, F. culmorum*, *F. equiseti, F. semtectumi* and *F. crookwellense* (Fink-Gremmels and Malekinejad 2007; Glenn 2007), is not strictly an animal toxin, rather is a biologically potent **mycoestrogen** (Bennett and Klich 2003) that structurally resembles the vertebrate oestrogen 17β-oestradiol. Zearalenone and its metabolic breakdown products, α- and β-zearalenol (ZOL), bind to oestrogen receptors of mammals, and disrupt the normal regulatory processes of endogenous oestrogen (Smith and Morris 2006). Zearalenone is also an anabolic agent that stimulates growth in cattle and sheep, and α- zearalenol has been developed as a growth promotant (Ralgro). Nevertheless, accidental ingestion of these compounds, particularly around mating time, can affect animal fertility severely. Pigs are particularly sensitive, and low concentrations cause (among other maladies) ovarian atrophy, prolonged oestrous intervals, decreased fertility and stillbirth or delivery of weak piglets (Fink-Gremmels and Malekinejad 2007). Some effects appear to be intergenerational (Schoevers et al. 2012). In ewes, reduced ovulation rate and fertilization of ovulated eggs collectively result in lower lambing percentages. Ingestion of as little as 1 mg/day over 5–7 days is sufficient to reduce lambing rate by about 5 %; 6 mg/day over a month will reduce oestrus in ewes by 60 % and ovulation by 40 % (Towers and Sprosen 1993). Endocrinology data in ewes indicate that exposure to zearalenone reduces levels of follicle-stimulating hormone in a dose-dependent manner (Smith and Morris 2006), which is consistent with impacts of zearalenone on fertility in mammals (Smith and Morris 2006). Higher doses of ZEA enlarge mammary glands and can cause sterility in prepubertal heifers (Coppock et al. 1990), and also reduce conception rate in heifers (Diekman and Green 1992).

While the principal mechanism of ZEA ingestion by animals is through infected grains, *Fusarium* species are also common contaminants of forage plants, particularly in the summer and autumn in New Zealand (DiMenna et al. 1987; Reed et al. 2004) and Australia (Reed et al. 2004). ZEA concentrations in summer through late autumn of up to 4 mg/kg of pasture herbage are not uncommon, are sufficient to reduce fertility in grazing ewes, and peak at the time when mating occurs (DiMenna et al. 1987; Reed et al. 2004). Individual samples as high as 25 mg/kg have been recorded (Towers and Sprosen 1993). More than 50 % of flocks tested in 1991 and 1992 in New Zealand had zearalenone contamination sufficient to reduce reproductive performance and lambing percentages by 5–50 % (Towers and Sprosen 1993).

Zearalenone is also implicated as a phytotoxin, and can affect photosynthesis and growth in some plants (Berestetskiy 2008; Kościelniak et al. 2011). Further research will clarify the implications of zearalenone biosynthesis for plant health in the long term.

2. Biosynthesis of Zearalenone

ZEA is a fungal macrocyclic polyketide of the **resorcylic acid lactone** family, a unique group of polyketides that possess a wide range of potent biological activities. "Subsequent to the sequencing of the *Fusarium graminearum* genome, targeted deletion of PKS genes in *F. graminearum* indicated that two open reading frames, PKS13 (or ZEA1) and PKS4 (or ZEA2),

are required for ZEA biosynthesis (Kim et al. 2005; Gaffoor and Trail 2006). PKS13 encodes a polypeptide with KS, AT, ACP and TE domains. None of the domains responsible for the reduction of polyketides (ER, DH and KR domains) are present, which is consistent with the structure of ZEA, where five ketone functions are not reduced (Kim et al. 2005). In contrast to PKS13, PKS4 resembles type1 PKS genes with a reducing function, and contains domains KS, AT, DH, ER, KE and ACP (Kim et al. 2005). The two PKS enzymes are thought to collaborate to synthesise ZEA. It has been proposed that PKS 4 produces a reduced hexaketide that is then transacylated to PKS13 where three non-reduced malonyl-CoAs are condensed, resulting in a nonaketide with both reduced and non-reduced units (Zhou et al. 2008). The TE domain of PKS13 catalyses the macrocyclisation of the backbone and the release of the product from the megasynthase (Wang et al. 2009). Genome analysis of *F. graminearum* indicates that PKS13 and PKS4 genes are at the same locus, are divergently transcribed (Kim et al. 2005; Gaffoor and Trail 2006), and are clustered with a further nine open reading frames (Kim et al. 2005; Gaffoor and Trail 2006). Deletion of the other ORFs at the ZEA locus indicated that only *zeb2*, which may be a transcription factor for the ZEA cluster, is also required for ZEA biosynthesis (Kim et al. 2005). However, in *zeb1* (a putative isoamyl alcohol oxidase gene) deletion strains β-ZOL accumulated rather than ZEA, indicating that ZEB1 catalyses the oxidation of β-ZOL to ZEA (Kim et al. 2005). The genes for PKS13, PKS4, *zeb2*, and *zeb1* are coordinately regulated (unlike the other genes at the same locus), and can therefore be considered a cluster (Kim et al. 2005; Lysøe et al. 2009). None of the four genes of the ZEA cluster are expressed in the zeb2 deletion strain, providing further evidence in support of its putative role as a transcriptional regulator of the ZEA cluster (Kim et al. 2005). Strains deficient in ZEA production were functionally identical with the wild-type strain in terms of virulence (head blight on whole barley plants), growth, pigmentation, conidiation, and perithecium formation, suggesting that ZEA has little or no role in these functions in *F. graminearum* (Kim et al. 2005)."

D. Other Polyketide Toxins

1. AK-toxin

The host-specific toxin **AK-toxin**, is an epoxy-decatrienoic acid ester produced by the Japanese pear pathotype of *Alternaria alternata* (Fr.:Fr.) Keissl., which causes black spots on susceptible cultivars of Japanese pear (*Pyrus pyrifolia* Nakai) (Nakashima et al. 1985; Otani et al. 1985; Tanaka 1933).

Although most host-specific toxins are diverse in structure, AK-toxin shares a common moiety with AF-toxin (Otani et al. 1972) and ACT-toxin (Kohmoto et al. 1979), of the strawberry and tangerine pathotypes of *A. alternata* respectively. This moiety, **9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid**, was identified as a precursor to all three toxins (Feng et al. 1990; Kohmoto et al. 1993; Nakatsuka et al. 1990) and exhibits a structure typical of polyketide or long chain fatty acid metabolites. AK-toxin occurs as two related molecular species, AK-toxin I and AK-toxin II (Nakashima et al. 1982, 1985). AK-toxin I is the most abundant and active species, exhibiting toxicity to only a very narrow range of susceptible pear cultivars (Otani et al. 1985). This all or nothing specificity matches exactly the host or non-host response to the pathogen. AK-toxin II is a dimethyl derivative of AK-toxin I and shows the same specificity, but with an activity 20 times less than that of AK-toxin I.

The site of action for AK-toxin is on the plasma membrane, near to plasmodesmata of susceptible cells (Park et al. 1987, 1992). An early affect includes the depolarization of membrane electropotential, typically within 5 min of toxin treatment (Namiki et al. 1986; Otani et al. 1989). This is followed by electrolyte loss, plasma membrane changes, and ROS-induced lipid peroxidation (Otani and Kohmoto 1992; Shimizu et al. 2006)

Four clustered genes involved in AK-toxin biosynthesis have been identified (Tanaka et al. 1999; Tanaka and Tsuge 2000). *AKT, AKT2*, and *AKT3* encode proteins with similarity to the carboxy-activating enzymes of the estelase–lipase (*AKT1, AKT2*) and the hydratase–isomerase (*AKT3*) families (Imazaki et al. 2010). *AKTR* encodes a transcriptional regulator (Tanaka and Tsuge 2000). All of the genes identified have been shown to be

located on a 4.1-Mb chromosome in the *A. alternata* Japanese pear pathotype (Tanaka and Tsuge 2000). AKT1, AKT2 and AKT3 localize to peroxisomes (Imazaki et al. 2010), and deletion of a gene encoding a peroxin protein essential for peroxisome biogenesis eliminated AK-toxin synthesis, indicating that peroxisomes are required for the synthesis of AK-toxin. (Imazaki et al. 2010)

2. Cercosporin

Cercosporin is a host non-specific perylenequinone phytotoxin, deep red in color, produced by several plant pathogenic species of the genus *Cercospora*. Cercosporin has been shown to be toxic in many laboratory models including mice, bacteria, many species of fungi, human tumor cells, and several species of plants (Chung et al. 1999; Foote 1976; Macri and Vianello 1979). Light has been demonstrated to be essential for disease in the pathosystem, an observation that suggested cercosporin is necessary for disease development. Mutants deficient in cercosporin production are nonpathogenic or incite fewer necrotic lesions on infected plants (Upchurch et al. 1991; Choquer et al. 2005; Daub et al. 2005).

Cercosporin is a photoactive compound that absorbs light energy and, in an electronically excited state, reacts with oxygen to produce active oxygen species (Berestetsky 2008; Daub et al. 2005). Both superoxide (O_2^-) and singlet oxygen ($^1O_2$) are produced, but $^1O_2$ is thought to be responsible for the toxicity (Daub and Hangarter 1983). Cercosporin-produced $^1O_2$ and O_2^- result in oxidation of fatty acids, sugars, cellulosic materials, guanine, and several amino acids, and has been demonstrated to cause DNA damage and lipid peroxidation of host cell membranes within minutes of exposure to light (Daub 1982; Daub et al. 2005). Production of cercosporin itself is induced by light, and is modulated by a number of nutritive and environmental variables (Jenns et al. 1989; You et al. 2008). Lousberg et al. (1971) and Yamazaki and Ogawa (1972), working independently, determined the structure of cercosporin to be 1,12-bis(2-hydroxy propyl)-2,11-dimethosy-6,7-metylenedioxy-4,9-dihydroxyperylene-3,10-puinone, with a molecular weight of 534. "The PKS gene required for cercosporin biosynthesis encodes a type 1 PKS containing a KS, AT, TE/CYC, and two ACP domains (Choquer et al. 2005). A cercosporin biosynthetic pathway comprised of eight genes has been proposed for *Cercospora nicotianae* (Chen et al. 2007). Since then, a further ABC transporter outside of this cluster has also been shown to be required for export of cercosporin from *C. nicotianae*."

IV. Proteinaceous Phytotoxins of Wheat

A. Proteinaceous Host Specific Toxins of Wheat Pathogens

The necrotrophic pathogens ***Pyrenophora tritici-repentis*** (Died.) Drechs. and ***Stagonospora nodorum*** (Berk.) Castell. and Germano cause **tan spot** and **stagonospora nodorum blotch** of wheat respectively, causing large losses in crops worldwide (Solomon et al. 2006; Shabeer and Bockus 1988). These fungi each produce multiple **proteinaceous host specific toxins** that are now considered as **necrotrophic effectors**, comprehensively reviewed in Friesen et al. 2008; Ciuffetti et al. 2010; Tan et al. 2010. These **effector proteins** trigger programmed cell death in host cells, manipulating the host defense response to benefit the necrotrophic lifestyle of the fungi.

1. ToxA

ToxA is a 13.2 kDa protein produced by both *P. tritici-repentis* and *S. nodorum*, and is the most thoroughly characterized of the proteinaceous necrotrophic effectors. Formerly known as **Ptr toxin** and Ptr Necrosis toxin (Ciuffetti et al. 1998), it was first detected in culture fluids in 1987 (Tomás and Bockus 1987). Early studies correlating presence or absence of the toxin with the disease susceptibility of host plants strongly suggested it was solely required for the induction of necrosis in plants by *P. tritici-repentis* (Lamari and Bernier 1989, 1991; Lamari et al. 1995). More recently, similar correlation analysis along with transformation of avirulent strains with *ToxA* in *S. nodorum* clearly demonstrate that ToxA is necessary and sufficient for the

induction of necrosis in host plants, and the level of expression of ToxA directly correlates with the amount of disease (Faris et al. 2011).

ToxA is produced by a gene that has apparently arisen in these fungi by **horizontal transfer**, initially to *S. nodorum,* perhaps within the last 350 years (Stukenbrock and McDonald 2007), and subsequently from *S. nodorum* to *P. tritici-repentis* (Friesen et al. 2006). Transfer of ToxA between *S. nodorum* and *P. tritici-repentis* is strongly implied by the presence in both species, but absence in closely related genera, of ~11 kb of identical sequence including the *toxA* gene, transposon-related sequence, and apparent non-coding DNA (Friesen et al. 2006). Further, a high degree of sequence polymorphism at this locus within *S. nodorum* isolates but little or no divergence between *P. tritici-repentis* strains suggests that the transfer was from *S. nodorum* to *P. tritici-repentis*. The authors suggest that this transfer is likely to have occurred around the early 1940s when *P. tritici-repentis* tan spot emerged for the first time as a devastating disease of wheat.

The translated amino acid sequence of ToxA is 178 residues long, including a 22 amino acid signal peptide and a 38-residue pro-peptide required for folding (Sarma et al. 2005). These peptides are cleaved during secretion of the mature 13.2 kDa protein. The ToxA three-dimensional structure was solved by X-ray crystallography showing the protein has a single-domain β sandwich fold consisting of two antiparallel β sheets (Sarma et al. 2005). The structure also revealed an arginine–glycine–aspartate (RGD) loop in which most previously determined loss-of-function mutations made in ToxA were located. This loop is required for uptake into plant cells (Manning et al. 2008), which is predicted to involve binding to plant integrins by analogy with the mode of uptake of mammalian fibronectin, which has a similar RGD motif loop (Manning et al. 2004). Once inside sensitive plant cells, ToxA has been shown to localise to chloroplasts (Manning and Ciuffetti 2005) and bind to **ToxABP1**, a Thf1 homologue possibly involved in photosystem (PS) II turnover (Manning et al. 2007). This interaction is likely to be involved in the light-dependent reactive oxygen species accumulation and reduced PS I and II observed in ToxA-sensitive plant cells.

The single dominant host gene necessary for sensitivity to ToxA-mediated disease, ***Tsn1***, is found in sensitive genotypes of wheat, and genotypes containing a non-functional copy are resistant to *P. tritici-repentis* and *S. nodorum* disease (Faris and Friesen 2009; Faris et al. 2010). *Tsn1* encodes a protein related to those that modulate resistance to pathogens in other sytems. The predicted Tsn1 protein sequence contains nucleotide-binding site/leucine rich repeat (NB-LRR) and serine/threonine protein kinase (S/TPK) domains, typical features of plant resistance proteins, although usually not found together on a single protein (Faris et al. 2010). Each of these regions of the protein are required for ToxA/disease susceptibility. Interestingly, although Tsn1 is required for cellular uptake of ToxA, the Tsn1 protein does not directly interact with ToxA, and appears to be cytoplasmic (Faris et al. 2010).

2. *Pyrenophora tritici-repentis* ToxB

Along with ToxA, *P. tritici-repentis* produces other host-specific toxins, including the necrotrophic effector protein **ToxB**. ToxB is unrelated to ToxA, and is responsible for disease susceptibility in different *P. tritici-repentis* races. Unlike ToxA, ToxB homologues are found in non-pathogenic races and in other dothideomycetes, and copy number and sequence varies among different races (Amaike et al. 2008; Andrie et al. 2008). The level of chlorosis induced by ToxB is dependent on light, expression level and sequence, with the most virulent races having multiple copies and high expression levels (Amaike et al. 2008; Aboukhaddour et al. 2012; Strelkov et al. 1998). ToxB appears to cause disease specifically in wheat; a homologue of the *ToxB* gene found in *P. bromi* is not required for disease of the fungus's host bromegrass (*Bromus inermis*) but heterologously expressed Pb ToxB can cause chlorosis of wheat (Andrie and Ciuffetti 2011). As for ToxB/Tsn1, a single dominant plant gene, *tsc2*, causes disease susceptibility in wheat (Friesen and Faris 2004).

3. Other Protein HSTs of *Stagonospora nodorum*

Along with ToxA, four other necrotrophic effectors are produced by *S. nodorum*; these are ***SnTox1***, ***SnTox2***, ***SnTox3*** and ***SnTox4***, which facilitate necrotic disease in host wheat containing corresponding susceptibility loci *Snn1*, *Snn2*, *Snn3*, and *Snn4* respectively (Friesen et al. 2008; Abeysekara et al. 2009). *SnTox1* and *SnTox3* have been cloned and characterised (Liu et al. 2009, 2012). Both *SnTox1* and *SnTox3* are small (~10 and ~18 kDa respectively), secreted and contain a large number of cysteine residues, similar features to biotrophic effector proteins. For both genes, disruption removed disease symptoms, and introduction of the gene into avirulent isolates caused those strains to induce disease on hosts containing respective susceptibility genes. Interestingly, as for *ToxA*, *SnTox1* and *SnTox3* genes are located adjacent to transposon-related sequences, although the greater region surrounding *SnTox1* is gene-rich (Friesen et al. 2006; Liu et al. 2009, 2012).

V. Sesquiterpenoid-Containing Toxins

A. Trichothecenes

Trichothecenes are a major group of small molecule toxins produced by a range of fungi from the order Hypocreales, including plant pathogens from the genera *Fusarium*, Myrothecium and *Trichoderma* (McCormick et al. 2011). Trichothecenes are structurally defined by an **epoxide-containing sesquiterpenoid** skeleton, and can be either macrocyclic or non-macrocyclic depending on whether the bridge between C-4 and C-15 contains a macrocyclic ester or an ester–ether (Bennett and Klich 2003; Mehrabi et al. 2011). Trichothecene toxins were originally chemically characterised by Japanese and American researchers investigating human and cattle toxicoses respectively which were associated with consumption of mouldy grain (Yates et al. 1968; Tatsuno et al. 1968; Yoshizawa and Morooka 1973).

1. Biological Significance of Trichothecenes

Their importance in agriculture is two-fold in that they not only contribute to pathogen virulence in some plant systems but also contaminate food, which has important implications for trade and consumption by humans and mammals. Toxicosis symptoms include feed refusal and reduced growth (particularly in pigs), vomiting, haematotoxicity, and immunosuppression (Rotter et al. 1994; Parent-Massin 2004; Pestka 2007).

Trichothecenes survive many cooking processes, and because of their toxicity and prevalence in crops such as wheat, barley, and maize, and their presence in finished products, are subject to maximum limits in a number of jurisdictions (Anonymous 2005; van Egmond et al. 2007). In a practical sense, trichothecene contamination of cereal products, although representing a major problem to millers and brewers; can be managed by careful monitoring of incoming grain products, and in the case of brewing during the malting process. Indeed, contaminated lots can be redeployed as feed for less sensitive mammals or diluted to within acceptable levels. In developing countries where toxin monitoring procedures or capacity to use contaminated products for alternative purposes may not exist, trichothecene toxicosis represents a significant risk to humans and livestock.

Trichothecenes are amphipathic molecules that move passively across membranes and have multiple modes of action. Early studies demonstrated that they block translation by binding the 60S ribosomal subunit and preventing peptide bond formation (Carrasco et al. 1973; McCormick et al. 2011). Consequently, altered ribosome structure imparts some resistance against trichothecenes (Fried and Warner 1981). More recently, chemical genomics screen in yeast revealed the importance of mitochondrial function in toxicity of these molecules, and the fact that the treatment of cells with trichothecenes resulted in perturbation of mitochondrial function (McLaughlin et al. 2009). Cellular responses to the toxin also include activation of **mitogen-activated protein kinase** cascades and induction of **programmed cell death** in both animals (Baltriukiene et al. 2007; Rocha et al. 2005) and plants (Desmond et al. 2008; Nishiuchi et al. 2006).

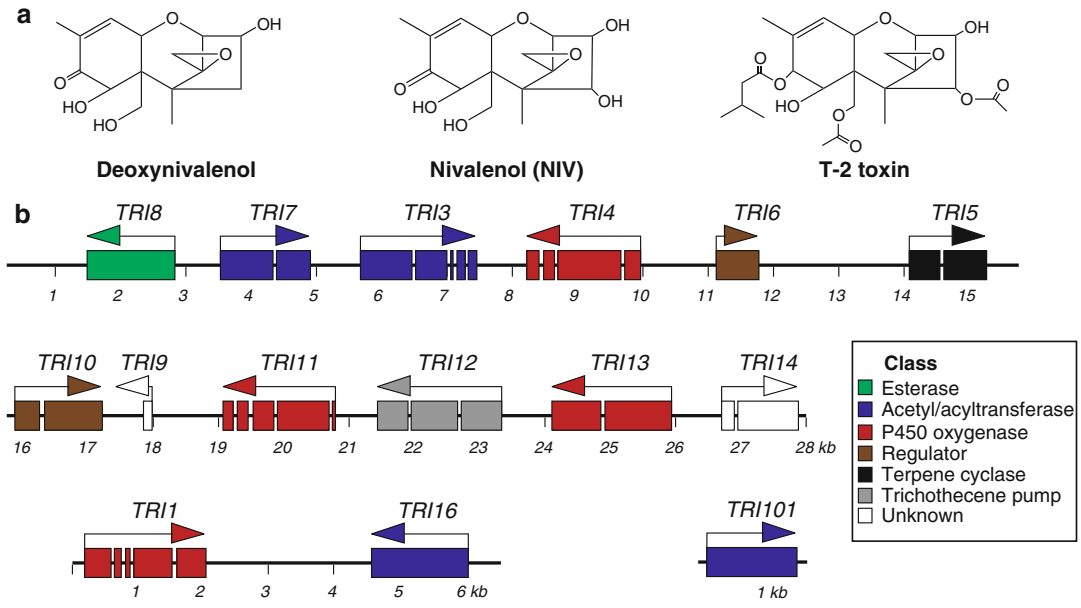

Fig. 4.5. Trichothecenes and associated gene loci from *Fusarium sporotrichioides*. (**a**) Three trichothecenes, deoxynivalenol, nivalenol and T-2 toxin; (**b**) the *TRI* loci from *F. sporotrichioides* required for trichothecene production (accession numbers AF359360, AY217783, and AF127176). A *black line* represents the gene sequence, exons of pathway-associated genes are *coloured boxes* on the sequence representing the different encoded gene classes, and an *arrow* above the exons indicates gene orientation

The role of trichothecenes in virulence towards plant hosts has been studied extensively in a number of systems. Early studies correlated virulence of fungal isolates with their capacity to produce trichothecenes (Desjardins et al. 1989). Unequivocal proof for the role of these toxins in plant disease was established using transformation-mediated disruption of the gene responsible for the first committed step in trichothecene biosynthesis in *F. sambucinum* causing dry rot of parsnip (Desjardins et al. 1992). Interestingly, the same strains showed unaltered virulence towards potato (Desjardins et al. 1992). Trichothecenes are also required for full virulence of *F. graminearum* in head blight disease on wheat (Proctor et al. 1995; Bai et al. 2002; Jansen et al. 2005), and for *F. culmorum* to cause crown/root rot of wheat (Scherm et al. 2011). In barley, trichothecene production appears to be dispensable for Fusarium head blight, and in maize ears, **nivalenol** but not **deoxynivalenol** contributes to disease (Maier et al. 2006). The differential contribution of trichothecenes to disease, despite the broad spectrum phytotoxicity of these molecules, is intriguing. In all cases reported to date, trichothecenes contribute quantitatively to disease on plants.

2. Biosynthesis of Trichothecenes

The founding member of the trichothecene class of mycotoxins is the antibiotic **trichothecin** isolated from ***Trichothecium roseum*** (Freeman and Morrison 1949). Subsequent to this discovery, over 40 different chemical variants have been described from various *Fusarium* spp. (Desjardins 2006). Three trichothecenes are shown in Fig. 4.5, from which the core moiety can be observed containing the expoxide ring, which is critical for toxicity. The various forms of trichothecenes from *Fusarium* spp. come about as a result of the patterns of oxygenation and esterification at C3, C4, C7, C8, and C15. These differing patterns of decoration can dramatically alter the toxicity of the molecule to both the producing organism and to hosts, and are often referred to as chemotypes (Nishiuchi et al. 2006).

As with many other fungal secondary metabolites, genes for the regulation and biosynthesis of trichothecenes are clustered. In *F. graminearum*, which produces deoxynivalenol (Fig. 4.5) or nivalenol (Fig. 4.5), the genes are found at four separate loci, while in ***Fusarium sporotrichioides*** three separate loci are known (Fig. 4.5). The majority of the genes are contained in a 10–12 gene cluster, with the remaining genes elsewhere either by themselves or in pairs. The exact number of genes is dependent on the chemotype of the individual strain, but almost every step in the biosynthesis of deoxynivalenol, nivalenol, and **T2 toxin** (Fig. 4.5) has now been assigned a specific gene. In different *Fusarium* spp., the arrangement of the genes differs considerably (Proctor et al. 2009). The arrangement of trichothecene biosynthesis genes differs again in *Trichoderma* spp. whereby the gene (***TRI5***) for the first commitment step is located outside of the main cluster (Cardoza et al. 2011). There also appears to be some divergence of gene function whereby at least two genes in *Trichoderma* spp. have different biochemical functions in relation to their homologues in *Fusarium* spp. (Cardoza et al. 2011). The implications of cluster organization on trichothecene production remain obscure.

Trichothecene mycotoxins present a major problem in agriculture, with wheat, barley, and maize being of particular concern. The absence of qualitative resistance to *Fusarium* spp. in these hosts challenges our capacity to grow trichothecene-free feed and food. In the light of this, novel strategies to control both the pathogens and trichothecene production are being pursued. For example, strategies include the expression of trichothecene modifying/degrading enzymes in the host (Ohsato et al. 2007; Okubara et al. 2002), modification of host targets such as ribosomal proteins (Harris and Gleddie 2001; Di et al. 2010), and over-expression of host defence response genes (Mackintosh et al. 2006; Shin et al. 2008). While these strategies are in various phases of development, none appear to provide absolute resistance or zero trichothecene contamination. A combination of approaches, including continued plant breeding and robust monitoring, will be required to minimise the presence of trichothecenes in the food chain into the future.

VI. Miscellaneous Fungal Toxins of Herbivores

Two classes of alkaloids produced via non-traditional secondary metabolite machinery are the **lolines** (saturated pyrolizidine) and **indole–diterpenes** (Fig. 4.6). Each of these metabolite classes have been well-studied within the **epichloae** as a result of the impact they have had on **protection against insects** (lolines) and **livestock toxicity** (indole–diterpenes).

A. Lolines

The lolines including ***N*$_1$-formylloline** (NFL) (Fig. 4.6) and ***N*$_1$-acetylnorloline** (NANL) are an abundantly produced class of alkaloid synthesized by epichloae endophytes in association with the grass host. The lolines can be found within both the aerial plant parts (pseudostem, leaf blades, and reproductive tissues) and within the roots, even though the endophyte does not associate in the grass roots, suggesting that lolines are readily translocated in the plant (Schardl et al. 2007; Patchett et al. 2008; Koulman et al. 2007). Using genetic based screening, **the lolines have been shown to exhibit the potent anti-insect properties** antibiosis (toxicity) and antixenosis (feeding deterrant), with activity against economically important pests such as the aphids *Rhopalosiphum padi* (bird cherry-oat aphid) and *Schizaphis graminum* (greenbug) (Wilkinson et al. 2000), and may also be toxic to *Chaetocnema pulicaria* (corn flea beetle) (Ball et al. 2011), *Trigonotylus caelestialium* (rice leaf bug) (Shiba and Sugawara 2009), *Listronotus bonariensis* (ASW) (Jensen et al. 2009), and nematodes (*Pratylenchus scribneri*) at high loline concentrations (Bacetty et al. 2009). Lolines are likely to impact the survival and reproduction of other insects, but often the results can be confounded with the presence of other compounds produced in association with the grass host (Schardl et al. 2007). The family of lolines is considered an important natural biocontrol agent for endophyte-infected forage grasses, tall fescue (*Lolium arundinaceum*), and meadow fescue (*Festuca pratensis*) (Ball and Tapper 1999; Latch and Christensen 1985; Easton 2007).

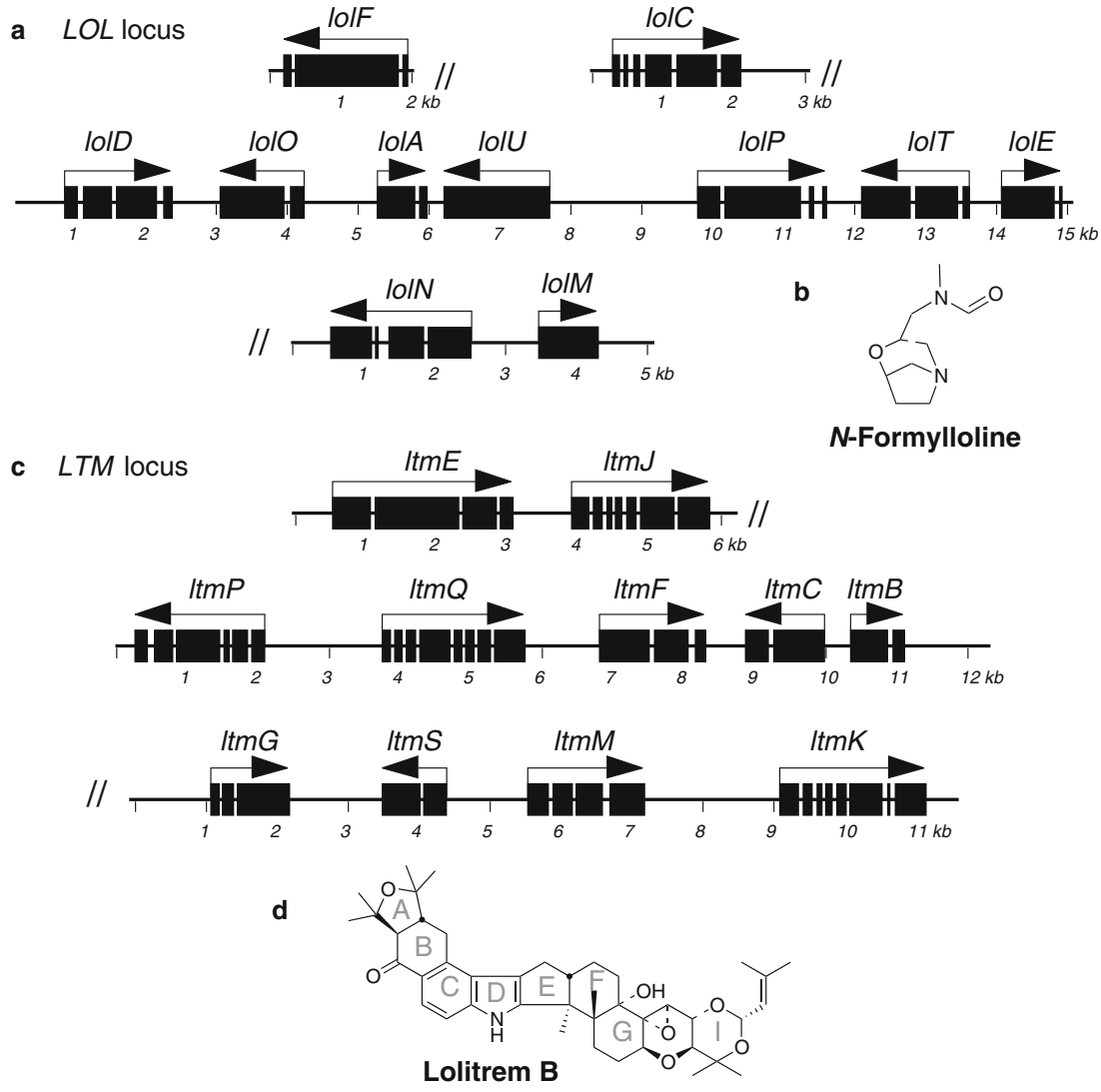

Fig. 4.6. Lolines and indole-diterpenes, miscellaneanous fungal toxins of herbivores. (**a**) The *LOL* gene locus from *Epichloë festucae* strain E2368 (accession numbers JF830815, JF830814 and JF830816); (**b**) the loline *N*-formylloline; (**c**) the *LTM* gene locus from *E. festucae* strain Fl1 (accession numbers JN613318, JN613319 and JN613320); (**d**) the indole-diterpene lolitrem B that causes perennial ryegrass staggers. A *black line* represents the gene sequence, exons of pathway-associated genes are *black boxes* on the sequence, and flanking genes are *grey boxes*. An *arrow* above the exons indicates gene orientation. The symbol // indicates repetitive sequence not included in locus map and indicates linkage between clusters

The loline biosynthetic pathway has been studied using a combination of feeding studies, genetic analyses, and genetic manipulation of key pathway genes (Blankenship et al. 2005; Spiering et al. 2005; Wilkinson et al. 2000). Generally, alkaloid production within the epichloae is more readily detected in planta, and to date effective culture conditions have been demonstrated only for lolines from ***Neotyphodium uncinatum*** (Blankenship et al. 2001). Effective incorporation of labeled proline and homoserine using the inducible culture system indicated these compounds were precursors and contributed to the formation of the lolines. (Blankenship et al. 2005)

The genes required for loline biosynthesis were discovered as a single locus (*LOL*) (Fig. 4.6) using a map-based cloning approach with ***Epichloë festucae*** (Wilkinson et al. 2000; Kutil et al. 2007) and suppression-subtractive hybridization with *N. uncinatum* (Spiering et al. 2002). In each case, the genes were

identified in a complex cluster that contains AT-rich repeats that are especially prominent in *E. festucae*. The hybrid endophyte, *N. uncinatum* contains two copies of ***LOL* genes**, where each has been inherited from the ancestral progenitors *Epichloë bromicola* and *Epichloë typhina* (Spiering et al. 2002, 2005). However, the *lolP* gene in *LOL* cluster 2 is truncated as a result of a deletion within the coding region of the gene, and is considered non-functional (Spiering et al. 2005). Manipulation of two genes within the cluster, *lolC* and *lolP*, was achieved in *N. uncinatum* using RNA interference (*lolC,* early pathway) or gene replacement (*lolP,* late pathway) (Spiering et al. 2005, 2008). Down-regulation of *lolC* resulted in reduced loline production, while deletion of the *lolP* gene resulted in accumulation of pathway intermediates NANL and *N*-methylloline (NML) confirming their roles in loline biosynthesis. At least 11 co-regulated genes (Fig. 4.6) are thought to be required for loline production, but this gene cluster does not contain the signature genes, such as NRPS, PKS, and DMATS-like genes, that usually define secondary metabolite gene clusters. (Spiering et al. 2005; Kutil et al. 2007; Schardl et al. 2012)

In the development of endophyte-infected tall fescue forage cultivars, the production of lolines along with the inability to produce ergot alkaloids are considered important criteria (Bouton et al. 2002; Hopkins et al. 2010, 2011). However, variation in the accumulation of pathway intermediates and end products, NFL, NANL, NML and N_1-acetylloline (NAL) can be found when surveying **endophytes** suitable for tall fescue (Ball et al. 2006, 2011; Ball and Tapper 1999). The impact that this variation may have on insect protection is not fully understood in these endophyte-infected tall fescue associations, but they often show more resistance to insects than endophyte-free lines (Timper et al. 2005; Bultman et al. 2006).

B. Indole–diterpenes

The **indole–diterpenes** represent a diverse array of compounds produced by fungi in the classes of *Eurotiomycetes* and *Sordariomycetes* that consist mainly of the **Clavicipitaceae**, *Aspergillus* spp., *Nodulisporium* spp., and *Penicillium* spp. This broad range of bioactive compounds has been shown to have mammalian and insect toxicity through activation of various ion channels (Gallagher et al. 1984; Knaus et al. 1994).

Lolitrem B (Fig. 4.6) is one of the most well-known indole–diterpenes to impact agriculture. This alkaloid, produced by the epichloid endophyte ***Neotyphodium lolii*** in association with perennial ryegrass, has been documented as the causative agent of **ryegrass staggers**, a disorder common to livestock grazing *N. lolii*-infected perennial ryegrass (*Lolium perenne*) (Gallagher et al. 1984). Tremors in cattle have also been observed when ingesting sclerotia produced by *Claviceps paspali* on dalligrass (*Paspalum dilatatum*) (Cole et al. 1977) and *Claviceps cynodontis*-infected Bermuda grass (*Cynodon dactylon*), probably as a result of the production of a related group of compounds, the paspalitrems (Uhlig et al. 2009; Cole et al. 1977). Livestock afflicted with ryegrass staggers display symptoms of ataxia and sustained tremors, and are easily startled (Fletcher and Harvey 1981; DiMenna et al. 1992). Although symptoms of indole–diterpene-induced staggers are generally reversible if the animal is removed from the source (Gallagher et al. 1982), they can dramatically reduce stock productivity (reduction in live weight gains), cause unintended stock loss from misadventure (including injuries and even death from falling) and require time-consuming management of affected stock (Fletcher 1986).

More recently, Kobe beef production in Japan was affected with lolitrem B (Fig. 4.6) toxicity as a result of feeding straw residue that originated from commercial grass seed production fields in Oregon (Craig 2009; Miyazaki et al. 2004). Once burning field residue was banned in the region, an opportunity to capitalize on the straw residue as a by-product of seed production was marketed. Unfortunately, much of the grass seed produced is endophyte-infected and the straw residue was extremely high in both lolitrem B and the ergot alkaloid ergovaline. In the case of afflicted Kobe beef, straw residue is now routinely tested for levels of lolitrem B and ergovaline, and must be under a toxicity threshold level of 2.0 μg/g lolitrem B prior to exporting (Craig 2009).

The indole–diterpenes, such as lolitrem B and other related compounds that have a paspaline intermediate, are probably synthesized from an indole donor (possibly indole-3-glycerol phosphate from tryptophan biosynthesis) and the 5 carbon isoprenyl diphosphate and

dimethylallyl diphosphate that form the cyclic diterepene moiety. Additional enzymatic decoration of the primary intermediate, paspaline, results in the array of indole–diterpenes that are detected both among and within different species (Young et al. 2009). Genes required for indole–diterpene production have now been cloned and characterized from a number of different species (Zhang et al. 2004; Young et al. 2006; Nicholson et al. 2009; Young et al. 2001), including the perennial ryegrass endophyte *N. lolii* (Young et al. 2006). Similar to the gibberellin biosynthesis gene cluster from *Fusarium fujikuroi* (synonym *Gibberella fujikuroi*) (Tudzynski and Hölter 1998), a gene encoding a pathway-specific **geranylgeranyl diphosphate synthase** was identified in *N. lolii* (Young et al. 2005). Identification of two or more copies of geranylgeranyl diphosphate synthases, of which one is located amongst other genes encoding functions required for secondary metabolite production, is now considered a key identifying feature for the identification of gene clusters required for the production of compounds with diterpene moieties. (Young et al. 2001, 2005; Tudzynski and Hölter 1998; Saikia and Scott 2009)

The ***LTM* locus** in *N. lolii* and related *E. festucae* contains 11 genes required for lolitrem B production as a complex gene cluster (Fig. 4.6) embedded with over 50 kb of AT-rich repeats (Young et al. 2006, 2009). Typically, strains that are unable to produce lolitrem B but retain the ability to produce simpler indole–diterpenes have lost two genes, *ltmE* and *ltmJ*, required for the formation of the AB ring (Fig. 4.6) (Young et al. 2009). Toxicity of the simpler indole–diterpenes such as paxilline and terpendole C are still tremorgenic, but the duration and intensity is reduced when compared to lolitrem B (Gatenby et al. 1999). Analysis of pathway intermediates and mutants within the pathway, both naturally occurring and genetically engineered, have helped elucidate the enzymatic steps. Lolitrem B biosynthesis is very complex because of promiscuity of enzymes, and as such is more like a metabolic grid that can produce a greater number of metabolites than expected from a linear pathway.

More recently, within the epichloae, an **epoxy–janthitrem**-producing isolate has been identified and incorporated into perennial ryegrass cultivar development (Hume et al. 2007). The epoxy–janthitrems are likely to be synthesized via a similar pathway to lolitrem B, but unknown enzymatic steps are responsible for the AB ring structure. Although janthitrems are known to be mildly tremorgenic, the association of the janthitrem-producing isolate within perennial ryegrass provided greater protection from devastating pasture insect pests such as African black beetle (*Heteronychus arator*), root aphid (*Aploneura lentisci*), and porina (*Wisceana cervinata*) than in other endophyte–grass combinations (Jensen and Popay 2004; Hume et al. 2007).

VII. Conclusions

The fungal secondary metabolites summarized in this chapter represent examples of different biochemical classes that contribute, through diverse mechanisms, to the outcomes of interactions between plants, other organisms and the environment in the agricultural setting. Genes involved in the biosynthesis of secondary metabolites are typically clustered in filamentous fungi, including plant pathogens (Fox and Howlett 2008; Keller 2011). The origins and evolutionary processes of these gene clusters, however, are largely unknown. In the past decade, advances in rapid and inexpensive sequencing of genomes has led to a revolution in the identification of novel secondary metabolite gene clusters from agronomically-important fungi. Comparative analyses of genes and loci indicate that secondary metabolite pathways, or sub-clusters thereof, can be shared by genetically diverse fungal genera, and there is now unequivocal evidence for the involvement of **horizontal gene transfer** (HGT) in the evolution of fungal secondary metabolite gene clusters (Ma et al. 2010; Mehrabi et al. 2011; Slot and Rokas 2011; Walton 2000).

Collectively, these developments demonstrate the phenomenal potential for fungi to acquire and modify secondary metabolite genes to facilitate the adoption of new traits.

While secondary metabolite gene clusters are readily identifiable in sequenced fungal genomes, the characterisation of the final biosynthetic products and their intermediates present a greater hurdle. Metabolomics techniques are now able to more rapidly identify novel compounds in the spectrum of secondary metabolites currently found in animal feed. However, this has been complicated by the fact that many metabolites are temporally and spatially regulated by factors that are not well-understood, and few are synthesised in axenic culture. Recent advances in

artificially up-regulating fungal secondary metabolite pathways will facilitate the identification of novel metabolites produced by many fungi.

Modern genomic and metabolic tools are rapidly advancing a deeper understanding of the metabolite potential of common fungal contaminants in forage, and of the role of these compounds in virulence, and will thereby reduce the risk of fungi to food security, and open up new avenues for the beneficial use of bioactive compounds in the future.

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References

Abe K, Iwanami H, Kotoda N, Moriya S, Takahashi S (2010) Evaluation of apple genotypes and *Malus* species for resistance to *Alternaria* blotch caused by *Alternaria alternata* apple pathotype using detached-leaf method. Plant Breed 129:208–218

Abeysekara NS, Friesen TL, Keller B, Faris JD (2009) Identification and characterization of a novel host-toxin interaction in the wheat-*Stagonospora nodorum* pathosystem. Theor Appl Genet 120: 117–126

Aboukhaddour R, Kim YM, Strelkov SE (2012) RNA-mediated gene silencing of ToxB in *Pyrenophora tritici-repentis*. Mol Plant Pathol 13:318–326

Ahn J-H, Walton JD (1996) Chromosomal organization of *TOX2*, a complex locus required for host-selective toxin biosynthesis in *Cochliobolus carbonum*. Plant Cell 8:887–897

Ahn J-H, Walton JD (1997) A fatty acid synthase gene in *Cochliobolus carbonum* required for production of HC-toxin, cyclo(D-prolyl-L-alanyl-D-alanyl-L-2-amino-9,10-epoxi-8-oxodecanoyl). Mol Plant Microbe Interact 10:207–214

Ahuja M, Chiang YM, Chang SL, Praseuth MB, Entwistle R et al (2012) Illuminating the diversity of aromatic polyketide synthases in *Aspergillus nidulans*. J Am Chem Soc 134(19):8212–8221

Akagi Y, Akamatsu H, Otani H, Kodama M (2009a) Horizontal chromosome transfer: a mechanism for the evolution and differentiation of a plant pathogenic fungus. Eukaryot Cell 8:1732–1738

Akagi Y, Taga M, Yamamoto M, Tsuge T, Fukumasa-Nakai Y, Otani H, Kodama M (2009b) Chromosome constitution of hybrid strains constructed by protoplast fusion between the tomato and strawberry pathotypes of *Alternaria alternata*. J Genet Plant Pathol 75:101–109

Akamatsu H, Itoh Y, Kodama M, Otani H, Kohmoto K (1997) AAL-toxin deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. Phytopathology 87:967–972

Akamatsu H, Taga M, Kodama M, Johnson R, Otani H, Kohmoto K (1999) Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. Curr Genet 35:647–656

Akamatsu H, Otani H, Kodama M (2003) Characterization of a gene cluster for host-specific AAL-toxin biosynthesis in the tomato pathotype of *Alternaria alternata*. Fungal Genet Newsl 50:355

Amaike S, Ozga JA, Basu U, Strelkov SE (2008) Quantification of ToxB gene expression and formation of appressoria by isolates of *Pyrenophora triticirepentis* differing in pathogenicity. Plant Pathol 57: 623–633

Andrie RM, Ciuffetti LM (2011) *Pyrenophora bromi*, causal agent of brownspot of bromegrass, expresses a gene encoding a protein with homology and similar activity to Ptr ToxB, a host-selective toxin of wheat. Mol Plant Microbe Interact 24: 359–367

Andrie RM, Schoch CL, Hedges R, Spatafora JW, Ciuffetti LM (2008) Homologs of ToxB, a host-selective toxin gene from *Pyrenophora tritici-repentis*, are present in the genome of sister-species *Pyrenophora bromi* and other members of the Ascomycota. Fungal Genet Biol 45:363–377

Anonymous (2005) Commission Regulation (EC) No 856/2005 of 6 June 2005 amending Regulation (EC) No 466/2001 as regards *Fusarium* toxins. Off J Eur Union L143:3–8

Bacetty A, Snook M, Glenn A, Noe J, Hill N, Culbreath A, Timper P, Nagabhyru P, Bacon C (2009) Toxicity of endophyte-infected tall fescue alkaloids and grass metabolites on *Pratylenchus scribneri*. Phytopathology 99:1336–1345

Bacon CW (1988) Procedure for isolating the endophyte from tall fescue and screening the isolates for ergot alkaloids. Appl Environ Microbiol 54: 2615–2618

Bacon CW (1995) Toxic endophyte-infected tall fescue and range grasses: historic perspectives. J Anim Sci 73:861–870

Bacon CW, Lyons PC, Porter JK, Robbins JD (1986) Ergot toxicity from endophyte-infected grasses: a review. Agron J 78:106–116

Bai GH, Desjardins AE, Plattner RD (2002) Deoxynivalenol non-producing *Fusarium graminearum* causes

initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153:91–98

Bailey AM, Kershaw MJ, Hunt BA, Paterson IC, Charnley K, Reynolds SE, Clarkson JM (1996) Cloning and sequence analysis of an intron-containing domain from a peptide synthetase-encoding gene of the entomopathogenic fungus *Metarhizium anisopliae*. Gene 173:195–197

Baker SE, Kroken S, Inderbitzin P, Asvarak T, Li B-Y et al (2006) Two polyketide synthase-encoding genes are required for biosynthesis of the polyketide virulence factor, T-toxin, by *Cochliobolus heterostrophus*. Mol Plant Microbe Interact 19(2):139–149

Ball OJ, Tapper BA (1999) The production of loline alkaloids in artificial and natural grass/endophyte associations. In: Proceedings of the 52nd New Zealand plant protection conference, Auckland, pp 264–269

Ball OJP, Coudron TA, Tapper BA, Davies E, Trently D, Bush LP, Gwinn KD, Popay AJ (2006) Importance of host plant species, *Neotyphodium* endophyte isolate, and alkaloids on feeding by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae. J Econ Entomol 99:1462–1473

Ball OJP, Gwinn KD, Pless CD, Popay AJ (2011) Endophyte isolate and host grass effects on *Chaetocnema pulicaria* (Coleoptera: Chrysomelidae) feeding. J Econ Entomol 104:665–672

Baltriukiene D, Kalvelyte A, Bukelskiene V (2007) Induction of apoptosis and activation of JNK and p38 MAPK pathways in deoxynivalenol-treated cell lines. Altern Lab Anim 35:53–59

Bennett JW, Klich M (2003) Mycotoxins. Clin Microbiol Rev 16(3):497–516

Berestetskiy A (2008) A review of fungal phytotoxins: from basic studies to practical use. Appl Biochem Microbiol 44(5):453–465

Bezuidenhout SC, Gelderblom WCA, Gorst-Allman CP, Horak RM, Marasas WFO, Spiteller G, Vleggaar R (1988) Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. J Chem Soc Chem Commun 11:743–745

Blankenship JD, Spiering MJ, Wilkinson HH, Fannin FF, Bush LP, Schardl CL (2001) Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media. Phytochemistry 58: 395–401

Blankenship JD, Houseknecht JB, Pal S, Bush LP, Grossman RB, Schardl CL (2005) Biosynthetic precursors of fungal pyrrolizidines, the loline alkaloids. Chembiochem 6:1016–1022

Böhnert HU, Fudal I, Dioh W, Tharreau D, Notteghem JL, Lebrun MH (2004) A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. Plant Cell 16: 2499–2513

Bojja RS, Cerny RL, Proctor RH, Du L (2004) Determining the biosynthetic sequence in the early steps of the fumonisin pathway by use of three gene-disruption mutants of *Fusarium verticillioides*. J Agric Food Chem 52:2855–2860

Bottini AT, Bowen JR, Gilchrist DG (1981) Phytotoxins II. Characterization of a phytotoxic fraction from *Alternaria alternata* f. sp. *lycopersici*. Tetrahedron Lett 22:2723–2726

Bouton JH, Latch GCM, Hill NS, Hoveland CS, McCann MA, Watson RH, Parish JA, Hawkins LL, Thompson FN (2002) Reinfection of tall fescue cultivars with non-ergot alkaloid-producing endophytes. Agron J 94:567–574

Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumonisin B1. Proc Natl Acad Sci USA 97:4961–4966

Braun CJ, Siedow JN, Williams ME, Levings CS III (1989) Mutations in the maize mitochondrial T-*urf13* gene eliminate sensitivity to a fungal pathotoxin. Proc Natl Acad Sci USA 86:4435–4439

Braun CJ, Siedow JN, Levings CS III (1990) Fungal toxins bind to the URF13 protein in maize mitochondria and *Escherichia coli*. Plant Cell 2:153–161

Brosch G, Ransom R, Lechner T, Walton JD, Loidl P (1995) Inhibition of maize histone deacetylases by HC-toxin, the host-selective toxin of *Cochliobolus carbonum*. Plant Cell 7:1941–1950

Brown DW, Butchko RAE, Busman M, Proctor RH (2007) The *Fusarium verticillioides FUM* gene cluster encodes a Zn(II)2cys6 protein that affects *FUM* gene expression and fumonisin production. Eukaryot Cell 6:1210–1218

Brown DW, Butchko RAE, Baker SE, Proctor RH (2012) Phylogenomic and functional domain analysis of polyketide synthases in *Fusarium*. Fungal Biol 116 (2):318–331

Bultman T, Pulas C, Grant L, Bell G, Sullivan T (2006) Effects of fungal endophyte isolate on performance and preference of bird cherry-oat aphid. Environ Entomol 35:1690–1695

Bushley KE, Turgeon BG (2010) Phylogenomics reveals subfamilies of fungal nonribosomal peptide synthetases and their evolutionary relationships. BMC Evol Biol 10:26

Butchko RAE, Plattner RD, Proctor RH (2003) *FUM13* encodes a short chain dehydrogenase/reductase required for C-3 carbonyl reduction during fumonisin biosynthesis in *Gibberella moniliformis*. J Agric Food Chem 51:3000–3006

Butchko RAE, Plattner RD, Proctor RH (2006) Deletion analysis of *FUM* genes involved in tricarballylic ester formation during fumonisin biosynthesis. J Agric Food Chem 54:9398–9404

Caldas ED, Jones AD, Winter CK, Ward B, Gilchrist DG (1995) Electrospray ionization mass spectrometry of sphinganine analog mycotoxins. Anal Chem 67:196–207

Caporael LR (1976) Ergotism: the satan loosed in Salem? Science 192:21–26

Cardoza RE, Malmierca MG, Hermosa MR, Alexander NJ, McCormick SP et al (2011) Identification of loci and functional dharacterization of trichothecene

biosynthesis genes in filamentous fungi of the genus *Trichoderma*. Appl Environ Microbiol 77:4867–4877

Carrasco L, Barbacid M, Vazquez D (1973) The trichodermin group of antibiotics, inhibitors of peptide bond formation by eukaryotic ribosomes. Biochim et Biophys Acta (BBA) Nucleic Acids Protein Synth 312:368–376

Challis GL, Ravel J, Townsend CA (2000) Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem Biol 7:211–224

Chang HR, Bronson CR (1996) A reciprocal translocation and possible insertion(s) tightly associated with host-specific virulence in *Cochliobolus heterostrophus*. Genome 39:549–557

Chen H, Lee M-H, Daub ME, Chung K-R (2007) Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*. Mol Microbiol 64 (3):755–770

Cheng Y-Q, Walton JD (2000) A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. J Biol Chem 275:4906–5008

Cheng Y-Q, Ahn J-H, Walton JD (1999) A putative branched-chain amino acid aminotransferase gene required for HC-toxin biosynthesis and pathogenicity in *Cochliobolus carbonum*. Microbiology 145:3539–3546

Choquer M, Dekkers KL, Chen H-Q, Cao L, Ueng PP et al (2005) The CTB1 gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of *Cercospora nicotianae*. Mol Plant Microbe Interact 18(5): 468–476

Chung KR, Jenns AE, Ehrenshaft M, Daub ME (1999) A novel gene required for cercosporin toxin resistance in the fungus *Cercospora nicotianae*. Mol Gen Genet 262:382–389

Ciuffetti LM, Pope MR, Dunkle LD, Daly JM, Knoche HW (1983) Isolation and structure of an inactive product from the host-specific toxin produced by *Helminthosporium carbonum*. Biochemistry 22: 3507–3510

Ciuffetti LM, Francl LJ, Ballance GM, Bockus WW, Lamari L, Meinhardt SW, Rasmussen JB (1998) Standardization of toxin nomenclature in the *Pyrenophora tritici-repentis*/wheat interaction. Can J Plant Pathol 20:421–424

Ciuffetti LM, Manning VA, Pandelova I, Betts MF, Martinez JP (2010) Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*-wheat interaction. New Phytol 187:911–919

Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am Nat 160:S99–S127

Cole RJ, Dorner JW, Lansden JA, Cox RH, Pape C, Cunfer BM, Nicholson SS, Bendell DM (1977) Paspalum staggers: isolation and identification of tremorgenic metabolites from sclerotia of *Claviceps paspali*. J Agric Food Chem 25:1197–1201

Comstock JC, Scheffer RP (1973) Role of host-selective toxin in colonization of corn leaves by *Helminthosporium carbonum*. Phytopathology 63:24–29

Coppock RW, Mostrom MS, Sparling CG, Jacobsen B, Ross SC (1990) Apparent zearalenone intoxication in a dairy herd from feeding spoiled acid-treated corn. Vet Hum Toxicol 32(3):246–248

Covert SF (1998) Supernumerary chromosomes in filamentous fungi. Curr Genet 33:311–319

Cox RJ (2007) Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. Org Biomol Chem 5(13):2010–2026

Craig AM (2009) Toxic effects of the endophyte in seed straw. In: Fribourg HA, Hannaway DB, West CP (eds) Tall fescue for the twenty-first century, vol 53, Agronomy monograph. ASA/CSSA/SSSA, Madison, pp 327–335

Cross DL (2009) Toxic effects of the endophyte in horses. In: Fribourg HA, Hannaway DB, West CP (eds) Tall fescue for the twenty-first century, vol 53, Agronomy monograph. ASA/SSA/SSSA, Madison, pp 311–326

Curtis MJ, Wolpert TJ (2002) The oat mitochondrial permeability transition and its implication in victorin binding and induced cell death. Plant J 29: 295–312

Curtis MJ, Wolpert TJ (2004) The victorin-induced mitochondrial permeability transition precedes cell shrinkage and biochemical markers of cell death, and shrinkage occurs without loss of membrane integrity. Plant J 38:244–259

Danko SJ, Kono Y, Daly JM, Suzuki Y, Takeuchi S, McCrery DA (1984) Structural and biological activity of a host-specific toxin produced by the fungal corn pathogen *Phyllosticta maydis*. Biochemistry 23:759–766

Daub ME (1982) Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. Plant Physiol 69:1361–1364

Daub ME, Hangarter RP (1983) Production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiol 73:855–857

Daub ME, Herrero S, Chung K-R (2005) Photoactivated perylenequinone toxins in fungal pathogenesis of plants. FEMS Microbiol Lett 252(2):197–206

De Costa C (2002) St Anthony's fire and living ligatures: a short history of ergometrine. Lancet 359: 1768–1770

de Zélicourt A, Montiel G, Pouvreau J-B, Thoiron S, Delgrange S, Simier P, Delavault P (2009) Susceptibility of *Phelipanche* and *Orobanche* species to AAL-toxin. Planta 230:1047–1055

Desjardins AE (2006) Fusarium mycotoxins: chemistry, genetics, and biology. The American Phytopathological Society, Minnesota

Desjardins AE, Spencer GF, Plattner RD, Beremand MN (1989) Furanocoumarin phytoalexins, trichothecene toxins, and infection of *Pastinaca sativa* by

Fusarium sprorotrichioides. Phytopathology 79: 170–175

Desjardins AE, Hohn TM, McCormick SP (1992) The effect of gene disruption of trichodiene synthase (*Tox5*) on the virulence of *Gibberella pulicaris*. Mol Plant Microbe Interact 5:214–222

Desjardins AE, Plattner RD, Nelsen TC, Leslie JF (1995) Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays)* seedlings. Appl Environ Microbiol 61:79–86

Desmond OJ, Manners JM, Stephens AE, Maclean DJ, Schenk PM et al (2008) The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat. Mol Plant Pathol 9:435–445

Dewey RE, Timothy DH, Levings CS III (1987) A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. Proc Natl Acad Sci USA 84:5374–5378

Dewey RE, Siedow JN, Timothy DH, Levings CS III (1988) A 13-kilodalton maize mitochondrial protein in *E. coli* confers sensitivity to *Bipolaris maydis* toxin. Science 239:293–295

Di R, Blechl A, Dill-Macky R, Tortora A, Tumer NE (2010) Expression of a truncated form of yeast ribosomal protein L3 in transgenic wheat improves resistance to Fusarium head blight. Plant Sci 178: 374–380

Diekman MA, Green ML (1992) Mycotoxins and reproduction in domestic livestock. J Anim Sci 70(5): 1615–1627

Diez B, Gutierrez S, Barredo JL, van Solingen P, van der Voort LH, Martin JF (1990) The cluster of penicillin biosynthetic genes. Identification and characterization of the pcbAB gene encoding the alpha-aminoadipyl-cysteinyl-valine synthetase and linkage to pcbC and penDE genes. J Biol Chem 265:16358–16365

DiMenna ME, Lauren DR, Poole PR, Mortimer PH, Hill RA et al (1987) Zearalenone in New Zealand pasture herbage and the mycotoxin-producing potential of *Fusarium* species from pasture. N Z J Agric Res 30(4):499–504

DiMenna ME, Mortimer PH, Prestidge RA, Hawkes AD, Sprosen JM (1992) Lolitrem B concentrations, counts of *Acremonium lolii* hyphae, and the incidence of ryegrass staggers in lambs on plots of *A. lolii*-infected perennial ryegrass. N Z J Agric Res 35:211–217

Ding Y, Bojja RS, Du L (2004) Fum3p, a 2-ketoglutarate-dependent dioxygenase required for C-5 hydroxylation of fumonisins in *Fusarium verticillioides*. Appl Environ Microbiol 70:1931–1934

Easton HS (2007) Grasses and *Neotyphodium* endophytes: co-adaptation and adaptive breeding. Euphytica 154:295–306

Evans BS, Robinson SJ, Kelleher NL (2011) Surveys of non-ribosomal peptide and polyketide assembly lines in fungi and prospects for their analysis in vitro and in vivo. Fungal Genet Biol 48(1):49–61

Faris JD, Friesen TL (2009) Reevaluation of a tetraploid wheat population indicates that the Tsnl-ToxA interaction is the only factor governing stagonospora nodorum blotch susceptibility. Phytopathology 99:906–912

Faris JD, Zhang Z, Lu H, Lu S, Reddy L, Cloutier S, Fellers JP, Meinhardt SW, Rasmussen JB, Xu SS et al (2010) A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. Proc Nat Acad Sci USA 107:13544–13549

Faris JD, Zhang Z, Rasmussen JB, Friesen TL (2011) Variable expression of the *Stagonospora nodorum* effector SnToxA among isolates is correlated with levels of disease in wheat. Mol Plant Microbe Interact 24:1419–1426

Feng BN, Nakatsuka S, Goto T, Tsuge T, Nishimura S (1990) Biosynthesis of host-selective toxins produced by *Alternaria alternata* pathogens. I. (8R,9S)-9,10-epoxy-8-hydroxy-9-methyl-deca-(2E,4Z,6E)-trienoic acid as a biological precursor of AK-toxins. Agric Biol Chem 54:845–848

Filajdic N, Sutton TB (1991) Identification and distribution of *Alternaria mali* on apples in North Carolina and susceptibility of different varieties of apples to Alternaria blotch. Plant Dis 75:1045–1048

Fink-Gremmels J, Malekinejad H (2007) Clinical effects and biochemical mechanisms associated with exposure to the mycoestrogen zearalenone. Anim Feed Sci Technol 137(3–4):326–341

Fleetwood DJ, Scott B, Lane GA, Tanaka A, Johnson RD (2007) A complex ergovaline gene cluster in Epichloë endophytes of grasses. Appl Environ Microbiol 73:2571–2579

Fletcher L (1986) *Lolium* endophyte and sheep performance on perennial ryegrass cultivars. Proc N Z Grassland Assoc 47:99–105

Fletcher LR (1999) "Non-toxic" endophytes in ryegrass and their effect on livestock health and production. In: Woodfield DR, Matthew C (eds) Ryegrass endophyte: an essential New Zealand symbiosis. New Zealand Grassland Association, Napier, pp 133–139

Fletcher LR, Harvey IC (1981) An association of a *Lolium* endophyte with ryegrass staggers. N Z Vet J 29:185–186

Foote CS (1976) Photosensitizing oxidation and singlet oxygen: consequences in biological systems. In: Pryor WA (ed) Free radicals in biology, vol II. Academic, New York, pp 85–133

Fox EM, Howlett BJ (2008) Secondary metabolism: regulation and role in fungal biology. Curr Opin Microbiol 11:481–487

Freeman GG, Morrison RI (1949) The isolation and chemical properties of trichothecin, an antifungal substance from *Trichothecium roseum* link. Biochem J 44:1–5

Fried HM, Warner JR (1981) Cloning of yeast gene for trichodermin resistance and ribosomal protein L3. Proc Natl Acad Sci 78:238–242

Friesen TL, Faris JD (2004) Molecular mapping of resistance to *Pyrenophora tritici-repentis* race 5 and sensitivity to Ptr ToxB in wheat. Theor Appl Genet 109:464–471

Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB, Solomon PS, McDonald BA, Oliver RP (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. Nat Genet 38:953–956

Friesen TL, Faris JD, Solomon PS, Oliver RP (2008) Host-specific toxins: effectors of necrotrophic pathogenicity. Cell Microbiol 10:1421–1428

Gaffoor I, Trail F (2006) Characterization of two polyketide synthase genes involved in zearalenone biosynthesis in *Gibberella zeae*. Appl Environ Microbiol 72(3):1793–1799

Gallagher RT, Campbell AG, Hawkes AD, Holland PT, McGaveston DA, Pansier EA (1982) Ryegrass staggers: the presence of lolitrem neurotoxins in perennial ryegrass seed. N Z Vet J 30: 183–184

Gallagher RT, Hawkes AD, Steyn PS, Vleggaar R (1984) Tremorgenic neurotoxins from perennial ryegrass causing ryegrass staggers disorder of livestock: structure elucidation of lolitrem B. J Chem Soc Chem Commun 9:614–616

Gatenby WA, Munday-Finch SC, Wilkins AL, Miles CO (1999) Terpendole M, a novel indole-diterpenoid isolated from *Lolium perenne* infected with the endophytic fungus *Neotyphodium lolii*. J Agric Food Chem 47:1092–1097

Gechev TS, Gadjev IZ, Hille J (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants. Cell Mol Life Sci 61:1185–1197

Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Horak MJ, Vleggaar R, Kriek NPJ (1988) Fumonisins – novel mycotoxins with cancer promoting activity produced by *Fusarium moniliforme*. Appl Environ Microbiol 54:1806–1811

Gilchrist DG, Grogan RG (1976) Production and nature of a host-specific toxin from *Alternaria alternata* f. sp. lycopersici. Phytopathology 66 (2): 165–171

Gilchrist DG, Wang H, Bostock RM (1995) Sphingosine-related mycotoxins in plant and animal disease. Can J Bot 73:S459–S467

Glenn AE (2007) Mycotoxigenic *Fusarium* species in animal feed. Anim Feed Sci Technol 137(3–4): 213–240

Glenn AE, Zitomer NC, Zimeri AM, Williams LD, Riley RT, Proctor RH (2008) Transformation-mediated complementation of a *FUM* gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. Mol Plant Microbe Interact 21:87–97

Grogan RG, Kimble KA, Misagi I (1975) A stem canker disease of tomato caused by *Alternaria alternata* f. sp. *lycopersici*. Phytopathology 65:880–886

Gross ML, McCrery D, Crow F, Tomer KB, Pope MR, Ciuffetti LM, Knoche HW, Daly JM, Dunkle LD (1982) The structure of the toxin from *Helminthosporium carbonum*. Tet Lett 23:5381–5384

Haarmann T, Machado C, Lubbe Y, Correia T, Schardl CL, Panaccione DG, Tudzynski P (2005) The ergot alkaloid gene cluster in *Claviceps purpurea*: extension of the cluster sequence and intra species evolution. Phytochemistry 66:1312–1320

Hack E, Lin C, Yang H, Horner HT (1991) T-URF13 protein from mitochondria of Texas male-sterile maize (*Zea mays* L.): its purification and submitochondrial localization, and immunogold labeling in anther tapetum during microsporogenesis. Plant Physiol 95:861–870

Haese A, Schubert M, Herrmann M, Zocher R (1993) Molecular characterization of the enniatin synthetase gene encoding a multifunctional enzyme catalysing N-methyldepsipeptide formation in *Fusarium scirpi*. Mol Microbiol 7:905–914

Han F, Kleinhofs A, Kilian A, Ullrich SE (1997) Cloning and mapping of a putative barley NADP-dependent HC-toxin reductase. Mol Plant Microbe Interact 10:234–239

Han Y, Liu X, Benny U, Kistler HC, VanEtten HD (2001) Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. Plant J 25:305–314

Harimoto Y, Hatta R, Kodama M, Yamamoto M, Otani H, Tsuge T (2007) Expression profiles of genes encoded by the supernumerary chromosome controlling AM-toxin biosynthesis and pathogenicity in *Alternaria alternata*. Mol Plant Microbe Interact 20:1463–1476

Harris LJ, Gleddie SC (2001) A modified Rpl3 gene from rice confers tolerance of the *Fusarium graminearum* mycotoxin deoxynivalenol to transgenic tobacco. Physiol Mol Plant Pathol 58:173–181

Hatta R, Ito K, Hosaki Y, Tanaka T, Tanaka A, Yamamoto M, Akimitsu K, Tsuge T (2002) A conditionally dispensable chromosome controls host-specific pathogenicity in the fungal plant pathogen *Alternaria alternata*. Genetics 161: 59–70

Hoat TX, Nakayashiki H, Tosa Y, Mayama S (2006) Specific cleavage of ribosomal RNA and mRNA during victorin-induced apoptotic cell death in oat. Plant J 46:922–933

Hopkins AA, Young CA, Panaccione DG, Simpson WR, Mittal S, Bouton JH (2010) Agronomic performance and lamb health among several tall fescue novel endophyte combinations in the south-central USA. Crop Sci 50:1552–1561

Hopkins AA, Young CA, Butler TJ, Bouton JH (2011) Registration of Texoma MaxQ II tall fescue. J Plant Registr 5:14–18

Hopwood DA, Khosla C (1992) Genes for polyketide secondary metabolic pathways in microorganisms and plants. Ciba F Symp 171:88–106

Hopwood DA, Sherman DH (1990) Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu Rev Genet 24:37–66

Huang J, Lee SH, Lin C, Medici R, Hack E, Myers AM (1990) Expression in yeast of the T-URF13 protein from Texas male-sterile maize mitochondria confers sensitivity to methomyl and to Texas-cytoplasm-specific fungal toxins. EMBO J 9:339–348

Hume D, Ryan D, Cooper B, Popay A (2007) Agronomic performance of AR37-infected ryegrass in northern New Zealand. Proc N Z Grassland Assoc 69: 201–205

Imazaki A, Tanaka A, Harimoto Y, Yamamoto M, Akimitsu K et al (2010) Contribution of peroxisomes to secondary metabolism and pathogenicity in the fungal plant pathogen *Alternaria alternata*. Eukaryot Cell 9(5):682–694

Inderbitzin P, Asvarak T, Turgeon BG (2010) Six new genes required for production of T-Toxin, a polyketide determinant of high virulence of *Cochliobolus heterostrophus* to maize. Molec Plant Microbe Interact 23(4):458–472

Ito K, Tanaka T, Hatta R, Yamamoto M, Akimitsu K, Tsuge T (2004) Dissection of the host range of the fungal plant pathogen *Alternaria alternata* by modification of secondary metabolism. Mol Microbiol 52:399–411

Jansen C, von Wettstein D, Schafer W, Kogel K-H, Felk A et al (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proc Natl Acad Sci USA 102:16892–16897

Jenns AE, Daub ME, Upchurch RG (1989) Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology 79:213–219

Jensen JG, Popay AJ (2004) Perennial ryegrass infected with AR37 endophyte reduces survival of porina larvae. N Z Plant Protect 57:323–328

Jensen J, Popay A, Tapper B (2009) Argentine stem weevil adults are affected by meadow fescue endophyte and its loline alkaloids. N Z Plant Protect 62:12–18

Johal GS, Briggs SP (1992) Reductase activity encoded by the *HM1* disease resistance gene in maize. Science 258:985–987

Johnson RD, Johnson L, Itoh Y, Kodama M, Otani H, Kohmoto K (2000a) Cloning and characterization of a cyclic peptide synthetase gene from *Alternaria alternata* apple pathotype whose product is involved in AM-toxin synthesis and pathogenicity. Mol Plant Microbe Interact 13:742–753

Johnson RD, Johnson L, Kohmoto K, Otani H, Lane CR, Kodama M (2000b) A polymerase chain reaction-based method to specifically detect *Alternaria alternata* apple pathotype (*A. mali*), the causal agent of *Alternaria* blotch of apple. Phytopathology 90:973–976

Johnson L, Johnson RD, Akamatsu H, Salamiah A, Otani H, Kohmoto K, Kodama M (2001) Spontaneous loss of a conditionally dispensable chromosome from *Alternaria alternata* apple pathotype leads to loss of toxin production and pathogenicity. Curr Genet 40:65–72

Johnson R, Voisey C, Johnson L, Pratt J, Fleetwood D, Khan A, Bryan G (2007) Distribution of NRPS gene families within the *Neotyphodium/Epichloë* complex. Fungal Genet Biol 44:1180–1190

Kaspi CI, Siedow JN (1993) Cross-linking of the cms-T mitochondrial pore-forming protein URF13 by N, N'-dicyclohexylcarbodiimide and its effect on URF13 sensitivity to fungal toxins. J Biol Chem 268:5828–5833

Kawai M, Rich DH (1983) Total synthesis of the cyclic tetrapeptide, HC-toxin. Tet Lett 24:5309–5312

Keller N (2011) The fungal treasure chest: spore origins? Fungal Biol Rev 25:73–77

Kijima M, Yosida M, Sugita K, Horinouchi S, Beppu T (1993) Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. J Biol Chem 268:553–560

Kim S-D, Knoche HW, Dunkle LD (1987) Essentiality of the ketone function for toxicity of the host-selective toxin produced by *Helminthosporium carbonum*. Physiol Mol Plant Pathol 30:433–440

Kim Y-T, Lee Y-R, Jin J, Han K-H, Kim H et al (2005) Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zeae*. Mol Microbiol 58(4):1102–1113

Kleinkauf H, von Döhren H (1990) Nonribosomal biosynthesis of peptide antibiotics. Eur J Biochem 236:335–351

Knaus H, McManus OB, Lee SH, Schmalhofer WA, Garcia-Calvo M, Helms LMH, Sanchez M, Giangiacomo K, Reuben JP, Smith AB, Kaczorowski GJ, Garcia ML (1994) Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated channels. Biochemistry 33: 5819–5828

Kodama M, Rose MS, Yang G, Yun SH, Yoder OC, Turgeon BG (1999) The translocation-associated *Tox1* locus of *Cochliobolus heterostrophus* is two genetic elements on two different chromosomes. Genetics 151:585–596

Kohmoto K, Otani H (1991) Host recognition by toxigenic plant pathogens. Experientia 47:755–764

Kohmoto K, Taniguchi T, Nishimura S (1974) Correlation between the susceptibility of apple cultivars to *Alternaria mali* and their sensitivity to AM-toxin I. Ann Phytopathol Soc Jpn 43:65–66

Kohmoto K, Khan ID, Renbutsu Y, Taniguchi T, Nishimura S (1976) Multiple host-specific toxins of *Alternaria mali* and their effect on the permeability of host cells. Physiol Plant Pathol 8:141–153

Kohmoto K, Scheffer RP, Whiteside JO (1979) Host-selective toxins from *Alternaria citri*. Phytopathology 69:667–671

Kohmoto K, Itoh Y, Shimomura N, Kondoh Y, Otani H, Kodama M, Nishimura S, Nakatsuka S (1993) Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. Phytopathology 83:495–502

Kono Y, Daly JM (1979) Characterization of the host-specific pathotoxin produced by *Helminthosporium maydis* race T affecting corn with Texas male sterile cytoplasm. Bioorg Chem 8:391–397

Kono Y, Danko SJ, Suzuki Y, Takeuchi S, Daly JM (1983) Structure of the host-specific pathotoxins produced by *Phyllosticta maydis*. Tet Lett 24: 3803–3806

Korth KL, Kaspi CI, Siedow JN, Levings CS III (1991) URF13, a maize mitochondrial pore-forming protein, is oligomeric and has a mixed orientation in *Escherichia coli* plasma membranes. Proc Natl Acad Sci USA 88:10865–10869

Kościelniak J, Ostrowska A, Biesaga-Kościelniak J, Filek W, Janeczko A et al (2011) The effect of zearalenone on PSII photochemical activity and growth in wheat and soybean under salt (NaCl) stress. Acta Physiol Plant 33(6):2329–2338

Koulman A, Lane GA, Christensen MJ, Fraser K, Tapper BA (2007) Peramine and other fungal alkaloids are exuded in the guttation fluid of endophyte-infected grasses. Phytochemistry 68:355–360

Kroken S, Glass NL, Taylor JW, Yoder OC, Turgeon BG (2003) Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc Natl Acad Sci USA 100(26):15670–15675

Kuo M-S, Scheffer RP (1970) Comparative effects of host-specific toxins and *Helminthosporium* infections on respiration and carboxylation by host tissue. Phytopathology 60:1391–1394

Kutil BL, Greenwald C, Liu G, Spiering MJ, Schardl CL, Wilkinson HH (2007) Comparison of loline alkaloid gene clusters across fungal endophytes: predicting the co-regulatory sequence motifs and the evolutionary history. Fungal Genet Biol 44: 1002–1010

Lamari L, Bernier CC (1989) Toxin of *Pyrenophora tritici-repentis*: host-specificity, significance in disease, and inheritance of host reaction. Phytopathology 79:740–744

Lamari L, Bernier CC (1991) Genetics of tan necrosis and extensive chlorosis in tan spot of wheat caused by *Pyrenophora tritici-repentis*. Phytopathology 81:1092–1095

Lamari L, Ballance GM, Orolaza NP, Kowatch R (1995) In planta production and antibody neutralization of the Ptr necrosis toxin from *Pyrenophora tritici-repentis*. Phytopathology 85:333–338

Latch GCM, Christensen MJ (1985) Artificial infection of grasses with endophytes. Ann Appl Biol 107: 17–24

Leach J, Tegtmeier KJ, Daly JM, Yoder OC (1982) Dominance at the *Tox1* locus controlling T-toxin production by *Cochliobolus heterostrophus*. Physiol Plant Pathol 21:327–333

Lee BN, Kroken S, Chou DY, Robbertse B, Yoder OC, Turgeon BG (2005) Functional analysis of all nonribosomal peptide synthetases in *Cochliobolus heterostrophus* reveals a factor, NPS6, involved in virulence and resistance to oxidative stress. Eukaryot Cell 4:545–555

Lee TV, Johnson LJ, Johnson RD, Koulman A, Lane GA, Lott JS, Arcus VL (2010) Structure of a eukaryotic nonribosomal peptide synthetase adenylation domain that activates a large hydroxamate amino acid in siderophore biosynthesis. J Biol Chem 285: 2415–2427

Leisch JM, Sweeley CC, Staffeld GD, Anderson MS, Weber DJ, Scheffer RP (1982) Structure of HC-toxin, a cyclic tetrapeptide from *Helminthosporium carbonum*. Tetrahedron 38:45–48

Levings CS III (1990) The Texas cytoplasm of maize: cytoplasmic male sterility and disease susceptibility. Science 250:942–947

Lim SM, Hooker AL (1971) Southern corn leaf blight: genetic control of toxin production in race T and race O of *Cochliobolus heterostrophus*. Genetics 69: 115–117

Lipmann F (1971) Attempts to map a process evolution of peptide biosynthesis. Science 173:875–884

Liu Z, Faris JD, Oliver RP, Tan K-C, Solomon PS, McDonald MC, McDonald BA, Nunez A, Lu S, Rasmussen JB et al (2009) SnTox3 acts in effector triggered susceptibility to induce disease on wheat carrying the *Snn3* Gene. PLoS Pathog 5:e1000581

Liu Z, Zhang Z, Faris JD, Oliver RP, Syme R, McDonald MC, McDonald BA, Solomon PS, Lu S, Shelver WL et al (2012) The cysteine rich necrotrophic effector SnTox1 produced by *Stagonospora nodorum* triggers susceptibility of wheat lines harboring Snn1. PLoS Pathog 8:e1002467

Lorang JM, Carkaci-Salli N, Wolpert TJ (2004) Identification and characterization of victorin sensitivity in *Arabidopsis thaliana*. Mol Plant Microbe Interact 17:577–582

Lorang JM, Sweat TA, Wolpert TJ (2007) Plant disease susceptibility conferred by a "resistance" gene. Proc Natl Acad Sci USA 104:14861–14866

Lorang J, Cuesta-Marcos A, Hayes PM, Wolpert TJ (2010) Identification and mapping of adult-onset sensitivity to victorin in barley. Mol Breed 26: 545–550

Lousberg RJJC, Weiss U, Salmink CA, Aronone A, Merlini L, Nasini G (1971) The structures of cercosporin, a naturally occurring quinone. Chem Commun 71:1463–1464

Lu S, Lyngholm L, Yang G, Bronson C, Yoder OC, Turgeon BG (1994) Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. Proc Natl Acad Sci USA 91:12649–12653

Lysøe E, Bone KR, Klemsdal SS (2009) Real-time quantitative expression studies of the zearalenone

biosynthetic gene cluster in *Fusarium graminearum*. Phytopathology 99(2):176–184
Ma LJ, van der Does HC, Borkovich KA et al (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464: 367–373
Mackintosh CA, Lewis J, Radmer LE, Shin S, Heinen SJ et al (2006) Overexpression of defense response genes in transgenic wheat enhances resistance to Fusarium head blight. Plant Cell Rep 26:479–488
Macri F, Vianello A (1979) Inhibition of K+uptake, H+ extrusion and K+activated ATPase, and depolarization of transmembrane potential plant tissues treated with *Cercospora beticola* toxin. Physiol Plant Pathol 15:161–170
Maier FJ, Miedaner T, Hadeler B, Felk A, Salomon S et al (2006) Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (*Tri5*) gene in three field isolates of different chemotype and virulence. Mol Plant Pathol 7:449–461
Manning VA, Ciuffetti LM (2005) Localization of Ptr ToxA produced by *Pyrenophora tritici-repentis* reveals protein import into wheat mesophyll cells. Plant Cell 17:3203–3212
Manning VA, Andrie RM, Trippe AF, Ciuffetti LM (2004) Ptr ToxA requires multiple motifs for complete activity. Mol Plant Microbe Interact 17:491–501
Manning VA, Hardison LK, Ciuffetti LM (2007) Ptr ToxA interacts with a chloroplast-localized protein. Mol Plant Microbe Interact 20:168–177
Manning VA, Hamilton SM, Karplus PA, Ciuffetti LM (2008) The Arg-Gly-Asp-containing, solvent-exposed loop of Ptr ToxA is required for internalization. Mol Plant Microbe Interact 21:315–325
Marahiel MA, Stachelhaus T, Mootz HD (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev 97:2651–2673
McCormick SP, Stanley AM, Stover NA, Alexander NJ (2011) Trichothecenes: from simple to complex mycotoxins. Toxins 2011 3(7):802–814
McLaughlin JE, Bin-Umer MA, Tortora A, Mendez N, McCormick S et al (2009) A genome-wide screen in *Saccharomyces cerevisiae* reveals a critical role for the mitochondria in the toxicity of a trichothecene mycotoxin. Proc Natl Acad Sci 106: 21883–21888
Meehan F, Murphy HC (1946) A new *Helminthosporium* blight of oats. Science 104:412–413
Meehan F, Murphy HC (1947) Differential toxicity of metabolic by-products of *Helminthosporium victoriae*. Science 106:270–271
Meeley RB, Walton JD (1991) Enzymatic detoxification of HC-toxin, the host-selective cyclic peptide from *Cochliobolus carbonum*. Plant Physiology 97(3): 1080–1086
Meeley RB, Johal GS, Briggs SP, Walton JD (1992) A biochemical phenotype for a disease resistance gene of maize. Plant Cell 4(1):71–77
Mehrabi R, Bahkali AH, Abd-Elsalam KA, Moslem M, M'Barek SB, Gohari AM, Jashni MK, Stergiopoulos I, Kema GHJ, de Wit PJGM (2011) Horizontal gene and chromosome transfer in plant pathogenic fungi affecting host range. FEMS Microbiol Rev 35:542–554
Merhoff GC, Porter JM (1974) Ergot intoxication: historical review and description of unusual clinical manifestations. Ann Surg 180:773
Miyazaki S, Ishizaki I, Ishizaka M, Kanbara T, Ishiguro-Takeda Y (2004) Lolitrem B residue in fat tissues of cattle consuming endophyte-infected perennial ryegrass straw. J Vet Diagn Invest 16:340
Mukherjee PK, Buensanteai N, Moran-Diez ME, Druzhinina IS, Kenerley CM (2012) Functional analysis of non-ribosomal peptide synthetases (NRPSs) in *Trichoderma virens* reveals a polyketide synthase (PKS)/NRPS hybrid enzyme involved in the induced systemic resistance response in maize. Microbiology 158:155–165
Mullen TD, Jenkins RW, Clarke CJ, Bielawski J, Hannun YA, Obeid LM (2011) Ceramide synthase-dependent ceramide generation and programmed cell death: involvement of salvage pathway in regulating postmitochondrial events. J Biol Chem 286:15929–15942
Multani DS, Meeley RB, Paterson AH, Gray J, Briggs SP, Johal GS (1998) Plant-pathogen microevolution: molecular basis for the origin of a fungal disease in maize. Proc Natl Acad Sci USA 95:1686–1691
Musser SM, Plattner RD (1997) Fumonisin composition in cultures of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium nygami*. J Agric Food Chem 45:1169–1173
Nakashima T, Ueno T, Fukami H (1982) Structure elucidation of AK-toxins, host-specific phytotoxic metabolites produced by *Alternaria kikuchiana* Tanaka. Tet Lett 23:4469–4472
Nakashima T, Ueno T, Fukami H, Taga T, Masuda H, Osaki K, Otani H, Kohomoto K, Hishimura S (1985) Isolation and structures of AK-toxin I and II, host-specific phytotoxic metabolites produced by *Alternaria alternata* Japanese pear pathotype. Agric Biol Chem 49:807–815
Nakatsuka S, Feng B-N, Goto T, Tsuge T, Nishimura S (1990) Biosynthetic origin of (8R,9S)-9,10-epoxy-8-hydroxy-9-methyl-deca-(2E,4Z,6E)-trienoic acid, a precursor of AK-toxins produced by *Alternaria alternata* Japanese pear pathotype. Phytochemistry 29:1529–1531
Namiki F, Okamoto H, Katou K, Yamamoto M, Nishimura S, Nakatsuka S, Goto T, Kohmoto K, Otani H, Novacky A (1986) Studies on host-specific AF-toxins produced by *Alternaria alternata* strawberry pathotype causing Alternaria black spot of strawberry. V. Effect of toxins on membrane potential of susceptible plants as assessed

by electrophysiological method. Ann Phytopathol Soc Jpn 52:610–619

Navarre DA, Wolpert TJ (1995) Inhibition of the glycine decarboxylase multienzyme complex by the host-selective toxin victorin. Plant Cell 7:463–471

Navarre DA, Wolpert TJ (1999) Victorin induction of an apoptotic/senescence-like response in oats. Plant Cell 11:237–249

Nelson RR, Ullstrup AJ (1961) The inheritance of pathogenicity in *Cochliobolus carbonum*. Phytopathology 51:1–2

Nicholson MJ, Koulman A, Monahan BJ, Pritchard BL, Payne GA, Scott B (2009) Identification of two aflatrem biosynthesis gene loci in *Aspergillus flavus* and metabolic engineering of *Penicillium paxilli* to elucidate their function. Appl Environ Microbiol 75:7469–7481

Nikolskaya AN, Panaccione DG, Walton JD (1995) Identification of peptide synthetase-encoding genes from filamentous fungi producing host-selective phytotoxins or analogs. Gene 165:207–211

Nishiuchi T, Masuda D, Nakashita H, Ichimura K, Shinozaki K et al (2006) *Fusarium* phytotoxin trichothecenes have an elicitor-like activity in *Arabidopsis thaliana*, but the activity differed significantly among their molecular species. Mol Plant Microbe Interact 19:512–520

Ohsato S, Ochiai-Fukuda T, Nishiuchi T, Takahashi-Ando N, Koizumi S et al (2007) Transgenic rice plants expressing trichothecene 3-O-acetyltransferase show resistance to the Fusarium phytotoxin deoxynivalenol. Plant Cell Rep 26:531–538

Okubara PA, Blechl AE, McCormick SP, Alexander NJ, Dill-Macky R et al (2002) Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. Theor Appl Genet 106:74–83

Okuno T, Ishita Y, Sawai K, Matsumoto T (1974) Characterization of alternariolide, a host-specific toxin produced by *Alternaria mali* Roberts. Chem Lett 1974:635–638

Otani H, Kohmoto K (1992) Host-specific toxins of *Alternaria* species. In: Chelkowski J, Visconti A (eds) *Alternaria*: biology, plant diseases and metabolites. Elsevier, Amsterdam, pp 123–156

Otani H, Nishimura S, Kohmoto K (1972) Nature of specific susceptibility to *Alternaria kikuchiana* in Nijisseiki cultivars among Japanese pears (I). J Fac Agric Tottori Univ 7:5–12

Otani J, Kohmoto K, Nishimura S, Nakashima T, Ueno T, Fukami H (1985) Biological activities of AK-toxins I and II, host-specific toxins from *Alternaria alternata* Japanese pear pathotype. Ann Phytopathol Soc Jpn 51:285–293

Otani H, Tomiyama K, Okamoto H, Nishimura S, Kohmoto K (1989) Effect of AK-toxin produced by *Alternaria alternata* Japanese pear pathotype on membrane potential of pear cells. Ann Phytopathol Soc Jpn 55:466–468

Panaccione DG (1996) Multiple families of peptide synthetase genes from ergopeptine-producing fungi. Mycol Res 100:429–436

Panaccione DG, Scott-Craig JS, Pocard JA, Walton JD (1992) A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. Proc Natl Acad Sci USA 89:6590–6594

Panaccione DG, Johnson RD, Wang J, Young CA, Damrongkool P, Scott B, Schardl CL (2001) Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. Proc Natl Acad Sci USA 98: 12820–12825

Parent-Massin D (2004) Haematotoxicity of trichothecenes. Toxicol Lett 153:75–81

Park P, Tsuda M, Hayashi Y, Ueno T (1977) Effect of a host-specific toxin (AM-toxin I) produced by *Alternaria mali*, an apple pathogen, on the ultrastructure of plasma membrane of cells in apple and Japanese pear leaves. Can J Bot 55:2383–2393

Park P, Nishimura S, Kohmoto K, Otani H, Tsujimoto K (1981) Two action sites of AM-toxin I produced by apple pathotype of *Alternaria alternata* in host cells: an ultrastructural study. Can J Bot 59: 301–310

Park P, Ohno T, Nishimura S, Kohmoto K, Otani H (1987) Leakage of sodium ions from plasma membrane modification, associated with permeability change, in host cells treated with a host-specific toxin from a Japanese pear pathotype of *Alternaria alternata*. Can J Bot 65:330–339

Park P, Lee SS, Ohno T, Tsuge T, Nishimura S (1992) Slightly damaged type of plasma membrane modification in strawberry leaves treated with AF-toxin I produced by *Alternaria alternata* strawberry pathotype. Ann Phytopathol Soc Jpn 58:431–445

Patchett B, Chapman R, Fletcher L, Gooneratne S (2008) Root loline concentration in endophyte-infected meadow fescue (*festuca pratensis*) is increased by grass grub (*Costelytra zealandica*) attack. N Z Plant Protect 61:210–214

Pedley KF, Walton JD (2001) Regulation of cyclic peptide biosynthesis in a plant pathogenic fungus by a novel transcription factor. Proc Natl Acad Sci USA 98:14174–14179

Pestka JJ (2007) Deoxynivalenol: toxicity, mechanisms and animal health risks. Anim Feed Sci Technol 137:283–298

Pitkin JW, Panaccione DG, Walton JD (1996) A putative cyclic peptide efflux pump encoded by the *TOXA* gene of the plant pathogenic fungus *Cochliobolus carbonum*. Microbiology 142:1557–1565

Pope MR, Ciuffetti LM, Knoche HW, McCrery D, Daly JM, Dunkle LD (1983) Structure of the host-specific toxin produced by *Helminthosporium carbonum*. Biochemistry 22:3502–3506

Porter JK, Bacon CW, Robbins JD, Betowski D (1981) Ergot alkaloid identification in Clavicipitaceae

systemic fungi of pasture grasses. J Agric Food Chem 29:653–657
Proctor RH, Hohn TM, McCormick SP (1995) Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Mol Plant Microbe Interact 8:593–601
Proctor RH, Desjardins AE, Plattner RD, Hohn TM (1999) A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberella fujikuroi* mating population A. Fungal Genet Biol 27:100–112
Proctor RH, Brown DW, Plattner RD, Desjardins AE (2003) Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genet Biol 38: 237–249
Proctor RH, Plattner RD, Desjardins AE, Busman M, Butchko RAE (2006) Fumonisin production in the maize pathogen *Fusarium verticillioides*: genetic basis of naturally occurring chemical variation. J Agric Food Chem 54:2424–2430
Proctor RH, McCormick SP, Alexander NJ, Desjardins AE (2009) Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. Mol Microbiol 74:1128–1142
Ransom R, Walton JD (1997) Histone hyperacetylation in maize in response to treatment with HC-toxin or infection by the filamentous fungus *Cochliobolus carbonum*. Plant Physiol 115:1021–1027
Rasmussen JB, Scheffer RP (1988) Isolation and biological activities of four selective toxins from *Helminthosporium carbonum*. Plant Physiol 86: 187–191
Rausch C, Weber T, Kohlbacher O, Wohlleben W, Huson DH (2005) Specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPS) using transductive support vector machines (TSVMs). Nucleic Acids Res 33: 5799–5808
Reed KFM, Walsh JR, Sprague M (2004) Zearalenone and its presence in pasture. Anim Prod Aust 25: 140–143
Rhoads DM, Burnner-Neuenschwander B, Levings CS III (1998) Characterization of the interaction between fungal pathotoxins and URF13, the *cms-T* maize mitochondrial T-toxin receptor. In: Yoder OC, Kohmoto K (eds) Molecular genetics of host specific toxins in plant disease. Kluwer, The Netherlands, pp 355–365
Rocha O, Ansari K, Doohan FM (2005) Effects of trichothecene mycotoxins on eukaryotic cells: a review. Food Addit Contam 22:369–378
Rose MS, Yun S-H, Asvarak T, Lu S-W, Yoder OC et al (2002) A decarboxylase encoded at the *Cochliobolus heterostrophus* translocation-associated *Tox1B* locus is required for polyketide (T-toxin) biosynthesis and high virulence on T-cytoplasm maize. Molec Plant Microbe Interact 15(9):883–893
Rotter BA, Thompson BK, Lessard M, Trenholm HL, Tryphonas H (1994) Influence of low-level exposure to Fusarium mycotoxins on selected immunological and hematological parameters in young swine. Toxicol Sci 23:117–124
Saikia S, Scott B (2009) Functional analysis and subcellular localization of two geranylgeranyl diphosphate synthases from *Penicillium paxilli*. Molec Genet Genom 282:257–271
Saito K, Niizeki K (1988) Fundamental studies on breeding of the apple. IX. Genetic analysis of resistance to Alternaria blotch in the interspecific crosses. Bull Fac Agric Hirosaki Univ 50:27–34
Sarma GN, Manning VA, Ciuffetti LM, Karplus PA (2005) Structure of Ptr ToxA: an RGD-containing host-selective toxin from *Pyrenophora tritici-repentis*. Plant Cell 17:3190–3202
Sawamura K (1966) Studies on spotted disease of apples. VI. On the host-specific toxin of *Alternaria mali* Roberts. Bull Hortic Res Stn Japan Ser C 4:43–59
Sawamura K (1990) Alternaria blotch. In: Jones AL, Aldwinckle H (eds) Compendium of apple and pear diseases. APS Press, St. Paul, pp 24–25
Schardl CL (1996) Epichloë species: fungal symbionts of grasses. Annu Rev Phytopathol 34:109–130
Schardl CL, Leuchtmann A, Spiering MJ (2004) Symbioses of grasses with seedborne fungal endophytes. Annu Rev Plant Biol 55:315–340
Schardl CL, Grossman RB, Nagabhyru P, Faulkner JR, Mallik UP (2007) Loline alkaloids: currencies of mutualism. Phytochemistry 68:980–996
Schardl CL, Young CA, Faulkner JR, Florea S, Pan J (2012) Chemotypic diversity of epichloae, fungal symbionts of grasses. Fungal Ecol 5:331–344
Scheffer RP, Ullstrup AJ (1965) A host-specific toxic metabolite from *Helminthosporium carbonum*. Phytopathology 55:1037–1038
Scheffer RP, Nelson RR, Ullstrup AJ (1967) Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae*. Phytopathology 57:1288–1291
Scherm B, OrrÙ M, Balmas V, Spanu F, Azara E et al (2011) Altered trichothecene biosynthesis in TRI6-silenced transformants of *Fusarium culmorum* influences the severity of crown and foot rot on durum wheat seedlings. Mol Plant Pathol 12: 759–771
Schoevers EJ, Santos RR, Colenbrander B, Fink-Gremmels J, Roelen BAJ (2012) Transgenerational toxicity of Zearalenone in pigs. Reprod Toxicol 34 (1):110–119
Schwarzer D, Finking R, Marahiel MA (2003) Nonribosomal peptides: from genes to products. Nat Prod Rep 20:275–287
Scott-Craig JS, Panaccione DG, Pocard JA, Walton JD (1992) The multifunctional cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7-kb open reading frame. J Biol Chem 67:26044–26049
Seshime Y, Juvvadi PR, Fujii I, Kitamoto K (2005) Discovery of a novel superfamily of type III polyketide

synthases in *Aspergillus oryzae*. Biochem Biophys Res Commun 331(1):253–260

Shabeer A, Bockus WW (1988) Tan spot effects on yield and yield components relative to growth stage in winter wheat. Plant Disease 72:599–602

Shiba T, Sugawara K (2009) Fungal loline alkaloids in grass–endophyte associations confer resistance to the rice leaf bug, *Trigonotylus caelestialium*. Entomol Exp Appl 130:55–62

Shimizu N, Hosogi N, Hyon GS, Jiang S, Inoue K et al (2006) Reactive oxygen species (ROS) generation and ROS-induced lipid peroxidation are associated with plasma membrane modifications in host cells in response to AK-toxin I from *Alternaria alternata* Japanese pear pathotype. J Gen Plant Pathol 72(1):6–15

Shimomura N, Otani H, Kodama M, Kohmoto K (1991) Two primary action sites for AM-toxin produced by *Alternaria alternata* apple pathotype and their pathological significance. Ann Phytopathol Soc Jpn 57:47–255

Shin S, Mackintosh CA, Lewis J, Heinen SJ, Radmer L et al (2008) Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*. J Exp Bot 59: 2371–2378

Siegel MR, Latch GCM, Bush LP, Fannin FF, Rowan DD, Tapper BA, Bacon CW, Johnson MC (1990) Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. J Chem Ecol 16:3301–3315

Slot JC, Rokas A (2011) Horizontal transfer of a large and highly toxic secondary metabolic gene cluster between fungi. Curr Biol 21:134–139

Smith JF, Morris CA (2006) Review of zearalenone studies with sheep in New Zealand. Proc New Zeal Soc An 66:306–310

Solomon PS, Lowe RGT, Tan K-C, Waters ODC, Oliver RP (2006) *Stagonospora nodorum*: cause of stagonospora nodorum blotch of wheat. Molec Plant Pathol 7:147–156

Spassieva SD, Markham JE, Hille J (2002) The plant disease resistance gene *Asc-1* prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant J 32: 561–572

Spiering MJ, Wilkinson HH, Blankenship JD, Schardl CL (2002) Expressed sequence tags and genes associated with loline alkaloid expression by the fungal endophyte *Neotyphodium uncinatum*. Fungal Genet Biol 36:242–254

Spiering MJ, Moon CD, Wilkinson HH, Schardl CL (2005) Gene clusters for insecticidal loline alkaloids in the grass-endophytic fungus *Neotyphodium uncinatum*. Genetics 169:1403–1414

Spiering MJ, Faulkner JR, Zhang DX, Machado C, Grossman RB, Schardl CL (2008) Role of the LolP cytochrome P450 monooxygenase in loline alkaloid biosynthesis. Fungal Genet Biol 45: 1307–1314

Stachelhaus T, Mootz HD, Marahiel MA (1999) The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem Biol 6:493–505

Stadler P, Stütz P (1975) The ergot alkaloids. Alkaloids Chem Physiol 15:1–40

Stein T, Vater J, Kruft V, Wittmann-Liebold B, Franke P, Panico M, McDowell R, Morris HR (1994) Detection of 4¢-phosphopantetheine as the thioester binding site for L-valine of gramicidin S synthetase 2. FEBS Lett 340:39–44

Strelkov SE, Lamari L, Ballance GM (1998) Induced chlorophyll degradation by a chlorosis toxin from *Pyrenophora tritici-repentis*. Can J Plant Pathol 20:428–435

Strickland JR, Aiken GE, Spiers DE, Fletcher LR, Oliver JW (2009) Physiological basis of fescue toxicosis. In: Fribourg HA, Hannaway DB, West CP (eds) Tall fescue for the twenty-first century, vol 53, Agronomy monograph. ASA/CSSA/SSSA, Madison, pp 203–228

Stukenbrock EH, McDonald BA (2007) Geographical variation and positive diversifying selection in the host-specific toxin SnToxA. Molec Plant Pathol 8:321–332

Sweat TA, Wolpert TJ (2007) Thioredoxin h5 is required for victorin sensitivity mediated by a CC-NBS-LRR gene in Arabidopsis. Plant Cell 19: 673–687

Sweat TA, Lorang JM, Bakker EG, Wolpert TJ (2008) Characterization of natural and induced variation in the *LOV1* gene, a CC-NB-LRR gene conferring victorin sensitivity and disease susceptibility in *Arabidopsis*. Mol Plant Microbe Interact 21:7–19

Tada Y, Hata S, Takata Y, Nakayashiki H, Tosa Y, Mayama S (2001) Induction and signaling of an apoptotic response typified by DNA laddering in the defense response of oats to infection and elicitors. Mol Plant Microbe Interact 14:477–486

Tada Y, Kusaka K, Betsuyaku S, Shinogi T, Sakamoto M, Ohura Y, Hata S, Mori T, Tosa Y, Mayama S (2005) Victorin triggers programmed cell death and the defense response via interaction with a cell surface mediator. Plant Cell Physiol 46:1787–1798

Tan K-C, Oliver RP, Solomon PS, Moffat CS (2010) Proteinaceous necrotrophic effectors in fungal virulence. Funct Plant Biol 37:907–912

Tanaka S (1933) Studies on black spot disease of Japanese pear (*Pyrus serotina* Rehd). Mem Coll Agric Kyoto Imp Univ 28:1–31

Tanaka A, Tsuge T (2000) Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity in the Japanese pear pathotype of *Alternaria alternata*. Mol Plant Microbe Interact 13:975–986

Tanaka A, Shiotani H, Yamamoto M, Tsuge T (1999) Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. Mol Plant Microbe Interact 12: 691–702

Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B (2005) A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol Microbiol 57: 1036–1050

Tatsuno T, Saito M, Enomoto M, Tsunoda H (1968) Nivalenol, a toxic principle of *Fusarium nivale*. Chem Pharm Bull(Tokyo) 16:2519–2520

Timper P, Gates RN, Bouton JH (2005) Response of *Pratylenchus* spp. in tall fescue infected with different strains of the fungal endophyte *Neotyphodium coenophialum*. Nematology 7: 105–110

Tkacz JS (2000) Polyketide and peptide products of endophytic fungi: variations on two biosynthetic themes of secondary metabolism. In: Bacon CW, White JF (eds) Microbial endophytes. Dekker Inc., New York, pp 263–294

Tomás A, Bockus WW (1987) Cultivar specific toxicity of culture filtrate of *Pyrenophora tritici-repentis*. Phytopathology 77:1337–1366

Towers NR, Sprosen JM (1993) Zearalenone-induced infertility in sheep and cattle in New Zealand. N Z Vet J 41:223–224

Tsai H, Wang H, Gebler JC, Poulter CD, Schardl CL (1995) The *Claviceps purpurea* gene encoding dimethylallyltryptophan synthase, the committed step for ergot alkaloid biosynthesis. Biochem Biophys Res Commun 216:119–125

Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagi Y, Egusa M, Yamamoto M, Otani H (2013) Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. FEMS Microbiol Rev 37(1):44–66

Tudzynski B, Hölter K (1998) Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. Fungal Genet Biol 25:157–170

Tudzynski P, Holter K, Correia T, Arntz C, Grammel N, Keller U (1999) Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*. Mol Gen Genet 261: 133–141

Turgay K, Marahiel MA (1994) A general approach for identifying and cloning peptide synthetase genes. Peptide Res 7:238–241

Turgeon BG, Baker SE (2007) Genetic and genomic dissection of the *Cochliobolus heterostrophus* Tox1 locus controlling biosynthesis of the polyketide virulence factor T-toxin. In: Dunlap JC (ed) Fungal genomics. San Diego, pp 219–261

Tzeng TH, Lyngholm LK, Ford CF, Bronson CR (1992) A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. Genetics 130:81–96

Ueno T, Nakashima T, Hayashi Y, Fukami H (1975) Structures of Am-toxin I and II, host-specific phytotoxic metabolites produced by *Alternaria mali*. Agric Biol Chem 39:1115–1122

Ueno T, Nakashima T, Uemoto M, Fukami H, Lee SN, Izumiya N (1977) Mass spectrometry of *Alternaria mali* toxins and related cyclodepsipeptides. Biomed Mass Spectr 4:134–142

Uhlig S, Botha CJ, Vrålstad T, Rolén E, Miles CO (2009) Indole–diterpenes and ergot alkaloids in *Cynodon dactylon* (Bermuda Grass) Infected with *Claviceps cynodontis* from an outbreak of tremors in Cattle. J Agric Food Chem 57:11112–11119

Ullstrup AJ (1941) Two physiologic races of *Helminthosporium maydis* in the corn belt. Phytopathology 31:508–521

Ullstrup AJ, Brunson AM (1947) Linkage relationships of a gene in corn determining susceptibility to a *Helminthosporium* leaf spot. J Am Soc Agron 39: 606–609

Upchurch RG, Walker DC, Rollins JA, Ehrenshaft M, Daub ME (1991) Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. Appl Environ Microbiol 57:2940–2945

van Egmond H, Schothorst R, Jonker M (2007) Regulations relating to mycotoxins in food. Anal Bioanal Chem 389:147–157

Vartapetian AB, Tuzhikov AI, Chichkova NV, Taliansky M, Wolpert TJ (2011) A plant alternative to animal caspases: Subtilisin-like proteases. Cell Death Differ 18:1289–1297

Verdin E, Dequiedt F, Kasler HG (2003) Class II histone deacetylases: versatile regulators. Trends Genet 19: 286–293

von Allmen JM, Rottmann WH, Gengenbach BG, Ahrvey AJ, Lonsdale DM (1991) Transfer of methomyl and HmT-toxin sensitivity from T-cytoplasm maize to tobacco. Mol Gen Genet 229:405–412

von Döhren H, Keller U, Vater J, Zocher R (1997) Multifunctional peptide synthetases. Chem Rev 97:2675–2705

Wakil SJ (1989) Fatty acid synthase, a proficient multifunctional enzyme. Biochemistry 28:4523–4530

Walton JD (1987) Two enzymes involved in biosynthesis of the host-selective phytotoxin HC-toxin. Proc Nat Acad Sci USA 84:8444–8447

Walton JD (2000) Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. Fungal Genet Biol 30: 167–171

Walton JD (2006) HC-toxin. Phytochemistry 67: 1406–1413

Walton JD, Earle ED (1983) The epoxide in HC-toxin is required for activity against susceptible maize. Physiol Plant Pathol 22:371–376

Walton JD, Holden FR (1988) Properties of two enzymes involved in the biosynthesis of the fungal pathogenicity factor HC-toxin. Mol Plant Microbe Interact 1:128–134

Walton JD, Earle ED, Gibson BW (1982) Purification and structure of the host-specific toxin from *Helminthosporium carbonum* race 1. Biochem Biophys Res Comm 107:785–794

Walton JD, Ahn JH, Pitkin JW, Cheng YQ, Nikolskaya AN, Ransom R, Wegener S (1998) Enzymology, molecular genetics, and regulation of biosynthesis of the host-selective toxin HC-toxin. In: Kohomoto K, Yoder OC (eds) Molecular genetics of

host-specific toxins in plant disease. Kluwer, The Netherlands, pp 25–34

Wang W, Jones C, Ciacci-Zanella J, Holt T, Gilchrist DG, Dickman MB (1996) Fumonisins and *Alternaria alternata lycopersici* toxins: sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. Proc Natl Acad Sci USA 93:3461–3465

Wang J, Machado C, Panaccione DG, Tsai H, Schardl CL (2004) The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fungal Genet Biol 41:189–198

Wang M, Zhou H, Wirz M, Tang Y, Boddy CN (2009) A thioesterase from an iterative fungal polyketide synthase shows macrocyclization and cross coupling activity and may play a role in controlling iterative cycling through product offloading. Biochemistry 48(27):6288–6290

Weber G, Schmörgendorfer K, Schneider-Scherzer E, Leitner E (1994) The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8 kilobase open reading frame. Curr Genet 26:120–125

Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL (2000) Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. Mol PlantMicrobe Interact 13:1027–1033

Williams LD, Glenn AE, Bacon CW, Smith MA, Riley RT (2006) Fumonisin production and bioavailability to maize seedlings grown from seeds inoculated with *Fusarium verticillioides* and grown in natural soils. J Agric Food Chem 54: 5694–5700

Wise RP, Fliss AE, Pring DR, Gengenbach BG (1987) *urf*13-*T* of T cytoplasm maize mitochondria encodes a 13 kD polypeptide. Plant Mol Biol 9: 121–126

Wolf SJ, Earle ED (1991) Effects of *Helminthosporium carbonum* race 1 toxin on host and non-host cereal protoplasts. Plant Sci 70:127–137

Wolpert TJ, Macko V, Acklin W, Juan B, Seibl J, Meili J, Arigoni D (1985) Structure of victorin C, the major host-selective toxin from *Cochliobolus victoriae*. Experientia 41:1524–1529

Wolpert TJ, Macko V, Acklin W, Juan B, Arigoni D (1986) Structure of minor host-selective toxins from *Cochliobolus victoriae*. Experientia 42:1296–1301

Wolpert TJ, Navarre DA, Moore DL, Macko V (1994) Identification of the 100 kD victorin binding protein from oats. Plant Cell 6:1145–1155

Wolpert TJ, Dunkle LD, Ciuffetti LM (2002) Host-selective toxins and avirulence determinants: what's in a name? Annu Rev Phytopathol 40:251–285

Yamagishi D, Akamatsu H, Otani H, Kodama M (2006) Pathological evaluation of host-specific AAL-toxins and fumonisin mycotoxins produced by *Alternaria* and *Fusarium* species. J Gen Plant Pathol 72:323–326

Yamazaki S, Ogawa T (1972) The chemistry and stereochemistry of cercosporin. Agric Biol Chem 36:1707–1718

Yang G, Rose MS, Turgeon BG, Yoder OC (1996) A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. Plant Cell 8:2139–2150

Yao N, Tada Y, Park P, Nakayashiki H, Tosa Y, Mayama S (2001) Novel evidence for apoptotic cell response and differential signals in chromatin condensation and DNA cleavage in victorin-treated oats. Plant J 28:13–26

Yates SG, Tookey HL, Ellis JJ, Burkhardt HJ (1968) Mycotoxins produced by *Fusarium nivale* isolated from tall fescue (*Festuca arundinacea* schreb.). Phytochemistry 7:139–146

Yi H, Bojja RS, Fu J, Du L (2005) Direct evidence for the function of *FUM13* in 3-ketoreduction of mycotoxin fumonisins in *Fusarium verticillioides*. J Agric Food Chem 53:5456–5460

Yoder OC (1998) A mechanistic view of the fungal/plant interaction based on host-specific toxin studies. In: Kohmoto K, Yoder OC (eds) Molecular genetics of host-specific toxins in plant disease. Kluwer, The Netherlands, pp 3–15

Yoder OC, Scheffer RP (1973a) Effects of *Helminthosporium carbonum* toxin on nitrate uptake and reduction by corn tissues. Plant Physiol 52:513–517

Yoder OC, Scheffer RP (1973b) Effects of *Helminthosporium carbonum* toxin on absorption of solutes by corn roots. Plant Physiol 52:518–523

Yoshida H, Sugita K (1992) A novel tetracyclic peptide, trapoxin, induces phenotypic change from transformed to normal in sis-oncogene-transformed NIH3T3 cells. Jpn J Cancer Res 83:324–328

Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 265:17174–17179

Yoshizawa T, Morooka N (1973) Deoxynivalenol and its monacetate: new mycotoxins from *Fusarium roseum* and moldy barley. Agric Biol Chem 37:2933–2934

You B-J, Lee M-H, Chung K-R (2008) Production of cercosporin toxin by the phytopathogenic Cercospora fungi is affected by diverse environmental signals. Can J Microbiol 54(4):259–269

Young CA, McMillan L, Telfer E, Scott B (2001) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. Mol Microbiol 39:754–764

Young CA, Bryant MK, Christensen MJ, Tapper BA, Bryan GT, Scott B (2005) Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a

mutualistic endophyte of perennial ryegrass. Mol Genet Genomics 274:13–29

Young CA, Felitti S, Shields K, Spangenberg G, Johnson RD, Bryan GT, Saikia S, Scott B (2006) A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Genet Biol 43:679–693

Young CA, Tapper BA, May K, Moon CD, Schardl CL, Scott B (2009) Indole-diterpene biosynthetic capability of epichloë endophytes as predicted by *ltm* gene analysis. Appl Environ Microbiol 75:2200–2211

Yun SH, Turgeon BG, Yoder OC (1998) REMI-induced mutants of *Mycosphaerella zeae-maydis* lacking the polyketide PM-toxin are deficient in pathogenesis to corn. Physiol Mol Plant Pathol 52:53–66

Zaleta-Rivera K, Xu C, Yu F, Butchko RAE, Proctor RH, Hidalgo-Lara ME, Raza A, Dussault PH, Du L (2006) A bidomain nonribosomal peptide synthetase encoded by *FUM14* catalyzes the formation of tricarballylic esters in the biosynthesis of fumonisins. Biochemistry 45:2561–2569

Zhang S, Monahan BJ, Tkacz JS, Scott B (2004) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Aspergillus flavus*. Appl Environ Microbiol 70:6875–6883

Zhou H, Zhan J, Watanabe K, Xie X, Tang Y (2008) A polyketide macrolactone synthase from the filamentous fungus *Gibberella zeae*. Proc Natl Acad Sci USA 105(17):6249–6254

5 Organopollutant Degradation by Wood Decay Basidiomycetes

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CONTENTS

I. Introduction

Wood decay fungi are obligate aerobes, deriving nutrients from the biological 'combustion' of wood, using molecular oxygen as terminal electron acceptor (Kirk and Farrell 1987; Blanchette 1991). Non-specific extracellular enzymes are generally viewed as key components in lignin depolymerization. The major enzymes implicated in lignin degradation are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. All three can act with low molecular weight mediators to bring about oxidation of lignin and various xenobiotics (Cullen 2002).

Saprotrophic Agaricomycotina, particularly ligninolytic 'white-rot fungi' have been extensively studied for their ability to degrade a wide range of organopollutants such as polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals, pentachlorophenol (PCP), pesticides, and explosives. The unique extracellular systems of the white-rot fungi have been repeatedly invoked to explain the extraordinary oxidation potential of these microbes, but the precise mechanisms remain elusive. In addition to secreted enzyme systems, intracellular metabolic processes are responsible for further transformation, degradation, and, often, mineralization of compounds. Cytochrome P450s have been implicated in several instances, and a hallmark of most Agaricomycotina is a large number of P450 encoding genes.

The nature and extent of white-rot genetic diversity have been more fully appreciated in recent years, with the rapid increase in genome sequencing and analysis. Among the white-rot species known to degrade recalcitrant PAHs, sequences of *Phanerochete chrysosporium* (Martinez et al. 2004), *Pleurotus ostreatus* (http://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.info.html) (Fig. 5.1), *Ceriporiopsis subvermispora* (Fernandez-Fueyo et al. 2012), *Trametes versicolor*, and *Dichomitus squalens* (Floudas et al. 2012) are now publicly available. This review provides a critical analysis of recent advances on the genetics and physiology of wood decay fungi as they relate to organopollutant degradation, and we place particular emphasis on the model experimental systems *P. chrysosporium* and *P. ostreatus*. This is not

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Fig. 5.1. A fruiting body of *Pleurotus ostreatus*, the oyster mushroom, a commercially important edible white-rot filamentous basidiomycete cultivated on lignocellulosic waste. This is a model fungus for the study of biochemical and molecular mechanisms involved in biodegradation of lignin and recalcitrant and toxic anthropogenic aromatic compounds. The dikaryon stage is required for the formation of the fruiting bodies

intended to be a comprehensive treatment of the voluminous physiological literature in this area. Interested readers are referred to earlier reviews (Kirk and Farrell 1987; Eriksson et al. 1990; Cullen and Kersten 2004).

II. Biochemistry of Lignin and Organopollutant Degradation

A. Lignin Peroxidase

Lignin peroxidase (LiP) will cleave Cα–Cβ bonds of lignin model compounds and partially depolymerize methylated lignin in vitro (Glenn et al. 1983; Tien and Kirk 1983, 1984; Gold et al. 1984). A variety of oxidations, all dependent on H_2O_2, have been demonstrated (Harvey et al. 1985; Kersten et al. 1985; Shoemaker et al. 1985; Hammel et al. 1986b). In short, LiP oxidizes aromatic substrates by a single electron, and the resulting aryl cation radicals undergo spontaneous degradation, yielding many different products dependent on substrate structure. The complex reactions and the role(s) of peroxidases in ligninolysis have been reviewed (Higuchi 1990; Hammel and Cullen 2008).

Given their low specificity and high oxidation potential, it is perhaps not surprising the LiPs oxidize a remarkable array of organopollutants (reviewed in Refs. Higson 1991; Hammel 1995a, b; Pointing 2001; Cullen 2002). Among these are the PAHs, pollutants from both geochemical and anthropogenic sources. PAHs consist of three or more benzene rings fused in a linear, angular, or cluster arrangement. As their molecular weight increases, water solubility and biodegradability decrease and genotoxicity increases. The biodegradation and bioremediation of these compounds have attracted much attention in recent decades. (reviewed in Refs. Peng et al. 2008; Gan et al. 2009; Haritash and Kaushik 2009; Lu et al. 2011)

***Phanerochaete chrysosporium* has been the most intensively studied white-rot fungus** for its extraordinary ability to oxidize and/or mineralize a broad range of PAHs. For example, *P. chrysosporium* degraded at least 22 PAHs, including all of the most abundant PAH components present in anthracene oil, and underwent 70–100 % disappearance during 27 days of incubation with nutrient nitrogen-limited cultures (Bumpus 1989). The mechanism(s) of degradation of PAHs is/are diverse, and can include those that are unique to ligninolytic fungi or exist in other microorganisms. Benzo (a) pyrene, anthracene, and pyrene have ionization potentials below 7.6 eV, and serve as substrates for LiP (Hammel et al. 1986a; Hammel 1995a). In

addition to PAHs, purified LiPs will transform chlorinated phenols (Hammel and Tardone 1988; Mileski et al. 1988; Valli and Gold 1991; Reddy and Gold 2000), tetrahydrofurans (Vazquez-Duhalt et al. 1994), dioxins (Hammel et al. 1986a; Valli et al. 1992b,), methoxybenzenes (Kersten et al. 1985), and various chloro- and nitro-methoxybenzenes (Valli and Gold 1991; Valli et al. 1992a, b; Teunissen et al. 1998).

B. Manganese Peroxidase

Like LiPs, **manganese peroxidases (MnPs) exhibit a typical peroxidase catalytic cycle, but with Mn(II) as the substrate.** The Mn(II) is chelated by bidentate organic acid chelators such as glycolate or oxalate. Chelation is thought to stabilize Mn(III) and allow its diffusion at some distance from the enzyme (Glenn et al. 1986; Paszczynski et al. 1986). MnPs lack sufficient oxidative potential to cleave the major non-phenolic units of lignin, but can oxidize phenolic structures. The resulting phenoxy radicals undergo a variety of reactions including polymer cleavage within certain units, e.g., between Cα and aromatic rings (Wariishi et al. 1991; Tuor et al. 1992). MnPs purified from *P. chrysosporium*, *Nematoloma frowardi*, and *Phlebia radiata* have been shown to oxidize pentachlorophenol and 2,4,6-trinitrotoluene (TNT) in a Mn-dependent manner (Scheibner and Hofrichter 1998; Van Aken et al. 1999; Reddy and Gold 2000), whereas decolorization of azo dye by *Pleurotus eryngii* and *Bjerkandera adusta* MnP isozymes is Mn-independent (Heinfling et al. 1998a).

Phanerochete chrysosporium cultures will efficiently degrade the PAHs phenanthrene and fluorine, an observation difficult to explain because neither serves as a LiP or MnP substrate (George and Neufield 1989; Hammel et al. 1992; Vazquez-Duhalt et al. 1994; Bogan et al. 1996c). Alternative mechanisms must be operative. Among these, peroxidation of unsaturated lipids has been shown to generate transient lipoxyradical intermediates that oxidize non-phenolic lignin model compounds. MnP/lipid peroxidation depolymerizes phenolic- and phenol-blocked (methylated) synthetic lignins (Bao et al. 1994), as well as β-O-4 linkages of lignin model compounds (Bao et al. 1994; Kapich et al. 1999). The system has also been shown to oxidize fluorine (Bogan et al. 1996a) and phenanthrene (Moen and Hammel 1994).

Certain peroxidases oxidize Mn(II) as well as non-phenolic substrates (e.g., veratryl alcohol) in the absence of manganese (Mester and Field 1998; Camarero et al. 1999). Designated **'versatile peroxidases'** (VPs), these enzymes typically feature Mn-binding residues as well as a conserved Trp involved in the electron transfer that enables oxidation of non-phenolic compounds. Recent work suggested a role for VP in the transformation of azo dyes (Salame et al. 2010, 2012) and carbamazepine (Golan-Rozen et al. 2011) (see below).

Less well-studied peroxidases, but potentially involved in degradation of lignin and organopollutants, are the **heme thiolate peroxidases (HTPs) and the dye decolorization peroxidases (DyPs)** (Hofrichter et al. 2010; Lundell et al. 2010). The HTPs include chloroperoxidases and peroxygenases which catalyze a wide range of reactions, including oxidations of various aliphatic and aromatic compounds (Ullrich and Hofrichter 2005; Gutierrez et al. 2011). DyPs and putative DYP-encoding genes have been identified in various fungi, and recent studies have attributed high redox potentials for the enzyme from the white-rot fungus *Auricularia auricula-judae* (Liers et al. 2010).

C. Laccase

Laccases catalyze the 1-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates. Their oxidation of the phenolic units in lignin generates phenoxy radicals, which can lead to aryl-Cα cleavage (Kawai et al. 1988). Non-phenolic lignin-related substrates are oxidized in the presence of certain auxiliary substrates such as ABTS (2,2'-azino-bis-3-ethylthiazoline-6-sulfonate) (Bourbonnais et al. 1997, 1998; Collins et al. 1999). For examples, in the presence of synthetic mediators, organophosphorous insecticides are degraded by *P. ostreatus* laccase (Amitai et al. 1998), and

high ionization potential PAHs are oxidized by *Coriolopsis gallica* and *T. versicolor* laccases (Johannes et al. 1996; Pickard et al. 1999). Cultures of the white-rot fungi *Pycnoporus cinnabarinus* and *Trametes versicolor* produce small molecular weight compounds thought to act as natural intermediaries for oxidation of non-phenolic lignin substructures (Eggert et al. 1996) and PAHs (Johannes and Majcherczyk 2000). Most white-rot fungi produce multiple laccase isozymes (Fukushima and Kirk 1995; Salas et al. 1995; Perie et al. 1998) but some, notably *P. chrysosporium*, produce none. Laccases and their applications have been reviewed (Giardina et al. 2010).

Both **peroxidases and laccases can degrade pentachlorophenol (PCP)**, a restricted-use wood preservative. The first step in the oxidation of such chlorophenols, the formation of para-quinones and release of a chlorine atom, can be carried out by several white-rot fungi. When *P. chrysosporium* LiP and MnP genes were expressed in *Amylomyces rouxii*, a zygomycete producing only phenoloxidases, the transformant exhibited increased activity (95 % depletion) in comparison to the wild type grown without the inducer tyrosine (45 % removal) (Montiel-Gonzalez et al. 2009).

Another mechanism for PCP detoxification could be humification of these xenobiotics via polymerization into soil organic matter. Polymerization of PCP and ferulic acid by manganese peroxidase, lignin peroxidase, and laccase converted a significant portion of the PCP into soil-bound transformation products that are not extractable with organic solvents (Ruttimann-Johnson and Lamar 1996). Using labeled PCP, highest binding to the humic substances was obtained with *P. ostreatus*, followed by *Irpex lacteus*, *T. versicolor*, and *Bjerkandera adusta*. The highest mineralization rate of 8.8 % was demonstrated for *T. versicolor*. Remediation of PCP-contaminated soils with ligninolytic fungi has been the focus of several studies, and inoculum formulation has been a central objective. (Lamar and Dietrich 1990; Lamar et al. 1990a, b, 1994; Lestan and Lamar 1996; Ford et al. 2007a, b)

In addition to PCP, **peroxidases and laccases will degrade a wide range of aromatic dyes** that can pose severe environmental problems. Their highly variable and complex chemical structures also make them difficult to remove by using conventional wastewater treatment systems. The most frequently used color-removal technologies are physical (adsorption, filtration, and flotation), chemical (coagulation, oxidation, reduction, and electrolysis), and biological (aerobic and anaerobic). Thus, color removal is one of the most difficult requirements to be faced by the textile finishing, dye manufacturing, pulp, and paper industries. These industries are major water consumers and are, therefore, a source of considerable pollution. Azo dyes, the largest class of synthetic dyes, are characterized by the presence of one or more azo bonds (-N = N-) in association with one or more aromatic systems, which may also carry sulfonic acid groups (Singh and Arora 2011).

Numerous wood-rotting fungi, including *P. chrysosporium*, *Coriolus versicolor*, *Irpex lacteus*, *P. ostreatus*, and *Ganoderma applanatum*, are able to degrade a wide range of synthetic dyes (Wesenberg et al. 2003; Kaushik and Malik 2009). Many of the decolorization studies indicate that wood degrading fungi have a potential to be developed further into industrial wastewater treatment technology. (Stolz 2001)

Dye degradation and decolorization are of particular relevance to this chapter as a result of the dual role they play in the study of ligninolytic fungi and their oxidative systems. On the one hand, dyes are targets for degradation and bioremediation as toxic pollutants, as described above. On the other hand, they have been used as model compounds to elucidate catalytic mechanisms of ligninolytic enzymes. Evidence suggests that lignin-degrading enzymes, MnP, VP, LiP, and laccases are directly involved not only in the degradation of lignin, their natural lignocellulosic substrates, but also in the degradation of dyes (Heinfling et al. 1998a, b).

Polymeric dye decolorization and ligninolytic activity of *P. chrysosporium* were correlated by comparing the effect of various physiological parameters, mutations, and inhibitors on both processes. Dye decolorization, like ligninolytic activity, appears to be a secondary metabolic process. It was repressed by nitrogen and only occurred after the nitrogen in the cultures had been consumed. Dye decolorization paralleled lignin degradation temporally. Thus it was concluded that some dyes are a good model to represent the ligninolytic system. (Glenn and Gold 1983)

Pleurotus eryngii VP-active sites were demonstrated by measuring oxidizing activity

towards high-redox-potential aromatic compounds and dyes such as Reactive Black 5 (RB5) (Camarero et al. 2005). This was later verified by site-directed mutagenesis, again using RB5 oxidation to show the importance of Trp164 in the reaction (Ruiz-Duenas et al. 2008).

Laccases are also important in dye decolorization (Giardina et al. 2010). Studies of anthraquinone and azo dye degradation by purified laccase from *Lentinus* sp., together with molecular docking of Acid Blue 80, RBBR, and Acid Red 37 onto the enzyme, confirmed the amino acid residues involved in dye oxidation (Hsu et al. 2012). The potential of *Trametes trogii* purified laccase for the decolorization of different types of recalcitrant dyes without the addition of redox mediators has been shown (Grassi et al. 2011), and random mutagenesis has been shown to improve performance of a *P. ostreatus* laccase (Miele et al. 2010).

D. Peroxide Generation

Several systems have been suggested as sources of **extracellular H_2O_2 necessary for peroxidase activity**. Considerable evidence implicates GLOX, a radical-copper oxidase (Whittaker et al. 1996) produced by *P. chrysosporium* (Kersten and Kirk 1987). GLOX utilizes a wide range of small aldehydes such as glyoxal and methylglyoxal (extracellular metabolites of *P. chrysosporium*), and transfers the electrons to O_2, generating H_2O_2. Glycolaldehyde, another substrate, is produced by the action of LiP on β-O-4 lignin substructures. This suggests a physiological connection between GLOX and LiP, and this is further supported by the reversible inactivation of GLOX in the absence of a peroxidase system (Kersten 1990). GLOX is reactivated, however, by reconstituting the complete peroxidase system, including both LiP and substrate. Thus, the supply of H_2O_2 by GLOX responds to the demand of the peroxidases, thereby providing an extracellular regulatory mechanism controlling the coupled enzyme systems. GLOX activity has been detected in several lignin-degrading fungi when grown on oak sawdust (Orth et al. 1993).

Detailed comparative studies with galactose oxidase of *Dactylium dendroides* have defined GLOX as a copper radical oxidase (Kersten and Cullen 1993; Whittaker et al. 1999; Whittaker 2002). Site-specific mutagenesis have confirmed essential residues including an internal Cys-Tyr radical forming a metalloradical complex and copper ligands Tyr135 Tyr377 and His378. (reviewed by Whittaker 2002)

Six additional copper radical oxidase genes were identified by BLAST searches of the *P. chrysosporium* genome. Residues coordinating copper and forming the radical redox site are conserved (Martinez et al. 2004; Vanden Wymelenberg et al. 2006b). Designated *cro1* through *cro6*, three of these genes (*cro3-5*) are predicted to encode repeats of an N-terminal WSC domains, which may be involved in carbohydrate binding (IPR002889; http://www.ebi.ac.uk/interpro/IEntry?ac=IPR002889). *Cro6* is most closely related to *glx* (47 % amino acid identity), but contains a ~200 amino acid N-terminal region absent from the other copper radical oxidases. The predicted *cro2* protein is only 28 % identical to GLOX but, in contrast to GLOX, the enzyme oxidized a glycolaldehyde dimer, but not methylglyoxal (Vanden Wymelenberg et al. 2006b).

Also possibly important in peroxide generation are the **glucose–methanol–choline oxidases (GMCs) which include aryl alcohol oxidase (AAO), methanol oxidase, and various sugar oxidases** (reviewed in Ref. (Hernandez-Ortega et al. 2012)). AAOs oxidize benzyl alcohols to aldehydes, transferring the electrons to O_2, producing H_2O_2 (Muheim et al. 1990; Asada et al. 1995). *P. ostreatus* secretes a mixture of benzyl alcohols that are oxidized by AAO (Sannia et al. 1991). The white-rot fungus *Bjerkandera adusta* secretes chlorinated benzyl alcohols that are substrates for AAO but not LiP. Since both LiP and AAO are produced in *B. adusta* cultures, such substrate preferences may have important physiological roles in ligninolysis.

Methanol oxidase may play an important role in generating H_2O_2 in both white-rot and brown-rot fungi. The enzyme is highly expressed and associated with hyphal cell walls in the brown-rot fungus, *Gloeophyllum trabeum* (Daniel et al. 2007). Brown-rot demethylation of lignin may provide the substrate, and the H_2O_2 produced is thought to participate in generation of highly reactive hydroxyl radical via a Fenton reaction, $H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \cdot OH$. This diffusible radical will mediate the rapid depolymerization of cellulose, a central feature of brown-rot decay. High expression of methanol oxidase has also been observed in the white-rot fungus *P. chrysosporium* when grown on media containing ground wood as sole carbon source (Vanden Wymelenberg et al. 2010).

Pyranose 2-oxidase oxidizes various monosaccharides at C-2, with transfer of electrons to O_2 to produce H_2O_2. This GMC enzyme has been identified in various fungi, including *P. chrysosporium*, *T. versicolor*, *Oudemansiella mucida*, and *Agaricus bisporus* (Daniel et al. 1994; Artolozaga et al. 1997), and the catalytic mechanism has received considerable attention in recent times (Tan et al. 2010; Sucharitakul et al. 2011; Tan et al. 2011; Wongnate et al. 2011). *P. chrysosporium* mycelium also exhibits glucose 1-oxidase activity (Kelley and Reddy 1986, 1988), but this enzyme appears to be less common than pyranose 2-oxidase (reviewed by Ander and Marzullo 1997).

Cellobiose dehydrogenase (CDH) is widely distributed among fungi, but its precise role in ligninolysis and/or organopollutant degradation, if any, remains uncertain (Henriksson et al. 2000; Zamocky et al. 2006). The enzyme contains a dehydrogenase domain and a heme prosthetic group (Hallberg et al. 2000). CDH oxidizes cellodextrins, mannodextrins, and lactose, and suitable electron acceptors include quinones, phenoxyradicals, and Fe^{3+}. Interestingly, recent studies have shown that CDH will enhance cellulose depolymerization by members of the glycoside hydrolase family 61 (Harris et al. 2010; Langston et al. 2011). Previously considered to be cellulases, the latter 'hydrolases' have been recently classified as copper-dependent monooxygenases (Quinlan et al. 2011; Westereng et al. 2011).

E. Other Oxidoreductases

In addition to the extracellular peroxidases and laccases, transformation and/or complete mineralization of organopollutants involve additional extracellular and intracellular processes. Examples include glycosyl conjugation of triclosan by *T. versicolor* cultures (Hundt et al. 2000), and O-methylation of PCP and triclosan by *P. chrysosporium* (Lamar et al. 1990a) and *P. cinnabarinus* (Hundt et al. 2000) cultures, respectively.

The role of **cytochrome P450s in organopollutant degradation** remains largely unexplored, but some progress has been made. The metabolic steps in PAH degradation have occurred in both N-limited and N-sufficient culture media, and are similar to those in nonligninolytic fungi, such as *Cunninghamella elegans* (Casillas et al. 1996) and N-sufficient cultures of *P. chrysosporium*. Apparently, *P. ostreatus* acts on PAHs like nonligninolytic fungi, but is also able to mineralize PAHs (Bezalel et al. 1996a, b). Since *P. ostreatus* does not contain lignin peroxidase, and since PAH metabolism did not correlate with laccase or MnP activities, it is possible that a cytochrome P450 monooxygenase is responsible for the initial step in the attack. The ligninolytic system of *P. ostreatus* may be involved in the later steps of metabolism, such as ring cleavage, which leads to CO_2 evolution. These conclusions were based on metabolite analyses, physiological and biochemical studies (Bezalel et al. 1997).

Additional investigations suggest that *P. chrysosporium* degradation of 2,4,6-trichlorophenol (Reddy et al. 1998) and PCP (Hammel and Tardone 1988; Mileski et al. 1988; Reddy and Gold 1999, 2000) involve oxidative dechlorination by extracellular peroxidases, followed by intracellular reductive dechlorination and hydroxylation reactions. White-rot cytochrome P450s reactions also include monooxygenase bioconversions of phenanthrene by *P. ostreatus* (Syed et al. 2010), benzo(a)

pyrene by *Pleurotus pulmonarius* (Masapahy et al. 1999) and by *P. chrysosporium* (Syed et al. 2011a), 4-methyldibenzothiophene by *T. versicolor* (Ichinose et al. 1999), and endosulfan and carbamazepine (CBZ) by *P. chrysosporium.* (Kullman and Matsumura 1996; Golan-Rozen et al. 2011)

Fungi are active in **biodegradation of a wide array of pharmaceuticals and hormones.** Analyses of the degradation pathways and metabolites formation was recently reviewed (Cruz-Morato et al. 2012). As mentioned above, **cytochrome P450s and MnPs** have been implicated in the transformation and metabolism of CBZ, a drug used in large quantities worldwide for the treatment of epilepsy, and increasingly used for various psychiatric treatments (Leclercq et al. 2009). Studies in Europe and North America have shown that CBZ and CBZ metabolites are among the most frequently detected pharmaceuticals in wastewater effluents, river water, and drinking water (Heberer 2002; Miao et al. 2005; Benotti et al. 2009). CBZ is an environmentally recalcitrant compound extremely stable in water and soil (Lienert et al. 2007), mainly as a result of its remarkably high stability towards bacterial degradation. Thus, because of its very slow degradation, it has been proposed as a tracer for anthropogenic activity and contamination originated from municipal waste water (Gasser et al. 2010).

Nevertheless, CBZ modification by different white-rot fungi has been reported (Kang et al. 2008). For example, the white-rot fungi *T. versicolor* and *G. lucidum* eliminated 57 % and 46 % respectively of CBZ after 7 incubation days (Marco-Urrea et al. 2009). Similar removal efficiency of CBZ was observed by *T. versicolor* in a solid-phase bioreactor containing sewage sludge and mycelium (Rodriguez-Rodriguez et al. 2010). *P. ostreatus* strains F6, N001 (dikaryons), and PC9 (monokaryon) degraded CBZ to levels ranging from 48 % to 99 % of the initial concentration. With strain PC9, CBZ concentration was reduced from 10 mg l^{-1} to 20 μg l^{-1} within 17 days of incubation (Golan-Rozen et al. 2011). To evaluate the potential use of *P. ostreatus* to remediate contaminated water, CBZ removal was studied at its environmental relevant concentration (~1 μg l^{-1}, 4.6 nM). When optimal conditions were obtained, CBZ concentration decreased by 97.9 % to 0.093 nM (22 ppt) within 8 days. Unlike the accumulation of the metabolite 10,11EPCBZ observed at high CBZ concentration, in this experiment 10,11EPCBZ disappeared, gradually reaching a minimal concentration of 0.1 nM. These results suggested that at environmentally relevant concentrations *P. ostreatus* can not only transform CBZ to 10,11EPCBZ, but may also continue its metabolism. (Golan-Rozen et al. 2011)

Several enzymatic mechanisms have been suggested to be involved in the oxidation of CBZ. When a cytochrome P450 inhibitor was added to the growth medium, CBZ elimination by *T. versicolor* was inhibited by more than half, indicating the possible involvement of cytochrome P450 in the biodegradation process (Montiel-Gonzalez et al. 2009). When *P. ostreatus* was grown in media supporting high levels of both cytochrome P450 and manganese peroxidase (MnP), 99 % of the added CBZ was eliminated from the solution (Golan-Rozen et al. 2011). High removal of CBZ was also obtained when either MnP or CYP450 was active. When both CYP450 and MnP were inactivated, only 10–30 % of the added CBZ was removed.

In-vitro reaction between CBZ and crude lignin peroxidase produced by the fungus *P. chrysosporium* in the presence of H_2O_2 and veratryl alcohol resulted in only 5–9 % elimination. Repeated treatment with laccase from *T. versicolor* and 1-hydroxybenzotriazole (used as a redox mediator) resulted in the elimination of 20 % of the CBZ after 24 h (Hata et al. 2010). Increased removal of CBZ (about 80 %) was only observed when a lignin-derived quinone was added to the growth medium of *T. versicolor* together with ferrous oxalate to form a Fenton-like reaction (Marco-Urrea et al. 2010). This reaction facilitates the formation of hydroxyl radicals, which oxidize the CBZ molecule faster than the enzymatic reactions.

III. Comparative Genome Analysis

Knowledge of the genomes of wood decay fungi is rapidly advancing, in large part because of the support of the U.S. Department of Energy's Joint Genome Institute. An interactive MycoCosm web portal (http://genome.jgi.doe.gov/programs/fungi/index.jsf) integrates all publicly accessible fungal genomes, including those featured in this review (Grigoriev et al. 2012). Throughout the following passages, we provide protein model identification numbers that allow searches of the JGI genome portal, and thereby link to detailed protein pages, alternative models, annotation, and comparisons to other databases.

A. Gene Structure, Phylogeny, and Expression

1. Peroxidases

Based on overall sequence conservation, a **Trp active site, and the absence of Mn-binding** residues, the LiP genes identified to date are confined to lignin-degrading fungi, with ten genes present in the genomes of *P. chrysosporium* and *T. versicolor*. However, of the ten white-rot genomes analyzed and published as of July 2012, most do not have LiP genes but feature two to sixteen MnP genes. *P. chrysosporium* and *T. versicolor* contain five and thirteen MnP genes respectively (Ohm et al. 2010; Fernandez-Fueyo et al. 2012; Floudas et al. 2012; Olson et al. 2012). Genome analysis has also identified at least four, two, and three VP genes in *P. ostreatus*, *T. versicolor*, and *D. squalens* respectively. Of the eight published brown-rot genomes, none contain genes encoding LiP, MnP, or VP (Martinez et al. 2009; Eastwood et al. 2011; Floudas et al. 2012).

Early studies had also identified *P. chrysosporium* MnP genes, *mnp1*, *mnp2*, and *mnp3* (Pease et al. 1989; Pribnow et al. 1989; Orth et al. 1994; Alic et al. 1997). The draft genome revealed two new MnP genes (Martinez et al. 2004), one of which, *mnp4*, was unexpectedly localized to a region 5 kb upstream of *mnp1*. A cytochrome P450 gene lies within the *mnp4*-*mnp*1 intergenic region. The *mnp5* predicted protein corresponds to the N-terminal amino acid sequence of a MnP long ago purified from *P. chrysosporium*-colonized wood pulp (Datta et al. 1991). Most intron positions are conserved within the MnP (Larrondo et al. 2005) and LiP (Stewart and Cullen 1999) gene families.

Deviations from these simple classifications have been noted. Certain MnPs vary in length and have been classified accordingly (Ruiz-Duenas et al. 2011; Fernandez-Fueyo et al. 2012). On the basis of homology modeling and the conservation of specific residues essential for catalysis, *C. subvermispora* protein models #118677 and #99382 were initially classified as LiP and VP genes respectively. Consistent with these designations, the corresponding proteins were capable of oxidizing nonphenolic model compounds and synthetic lignin. However, the putative VP was unable to oxidize Mn as predicted. Moreover, both enzymes exhibited catalytic properties intermediate between conventional LiPs and MnP (Fernandez-Fueyo et al. 2012).

Clustering of *P. chrysosporium* genes, especially those encoding LiPs and MnPs, is a well-known phenomenon. Prior to genome sequencing, the ten *P. chrysosporium* LiP genes had been designated *lipA* through *lipJ* (Gaskell et al. 1994), and eight of these LiP genes were mapped within 3 % recombination (Gaskell et al. 1994; Stewart and Cullen 1999). The genome sequence confirmed the genetic multiplicity and verified the overall organization, with the eight LiP genes located within 100 Kb on scaffold 19. Genes designated *lipD* and *lipF* were localized to scaffolds 11 and 9 respectively.

Beyond *P. chrysosporium*, genome sequence analysis has revealed clustering of LiP and MnP genes in most white-rot fungi. Typically, this involves simple tandem arrangements and occasionally a third or fourth gene more distantly positioned. For examples, *T. versicolor* LiP genes encoding protein models #133326, #134250, and #52333 are located on scaffold 12, and models #43576, #43578, #114944, and #112835 lie on scaffold 2. In both instances, the genes are located within a ~15 kb region. *T. versicolor* MnP genes encoding proteins #51455, #74179, and #51457 are tightly clustered within 7 Kb on scaffold 10. The VP-encoding genes of *T. versicolor* are located on scaffold 2, but very distant from LiP genes. No remarkable linkage is observed among the nine, thirteen and five MnP genes of *D. squalens*, *C. subvermispora*, and *P. ostreatus* respectively. As in the case of *T. versiolor*, the *D. squalens* and *P. ostreatus* VPs show no significant linkage to each other or to the MnP genes. In contrast, the abovementioned intermediary LiP–MnP genes of *C. subvermispora* lie within 9 Kb on scaffold 20.

The **regulation of LiP gene expression**, particularly in *P. chrysosporium*, has received considerable attention. Culture conditions substantially influence *lip* transcript levels (Holzbaur and Tien 1988; Stewart et al. 1992; Reiser et al. 1993; Janse et al. 1998; Vallim et al. 1998; Stewart and Cullen 1999; Belinky et al. 2003; Vanden Wymelenberg et al. 2009; Hiscox et al. 2010; Sakamoto et al. 2010). LiP genes within clusters may be differentially regulated but, to date, no clear relationship between organization and regulation has been reported (Stewart et al. 1992; Stewart and Cullen 1999; Macdonald

et al. 2011). In *P. chrysosporium* soil cultures, LiP transcript patterns shift depending upon the pollutant, e.g., anthracene versus PCP (Lamar et al. 1995; Bogan et al. 1996b).

In recent years, **LC-MS/MS and high throughput transcript analyses** have provided insight into the expression of specific LiP genes. Extracellular proteins corresponding to *lipC* were identified only in nitrogen-limited medium, whereas the *lipD* and *lipE* products were more abundant in carbon-limited cultures (Vanden Wymelenberg et al. 2006a, 2009). These results were consistent with transcript levels measured by Northern blots (Holzbaur and Tien 1988), by quantitative RT-PCR (qRT-PCR) (Stewart and Cullen 1999) and, more recently, by whole-genome expression microarrays (Vanden Wymelenberg et al. 2009). None of the *P. chrysosporium* LiP genes exhibited elevated transcript levels in medium containing microcrystalline cellulose (Avicel, Fluka Chemical) relative to glucose-grown cultures, but peptides corresponding to *lipD* were detected in the cellulose medium (Vanden Wymelenberg et al. 2005). In submerged medium containing ball-milled aspen (BMA) as sole carbon source, transcripts of *lipA* and *lipH* accumulated relative to glucose medium, but no extracellular peroxidase was detected by LC-MS/MS (Vanden Wymelenberg et al. 2010). On the other hand, significant transcript levels of *lipD, lipE* and *lipB* were measured in similar experiments using red oak (Sato et al. 2009). More perplexing, *lipD* and *lipE* transcript levels were lowest among all LiP genes in colonized aspen wood chips (Janse et al. 1998). Transcriptome studies of *Phanerochaete carnosa* suggest that wood species substantially influence LiP transcript levels (Macdonald et al. 2011; Macdonald and Master 2012; MacDonald et al. 2012).

As in the case of LiP-encoding genes, **media composition, especially manganese concentration, has a dramatic effect on MnP regulation** (Bonnarme and Jeffries 1990; Brown et al. 1990, 1991; Pease and Tien 1992; Lobos et al. 1994). Most studies have focused on transcriptional control, but recent results using *C. subvermispora* suggest that Mn concentration may also influence MnP secretion (Mancilla et al. 2010). Mechanisms of transcriptional regulation remain uncertain, but much attention has focused on promoters and the putative role of metal response elements (MREs) (Godfrey et al. 1990, 1994; Alic and Gold 1991; Brown et al. 1993; Alic et al. 1997). Gettemy et al. (1998) reported that *P. chrysosporium mnp1* and *mnp2* were substantially upregulated in response to Mn^{2+} concentration, and later deletion analysis identified an upstream Mn-responsive element (Ma et al. 2004). In contrast, dramatic upregulation of *T. versicolor mnp2* by Mn appears not to involve any MREs (Johansson et al. 2002). Transcripts of *P. chrysosporium mnp1* accumulate in carbon- or nitrogen-starved defined medium relative to replete medium, and the corresponding protein has been identified by LC-MS/MS under such nutrient limitation (Ravalason et al. 2008; Vanden Wymelenberg et al. 2009). Upregulation of *mnp2* transcripts has been observed in cultures that were nitrogen-starved but not carbon-starved (Vanden Wymelenberg et al. 2009).

Complex patterns of *P. chrysosporium* MnP gene expression have been observed in colonized wood and soil. *mnp4* is actively transcribed when *P. chrysosporium* is grown on wood-containing soil samples (Stuardo et al. 2004), and *mnp1*, *mnp2*, and *mnp3* transcripts are easily detected in colonized aspen wood chips (Janse et al. 1998). Transcripts corresponding to *mnp1* were detected in red oak medium (Sato et al. 2009). In *P. chrysosporium* soil cultures, the depletion of fluorine roughly correlates with transcript levels of *mnp1*, *mnp2* and *mnp3* (Bogan et al. 1996c). Degradation of this high oxidation potential PAH is consistent with a mechanism involving lipid peroxidation.

Simultaneous accumulation of transcripts corresponding to *C. subvermispora* MnPs and genes putatively involved in lipid biosynthesis (Watanabe et al. 2010) also support a **role for lipid peroxidation** (Fernandez-Fueyo et al. 2012). In line with this view, recent microarray and mass spectrometry data (Fernandez-Fueyo et al. 2012) revealed simultaneous upregulation of genes encoding MnP genes as well as those involved in lipid biosynthesis after 5 days growth in medium containing BMA. Interestingly, the *C. subvermispora* MnP genes exhibiting significant accumulation of transcripts in BMA relative to glucose (models #117436 and #49863) or secreting detectable protein (models #157986, #116608, #50297) were all classified as

Table 5.1. Number of genes encoding oxidoreductases implicated in ligninolysis and degradation of various organopollutants

	White-rot fungi					
	Phach	Pleos	Cersu	Trave	Dicsq	Brown-rot[a]
LiP	10	0	0	10	0	0
MnP	5	5[b]	13[c]	13	9	0
VP	0	4[b]	2	2[c]	3	0
HTP	3	3[b]	9	3	4	5.2 (2–6)
DYP	0	4[b]	0	2[c]	1[c]	0.3 (0–2)
Laccase	0	12[d]	7[c]	7[c]	11[c]	4.5 (3–6)[c]
GLOX	1	4	0	5[c]	5[c]	0
CRO1	1	1	0	1	1	0.8 (0–2)
CRO2	1	2	1	1[c]	1[c]	1.5 (0–4)[c]
CRO3-5	3	3	1	1[c]	1[c]	0.7 (0–1)[c]
CRO6	1	6	1	1	1	1
CDH	1	1	1	1[c]	1[c]	0.7 (0–2)[c]
GH61[e]	15[c]	29	9	18[c]	15[c]	4.5 (2–10)[c]
P450s	149	141[f]	222[c]	190	187	196

[a]The average number and range of genes in six phylogenetically related brown-rot fungi included as point of comparison. Brown-rot genomes analyzed were: *Serpula lacrymans* (Eastwood et al. 2011), *Postia placenta* (Martinez et al. 2009), *Coniophora puteana, Wolfiporia cocos, Gloeophyllum rabeum*, and *F. pinicola* (Fernandez-Fueyo et al. 2012). White-rot abbreviations: Phach, *Phanerochaete chrysosporium* (Martinez et al. 2004); Pleos, *Pleurotus ostreatus* (http://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html); Cersu, *Ceriporiopsis subvermispora* (Fernandez-Fueyo et al. 2012); Trave, *Trametes versicolor* and Dicsq, *Dichomitus squalens* (Floudas et al. 2012)

[b]See reference (Ruiz-Duenas et al. 2011)

[c]NanoLC-MS/MS unambiguously identified at least one protein in media containing ground aspen as sole carbon source. See Supplemental files in published accounts for *C. subvermispora and P. chrysosporium* (Fernandez-Fueyo et al. 2012), and for *T. versicolor*, *D. squalens*, and the six brown-rot fungi mentioned above (Floudas et al. 2012)

[d]See reference (Castanera et al. 2012)

[e]Lytic polysaccharide monooxygenase

[f]Enumerated using Cytochrome P450 Database http://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html. All others derived from published accounts

'extra long' MnPs (Fernandez-Fueyo et al. 2012). Two 'short' MnP proteins were detected by LC-MS/MS in the extracellular filtrates of *T. versicolor* (Floudas et al. 2012).

VP-encoding genes have not been identified in the genomes of any brown-rot fungi, but 2–4 genes are present in *P. ostreatus* (Ruiz-Duenas et al. 2011), *C. subvermispora* (Fernandez-Fueyo et al. 2012), *T. versicolor* and *D. squalens* (Floudas et al. 2012) (Table 5.1). Global transcriptome studies have not yet been reported for these fungi, although it is clear that *P. ostreatus* VP transcription is modulated by Mn (Cohen et al. 2001, 2002a, b), and recent genetic studies persuasively show its importance in dye decolorization (below). The four *P. ostreatus* genes are not tightly linked. The abovementioned 'transitional' or 'intermediary' LiP-MnP genes of *C. subvermispora* are closely linked on scaffold 20, but regulated expression and protein secretion have not been observed in a shake flask containing BMA suspensions (Fernandez-Fueyo et al. 2012). The VP genes of *T. versicolor* are unlinked, and peptides corresponding to protein model #26239 have been detected in BMA cultures. The three *D. squalen* VP genes are unlinked and LC-MS/MS failed to detect the proteins in the extracellular filtrates of the same medium (Floudas et al. 2012).

Genes encoding **putative heme-thiolate peroxidases (HTPs) are widespread** within the genomes of white-rot and brown-rot fungi (Table 5.1) although experimental affirmation of their expression is limited. *C. subvermispora, D. squalens*, and *T. versicolor* genomes feature nine, four, and three HTP genes respectively, but systematic studies of transcriptional regulation have not been reported, and LC-MS/MS has not detected any of the corresponding peptides in media containing BMA (Fernandez-Fueyo

et al. 2012; Floudas et al. 2012). Similarly, no extracellular protein has been observed for *P. chrysosporium* models #1710, #3274, and #34295. Transcripts corresponding to #34295 are upregulated in Avicel medium relative to glucose (Vanden Wymelenberg et al. 2009). Interestingly, relative to glucose medium, transcripts encoding #34295 accumulate in BMA medium but not in ball-milled pine medium. Possibly reflecting substrate-based differential regulation, transcripts from #3274 show the opposite pattern, i.e., upregulated in pine but not aspen (Vanden Wymelenberg et al. 2011). With the exception of tandemly arranged *T. versicolor* genes encoding protein models #154915 and #23785, none of the above mentioned HTP genes exhibit close linkage.

The **dye-decolorizing peroxidases (DyPs) are sporadically distributed among genomes of wood decay fungi**. With the exception of *Gloeophyllum trabeum* no DyP genes have been detected in brown-rot genomes. Among white-rot fungi, HTP genes are absent from *P. chrysosporium* and *C. subvermispora* whereas *P. ostreatus*, *T. versicolor*, and *D. squalens* contain four, two and one gene respectively. The *T. versicolor* gene encoding protein #48874 and #48870 lie within a ~15 kb region of scaffold 7. LC-MS/MS analysis of filtrates from BMA medium indicates that *D. squalens* protein #150405 and *T. versicolor* #48870 are particularly abundant, making up 1.3 % and 2.2 % of the total spectra (Fernandez-Fueyo et al. 2012).

Development of efficient systems for production of **recombinant peroxidases has been a key factor** in furthering biochemical investigations improving enzyme properties and assessing applications related to organopollutant degradation. Until relatively recently, production of active LiP in foreign hosts has been challenging. Yields were low in Baculovirus (Johnson and Li 1991; Johnson et al. 1992). Successful expression has also been reported using *Pichia* (Wang et al. 2004; Wang and Wen 2009) and *S. cerevisiae* (Ryu et al. 2008a, b) and, in the former reports, the catalytic ability using 2,4-dichlorophenol (DCP) has been examined. Although complicated by inclusion bodies, techniques for recovering fully active enzyme from *E. coli* have been developed (Doyle and Smith 1996; Nie et al. 1998), and the approach is now well-established for LiP, VP, and MnP (Miki et al. 2009; Ruiz-Duenas et al. 2009).

In addition to *E. coli*, *P. chrysosporium* MnP has been successfully produced by *A. oryzae* (Stewart et al. 1996) and *A. niger* (Conesa et al. 2000) transformants. Peroxidases from *C. subvermispora* (Larrondo et al. 2001), *P. eryngii* (Ruiz-Duenas et al. 1999), *P. eryngii* (Eibes et al. 2009) and from *Geotrichym candidum* (Sugano et al. 2000) have also been expressed in *Aspergillus*. In one case (Cortes-Espinosa et al. 2011), a MnP-expressing *A. niger* transformant exhibited enhanced phenanthrene degradation in soil relative to the parent strain.

Expression involving **native promoters has proven useful for production of peroxidases** for several white-rot fungi. For examples, significant increases in VP expression were achieved in *P. ostreatus* transformants relative to the parental strain (Tsukihara et al. 2006, 2008). A similar strategy of homologous expression was previously reported for producing *P. chrysosporium* MnP (Ma et al. 2003) and LiP (Sollewijn Gelpke et al. 1999, 2002).

2. Laccases

Among the multicopper oxidases, **multiple genes encoding laccase *sensu stricto*** (Hoegger et al. 2006) are, with the exception of *P. chrysosporium*, a common feature of white-rot genomes (Kojima et al. 1990; Saloheimo et al. 1991; Coll et al. 1993; Yaver and Golightly 1996; Yaver et al. 1996; Karahanian et al. 1998; Giardina et al. 1999; Temp et al. 1999). Laccase multiplicity is somewhat reduced in brown-rot fungi, and none have been detected in *Dacryopinax* sp. (Floudas et al. 2012). Prior to genome sequence, relatively little information was available on the organization of laccases, but *Trametes villosa* pulsed field gels suggested the possible linkage of certain laccase genes (Yaver and Golightly 1996). Seven laccase genes were identified in the *T. versicolor* genome, and close linkage was observed for those encoding proteins #47314 and #37188. Based on the percentage of total mass spectra (2.8 %), the latter protein is the most abundant

of 218 proteins identified in the extracellular filtrate of BMA medium. *D. squalens* laccases #67925, #169869 and #176907 were detected at more modest levels in the same medium, and no close linkage was observed among the 11 genes (Table 5.1). The seven genes of *C. subvermispora* are distantly linked, and transcripts corresponding to protein model #118801 were significantly upregulated in BMA medium relative to glucose. The protein was detected by LC-MS/MS analysis of BMA medium filtrates. The *P. ostreatus* strain PC15 genome contains 12 laccase genes (Castanera et al. 2012) and, with the exception of the adjacent models #1077328 and #1119530, all are distantly linked.

Laccase genes are often differentially regulated in response to culture conditions, and the patterns of regulation differ substantially between fungal species (Wahleithmer et al. 1995; Yaver and Golightly 1996; Yanai et al. 1996; Smith et al. 1998; Palmieri et al. 2000). Their regulation has been recently reviewed (Piscitelli et al. 2011b). Potential ACE response elements have been identified in *C. subvermispora* and may be responsible, in part, for copper induction of genes encoding laccases and MnP. (Alvarez et al. 2009)

Several systems have proven useful for production of recombinant laccases. For example, *P. ostreatus* laccase is produced in *Kluyveromyces lactis* and *S. cerevisiae* (Piscitelli et al. 2011b), and the latter system led to improved temperature and pH stability via mutagenesis (Piscitelli et al. 2011a). *Aspergillus* systems have been used to produce laccases from *C. subvermispora* (Larrondo et al. 2003), *T. villosa* (Yaver et al. 1996), and *Coprinus cinereus* (Yaver et al. 1999). More recently, *Pichia* spp. expression has been used to investigate the potential of various white-rot laccases for dye decolorization and PAH degradation (Guo et al. 2008; Lu et al. 2009; Wong et al. 2012).

3. Peroxide-Generating Copper Radical Oxidases

Discovered in *P. chrysosporium* (Kersten and Kirk 1987; Kersten 1990), **glyoxal oxidase (GLX) is encoded by a single gene** (Kersten and Cullen 1993; Kersten et al. 1995). Supporting an important role in ligninolysis, GLX homologs have been identified in the genomes of most white-rot fungi, but not in the brown-rotters (Fernandez-Fueyo et al. 2012; Floudas et al. 2012) (Table 5.1). In line with a physiological connection between peroxidases and GLX, coordinate increases in their transcript levels and/or protein secretion are observed under nutrient starvation (Stewart et al. 1992; Vanden Wymelenberg et al. 2006a, 2009), in colonized wood (Janse et al. 1998; Sato et al. 2009), and in soil (Bogan et al. 1996b).

Little is known concerning the expression of GLX genes from other white-rot fungi. Three of the five *T. versicolor* genes are distantly linked on scaffold #3, of which peptides corresponding to GLX protein model #118266 have been identified by LC-MS/MS in BMA-containing medium. In *D. squalens*, genes encoding GLX proteins #104366 and #126455 are tandemly arranged on scaffold 10, but none of the five GLX proteins have been detected in BMA medium. No linkage has been observed among the five *P. ostreatus* GLX genes and, to date, nothing has been published regarding their expression.

Beyond GLX, a total of six CRO genes have been identified in the *P. chrysosporium* genome. Interestingly, the WSC-containing genes *cro3*, *cro4*, and *cro5* lie within the LiP gene cluster on scaffold 19 (Cullen and Kersten 2004). The clustering of *lip* and *cro* genes seems consistent with a physiological connection between peroxidases and peroxide-generating oxidases. Relatively little data is available on the expression of *P. chrysosporium* GLX genes, although transcripts of all CRO genes have been measured over time in colonized wood wafers (Vanden Wymelenberg et al. 2006b), and the CRO2 protein has been shown in medium containing ball-milled pine (Vanden Wymelenberg et al. 2011).

Systems for heterologous expression of CROs include *Aspergillus nidulans* and *Pichia pastoris*. These have been used to confirm catalytic residues (Kersten et al. 1995; Whittaker et al. 1999) of GLX, and *A. nidulans* production of *P. chrysosporium cro2* revealed differences in the substrate preferences of GLX and CRO2 (Vanden Wymelenberg et al. 2006b).

This observation may explain how the highly efficient and **selective lignin degrader, *C. subvermispora*, lacks a clear GLX homolog** (Fernandez-Fueyo et al. 2012) (Table 5.1). Possibly, functionally related CROs fulfill the same role and/or are better suited for a spectrum of small molecular weight substrates unique to the ligninolytic system of *C. subvermispora*. Experimental support for this hypothesis is limited, but genome analysis has identified CROs in various fungi (Table 5.1), including at least three in the *C. subvermispora* genome. Further, transcriptome analysis showed upregulation of a *cro2*-like gene as well as several MnP genes in *C. subvermispora* cultures containing BMA as sole carbon source (Fernandez-Fueyo et al. 2012). Separate LC-MS/MS studies have identified *cro2* and WSC-containing CRO genes (*cro4*, *cro5*) in BMA culture filtrates of *T. versicolor* and *D. squalens* (Floudas et al. 2012).

4. Peroxide-Generating GMC Oxidoreductases

Genome analysis has greatly expanded knowledge of the distribution and diversity of GMC oxidases, particularly AAO-encoding genes. Based largely on recently published genomes (Fernandez-Fueyo et al. 2012; Floudas et al. 2012), Hernandez-Ortega et al. (2012) and coworkers describe relationships among 40 genes from various wood decay fungi. The **AAO genes were widely distributed among white-rot and brown-rot taxa**, although at least one white-rot fungus, *Auricularia delicata*, and three brown-rot fungi, *Coniophora puteana*, *Wolfiporia coccos*, and *Dacryopinax* sp. have no detectable AAO gene (Floudas et al. 2012). Transcript levels in nutrient-starved medium, Avicel medium, and BMA medium were modest for *C. subvermispora* and *P. chrysosporium* (Vanden Wymelenberg et al. 2009). One of the three *P. chrysosporium* putative AAO proteins (#135972) and two of the five *C. subvermispora* proteins (#117387, #84544) were detected by LC-MS/MS in glucose-replete media, but not in BMA. On the other hand, AAO-derived peptides were unambiguously identified in BMA cultures of *T. versicolor* (#133945 and #176148) and *D. squalens* (#160546 and #171752) (Floudas et al. 2012). Little is known regarding the expression of the *P. ostreatus* AAO genes, but sequence comparisons with other wood-decay fungi have been reported (Hernandez-Ortega et al. 2012). With the exception of the tandemly arranged *D. squalens* genes encoding proteins #102587 and #153908, close linkage has not been observed within the AAO gene families.

Recent genome analysis of **methanol oxidase (MOX), another potentially important GMC oxidase, shows a wide distribution among white and brown-rot fungi**. Among the white-rot fungi considered here (Table 5.1), *D. squalens*, *T. versicolor*, and *P. ostreatus* genomes each contained at least four unlinked genes. The *P. ostreatus* secretome has not yet been reported, and none of the *D. squalens* and *T. versicolor* MOX proteins were detected in by LC-MS/MS in BMA medium (Floudas et al. 2012). In contrast, *P. chrysosporium* MOX protein #126879 was identified in BMA culture filtrates, and the corresponding transcripts were significantly upregulated relative to glucose medium (Vanden Wymelenberg et al. 2010). Surprisingly, the same medium showed decreased transcript levels of *C. subvermispora* MOX #80773, and no LC-MS/MS evidence for MOX in BMA medium (Fernandez-Fueyo et al. 2012). The apparent absence of soluble MOX protein in filtrates should be carefully interpreted, as cell-wall associations seem likely (Daniel et al. 2007).

Several studies implicate pyranose 2-oxidase in lignin degradation. The corresponding gene has been isolated from *T. versicolor* (Nishimura et al. 1996), *P. chrysosporium* (de Koker et al. 2004), and *G. trabeum* (Dietrich and Crooks 2009), and obvious homologs are absent from most of the recently sequenced genomes. In *P. chrysosporium*, transcripts are upregulated under ligninolytic conditions (de Koker et al. 2004; Vanden Wymelenberg et al. 2009, 2010), and the extracellular protein has been identified in culture filtrates carbon-starved cultures (Vanden Wymelenberg et al. 2010) and in BMA medium (Vanden Wymelenberg et al. 2011). The *P. chrysosporium* and *G. trabeum*

pyranose 2-oxidases have been successfully expressed in *E. coli*. (Dietrich and Crooks 2009; Pisanelli et al. 2009).

Prior to the increase in genome data, genes encoding CDH were cloned from several fungi, including the white-rot fungi *P. chrysosporium* (Raices et al. 1995; Li et al. 1996), *T. versicolor* (Dumonceaux et al. 1998), and *P. cinnabarinus* (Moukha et al. 1999) All **white-rot genomes have a single CDH gene**. On the other hand, brown-rot fungal genomes have none (*P. placenta*, *F. pinicola*, *W. cocos*), one (*Coniophora puteana*, *G. trabeum*), or two (*S. lacrymans*) copies of the CDH gene. Sequences are highly conserved, and share a common architecture with separate FAD, heme, and cellulose-binding domains (CBD).

Northern blots had shown upregulation of *cdh* in cellulose-containing media (Li et al. 1996; Moukha et al. 1999), and competitive RT-PCR revealed transcripts in *P. chrysosporium* colonized wood (Vallim et al. 1998). Later microarray and LC-MS/MS investigations have shown that *P. chrysosporium* CDH transcripts accumulated in media containing Avicel relative to glucose as the sole carbon source (Vanden Wymelenberg et al. 2009). Transcripts have also been detected in red oak medium (Sato et al. 2009), and upregulation observed in BMA medium relative to glucose medium (Vanden Wymelenberg et al. 2010). The CDH protein has been identified in various culture filtrates, including those that are nutrient-starved (Vanden Wymelenberg et al. 2009), contain microcrystalline cellulose, or contain complex lignocellulose (Sato et al. 2009; Vanden Wymelenberg et al. 2011). The wood species used as substrate alters expression, with higher transcript and protein levels in media containing ball-milled pine relative to ball-milled aspen. (Vanden Wymelenberg et al. 2011)

Irrespective of the *P. chrysosporium* culture conditions, CDH transcripts and secretion are typically mirrored by expression of aldose 1-epimerase (#138479) (Vanden Wymelenberg et al. 2005; Sato et al. 2009; Vanden Wymelenberg et al. 2011). This coordinate expression may indicate a physiological coupling via generation of the cellobiose b-anomer, the preferred CDH substrate (Higham et al. 1994). Co-expression of CDH and genes encoding members of the CAZy glycoside 'hydrolase' family GH61 has also been observed. Now classified as copper-dependent monooxygenases (Quinlan et al. 2011; Westereng et al. 2011), GH61s will boost cellulose depolymerization by CDH (Harris et al. 2010; Langston et al. 2011). In addition to cellulose, xylan has been shown to increase secretion of CDH and GH61 (Hori et al. 2011). Further supporting these associations, of five recently sequenced wood decay fungi (*T. versicolor*, *D. squalens*, *Punctularia strigoso-zonata*, *Stereum hirsutum*, *Coniophora puteana*), all but *P. strigoso-zonata* simultaneously secreted ALE and CDH in BMA medium. Excluding the brown-rotter *C. puteana*, at least one GH61 monooxygenase was secreted by each of these same fungi (Floudas et al. 2012). The roles(s) and interaction(s) between these genes, if any, remain unclear.

Several systems are available for heterologous expression. Homologous expression of *P. chrysosporium* CDH was achieved by fusing *cdh* with the promoter of the highly expressed glyceraldehyde-3-phosphate dehydrogenase gene (Li et al. 2000). Expression in *Pichia* spp. has also been reported (Yoshida et al. 2001; Zamocky et al. 2008; Bey et al. 2011), and *E. coli* was used to isolate the flavin domain.

5. Cytochrome P450s

Prior to 2004, the involvement of cytochrome 450s in xenobiotic degradation by *P. chrysosporium* was well-established, but the extent of genetic diversity was not fully appreciated until the genome became available (reviewed by (Syed and Yadav 2012)). Approximately **150 *P. chrysosporium* P450 genes were identified**, and close linkage and tandem arrangements were observed (Martinez et al. 2004). Such organizational tendencies were subsequently shown among many of the 222 and 254 P450 genes of *C. subvermispora* (Fernandez-Fueyo et al. 2012) and *P. placenta* (Martinez et al. 2009) respectively. Most recent analyses of Agaricomycotina genomes reiterate the impressive genetic diversity and complex organization (Floudas et al. 2012). The distribution into families and clans has shown no clear trends related to phylogeny or to ecological role, i.e., brown-rot versus white-rot.

Functional analyses of P450s, especially those derived from *P. chrysosporium*, have advanced significantly in recent years. Syed and coworkers reported the identification and functional characterization of **P450 monooxygenases capable of oxidizing different ring-size PAHs using a genome-to-function strategy** (Syed et al. 2010). A P450 microarray screen (Doddapaneni and Yadav 2005), first identified six P450 genes (Pc-pah1–Pc-pah6) induced by PAHs of varying ring size. The cDNAs of the six P450 monooxygenases were cloned and co-expressed in *Pichia pastoris* along with a P450 reductase partner. Each of the six recombinant P450 monooxygenases showed PAH-oxidizing activity (Syed et al. 2010). In separate studies, the P450 monooxygenase CYP5136A3 showed common responsiveness and catalytic versatility towards endocrine-disrupting alkylphenols and PAHs. The recombinant CYP5136A3 possessed oxidation activity towards alklyphenols with varying alkyl side-chain length (C3–C9), in addition to PAHs (3–4 ring size) (Syed et al. 2011b).

A P450 monooxygenase involved in anthracene metabolism by *P. chrysosporium* was identified by a combination of functional screening and a microarray system (Chigu et al. 2010). A wide variety of compounds were screened, and resulted in characterization of novel cytochrome P450 functions and discovery of a versatile cytochrome P450 that exhibit broad substrate profiles. The authors (Hirosue et al. 2011) suggested that multifunctional properties of the versatile cytochrome P450s would play crucial roles in diversification of fungal metabolic systems involved in xenobiotic degradation.

B. Experimental Systems

Comparative analysis of the genomes of wood decay fungi has provided considerable insight into oxidative systems. Interpretations are relatively clear in some cases, such as the importance of class II ligninolytic peroxidases (LiP, MnP, VP) in white-rot, but not brown-rot, decay. Likewise, the diminished repertoire of cellulases in brown-rot relative to white-rot genomes is consistent with a mechanism of cellulose depolymerization involving hydroxyl radicals. Nevertheless, the roles and interactions of thousands of genes remain uncertain.

High throughput transcriptome and secretome approaches rarely provide complete functional understanding. Instead, the methods allow the number of gene models to be filtered to a more manageable subset that is worthy of more detailed investigations. Whole genome microarrays and RNAseq have been used extensively to assess transcript levels and regulation, principally under conditions favoring lignocellulose degradation. Microarrays representing *P. chrysosporium* P450 genes have identified those induced by various organopollutants (Doddapaneni and Yadav 2005). Proteome analysis has involved mass spectrometry-based identification of 2DE-separated proteins (Abbas et al. 2004; Shimizu et al. 2005; Ravalason et al. 2008; Hori et al. 2011), of peptides tagged for iTRAQ quantitation (Manavalan et al. 2011), and of concentrated total extracellular proteins (Vanden Wymelenberg et al. 2005, 2006a, 2009, 2010, 2011).

A disconcerting aspect of these studies has been the imposing numbers of highly expressed and/or regulated genes encoding proteins of unknown function. Considering *P. chrysosporium* grown under nutrient starvation or in Avicel medium, 193 upregulated genes are predicted to encode 'hypothetical proteins'. Of these, 54 were unambiguously detected in extracellular filtrates by nanoLC-MS/MS (Vanden Wymelenberg et al. 2009). A total of 55, 32, and 14 'unknown proteins' were also identified in cultures of *P. chrysosporium, P. placenta*, and *C. subvermispora* respectively, containing complex lignocellulose substrates. Functional analysis of these hypothetical proteins represents a daunting challenge.

1. Genetic Tools

A major obstacle to research has been the lack of refined genetic tools for functional analysis and, potentially, for strain improvement. In the absence of monokaryons, genome assembly and annotation can be substantially

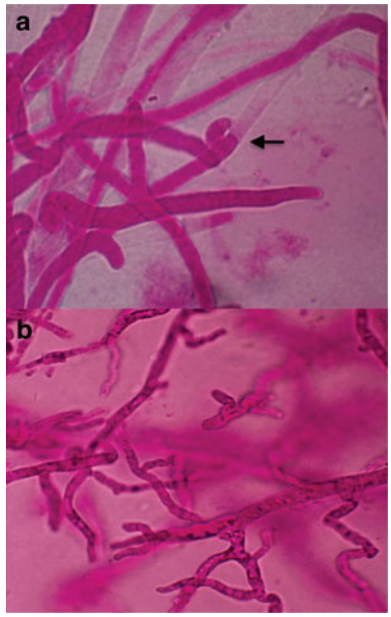

Fig. 5.2. Light microscopy of phloxine-stained *Postia placenta* hyphae. *Panel A*: sequenced parental dikaryon showing a clamp connection (*arrow*) that are commonly, but not always, observed in dikaryons. *Panel B*: typical of monokaryotic derivatives, no clamp connections are observed in the single basidiospore progeny

complicated by dikaryosis (Martinez et al. 2009), the typical nuclear conditions of agaricomycetes (Fig. 5.2). Genetic transformation for *P. chrysosporium* includes auxotroph complementation (Alic et al. 1989, 1990, 1991; Alic 1990; Randall et al. 1991; Akileswaran et al. 1993; Zapanta et al. 1998) and by drug resistance markers (Randall et al. 1989, 1991; Randall and Reddy 1992; Gessner and Raeder 1994). Transformation efficiencies are low, and gene targeting difficult (Alic et al. 1993). Still, reporters for studying gene expression have been described (Gettemy et al. 1997; Birch et al. 1998; Ma et al. 2001), homologous gene expression has proven useful, and RNA interference has been used to suppress Mn-dependent superoxide dismutase gene expression (Matityahu et al. 2008).

Beyond *P. chrysosporium*, ***P. ostreatus* offers transformation protocols** (Yanai et al. 1996; Honda et al. 2000; Irie et al. 2001; Sunagawa and Magae 2002) as well as methodology for physical (Larraya et al. 1999) and genetic mapping (Eichlerova-Volakova and Homolka 1997; Eichlerova and Homolka 1999; Larraya et al. 2000, 2002). *Trametes versicolor* has also been transformed with drug resistance vectors (Bartholomew et al. 2001; Kim et al. 2002), and gene disruptions have been reported (Dumonceaux et al. 2001). Recently, RNAi targeting of *P. ostreatus mnp3* was shown to suppress azo dye decolorization (Salame et al. 2010, 2011). RNAi provides a powerful tool, but suppression is often incomplete and the results further confounded by ectopic integration events.

In contrast to yeasts and many ascomycetes, filamentous basidiomycetes generally give low frequencies of homologous recombination. Recent advances have been made with *C. cinereus* (Nakazawa et al. 2011) and *Schizophyllum commune* (de Jong et al. 2010) strains in which nonhomologous end joining has been impaired by Ku knockouts. This approach substantially enhances the efficiency of gene targeting, but the species are inefficient lignin degraders.

Addressing this issue, **a Δ*ku80* strain has been constructed in *P. ostreatus*** (Salame et al. 2012). The recipient strain is similar to the parent with respect to growth, ligninolytic potential, and mating ability. Gene replacement showed 100 % homologous recombination, and the transformants remained stable in the absence of drug selection (Salame et al. 2012). By inactivation of a VP (*mnp4*), the enzyme was proven to be a major component of the ligninolytic system under Mn limitation. Thus, the system facilitates the efficient gene replacement in *P. ostreatus* and complements RNAi approaches.

2. Biochemical Tools

Efficient heterologous expression systems have been key to advancing our understanding of gene function, especially those encoding low levels of closely related proteins in the native systems. In particular, *E. coli* production and activation of peroxidases have been critical for evaluating catalytic properties (Doyle and Smith 1996; Nie et al. 1998). Similarly, co-expression of membrane-bound P450 monooxygenases with a reductase partner in *Pichia* has been essential for identifying P450s with activity against PAHs and

other compounds (Syed et al. 2010). Garcia-Ruiz and co-workers demonstrated the usefulness of *S. cerevisiae* for directed evolution of *P. eryngii* VP, and achieved improvements in secretion and activity. Additional rounds of evolution have enhanced VP stability in terms of temperature-, peroxide- and alkaline pH-tolerance (Garcia-Ruiz et al. 2012). Also using *S. cerevisiae* as host, directed evolution has improved a basidiomycete laccase. Enzyme stability related to temperature, pH, and organic solvents has been enhanced through a strategy that combines directed evolution with rational approaches (Mate et al. 2010).

IV. Current Research and Future Prospects

The advances made in recent years in biochemistry, genomics, and genome function studies of white-rot fungi provide a large amount of information on the mechanisms of degradation of a wide range of natural and xenobiotic aromatic hazardous compounds.

However, the conversion of this vast theoretical knowledge into practical biotechnology is limited. It is thus challenging to bridge this gap by identifying and resolving bottlenecks. The availability of increasing numbers of fungal genomes is an important step forward, but at the same time presents new challenges related to gene modeling, annotation, and meaningful phylogenetic comparisons. **Functional analyses of the hypothetical proteins remain a particularly daunting task.** Genetic approaches and possibly biochemical analysis of purified protein might be helpful, but the latter approach generally assumes that assays are available.

In addition to new discoveries related to the pathways of xenobiotic degradation, it could be important to identify new genes with traits that can support growth and activity of the fungi under environmental stress, and provide the ability to compete with bacteria. This may help moving from controlled sterile conditions to natural environments. In this connection, metatranscriptomics offer exciting new opportunities for identifying microbes and genes in organopollutant-contaminated soils (Damon et al. 2012; de Menezes et al. 2012).

Such investigations could lead to new strategies for improving the fitness of bioremediation strains via specialized inoculum preparation and/or genetic alterations. The latter might be augmented by altering expression of genes directly involved in xenobiotic degradation, such as specific peroxidases and CyP genes. Further strain improvements might focus on the expression of genes indirectly influencing oxidative enzyme systems. Examples include H_2O_2-generating enzymes that could enhance peroxidase catalysis or supply reactants for Fenton chemistry.

Genome analysis of wood-decay fungi contributes to our fundamental understanding of lignin degradation, a pivotal but incompletely understood, element of the carbon cycle. Ultimately, increasing genome resources will elucidate mechanisms of ligninolysis, and simultaneously serve as a framework for development of effective bioremediation and related bioprocesses.

References

Abbas A, Koc H et al (2004) Fungal degradation of wood: initial proteomic analysis of extracellular proteins of *Phanerochaete chrysosporium* grown on oak substrate. Curr Genet 47:49–56

Akileswaran L, Alic M et al (1993) Isolation and transformation of uracil auxotrophs of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Curr Genet 23:351–356

Alic MM (1990) Mating system and DNA transformation of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Diss Abstr Int B 51(8):3681

Alic M, Gold M (1991) Genetics and molecular biology of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. In: Bennett J, Lasure L (eds) More gene manipulations in fungi. Academic, New York, pp 319–341

Alic M, Kornegay JR et al (1989) Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl Environ Microbiol 55:406–411

Alic M, Clark EK et al (1990) Transformation of *Phanerochaete chrysosporium* and *Neurospora crassa* with adenine biosynthetic genes from *Schizophyllum commune*. Curr Genet 17:305–311

Alic M, Mayfield MB et al (1991) Homologous transformation of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Curr Genet 19:491–494

Alic M, Akileswaran L et al (1993) Gene replacement in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Gene 136:307–311

Alic M, Akileswaran L et al (1997) Characterization of the gene encoding manganese peroxidase isozyme 3 from *Phanerochaete chrysosporium*. Biochim Biophys Acta 1338(1):1–7

Alvarez JM, Canessa P et al (2009) Expression of genes encoding laccase and manganese-dependent peroxidase in the fungus *Ceriporiopsis subvermispora* is mediated by an ACE1-like copper-fist transcription factor. Fungal Genet Biol 46(1):104–111

Amitai G, Adani R et al (1998) Oxidative biodegradation of phosphorothiolates by fungal laccase. FEBS Lett 438(3):195–200

Ander P, Marzullo L (1997) Sugar oxidoreductases and veratryl alcohol oxidase as related to lignin degradation. J Biotechnol 53(2–3):115–131

Artolozaga MJ, Kubatova E et al (1997) Pyranose 2-oxidase from *Phanerochaete chrysosporium*–further biochemical characterisation. Appl Microbiol Biotechnol 47(5):508–514

Asada Y, Watanabe A et al (1995) Purification and characterization of an aryl-alcohol oxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Biosci Biotech Biochem 59:1339–1341

Bao W, Fukushima Y et al (1994) Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. FEBS Lett 354:297–300

Bartholomew K, Dos Santos G et al (2001) Genetic transformation of *Trametes versicolo*r to phleomycin resistance with the dominant selectable marker shble. Appl Microbiol Biotechnol 56(1–2):201–204

Belinky PA, Flikshtein N et al (2003) Reactive oxygen species and induction of lignin peroxidase in *Phanerochaete chrysosporium*. Appl Environ Microbiol 69(11):6500–6506

Benotti MJ, Trenholm RA et al (2009) Pharmaceuticals and endocrine disrupting compounds in U. S. drinking water. Environ Sci Technol 43 (3):597–603

Bey M, Berrin JG et al (2011) Heterologous expression of *Pycnoporus cinnabarinus* cellobiose dehydrogenase in *Pichia pastoris* and involvement in saccharification processes. Microb Cell Fact 10:113

Bezalel L, Hadar Y et al (1996a) Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 62(1):292–295

Bezalel L, Hadar Y et al (1996b) Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 62(7):2547–2553

Bezalel L, Hadar Y et al (1997) Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 63(7):2495–2501

Birch PR, Sims PF et al (1998) A reporter system for analysis of regulatable promoter functions in the basidiomycete fungus *Phanerochaete chrysosporium*. J Appl Microbiol 85(3):417–424

Blanchette R (1991) Delignification by wood-decay fungi. Ann Rev Phytopath 29:381–398

Bogan B, Lamar R et al (1996a) Fluorene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. Appl Environ Microbiol 62:1788–1792

Bogan BW, Schoenike B et al (1996b) Expression of *lip* genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium*. Appl Environ Microbiol 62(10):3697–3703

Bogan B, Schoenike B et al (1996c) Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. Appl Environ Microbiol 62:2381–2386

Bonnarme P, Jeffries T (1990) Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidase from lignin-degrading white-rot fungi. Appl Environ Microbiol 56:210–217

Bourbonnais R, Paice MG et al (1997) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol 63:4627–4632

Bourbonnais R, Leech D et al (1998) Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochim Biophys Acta 1379(3):381–390

Brown JA, Glenn JK et al (1990) Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. J Bacteriol 172(6):3125–3130

Brown J, Alic M et al (1991) Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese. J Bacteriol 173:4101–4106

Brown J, Li D et al (1993) Heat shock induction of manganese peroxidase gene transcription in *Phanerochaete chrysosporium*. Appl Environ Microbiol 59:4295–4299

Bumpus JA (1989) Biodegradation of polycyclic hydrocarbons by *Phanerochaete chrysosporium*. Appl Environ Microbiol 55(1):154–158

Camarero S, Sarkar S et al (1999) Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J Biol Chem 274(15):10324–10330

Camarero S, Ibarra D et al (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl Environ Microbiol 71(4):1775–1784

Casillas RP, Crow SA Jr et al (1996) Initial oxidative and subsequent conjugative metabolites produced during the metabolism of phenanthrene by fungi. J Ind Microbiol 16(4):205–215

Castanera R, Perez G et al (2012) Transcriptional and enzymatic profiling of *Pleurotus ostreatus* laccase genes in submerged and solid-state fermentation cultures. Appl Environ Microbiol 78(11):4037–4045

Chigu NL, Hirosue S et al (2010) Cytochrome P450 monooxygenases involved in anthracene metabolism by

the white-rot basidiomycete Phanerochaete chrysosporium. Appl Microbiol Biotechnol 87(5): 1907–1916

Cohen R, Hadar Y et al (2001) Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by Mn^{2+}. Environ Microbiol 3(5):312–322

Cohen R, Persky L et al (2002a) Mn^{2+} alters peroxidase profiles and lignin degradation by the white-rot fungus Pleurotus ostreatus under different nutritional and growth conditions. Appl Biochem Biotechnol 102–103(1–6):415–429

Cohen R, Yarden O et al (2002b) Lignocellulose affects Mn^{2+} regulation of peroxidase transcript levels in solid-state cultures of *Pleurotus ostreatus*. Appl Environ Microbiol 68(6):3156–3158

Coll P, Tabernero C et al (1993) Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic basidiomycete PM1 (CECT 2971). Appl Environ Microbiol 59:4129–4135

Collins PJ, O'Brien MM et al (1999) Cloning and characterization of a cDNA encoding a novel extracellular peroxidase from *Trametes versicolor*. Appl Environ Microbiol 65(3):1343–1347

Conesa A, van den Hondel CA et al (2000) Studies on the production of fungal peroxidases in *Aspergillus niger*. Appl Environ Microbiol 66(7):3016–3023

Cortes-Espinosa DV, Absalon AE et al (2011) Heterologous expression of manganese peroxidase in *Aspergillus niger* and its effect on phenanthrene removal from soil. J Mol Microbiol Biotechnol 21(3–4):120–129

Cruz-Morato C, Rodriguez-Rodriguez CE et al (2012) Biodegradation of pharmaceuticals by fungi and metabolites identification. In: Vincent T (ed) Emerging organic contaminants in sludges: analysis, fate and biological treatment. Springer, Berlin, pp 1–49

Cullen D (2002) Molecular genetics of lignin-degrading fungi and their application in organopollutant degradation. In: Kempken F (ed) The Mycota, vol XI. Springer, Berlin, pp 71–90

Cullen D, Kersten PJ (2004) Enzymology and molecular biology of lignin degradation. In: Brambl R, Marzulf GA (eds) The Mycota III biochemistry and molecular biology. Springer, Berlin, pp 249–273

Damon C, Lehembre F et al (2012) Metatranscriptomics reveals the diversity of genes expressed by eukaryotes in forest soils. PLoS One 7(1):e28967

Daniel G, Volc J et al (1994) Pyranose oxidase, a major source of H_2O_2 during wood degradation by *Phanerochaete chrysosporium, Trametes versicolor*, and *Oudemansiella mucida*. Appl Environ Microbiol 60:2524–2532

Daniel G, Volc J et al (2007) Characteristics of *Gloeophyllum trabeum* alcohol oxidase, an extracellular source of H_2O_2 in brown rot decay of wood. Appl Environ Microbiol 73(19):6241–6253

Datta A, Bettermann A et al (1991) Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. Appl Environ Microbiol 57:1453–1460

de Jong JF, Ohm RA et al (2010) Inactivation of ku80 in the mushroom-forming fungus *Schizophyllum commune* increases the relative incidence of homologous recombination. FEMS Microbiol Lett 310(1):91–95

de Koker TH, Mozuch MD et al (2004) Pyranose 2-oxidase from *Phanerochaete chrysosporium*: isolation from solid substrate, protein purification, and characterization of gene structure and regulation. Appl Environ Microbiol 70:5794–5800

de Menezes A, Clipson N et al (2012) Comparative metatranscriptomics reveals widespread community responses during phenanthrene degradation in soil. Environ Microbiol 14(9):2577–2588

Dietrich D, Crooks C (2009) Gene cloning and heterologous expression of pyranose 2-oxidase from the brown-rot fungus, *Gloeophyllum trabeum*. Biotechnol Lett 31(8):1223–1228

Doddapaneni H, Yadav JS (2005) Microarray-based global differential expression profiling of P450 monooxygenases and regulatory proteins for signal transduction pathways in the white rot fungus *Phanerochaete chrysosporium*. Mol Genet Genomics 274:454–466

Doyle WA, Smith AT (1996) Expression of lignin peroxidase H8 in *Escherichia coli*: folding and activation of the recombinant enzyme with Ca^{2+} and haem. Biochem J 315(Pt 1):15–19

Dumonceaux TJ, Bartholomew KA et al (1998) Cloning and sequencing of a gene encoding cellobiose dehydrogenase from *Trametes versicolor*. Gene 210:211–219

Dumonceaux T, Bartholomew K et al (2001) Cellobiose dehydrogenase is essential for wood invasion and nonessential for kraft pulp delignification by *Trametes versicolor*. Enzyme Microb Technol 29:478–489

Eastwood DC, Floudas D et al (2011) The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. Science 333(6043):762–765

Eggert C, Temp U et al (1996) A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Lett 391:144–148

Eibes GM, Lu-Chau TA et al (2009) Effect of culture temperature on the heterologous expression of *Pleurotus eryngii* versatile peroxidase in *Aspergillus* hosts. Bioprocess Biosyst Eng 32(1):129–134

Eichlerova I, Homolka L (1999) Preparation and crossing of basidiospore-derived monokaryons–a useful tool for obtaining laccase and other ligninolytic enzyme higher- producing dikaryotic strains of Pleurotus ostreatus. Antonie Van Leeuwenhoek 75(4):321–327

Eichlerova-Volakova I, Homolka L (1997) Variability of ligninolytic enzyme activities in basidiospore isolates of the fungus *Pleurotus ostreatus* in comparison with that of protoplast-derived isolates. Folia Microbiol 42(6):583–588

Eriksson K-EL, Blanchette RA et al (1990) Microbial and enzymatic degradation of wood and wood components. Springer, Berlin

Fernandez-Fueyo E, Ruiz-Duenas FJ et al (2012) Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc Natl Acad Sci USA 109(14):5458–5463

Floudas D, Binder M et al (2012) The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336(6089):1715–1719

Ford CI, Walter M et al (2007a) Fungal inoculum properties: extracellular enzyme expression and pentachlorophenol removal by New Zealand *trametes* species in contaminated field soils. J Environ Qual 36(6):1749–1759

Ford CI, Walter M et al (2007b) Fungal inoculum properties: extracellular enzyme expression and pentachlorophenol removal in highly contaminated field soils. J Environ Qual 36(6):1599–1608

Fukushima Y, Kirk TK (1995) Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. Appl Environ Microbiol 61:872–876

Gan S, Lau EV et al (2009) Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). J Hazard Mater 172(2–3):532–549

Garcia-Ruiz E, Gonzalez-Perez D et al (2012) Directed evolution of a temperature-, peroxide- and alkaline pH-tolerant versatile peroxidase. Biochem J 441(1):487–498

Gaskell J, Stewart P et al (1994) Establishment of genetic linkage by allele-specific polymerase chain reaction: application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. Biotechnology 12:1372–1375

Gasser G, Rona M et al (2010) Quantitative evaluation of tracers for quantification of wastewater contamination of potable water sources. Environ Sci Technol 44(10):3919–3925

George EJ, Neufield RD (1989) Degradation of fluorene in soil by fungus *Phanerochaete chrysosporium*. Biotechnol Bioengineer 33:1306–1310

Gessner M, Raeder U (1994) A histone promoter for expression of a phleomycin-resistance gene in *Phanerochaete chrysosporium*. Gene 142:237–241

Gettemy JM, Li D et al (1997) Truncated-gene reporter system for studying the regulation of manganese peroxidase expression. Curr Genet 31(6):519–524

Gettemy JM, Ma B et al (1998) Reverse transcription-PCR analysis of the regulation of the manganese peroxidase gene family. Appl Environ Microbiol 64(2):569–574

Giardina P, Palmieri G et al (1999) Protein and gene structure of a blue laccase from *Pleurotus ostreatus1*. Biochem J 341(Pt 3):655–663

Giardina P, Faraco V et al (2010) Laccases: a never-ending story. Cell Mol Life Sci 67(3):369–385

Glenn JK, Gold MH (1983) Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl Environ Microbiol 45(6):1741–1747

Glenn JK, Morgan MA et al (1983) An extracellular H_20_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. Biochem Biophys Res Comm 114:1077–1083

Glenn JK, Akileswaran L et al (1986) Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch Biochem Biophys 251:688–696

Godfrey BJ, Mayfield MB et al (1990) Characterization of a gene encoding a manganese peroxidase from *Phanerochaete chrysosporium*. Gene 93(1):119–124

Godfrey B, Akileswaran L et al (1994) A reporter gene construct for studying the regulation of manganese peroxidase gene expression. Appl Environ Microbiol 60:1353–2358

Golan-Rozen N, Chefetz B et al (2011) Transformation of the recalcitrant pharmaceutical compound carbamazepine by *Pleurotus ostreatus*: role of cytochrome P450 monooxygenase and manganese peroxidase. Environ Sci Technol 45(16):6800–6805

Gold MH, Kuwahara M et al (1984) Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete, *Phanerochaete chrysosporium*. Archives Biochem Biophys 234:353–362

Grassi E, Scodeller P et al (2011) Potential of *Trametes trogii* culture fluids and its purified laccase for the decolorization of different types of recalcitrant dyes without the addition of redox mediators. Biodeterior Biodegradation 65:635–643

Grigoriev IV, Nordberg H et al (2012) The genome portal of the Department of Energy Joint Genome Institute. Nucleic Acids Res 40(Database issue): D26–D32

Guo M, Lu F et al (2008) Purification of recombinant laccase from *Trametes versicolor* in *Pichia methanolica* and its use for the decolorization of anthraquinone dye. Biotechnol Lett 30(12):2091–2096

Gutierrez A, Babot ED et al (2011) Regioselective oxygenation of fatty acids, fatty alcohols and other aliphatic compounds by a basidiomycete heme-thiolate peroxidase. Arch Biochem Biophys 514(1–2):33–43

Hallberg BM, Bergfors T et al (2000) A new scaffold for binding haem in the cytochrome domain of the extracellular flavocytochrome cellobiose dehydrogenase. Structure Fold Des 8(1):79–88

Hammel KE (1995a) Mechanisms for polycyclic aromatic hydrocarbon degradation by ligninolytic fungi. Environ Health Perspect 103(Suppl 5):41–43

Hammel KE (1995b) Organopollutant degradation by fungi. In: Young LY, Cerniglia CE (eds) Microbial transformation and degradation of toxic organic chemical. Wiley-Liss, New York, pp 331–346

Hammel KE, Cullen D (2008) Role of fungal peroxidases in biological ligninolysis. Curr Opin Plant Biol 11(3):349–355

Hammel KE, Tardone PJ (1988) The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidases. Biochemistry 27:6563–6568

Hammel KE, Kalyanaraman B et al (1986a) Oxidation of polycyclic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium* ligninase. J Biol Chem 261:16948–16952

Hammel KE, Kalyanaraman B et al (1986b) Substrate free radicals are intermediates in ligninase catalysis. Proc Natl Acad Sci USA 83:3808–3812

Hammel KE, Gai WZ et al (1992) Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 58(6):1832–1838

Haritash AK, Kaushik CP (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J Hazard Mater 169(1–3):1–15

Harris PV, Welner D et al (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49(15):3305–3316

Harvey PJ, Schoemaker HE et al (1985) Single-electron transfer processes and the reaction mechanism of enzymic degradation of lignin. FEBS Lett 183:13–16

Hata T, Shintate H et al (2010) Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole. J Hazard Mater 181(1–3):1175–1178

Heberer T (2002) Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett 131(1–2):5–17

Heinfling A, Martinez MJ et al (1998a) Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta*. FEMS Microbiol Lett 165(1):43–50

Heinfling A, Martinez MJ et al (1998b) Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. Appl Environ Microbiol 64(8):2788–2793

Henriksson G, Johansson G et al (2000) A critical review of cellobiose dehydrogenases. J Biotechnol 78(2):93–113

Hernandez-Ortega A, Ferreira P et al (2012) Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation. Appl Microbiol Biotechnol 93(4):1395–1410

Higham CW, Gordon-Smith D et al (1994) Direct 1H NMR evidence for conversion of beta-D-cellobiose to cellobionolactone by cellobiose dehydrogenase from *Phanerochaete chrysosporium*. FEBS Lett 351 (1):128–132

Higson FK (1991) Degradation of xenobiotics by white rot fungi. Rev Environ Contam Toxicol 122:111–152

Higuchi T (1990) Lignin biochemistry: biosynthesis and biodegradation. Wood Sci Technol 24(1):23–63

Hirosue S, Tazaki M et al (2011) Insight into functional diversity of cytochrome P450 in the white-rot basidiomycete *Phanerochaete chrysosporium*: involvement of versatile monooxygenase. Biochem Biophys Res Commun 407(1):118–123

Hiscox J, Baldrian P et al (2010) Changes in oxidative enzyme activity during interspecific mycelial interactions involving the white-rot fungus *Trametes versicolor*. Fungal Genet Biol 47(6):562–571

Hoegger PJ, Kilaru S et al (2006) Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS J 273 (10):2308–2326

Hofrichter M, Ullrich R et al (2010) New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 87(3):871–897

Holzbaur E, Tien M (1988) Structure and regulation of a lignin peroxidase gene from *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 155:626–633

Honda Y, Matsuyama T et al (2000) Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. Curr Genet 37(3):209–212

Hori C, Igarashi K et al (2011) Effects of xylan and starch on secretome of the basidiomycete *Phanerochaete chrysosporium* grown on cellulose. FEMS Microbiol Lett 321(1):14–23

Hsu CA, Wen TN et al (2012) Biological degradation of anthroquinone and azo dyes by a novel laccase from *Lentinus* sp. Environ Sci Technol 46 (9):5109–5117

Hundt K, Martin D et al (2000) Transformation of triclosan by *Trametes versicolor* and *Pycnoporus cinnabarinus*. Appl Environ Microbiol 66(9):4157–4160

Ichinose H, Wariishi H et al (1999) Bioconversion of recalcitrant 4-methyldibenzothiophene to water-extractable products using lignin-degrading basidiomycete *Coriolus versicolor*. Biotechnol Prog 15 (4):706–714

Irie T, Honda Y et al (2001) Efficient transformation of filamentous fungus *Pleurotus ostreatus* using single-strand carrier DNA. Appl Microbiol Biotechnol 55(5):563–565

Janse BJH, Gaskell J et al (1998) Expression of *Phanerochaete chrysosporium* genes encoding lignin peroxidases, manganese peroxidases, and glyoxal oxidase in wood. Appl Environ Microbiol 64 (9):3536–3538

Johannes C, Majcherczyk A (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol 66(2):524–528

Johannes C, Majcherczyk A et al (1996) Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. Appl Microbiol Biotechnol 46(3):313–317

Johansson T, Nyman PO et al (2002) Differential regulation of mnp2, a new manganese peroxidase-encoding gene from the ligninolytic fungus

Trametes versicolor PRL 572. Appl Environ Microbiol 68(4):2077–2080

Johnson TM, Li JK (1991) Heterologous expression and characterization of an active lignin peroxidase from *Phanerochaete chrysosporium* using recombinant baculovirus. Arch Biochem Biophys 291:371–378

Johnson T, Pease E et al (1992) Production and characterization of recombinant lignin peroxidase isozyme H2 from *Phanerochaete chrysosporium* using recombinant baculovirus. Arch Biochem Biophys 296:660–666

Kang SI, Kang SY et al (2008) Identification of fungal metabolites of anticonvulsant drug carbamazepine. Appl Microbiol Biotechnol 79(4):663–669

Kapich AN, Jensen KA et al (1999) Peroxyl radicals are potential agents of lignin biodegradation. FEBS Lett 461(1–2):115–119

Karahanian E, Corsini G et al (1998) Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochim Biophys Acta 1443(1–2):65–74

Kaushik P, Malik A (2009) Fungal dye decolourization: recent advances and future potential. Environ Int 35(1):127–141

Kawai S, Umezawa T et al (1988) Degradation mechanisms of phenolic b-1 lignin substructure and model compounds by laccase of *Coriolus versicolor*. Arch Biochem Biophys 262:99–110

Kelley RL, Reddy CA (1986) Purification and characterization of glucose oxidase from ligninolytic cultures of *Phanerochaete chrysosporium*. J Bacteriol 166(1):269–274

Kelley RL, Reddy CA (1988) Glucose oxidase of *Phanerochaete chrysosporium*. Methods Enzymol 161:307–316

Kersten PJ (1990) Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc Natl Acad Sci USA 87 (8):2936–2940

Kersten P, Cullen D (1993) Cloning and characterization of a cDNA encoding glyoxal oxidase, a peroxide-producing enzyme from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Proc Natl Acad Sci USA 90:7411–7413

Kersten PJ, Kirk TK (1987) Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J Bacteriol 169:2195–2201

Kersten PJ, Tien M et al (1985) The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxybenzenes. J Biol Chem 260:2609–2612

Kersten PJ, Witek C et al (1995) *Phanerochaete chrysosporium* glyoxal oxidase is encoded by two allelic variants: structure, genomic organization and heterologous expression of *glx1* and *glx2*. J Bacteriol 177:6106–6110

Kim K, Leem Y et al (2002) Transformation of the medicinal basidiomycete *Trametes versicolor* to hygromycin B resistance by restriction enzyme mediated integration. FEMS Microbiol Lett 209 (2):273–276

Kirk TK, Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41:465–505

Kojima Y, Tsukuda Y et al (1990) Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J Biol Chem 256:15224–15230

Kullman SW, Matsumura F (1996) Metabolic pathways utilized by *Phanerochaete chrysosporium* for degradation of the cyclodiene pesticide endosulfan. Appl Environ Microbiol 62:593–600

Lamar R, Dietrich D (1990) In situ depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. Appl Environ Microbiol 56:3093–3100

Lamar RT, Glaser JA et al (1990a) Fate of pentachlorophenol (PCP) in sterile soils inoculated with the white-rot basidiomycete *Phanerochaete chrysosporium*; mineralization, volatilization and depletion of PCP. Soil Biol Biochem 22(4):433–440

Lamar RT, Larsen MJ et al (1990b) Sensitivity to and degradation of pentachlorophenol by *Phanerochaete* spp. Appl Environ Microbiol 56(11):3519–3526

Lamar RT, Davis MW et al (1994) Treatment of a pentachlorophenol- and creosote-contaminated soil using the lignin-degrading fungus *Phanerochaete chrysosporium*: a field demonstration. Soil Biol Biochem 26:1603–1611

Lamar RT, Schoenike B et al (1995) Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. Appl Environ Microbiol 61:2122–2126

Langston JA, Shaghasi T et al (2011) Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. Appl Environ Microbiol 77(19):7007–7015

Larraya LM, Perez G et al (1999) Molecular karyotype of the white rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 65(8):3413–3417

Larraya LM, Perez G et al (2000) Genetic linkage map of the edible basidiomycete *Pleurotus ostreatus*. Appl Environ Microbiol 66(12):5290–5300

Larraya LM, Idareta E et al (2002) Quantitative trait loci controlling vegetative growth rate in the edible basidiomycete *Pleurotus ostreatus*. Appl Environ Microbiol 68(3):1109–1114

Larrondo LF, Lobos S et al (2001) Isoenzyme multiplicity and characterization of recombinant manganese peroxidases from *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium*. Appl Environ Microbiol 67(5):2070–2075

Larrondo LF, Avila M et al (2003) Heterologous expression of laccase cDNA from *Ceriporiopsis*

subvermispora yields copper-activated apoprotein and complex isoform patterns. Microbiology 149 (Pt 5):1177–1182

Larrondo L, Vicuna R et al (2005) *Phanerochaete chrysosporium* genomics. In: Arora DK, Berka R (eds) Applied mycology and biotechnology, vol 5. Elsevier, Amsterdam, pp 315–352

Leclercq M, Mathieu O et al (2009) Presence and fate of carbamazepine, oxcarbazepine, and seven of their metabolites at wastewater treatment plants. Arch Environ Contam Toxicol 56(3):408–415

Lestan D, Lamar RT (1996) Development of fungal inocula for bioaugmentation of contaminated soils. Appl Environ Microbiol 62(6):2045–2052

Li B, Nagalla SR et al (1996) Cloning of a cDNA encoding cellobiose dehydrogenase, a hemoflavoenzyme from *Phanerochaete chrysosporium*. Appl Environ Microbiol 62(4):1329–1335

Li B, Rotsaert FA et al (2000) Homologous expression of recombinant cellobiose dehydrogenase in *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 270(1):141–146

Lienert J, Gudel K et al (2007) Screening method for ecotoxicological hazard assessment of 42 pharmaceuticals considering human metabolism and excretory routes. Environ Sci Technol 41 (12):4471–4478

Liers C, Bobeth C et al (2010) DyP-like peroxidases of the jelly fungus *Auricularia auricula-judae* oxidize nonphenolic lignin model compounds and high-redox potential dyes. Appl Microbiol Biotechnol 85(6):1869–1879

Lobos S, Larrain J et al (1994) Isozymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. Microbiology 140:2691–2698

Lu L, Zhao M et al (2009) Production and synthetic dyes decolourization capacity of a recombinant laccase from *Pichia pastoris*. J Appl Microbiol 107 (4):1149–1156

Lu XY, Zhang T et al (2011) Bacteria-mediated PAH degradation in soil and sediment. Appl Microbiol Biotechnol 89(5):1357–1371

Lundell TK, Makela MR et al (2010) Lignin-modifying enzymes in filamentous basdiomycetes-ecological, functional and phylogenetic review. J Basic Microbiol 50:4–20

Ma B, Mayfield MB et al (2001) The green fluorescent protein gene functions as a reporter of gene expression in *Phanerochaete chrysosporium*. Appl Environ Microbiol 67(2):948–955

Ma B, Mayfield MB et al (2003) Homologous expression of *Phanerochaete chrysosporium* manganese peroxidase, using bialaphos resistance as a dominant selectable marker. Curr Genet 43(6):407–414

Ma B, Mayfield MB et al (2004) Novel promoter sequence required for manganese regulation of manganese peroxidase isozyme 1 gene expression in *Phanerochaete chrysosporium*. Eukaryot Cell 3(3):579–588

Macdonald J, Master ER (2012) Time-dependent profiles of transcripts encoding lignocellulose-modifying enzymes of the white rot fungus *Phanerochaete carnosa* grown on multiple wood substrates. Appl Environ Microbiol 78(5):1596–1600

Macdonald J, Doering M et al (2011) Transcriptomic responses of the softwood-degrading white-rot fungus *Phanerochaete carnosa* during growth on coniferous and deciduous wood. Appl Environ Microbiol 77:3211–3218

MacDonald J, Suzuki H et al (2012) Expression and regulation of genes encoding lignocellulose-degrading activity in the genus *Phanerochaete*. Appl Microbiol Biotechnol 94(2):339–351

Manavalan A, Adav SS et al (2011) iTRAQ-based quantitative secretome analysis of *Phanerochaete chrysosporium*. J Proteomics 75(2):642–654

Mancilla RA, Canessa P et al (2010) Effect of manganese on the secretion of manganese-peroxidase by the basidiomycete *Ceriporiopsis subvermispora*. Fungal Genet Biol 47(7):656–661

Marco-Urrea E, Perez-Trujillo M et al (2009) Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. Chemosphere 74(6):765–772

Marco-Urrea E, Radjenovic J et al (2010) Oxidation of atenolol, propranolol, carbamazepine and clofibric acid by a biological Fenton-like system mediated by the white-rot fungus *Trametes versicolor*. Water Res 44(2):521–532

Martinez D, Larrondo LF et al (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol 22:695–700

Martinez D, Challacombe J et al (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci USA 106(6):1954–1959

Masapahy S, Lamb DC et al (1999) Purification and characterization of a benzo[a]pyrene hydroxylase from *Pleurotus pulmonarius*. Biochem Biophys Res Commun 266(2):326–329

Mate D, Garcia-Burgos C et al (2010) Laboratory evolution of high-redox potential laccases. Chem Biol 17(9):1030–1041

Matityahu A, Hadar Y et al (2008) Gene silencing by RNA Interference in the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 74 (17):5359–5365

Mester T, Field JA (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J Biol Chem 273:15412–15417

Miao XS, Yang JJ et al (2005) Carbamazepine and its metabolites in wastewater and in biosolids in a

municipal wastewater treatment plant. Environ Sci Technol 39(19):7469–7475

Miele A, Giardina P et al (2010) Random mutants of a *Pleurotus ostreatus* laccase as new biocatalysts for industrial effluents bioremediation. J Appl Microbiol 108(3):998–1006

Miki Y, Morales M et al (2009) *Escherichia coli* expression and in vitro activation of a unique ligninolytic peroxidase that has a catalytic tyrosine residue. Protein Expr Purif 68(2):208–214

Mileski GJ, Bumpus JA et al (1988) Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 54(12):2885–2889

Moen M, Hammel K (1994) Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl Environ Microbiol 60:1956–1961

Montiel-Gonzalez AM, Fernandez FJ et al (2009) Increased PCP removal by *Amylomyces rouxii* transformants with heterologous *Phanerochaete chrysosporium* peroxidases supplementing their natural degradative pathway. Appl Microbiol Biotechnol 84(2):335–340

Moukha SM, Dumonceaux TJ et al (1999) Cloning and analysis of *Pycnoporus cinnabarinus* cellobiose dehydrogenase. Gene 234(1):23–33

Muheim A, Leisola MSA et al (1990) Aryl-alcohol-oxidase and lignin-peroxidase from the white-rot fungus *Bjerkandera adusta* comparison with *Phanerochaete chrysosporium* lignin-peroxidase for reactivity with veratryl alcohol, homoveratric acid and alpha-benzyl veratryl alcohol. J Biotechnol 13(2–3):159–167

Nakazawa T, Ando Y et al (2011) Efficient gene targeting in DeltaCc.ku70 or DeltaCc.lig4 mutants of the agaricomycete *Coprinopsis cinerea*. Fungal Genet Biol 48(10):939–946

Nie G, Reading NS et al (1998) Expression of the lignin peroxidase H2 gene from *Phanerochaete chrysosporium* in *Escherichia coli*. Biochem Biophys Res Commun 249(1):146–150

Nishimura I, Okada K et al (1996) Cloning and expression of pyranose oxidase cDNA from *Coriolus versicolor* in *E. coli*. J Biotechnol 52:11–20

Ohm RA, de Jong JF et al (2010) Genome sequence of the model mushroom *Schizophyllum commune*. Nat Biotechnol 28(9):957–963

Olson A, Aerts A et al (2012) Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. New Phytol 194(4):1001–1013

Orth A, Royse D et al (1993) Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Appl Environ Microbiol 59:4017–4023

Orth A, Rzhetskaya M et al (1994) Characterization of a cDNA encoding a manganese peroxidase from *Phanerochaete chrysosporium*: genomic organization of lignin and manganese peroxidase genes. Gene 148:161–165

Palmieri G, Giardina P et al (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66 (3):920–924

Paszczynski A, Huynh V-B et al (1986) Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. Arch Biochem Biophys 244:750–765

Pease E, Tien M (1992) Heterogeneity and regulation of manganese peroxidases from *Phanerochaete chrysosporium*. J Bacteriol 174:3532–3540

Pease EA, Andrawis A et al (1989) Manganese-dependent peroxidase from *Phanerochaete chrysosporium*. Primary structure deduced from complementary DNA sequence. J Biol Chem 264 (23):13531–13535

Peng RH, Xiong AS et al (2008) Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev 32(6):927–955

Perie FH, Reddy GV et al (1998) Purification and characterization of laccases from the white-rot basidiomycete *Dichomitus squalens*. Arch Biochem Biophys 353(2):349–355

Pickard MA, Roman R et al (1999) Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Appl Environ Microbiol 65(9):3805–3809

Pisanelli I, Kujawa M et al (2009) Pyranose 2-oxidase from *Phanerochaete chrysosporium*–expression in *E. coli* and biochemical characterization. J Biotechnol 142(2):97–106

Piscitelli A, Del Vecchio C et al (2011a) Fungal laccases: versatile tools for lignocellulose transformation. C R Biol 334(11):789–794

Piscitelli A, Giardina P et al (2011b) Induction and transcriptional regulation of laccases in fungi. Curr Genomics 12(2):104–112

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57(1–2):20–33

Pribnow D, Mayfield MB et al (1989) Characterization of a cDNA encoding a manganese peroxidase, from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J Biol Chem 264(9):5036–5040

Quinlan RJ, Sweeney MD et al (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc Natl Acad Sci USA 108(37):15079–15084

Raices M, Paifer E et al (1995) Cloning and characterization of a cDNA encoding a cellobiose dehydrogenase from the white rot fungus *Phanerochaete chrysosporium*. FEBS Lett 369(2–3):233–238

Randall TA, Reddy CA (1992) The nature of extrachromosomal maintenance of transforming plasmids in the filamentous basidiomycete *Phanerochaete chrysosporium*. Curr Genet 21:255–260

Randall T, Rao TR et al (1989) Use of a shuttle vector for the transformation of the white-rot basidiomycete, *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 161:720–725

Randall T, Reddy CA et al (1991) A novel extrachromosomally maintained transformation vector for the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J Bacteriol 173(2):776–782

Ravalason H, Jan G et al (2008) Secretome analysis of *Phanerochaete chrysosporium* strain CIRM-BRFM41 grown on softwood. Appl Microbiol Biotechnol 80(4):719–733

Reddy GV, Gold MH (1999) A two-component tetrachlorohydroquinone reductive dehalogenase system from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 257(3):901–905

Reddy GV, Gold MH (2000) Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved. Microbiology 146(Pt 2):405–413

Reddy GV, Gelpke MD et al (1998) Degradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*: involvement of reductive dechlorination. J Bacteriol 180(19):5159–5164

Reiser J, Walther I et al (1993) Methods to investigate the expression of lignin peroxidase genes by the white-rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 59:2897–2903

Rodriguez-Rodriguez CE, Marco-Urrea E et al (2010) Degradation of naproxen and carbamazepine in spiked sludge by slurry and solid-phase *Trametes versicolor* systems. Bioresour Technol 101(7):2259–2266

Ruiz-Duenas FJ, Martinez MJ et al (1999) Heterologous expression of *Pleurotus eryngii* peroxidase confirms its ability to oxidize Mn(2+) and different aromatic substrates. Appl Environ Microbiol 65 (10):4705–4707

Ruiz-Duenas FJ, Morales M et al (2008) Site-directed mutagenesis of the catalytic tryptophan environment in *Pleurotus eryngii* versatile peroxidase. Biochemistry 47(6):1685–1695

Ruiz-Duenas FJ, Morales M et al (2009) Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J Exp Bot 60(2):441–452

Ruiz-Duenas FJ, Fernandez E et al (2011) *Pleurotus ostreatus* heme peroxidases: an in silico analysis from the genome sequence to the enzyme molecular structure. C R Biol 334(11):795–805

Ruttimann-Johnson C, Lamar RT (1996) Polymerization of pentachlorophenol and ferulic acid by fungal extracellular lignin-degrading enzymes. Appl Environ Microbiol 62(10):3890–3893

Ryu K, Hwang SY et al (2008a) Functionality improvement of fungal lignin peroxidase by DNA shuffling for 2,4-dichlorophenol degradability and H2O2 stability. J Biotechnol 133(1):110–115

Ryu K, Kang JH et al (2008b) Expression in yeast of secreted lignin peroxidase with improved 2,4-dichlorophenol degradability by DNA shuffling. J Biotechnol 135(3):241–246

Sakamoto T, Kitaura H et al (2010) Transcriptional effect of a calmodulin inhibitor, W-7, on the ligninolytic enzyme genes in *Phanerochaete chrysosporium*. Curr Genet 56(5):401–410

Salame TM, Yarden O et al (2010) *Pleurotus ostreatus* manganese-dependent peroxidase silencing impairs decolourization of Orange II. Microb Biotechnol 3(1):93–106

Salame TM, Ziv C et al (2011) RNAi as a potential tool for biotechnological applications in fungi. Appl Microbiol Biotechnol 89(3):501–512

Salame TM, Knop D et al (2012) Predominance of a Versatile-Peroxidase-Encoding Gene, *mnp4*, as Demonstrated by Gene Replacement via a Gene Targeting System for *Pleurotus ostreatus*. Appl Environ Microbiol 78(15):5341–5352

Salas C, Lobos S et al (1995) Properties of laccase isoenzymes produced by the basidiomycete *Ceriporiopsis subvermispora*. Biotechnol Appl Biochem 21:323–333

Saloheimo M, Niku-Paavola M et al (1991) Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. J Gen Microbiol 137:1537–1544

Sannia G, Limongi P et al (1991) Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. Biochim Biophys Acta 1073:114–119

Sato S, Feltus FA et al (2009) The first genome-level transcriptome of the wood-degrading fungus *Phanerochaete chrysosporium* grown on red oak. Curr Genet 55(3):273–286

Scheibner K, Hofrichter M (1998) Conversion of aminonitrotoluenes by fungal manganese peroxidase. J Basic Microbiol 38(1):51–59

Shimizu M, Yuda N et al (2005) Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. Proteomics 5(15):3919–3931

Shoemaker HE, Harvey PJ et al (1985) On the mechanism of enzymatic lignin breakdown. FEBS Lett 183:7–12

Singh K, Arora S (2011) Removal of synthetic textile dyes from wastewaters: a critical review on present treatment technologies. Crit Rev Environ Sci Technol 41:807–878

Smith M, Shnyreva A et al (1998) Tandem organization and highly disparate expression of the two laccase genes *lcc1* and *lcc2* in the cultivated mushroom *Agaricus bisporus*. Microbiology 144(Pt 4):1063–1069

Sollewijn Gelpke MD, Mayfield-Gambill M et al (1999) Homologous expression of recombinant lignin peroxidase in *Phanerochaete chrysosporium*. Appl Environ Microbiol 65(4):1670–1674

Sollewijn Gelpke MD, Lee J et al (2002) Lignin peroxidase oxidation of veratryl alcohol: effects of the mutants H82A, Q222A, W171A, and F267L. Biochemistry 41(10):3498–3506

Stewart P, Cullen D (1999) Organization and differential regulation of a cluster of lignin peroxidase genes of *Phanerochaete chrysosporium*. J Bacteriol 181:3427–3432

Stewart P, Kersten P et al (1992) The lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation, and the identification of a second dimorphic chromosome. J Bacteriol 174:5036–5042

Stewart P, Whitwam RE et al (1996) Efficient expression of a *Phanerochaete chrysosporium* manganese peroxidase gene in *Aspergillus oryzae*. Appl Environ Microbiol 62(3):860–864

Stolz A (2001) Basic and applied aspects in the microbial degradation of azo dyes. Appl Microbiol Biotechnol 56(1–2):69–80

Stuardo M, Vasquez M et al (2004) Molecular approach for analysis of model fungal genes encoding ligninolytic peroxidases in wood-decaying soil systems. Lett Appl Microbiol 38(1):43–49

Sucharitakul J, Wongnate T et al (2011) Hydrogen peroxide elimination from C4a-hydroperoxyflavin in a flavoprotein oxidase occurs through a single proton transfer from flavin N5 to a peroxide leaving group. J Biol Chem 286(19):16900–16909

Sugano Y, Nakano R et al (2000) Efficient heterologous expression in *Aspergillus oryzae* of a unique dye-decolorizing peroxidase, DyP, of *Geotrichum candidum*. Appl Environ Microbiol 66(4):1754–1758

Sunagawa M, Magae Y (2002) Transformation of the edible mushroom *Pleurotus ostreatus* by particle bombardment. FEMS Microbiol Lett 211 (2):143–146

Syed K, Yadav JS (2012) P450 monooxygenases (P450ome) of the model white rot fungus *Phanerochaete chrysosporium*. Crit Rev Microbiol 38 (4):339–363

Syed K, Doddapaneni H et al (2010) Genome-to-function characterization of novel fungal P450 monooxygenases oxidizing polycyclic aromatic hydrocarbons (PAHs). Biochem Biophys Res Commun 399(4):492–497

Syed K, Kattamuri C et al (2011a) Cytochrome b(5) reductase-cytochrome b(5) as an active P450 redox enzyme system in *Phanerochaete chrysosporium*: atypical properties and in vivo evidence of electron transfer capability to CYP63A2. Arch Biochem Biophys 509(1):26–32

Syed K, Porollo A et al (2011b) A fungal P450 (CYP5136A3) capable of oxidizing polycyclic aromatic hydrocarbons and endocrine disrupting alkylphenols: role of Trp(129) and Leu(324). PLoS One 6(12):e28286

Tan TC, Pitsawong W et al (2010) H-bonding and positive charge at the N5/O4 locus are critical for covalent flavin attachment in *Trametes* pyranose 2-oxidase. J Mol Biol 402(3):578–594

Tan TC, Haltrich D et al (2011) Regioselective control of beta-d-glucose oxidation by pyranose 2-oxidase is intimately coupled to conformational degeneracy. J Mol Biol 409(4):588–600

Temp U, Zierold U et al (1999) Cloning and characterization of a second laccase gene from the lignin-degrading basidiomycete *Pycnoporus cinnabarinus*. Gene 236(1):169–177

Teunissen PJ, Sheng D et al (1998) 2-Chloro-1,4-dimethoxybenzene cation radical: formation and role in the lignin peroxidase oxidation of anisyl alcohol. Arch Biochem Biophys 360(2):233–238

Tien M, Kirk TK (1983) Lignin-degrading enzyme from the Hymenomycete *Phanerochaete chrysosporium* Burds. Science (Washington, DC) 221:661–663

Tien M, Kirk TK (1984) Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc Natl Acad Sci USA 81:2280–2284

Tsukihara T, Honda Y et al (2006) Exclusive overproduction of recombinant versatile peroxidase MnP2 by genetically modified white rot fungus, *Pleurotus ostreatus*. J Biotechnol 126(4):431–439

Tsukihara T, Honda Y et al (2008) Mechanism for oxidation of high-molecular-weight substrates by a fungal versatile peroxidase, MnP2. Appl Environ Microbiol 74(9):2873–2881

Tuor U, Wariishii H et al (1992) Oxidation of phenolic b-aryl ether lignin model compounds by manganssese peroxidase from *Phanerochaete chrysosporium:* oxidative cleavage of an a-carbonyl model compound. Biochemistry 31:4986–4995

Ullrich R, Hofrichter M (2005) The haloperoxidase of the agaric fungus *Agrocybe aegerita* hydroxylates toluene and naphthalene. FEBS Lett 579(27):6247–6250

Valli K, Gold MH (1991) Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium*. J Bacteriol 173(1):345–352

Valli K, Brock J et al (1992a) Degradation of 2,4-dinitrotoluene by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 58:221–228

Valli K, Wariichi H et al (1992b) Degradation of 2,7-dichlorodibenzo-p-dioxin by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J Bacteriol 174:2131–2137

Vallim MA, Janse BJ et al (1998) *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts in wood. Appl Environ Microbiol 64(5):1924–1928

Van Aken B, Hofrichter M et al (1999) Transformation and mineralization of 2,4,6-trinitrotoluene (TNT) by manganese peroxidase from the white-rot basidiomycete *Phlebia radiata*. Biodegradation 10(2):83–91

Vanden Wymelenberg A, Sabat G et al (2005) The *Phanerochaete chrysosporium* secretome: database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. J Biotechnol 118(1):17–34

Vanden Wymelenberg A, Minges P et al (2006a) Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveals complex mixtures of secreted proteins. Fungal Genet Biol 43:343–356

Vanden Wymelenberg A, Sabat G et al (2006b) Structure, organization, and transcriptional regulation of a family of copper radical oxidase genes in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl Environ Microbiol 72:4871–4877

Vanden Wymelenberg A, Gaskell J et al (2009) Transcriptome and secretome analysis of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. Appl Environ Microbiol 75:4058–4068

Vanden Wymelenberg A, Gaskell J et al (2010) Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. Appl Environ Microbiol 76:3599–3610

Vanden Wymelenberg A, Gaskell J et al (2011) Significant alteration of gene expression in wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium* by plant species. Appl Environ Microbiol 77(13):4499–4507

Vazquez-Duhalt R, Westlake DWS et al (1994) Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. Appl Envrion Microbiol 60:459–466

Wahleithmer JA, Xu F et al (1995) The identification and characterization of four laccase genes from the plant pathogenic fungus *Rhizoctonia solani*. Curr Genet 29:395–403

Wang W, Wen X (2009) Expression of lignin peroxidase H2 from *Phanerochaete chrysosporium* by multicopy recombinant *Pichia* strain. J Environ Sci (China) 21(2):218–222

Wang H, Lu F et al (2004) Heterologous expression of lignin peroxidase of *Phanerochaete chrysosporium* in *Pichia methanolica*. Biotechnol Lett 26(20):1569–1573

Wariishi H, Valli K et al (1991) In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem Biophys Res Comm 176:269–275

Watanabe T, Tsuda S et al (2010) Characterization of a Delta12-fatty acid desaturase gene from *Ceriporiopsis subvermispora*, a selective lignin-degrading fungus. Appl Microbiol Biotechnol 87(1):215–224

Wesenberg D, Kyriakides I et al (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22(1–2):161–187

Westereng B, Ishida T et al (2011) The putative endoglucanase PcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. PLoS One 6(11): e27807

Whittaker J (2002) Galactose oxidase. Adv Protein Chem 60:1–49

Whittaker MM, Kersten PJ et al (1996) Glyoxal oxidase from *Phanerochaete chrysosporium* is a new radical-copper oxidase. J Biol Chem 271(2):681–687

Whittaker MM, Kersten PJ et al (1999) Identification of catalytic residues in glyoxal oxidase by targeted mutagenesis. J Biol Chem 274(51):36226–36232

Wong KS, Huang Q et al (2012) Biodegradation of dyes and polyaromatic hydrocarbons by two allelic forms of *Lentinula edodes* laccase expressed from *Pichia pastoris*. Bioresour Technol 104:157–164

Wongnate T, Sucharitakul J et al (2011) Identification of a catalytic base for sugar oxidation in the pyranose 2-oxidase reaction. Chembiochem 12 (17):2577–2586

Yanai K, Yonekura K et al (1996) The integrative transformation of *Pleurotus ostreatus* using bialaphos resistance as a dominant selectable marker. Biosci Biotechnol Biochem 60(3):472–475

Yaver D, Golightly E (1996) Cloning and characterization of three laccase genes from the white-rot basidiomycete *Trametes villosa*: genomic organization of the laccase gene family. Gene 181:95–102

Yaver D, Xu F et al (1996) The purification, characterization, molecular cloning and expression of two laccase genes from the white-rot basidiomycete *Trametes villosa*. Appl Environ Microbiol 62:834–841

Yaver DS, Overjero MD et al (1999) Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase *lcc1*. Appl Environ Microbiol 65 (11):4943–4948

Yoshida M, Ohira T et al (2001) Production and characterization of recombinant *Phanerochaete chrysosporium* cellobiose dehydrogenase in the methylotrophic yeast *Pichia pastoris*. Biosci Biotechnol Biochem 65(9):2050–2057

Zamocky M, Ludwig R et al (2006) Cellobiose dehydrogenase–a flavocytochrome from wood-degrading, phytopathogenic and saprotropic fungi. Curr Protein Pept Sci 7(3):255–280

Zamocky M, Schumann C et al (2008) Cloning, sequence analysis and heterologous expression in *Pichia pastoris* of a gene encoding a thermostable cellobiose dehydrogenase from *Myriococcum thermophilum*. Protein Expr Purif 59(2):258–265

Zapanta LS, Hattori T et al (1998) Cloning of *Phanerochaete chrysosporium leu2* by complementation of bacterial auxotrophs and transformation of fungal auxotrophs. Appl Environ Microbiol 64 (7):2624–2629

Biology, Disease Control and Management

6 Biological Control of Weeds with Fungi

Harry C. Evans[1]

CONTENTS

I. General Introduction

Biological control of weeds with fungi was reviewed in the original edition of The Mycota XI (Evans 2002a), as well as in contemporary publications (Evans et al. 2002a, b). Essentially, therefore, this chapter concentrates on more recent events in an attempt to bring the subject up-to-date; reporting the progress achieved over the past decade, without dwelling on the early (pre-2000) history. However, for continuity and clarity—especially for the reader unfamiliar with this field of applied mycology—some repetition is both necessary and unavoidable.

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Since the start of the new millennium, the issues of **climate change and globalisation** have come much more to the fore, occupying not only political and scientific mainstream thinking but also assuming centre stage in the public arena. With increasing globalisation, and a world without borders, comes the burgeoning threat from human-vectored alien species, as geographical barriers are by-passed and exotic plant species, in particular, begin to displace native floras and disrupt both natural and agricultural ecosystems (Cox 1999; Mack et al. 2000; Baskin 2002; Simberloff 2004; Newcombe and Dugan 2010; Trueman et al. 2010; Lambetini et al. 2011), leading to a more homogenised world dominated by 'adaptive generalists' (Meyer 2006). According to some, we are already embedded irrevocably in the so-called **Anthropocene or Homogocene era** (Rosenzweig 2001; Olden et al. 2004; Steffen et al. 2011). However, in reality, this epoch was foreseen by the pioneering ecologist Charles Elton many decades before: "We must make no mistake: we are seeing one of the great historical convulsions in the world's fauna and flora" (Elton 1958). Unfortunately, the situation could get even worse since climate change has the potential to further increase the ecological impact and widen the geographic range of some of these alien weeds. The first hard evidence comes from a recent pioneering study of endemic and exotic plant species in California which supports the prediction that **global warming will lead to increased species invasions over the next century** (Sandel and Dangremond 2011). This evidence, based on both factual and theoretical (modelling) data, shows that increasing temperatures favour the traits possessed by alien plant species, especially

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in the Gramineae. Indeed, invasive African grasses could themselves be a future driver of climate change, as they replace the C3 woody plants of the Amazon basin (Mack et al. 2000).

In non-agricultural situations, **invasive alien weeds** pose the major threat to biodiversity after anthropomorphic habitat destruction (Cronk and Fuller 1995; Lambetini et al. 2011). However, in such natural ecosystems—typically, covering vast areas and with low labour input—weed management is problematic because of the logistics and costs involved. Moreover, the use of chemical herbicides in these environmentally-sensitive areas is often not an option, ecologically or economically. The prevailing weed management strategy—if, indeed, one were to be in place—relies on cultural or manual control which, at best, aims at containment, and usually only in designated priority sites because of the aforementioned logistical and economic constraints. Perversely, there is now a cadre of ecologists and conservationists who are advocating that control of alien weeds is strategically useless and ecologically undesirable (Davis et al. 2011)—even in World Heritage sites such as the Galápagos Islands (Vince 2011)—although there has been a strong backlash by others committed to trying to stem the tide of **invasive plant species** (Simberloff et al. 2011). Thus, this has tended to polarise opinion, particularly in the conservation world, and there would appear to be no common ground between the two extreme approaches: accepting invasive alien invasive plants as part of an ever-changing ecosystem, or eradicating them to restore the original ecosystem. Within this potent mix, however, another approach—largely ignored by conservation policy-makers and ecologists—is **biological control through the use of coevolved natural enemies for the sustainable management of invasive alien weeds.**

In contrast, agricultural weeds in modern times have been effectively managed through the use of chemical herbicides, with little or no need for, or consideration of, alternative strategies. Nevertheless, that situation is changing rapidly, as increasing numbers of herbicides are removed from the global market following closer monitoring of their negative impacts on the environment, in general, and on human health, in particular, as well as the increasing influence of organic agriculture. The situation has been compounded by the steadily **escalating number of weeds developing resistance to chemical herbicides** over the past decade, reducing even further the choice of products available to the farmer (Service 2007; Powles and Duke 2008). Most alarming of all, perhaps, is the appearance of **glyphosate-resistant 'superweeds'**, since this product alone accounts for 30 % of global herbicide sales which hover around US$ 20–25 billion per annum (Carpenter and Gianessi 2010). The most recent pronouncements concerning its potential to cause vertebrate birth defects, as well as impacts on amphibian food chains (Paganelli et al. 2010; Relyea 2012), should accelerate the movement towards alternative methods of weed control. The world **biopesticide** market is projected to reach nearly US$ 3.5 billion within 5 years (Anon 2012), driven by environmental concerns and regulatory riders on traditional pesticides. However, nearly all the products are bioinsecticides and biofungicides, based on *Bacillus*, *Beauveria*, *Metarhizium,* and *Trichoderma*, and, patently, **bioherbicides** based on plant pathogens have failed to penetrate this rapidly expanding market (Glare et al. 2012).

In this chapter, the option of exploiting plant-pathogenic fungi as an alternative weed management approach is discussed, with the focus on recent developments and future potential. The introduction and release of exotic fungal pathogens—known as **classical or inoculative biological control (CBC)**—is the strategy most frequently associated with natural ecosystems because of its low-tech, not-for-profit concept. Ironically, however, some of the greatest successes of the CBC tactic have been in agricultural ecosystems, despite the fact that **inundative biological control**

(IBC), through the application of a formulated fungal product or **mycoherbicide**, is the approach often considered most suitable for the high-tech agricultural situation.

II. Classical Biological Control (CBC)

A. The Concept

CBC has been in practice for over a century: the concept having been conceived, developed, and refined by entomologists for the management of invasive alien species, principally arthropod pests and weeds. However, the theory that underpins the practice has only recently been conceptualised formally through the **enemy release hypothesis** (Keane and Crawley 2002); so recent, in fact, that it failed to make the original chapter (Evans 2002a). In essence, **the theory posits that exotic plant species become invasive because of increased fitness in the absence of their coevolved natural enemies**. And, therefore, the solution to address the problem of invasive neophytes became obvious: source, import, and release the natural enemies, or CBC agents, from the centre or region of origin of the target weed species into the invaded ecosystem or country to reduce its fitness and, thus, increase the competitive ability of the indigenous flora. Supportive data quickly followed, based on a desk study that analysed biotrophic plant pathogens (predominantly, rusts, smuts, and powdery mildews) associated with over 470 plant species from Europe naturalised in the USA (Mitchell and Power 2003). The results showed that, on average, the exotic plants had 84 % fewer fungi than in their native European range and, significantly, those with the least fungal natural enemies proved to be the most invasive and troublesome.

It could be argued, of course, that plant invasiveness cannot be explained in such simplistic terms, and that multiple factors are involved: release from natural-enemy pressure being one of many components, such as resource availability (Blumenthal 2006). In the recently proposed **endophyte-enemy release hypothesis**, for example, it is posited that exotic plant species may also lack their **coevolved endophytic fungi** (Evans 2008). Those endophytes that form beneficial associations with their coevolved hosts—such as increasing plant resistance to abiotic and biotic stresses (Rudgers et al. 2004; Schulz and Boyle 2005)—and thus act as bodyguards, would not be a necessity in a new environment with little or no natural-enemy pressure. Indeed, neophyte hosts arriving without their coevolved endophytes would be fitter and, therefore, more invasive, as nutrients are not sequestered by or diverted to these fungal mutualists, thereby freeing-up more resources for growth and reproduction. Nevertheless, if their coevolved natural enemies should ever catch up with such vulnerable, endophyte-deficient plants, the consequences could be disastrous. This may help to explain not only the **'silver-bullet' phenomenon—whereby, the release of a single CBC agent results in spectacular and sustainable control of the target weed** (Van Wilgen et al. 2004; Page and Lacey 2006; Barton et al. 2007)—but, also, the devastating impact of newly-arrived pathogens on their coevolved crop hosts (Large 1940) more recently, referred to as *pathogen pollution* and tagged with the label 'emerging infectious diseases' (Anderson et al. 2004; Evans and Waller 2010). If there are any doubters about the devastating impact of coevolved fungal pathogens on exotic plant species (and, therefore, the potential efficacy of CBC as a management strategy for invasive alien weeds), examples in the agricultural sector are all too common (Large 1940; Quimby 1982; Evans 2002b; Agrios 2005).

CBC practitioners had, in fact, been adhering to this simple concept encapsulated in the enemy release hypothesis since the late nineteenth century, with the aim of reducing the competitiveness of invasive alien weeds through the release of their coevolved natural enemies and thus restoring the balance of nature. However, this remained the domain of entomologists until the 1970s (McFadyen 1998), when plant pathologists first entered the field to immediate and spectacular effect (Cullen et al. 1973; Burdon et al. 1981). Nevertheless, to most conservation policy-makers the CBC strategy is either unknown or viewed

with scepticism, bordering on hostility. As touched upon in the General Introduction on the management of invasive alien plants in the Galápagos Islands, the debate has centred primarily on an eradication policy versus the other extreme of 'embracing' them within the island ecosystems (Vince 2011). A third option, the use of CBC, has received little or no attention.

Ironically—in the light of this recent Galápagos controversy—it was Charles Darwin who first hinted at the controlling impact of natural enemies after observing at first-hand the invasiveness of alien plant species in island ecosystems during the voyage of the Beagle: "cases could be given of introduced plants which have become common throughout whole islands in a period of less than 10 years..... the geometrical tendency to increase must be checked by destruction at some period of life.....lighten any check, mitigate the destruction ever so little, and the number of the species will almost instantaneously increase to any amount" (Darwin 1859, pp 118–119).

B. The Practice

Best practices must always be followed if this still-evolving field of weed management is to be accepted by both conservationists and farmers alike, but also, more crucially, by the decision-makers holding the purse strings. Because of its very nature—no saleable product, for example—**CBC is funded invariably by central or local government agencies**. Thus, both political and public concerns need to be addressed. Fortunately, scientific and safety standards—until recently set and regulated by the CBC practitioners themselves—have been high; negative impacts have been minimal, and all were entirely predictable (Evans 2000; Culliney 2005). Unfortunately, in sharp contrast, the spurious and ill-considered attempts at biological control by non-specialists—typically, involving the importation of generalist predators (snails, snakes and toads, for example, in a parody of the song 'The woman who swallowed a fly') and, therefore, doomed to failure and potential ecological disaster—are the ones that have most attracted press coverage and hence caught the public imagination. Thus, CBC—or, **"the intelligent introduction of counter-pests" (= natural enemies)** (Elton 1958)—needs to be distanced from this form of unregulated, unintelligent, unscientific biological control and from the perception of the general public that it is a hazardous option for the management of invasive alien species, and of exotic weeds, in particular.

1. Selecting the Agents

Because only **coevolved natural enemies** should ever be considered for CBC, fungal pathogens must be sourced from the centre of origin or diversity of the exotic weed target. Invariably, these potential agents are **obligate biotrophs**—most typically, **rust fungi**—although **hemibiotrophs** can also be, and, indeed, have been, taken into consideration (Evans 2002a). Thus, the first step is a literature and herbarium survey—both botanical and mycological—in order to determine the natural distribution of the plant and thereby to delimit the target area for the field survey and agent collection, as well as to identify and collate any associated **mycobiota**. This is not always straightforward, since the plant host may be rare or of no economic interest in its native range, and so poorly-studied and collected. Typically, the plant species may have significantly more site and fungal records from its invasive than from its native range. This is exemplified by lantana weed (*Lantana camara*): as an exotic ubiquitous plant in India, 30 fungal pathogens have been recorded—some with genus or species-specific epithets, erroneously suggesting coevolutionary traits—whilst in its neotropical native range, only nine pathogens have been recorded following comprehensive surveys in Brazil (Barreto et al. 1995). These Indian fungi constitute an assemblage of non-specific opportunistic pathogens with no impact on the fitness of *L. camara*—hence, its invasive status—compared to the damaging biotrophic pathogens found in its natural range (see Fig. 6.2d). Conversely, for some exotic plant species there is a complete absence or scarcity of published records of fungal pathogens, in both their invasive and natural ranges. As a bonus, this preliminary 'desk-bound' survey can often uncover rare and even new, previously unpublished records of fungal

pathogens before the field surveys even begin (Evans 1987a).

An assessment of the data collated from the literature, herbarium, and field surveys can also provide useful indications as to the specificity of the associated fungal pathogens and the damage that they inflict on their hosts, from which early decisions can be reached concerning their potential as CBC agents. This information is also helpful to prioritise those agents warranting further study, or, in certain cases, to discontinue the programme if suitable agents are not identified.

2. Screening the Agents

Potential agents can be screened either in the country of origin—with no quarantine measures—or, in an intermediate country where the target weed is absent—with low-level quarantine—or, in the weed-affected country—with high-level quarantine. **Host-specificity testing is the most time-consuming and, thus, the most expensive phase of a CBC programme.** In parallel with this screening, the taxonomic position, life cycle, and infection parameters of the selected agent(s) need to be established. Often, this is not a straightforward process because the pathogen may be taxonomically difficult or new to science (Evans and Ellison 2005), and, especially in rust fungi, the life cycle may be unique and the infection parameters challenging (Evans 1987b; Ellison et al. 2006; Seier et al. 2009).

The protocol that underpins specificity screening—and so provides the main data for the risk assessment—follows the **centrifugal phylogenetic testing sequence**, initially developed for arthropods (Wapshere 1974a, b), and is based on genetic relatedness, in contrast to earlier, more emotive ones that concentrated on threats posed to crop plants. Indeed, the very rigorousness of the test—for example, the frequency of false positives due to optimal infection conditions and artificially high inoculum loads—even led to the claim that potentially beneficial agents could be rejected (Wapshere 1989). Additional techniques can be used to better interpret, and give added value to the test results. A clear-staining methodology (Bruzzese and Hasan 1983) has been used to identify resistance factors to the agent both on and within the challenged test plant species, and to clarify any 'suspect' symptoms, such as hypersensitive reactions (Evans and Tomley 1996; Evans 2000).

3. Risk Assessment

Once a potential agent has been given the 'all clear', in the sense that it has demonstrated a sufficiently high level of specificity to the target weed so as not to pose a threat to non-targets in the country of release, a document—usually called a **pest risk assessment** (PRA)—is prepared detailing all the scientific data generated from the field, greenhouse, and laboratory studies. This is presented to the relevant quarantine authorities in the receiving country and, after peer review (at the government, state, or even public level), the decision whether or not to import and release the agent is reached independent of the stakeholders. Additional testing—typically, to include rare and locally-unique plant species or to 'tweak' infection parameters—may be required at this stage. **Conflicts of interest** inevitably occur, of course, that can delay or even lead to the abandonment of CBC programmes. The **Australian Biological Control Act of 1984**—based on socio-economic and ecological evidence and still **the only example of legislation of CBC in the world** (Sheppard et al. 2003)—was invoked specifically to minimise the threats from lobby groups using the **Rule of Law principle**, such as bee-keepers. Conflicts continue, however (Chew 2009).

C. Case Studies

1. Past: Updated

Several of the programmes covered previously (Evans 2002a) are updated and analysed in greater detail to reflect the progress made in this continuously-evolving specialist field. In particular, the increasing ecological and economic awareness and the associated benefits of

CBC are highlighted, with a summary of the lessons learned from each case study.

a) Mistflower: *Ageratina riparia* (*Asteraceae*)
One of the earliest CBC initiatives targeting the use of fungal pathogens, the mistflower story has been long and eventful. From its beginnings during the early 1970s in Hawaii (Trujillo 1985)—with the unexpected and highly successful control of an invasive weed by a CBC agent pertaining to a fungal family that, hitherto, had been a 'minor' player on the plant pathology stage—there have been many twists and turns reflecting advances in the field to improve the science and safety of CBC. Thus, our knowledge of the taxonomy, biology, ecology, and pathogenicity of the fungal agent, the white smut *Entyloma ageratinae* (*Entylomataceae*, *Exobasidiomycetes*)—as well as of its impacts on and benefits to invaded ecosystems in Hawaii, New Zealand, and South Africa—has changed and expanded immeasurably since these pioneering days (Barreto and Evans 1988; Morin et al. 1997; Trujillo 2005; Barton et al. 2007; Barton and Fowler 2008; Waipara et al. 2009; Heystek et al. 2011). To aid identification of the mistflower fungus, the use of more advanced microscopy, such as SEM, was called upon (Barreto and Evans 1988) and, of course, in more recent times, **molecular systematics** have come into prominence, which can help to resolve the kind of taxonomic controversy that initially plagued this CBC programme. Similarly, improvement of the **host-range screening protocol**, especially the quantity and quality of the plant test list has been a priority.

In the case of the mistflower fungus, for example, 44 plant species were tested in Hawaii; whilst for South Africa, a further 18 species were included and for New Zealand, an additional 34 species were screened (Barton 2012). Moreover, the success story continues with the news that the white smut has 'arrived' recently in Australia—transported either accidentally or deliberately—and is already having a significant impact on mistflower populations in New South Wales (McFadyen, 2012, personal communication).

Lessons learned: the over-riding message from this success story is that even the most seemingly ineffectual natural enemy in its native range can exert significant control over its host in the exotic range, and that seemingly fragile, habitat-restricted indigenous plant species can become dominant and invasive in new ecosystems. Thus, mistflower is almost an endangered species in Mexico, proving difficult to locate and apparently confined to fast-flowing mountainous rivers in Veracruz State, with white smut appearing as a minor leaf disease (Barreto and Evans 1988). Indeed, in a dictionary of plant pathology, *Entyloma* has only a short entry, concluding with the statement: "no serious diseases caused" (Holliday 1989); whilst there is only a passing mention of the genus in the most modern and comprehensive text on plant pathology (Agrios 2005). Therefore, perhaps more than any other, this CBC programme highlights both the dangers of the indiscriminate movement of plant species and the elegant yet simple solution to their management if they should ever become invasive. Finally, it also offers an insight into **'natural control'** by natural enemies, since it is one, if not the only documented example of an exotic plant species introduced together with a coevolved fungal pathogen. When it was first collected in Jamaica in the 1970s, both the fungus identified as a *Cercosporella* sp. and the host plant were thought to be indigenous (Leather 1967). However, almost certainly, mistflower was imported from its Mexican home as a living specimen shortly after its discovery in the mid-nineteenth century—probably in the then-fashionable Wardian case—together with its pathogen, and escaped the botanical garden to become naturalised. Such a scenario has been observed in the Central Highlands of Sri Lanka, where natural habitats around a botanical garden (Hakgala) are heavily invaded by mistflower (Author 1999, personal observation). However, in sharp contrast to Sri Lanka and other recipient countries, it never became an aggressive invader

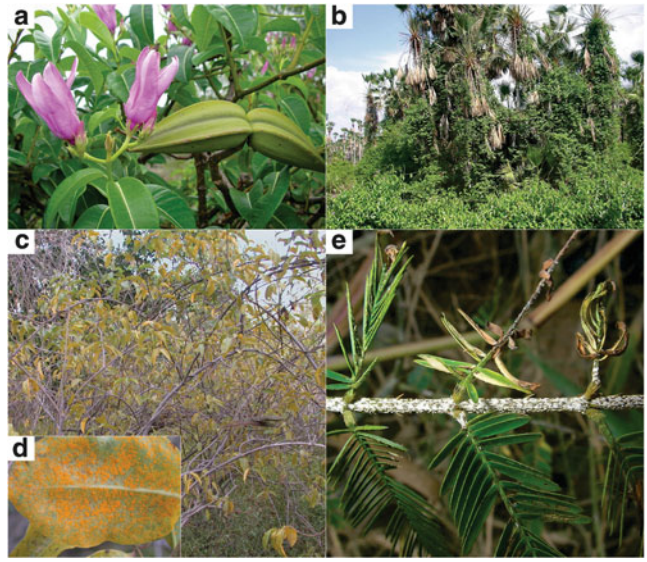

Fig. 6.1. (**a**) Flowers and fruit of rubber-vine weed (*Cryptostegia madagascariensis*), Ceará State, NE Brazil—note the 'clean', pest-free foliage. (**b**) Rubber-vine weed forming dense stands under and growing over the endemic 'carnaúba' palm, *Copernicia prunifera*—an ecologically and economically important plant in the region. (**c**) Impact of the biotrophic rust *Maravalia cryptostegiae* on rubber-vine weed (*Cryptostegia grandiflora*) in northern Queensland (Australia), shortly after its release. (**d**) Inset of the rust forming uredinial pustules on the lower leaf surface. (**e**) White asexual fruiting structures (acervuli) of the hemibiotrophic ascomycete, *Sphaerulina* (*Phloeospora*) *mimosa-pigrae*, on the giant sensitive plant, *Mimosa pigra* in its native Mexican range—the fungus has since been released in the Northern Territory of Australia

in Jamaica, especially in the favourable upland ecosystems, because of the presence of the white smut: a perfect example, perhaps, of accidental CBC.

b) Rubber-vine: *Cryptostegia grandiflora* (*Asclepiadaceae*)

After nearly a decade of research (Evans 2000), the coevolved rust *Maravalia cryptostegiae* from Madagascar was released into the northern region of Queensland in 1994–1995 against its woody climbing host *Cryptostegia grandiflora*, described as the greatest single threat to biodiversity in tropical Australia (McFadyen and Harvey 1990). Preliminary impact assessments were promising (Fig. 6.1c, d), leading to the confident prediction "that rubber-vine weed will be brought under substantial if not complete control within the next decade" (Evans 2002a). Data from independent, long-term monitoring studies were released shortly afterwards, which showed over 40 % reduction in weed populations with almost zero seedling recruitment (Vogler and Lindsay 2002; Tomley and Evans 2004). This was followed up by an economic impact assessment—made possible because of the weed's agricultural as well as ecological significance—which put the net benefit of the programme up to 2005 at over AU$ 230 million, with a cost:benefit ratio of 1:108 (Page and Lacey 2006). This makes it one of the most successful CBC weed programmes in the long history of Australian CBC initiatives (Palmer et al. 2010), and, therefore, in the world. The same weed is also becoming problematic in Mexico (Rodriguez-Estrella et al. 2010) and Curaçao in the Netherlands Antilles (Author 2003, personal observation), and a sister species, *C. madagascariensis*, is having a major impact on biodiversity in north-east Brazil (Herrera and Major 2006; Alves et al. 2008; Fig. 6.1a, b). Since a tried, tested, and successful 'technology' for

management of rubber-vine weed is already in place, it should be a relatively simple and inexpensive process to transfer this to the affected regions; provided, of course, that stakeholders and officials in those countries share and buy into the CBC strategy.

Lessons learned: biotrophic fungi, such as rusts, have coevolved with their hosts over millennia, and this is reflected in their complex life histories, intimately linked with and adapted to a particular host. Thus, for each potential CBC fungal agent of weeds, there is an immediate challenge: to sort out the taxonomy of an under-studied pathogen and to elucidate its life cycle. Indeed, the mistflower study also threw up some intriguing taxonomic and evolutionary dilemmas—such as the presence of pycnia in the Ustilagomycotina (Barreto and Evans 1988)—which have not been pursued since, probably because the subject (white smuts) and the context (weed pathology) are not in the mainstream of mycology. Invariably, more cutting-edge science is now required to solve these problems, providing new insights into fungal biology. Until this 'academic' phase of the CBC programme is completed to the satisfaction of the stakeholders, investment in the more applied phases—determining infection protocols, extended host-range screening—may not be forthcoming. For the rubber-vine rust, the challenge was especially daunting, since the taxonomy and life cycle of one of its closest relatives, coffee rust (*Hemileia vastatrix*), has remained unresolved for over a century, despite the best efforts of plant pathologists and mycologists (Agrios 2005; Ayres 2005). However, based on the history of coffee rust—as it spread inexorably, and human-assisted, around the globe from its East African roots, devastated coffee plantations (McCook 2006)—it was a challenge worth pursuing because the chances of achieving successful weed control through the release of a close rust relative were almost assured. Both rusts produce the characteristic half-rough, half-smooth urediniospores and, rarely, the ephemeral, thin-walled teliospores forming 'non-infective' basidiospores. Thus, the prevailing theory has been that these *Hemileia*-like rusts on higher dicotyledonous hosts are advanced and long-cycled—based on the hypothesis that primitive rusts with unexpanded life cycles occur only on primitive hosts (Savile 1976)—and, therefore, that they are heteroecious (Ritschel 2005). However, using standard cytology, the nuclear events in the urediniospores were interpreted as meiotic, and it was posited that both *M. cryptostegiae and H. vastatrix* are primitive, unexpanded rusts in which the teliospores are vestigial and only uredinioid teliospores function in the life cycle (Evans 1993). Fortunately, this interpretation was accepted by the Australian authorities reviewing the proposal to introduce and release the rust. Thanks to recent advances in molecular systematics and **cytometry**, these rusts have now been shown to share a common ancestry; both genera occupy a basal position in *Pucciniales* phylogenetics (Wingfield et al. 2004), within the *Mikronegeriaceae* (Aime 2006), and, as posited, represent the most primitive lineages (Hart 1988; Evans 1993), whilst **computer-assisted DNA image cytometry** has proved unequivocally that the 'urediniospores' of *H. vastatrix*—and, by implication, those of *M. cryptostegiae*—are, indeed, meiospores (Carvalho et al. 2011).

c) Giant Sensitive Plant: *Mimosa pigra (Mimosaceae)*

The current analysis of the long-running programme against one of the Weeds of National Significance in the Northern Territory of Australia—following the introduction of 15 CBC agents from the Neotropics, 13 insect species and two fungal pathogens—is that at least two of the insect agents are having an impact but the fungi have been deemed a failure (Palmer et al. 2010). Both of the pathogens, collected in Mexico, are of particular taxonomic interest: the rust *Diabole cubensis*—typified by subcuticular telia producing powdery, unicellular, paired teliospores—is the only species in a genus of uncertain phylogeny (Evans et al. 1995, 2002b; Cannon 2007), and a new hemibiotrophic ascomycete, *Sphaerulina* (*Phloeospora*) *mimosa-pigrae*, which produces both teleomorph and anamorph on living woody tissues (Evans et al. 1993; Fig. 6.1e). Hennecke (2006) reported that the rust had

failed to establish, and similar doubts remain about the fate of the ascomycete fungus after the high expectations from them, based on field observations in the Neotropics and experimental work in UK quarantine (Seier and Evans 1996). However, a recent report has found the rust to be widespread in the Northern Territory, with spread up to 100 km from the release sites (Burrows et al. 2012). The question remains, however: is this a temporary resurgence of an otherwise ineffectual disease due to favourable but abnormal climatic factors, or is the rust beginning to have an impact after an extended lag phase of adaptation?

Lessons learned: this case study exemplifies some of the lessons learned, and still to be learned, concerning the CBC approach to the management of invasive alien weeds, particularly since *M. pigra* continues to be a major problem not only in Australia but also in Africa and Asia (Heard and Paynter 2009). In contrast to the two previous case studies, there would appear to be no 'silver-bullet' solution whereby a single CBC agent can provide relatively rapid, effective, and sustainable control. Thus, a guild of natural enemies—targeted at different plant tissues and climatic regimes—combined with more traditional management practices, seemingly, have not yet had a significant impact on weed infestations. The argument has often been made that woody plants such as *M. pigra* are especially difficult targets for CBC, but there are several outstanding examples in South Africa where invasive Australian *Acacia* trees have effectively been controlled by both fungal and insect CBC agents (Dennill et al. 1999; Wood and Morris 2007). Perhaps it is more relevant to analyse in-depth these perceived failures by asking the question: "What would the situation have been without any biological control?" (Hoffmann and Moran 2008). Through retrospective analysis of a 'failed' South African CBC programme—and the use of modelling to predict the scale of infestation without the presence of a CBC agent—these authors showed that significant but cryptic benefits had accrued. Such could be the case with the *M. pigra* programme, and the 'reappearance' of *Diabole cubensis* demonstrates clearly **the need for and the under-investment in long-term, post-release monitoring,** as highlighted recently by Morin et al. (2009). In addition, this target weed has an exceptionally wide native range—from the southern USA to northern Argentina—and, as a consequence, is highly variable (Barneby 1989). As discovered with rubber-vine—variants of which occur along its western range from north to south Madagascar (Marohasy and Forster 1991)—**if the correct rust pathotype is not matched with the invasive biotype, then full pathogenicity will not be expressed in the field**, even if greenhouse screening indicates otherwise (Evans and Tomley 1996; Evans 2000). Thus, throughout the native range of *M. pigra*, variations in the host will be mirrored by those in its coevolved natural enemies. Almost certainly, therefore, the pathotypes of the two fungal CBC agents are not matched to the weed biotype(s) in Australia, because surveys were concentrated in only a small sector of the range: this could be the main contributing factor to their apparent ineffectiveness. Of course, these past programmes were undertaken before molecular systematics was in common use, but the clear message for future CBC programmes is that this tool should be an integral component from the beginning, in order to better determine the geographical source of the invasive weed target (Morin et al. 2006; Le Roux and Wieczorek 2009)—and, as a result, to identify the best-suited natural enemies at an early stage, and thereby avoid the waste of often limited resources. Earlier, in fact, chemotaxonomy was used to distinguish between populations of leafy spurges (*Euphorbia* spp.): analysis of latex profiles revealed differences in spurge populations, and helped to pinpoint sources of potential CBC agents, particularly rust pathotypes, in Europe for screening against compatible biotypes in the North American invasive range (Holden and Mahlberg 1992).

2. Present

The history of CBC of weeds has mainly been played out in Australasia, South Africa, and the Americas, as past case studies clearly show. In the original review, CBC, or the lack of

it, in the Palaeotropics and Europe was discussed and bemoaned in the section 'Future Targets'. It is apposite, therefore, to report here that CBC programmes are now underway against invasive weeds in these regions and, moreover, are targeted at some of the prioritised weed species (Evans 2002a). With nascent interest in this 'novel' weed management now growing in both Asia and Europe, the present case studies focus on on-going programmes in these continents; in particular by analysing whether the past lessons learned are being put into practice and, indeed, what lessons still need to be learned.

a) Mikania Weed: *Mikania micrantha (Asteraceae)*

Alternatively known as 'mile-a-minute weed'—a name it shares with several other invasive weeds—mikania weed is one of a suite of neotropical asteraceous plants causing havoc in Old World ecosystems: *Ambrosia artemisiifolia*, *Ageratina* spp., *Chromolaena odorata*, *Parthenium hysterophorus*, *and Xanthium* spp., to name but a few of the principal culprits listed amongst the world's worst weeds (Holm et al. 1977). As with *Mimosa pigra*, *Mikania micrantha* is extremely widely distributed in the Neotropics and, from the beginning of the programme, surveys were conducted throughout the native range, where it is an inconspicuous plant in riverine and marshy habitats (Barreto and Evans 1995): contrast this with India, where it overgrows forest ecosystems in both the Western Ghats of Kerala and the north-east of Assam (Muniappan and Viraktamath 1993; Cock et al. 2000). In addition, the weed is impacting livelihoods in small-farming systems in the Western Ghats region (Ellison 2001), and is now one of the major constraints to tea production in Assam (Ellison 2004).

Towards the beginning of the CBC programme, the previous lessons learned were put into action. Plant samples were collected throughout Latin America and **DNA profiling** of over 50 plant accessions from both the native and introduced ranges was used to demonstrate wide host variation in the Neotropics, contrasting with a narrow genetic base in India (Ellison et al. 2004). Unfortunately, however, there were no close matches between these neotropical and palaeotropical collections, and thus the source (s) of the exotic introductions still remains a mystery. Emphasis was also placed on collecting multiple isolates of the most widespread and damaging fungal pathogen—the microcyclic rust *Puccinia spegazzinii*, that induces gross distortion of leaf and stem tissues—from Mexico through to Argentina, and these were cultured and maintained in UK quarantine for screening against Indian weed biotypes (Ellison et al. 2008). Great variation was found in the isolates, both in macro-morphology and symptomatology (Evans and Ellison 2005; Fig. 6.2a–c). Of the short list of seven rust pathotypes screened, one from Trinidad was selected for further host-range testing against more than 60 related plant species, and found to be highly specific. Amongst all the neotropical *Mikania* species evaluated, the rust isolates infected only *M. micrantha*, whilst, unexpectedly, two *Mikania* species from this poorly-represented genus in the Palaeotropics exhibited some degree of susceptibility to the rusts (Ellison et al. 2008). This may suggest an ancient Gondwanaland association. As well as throwing up intriguing conundrums in plant genetics, this programme also had its share of paradoxes in rust evolution to solve. In addition to *Puccinia spegazzinii*, two rust species in the genus *Dietelia*, typified by aecioid teliospores in columns, were encountered, and subsequently cultured and studied in quarantine. Closer examination of their biology showed that one species had non-functional spermogonia and 2-spored metabasidia, whilst the other—the novel species *D. mesoamericana*—possessed abundant functional spermogonia and 4-spored metabasidia, indicating some fundamental shift in host-pathogen genetics: one species infecting via basidiospores in the diplophase, the other in the haplophase. Of course, being pathogens of plants of non-economic relevance in the native range, these rusts and the subtlety of their life cycles had previously never warranted further investigation. Moreover, based on these greenhouse studies, it was also posited that: "The fact that all three species share a fundamentally similar symptomatology suggests a common

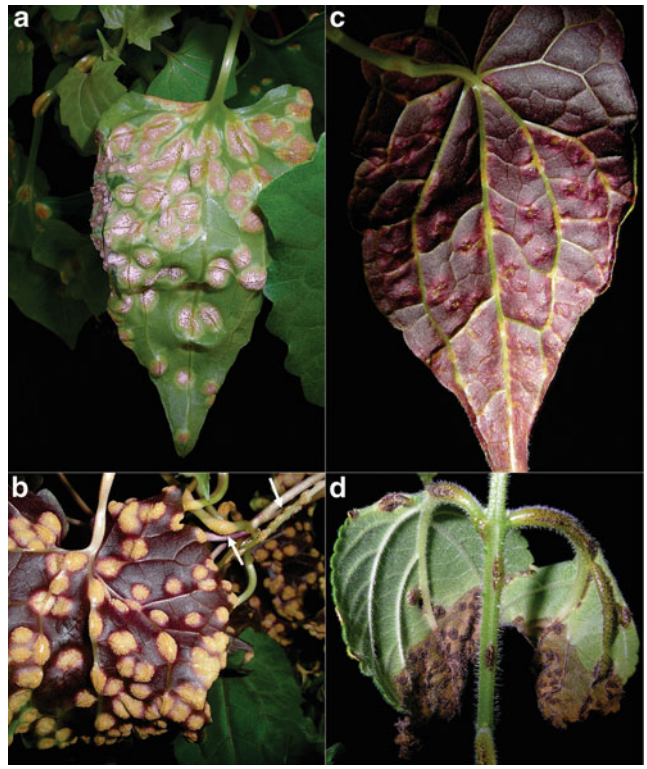

Fig. 6.2. (**a**) Pustules (telia) of the microcyclic rust, *Puccinia spegazzinii*, forming on the underside of leaves of *Mikania micrantha*—rust pathotype ex Trinidad, weed biotype ex SW India—the *white areas* indicate where teliospores have germinated to produce the infective basidiospores. (**b**) Telial pustules of rust pathotype ex Ecuador forming on and causing hypertrophy on petioles and stems (*arrows*), leading to leaf/plant death. (**c**) Incompatible reaction of rust pathotype ex Trinidad against weed biotype ex NE India, showing abortive pustules. (**d**) The microcyclic rust, *Puccinia lantanae*, forming telial pustules on a biotype of *Lantana camara* from Australia—this promising pathotype from Peru induces growth disorders on both leaves and stems, leading to die-back

origin" (Evans and Ellison 2005). This conjecture has now been confirmed with *Dietelia* aligning alongside *Puccinia* in the *Pucciniaceae* lineage rather than in the *Pucciniosiraceae*, where endocyclic rusts have traditionally been assigned (Wingfield et al. 2004; Aime 2006; Maier et al. 2007).

The document detailing all the findings from the UK quarantine studies was peer-reviewed and approved by Indian authorities, and permission to import *P. spegazzinii* into quarantine in New Delhi was granted in 2003. However, even the seemingly straightforward task of transporting rust inoculum presented problems. In common with some other microcyclic rusts exploited for CBC (Barreto et al. 1995; Seier et al. 2009), *P. spegazzinii* loses viability or enters dormancy when dried (Ellison et al. 2008). Repeated attempts to develop a cryopreservation protocol were only partially successful: teliospores removed from liquid nitrogen germinated, but the basidiospores proved to be non-infective (Ryan and Ellison 2002). Finally, rust-infected, living plants were sent to India as inoculum, where an additional 70 plant species of local importance were screened in quarantine. Once specificity had been confirmed, the rust was released in south-west India in 2005, and later in Assam (Kumar et al. 2008; Sankaran et al. 2008); making it not only a flagship programme for India, but also **the first example of a fungal pathogen being introduced as a CBC agent against an invasive alien weed in Asia** (Ellison et al. 2008). Although the rust has not had

sufficient time to be assessed critically, it is acknowledged that there have been problems in its establishment. However, other weed-affected countries in Asia and the Pacific—notably, China, Papua New Guinea and Fiji—have 'piggy-backed' on the programme and significant impact of the rust has already been reported from at least one of them (PNG), less than 2 years after release (Day et al. 2011; Ellison and Day 2011).

Lessons still to be learned: in sharp contrast to CBC programmes aimed at invasive alien insect pests—where frequently millions rather than thousands of dollars are involved, especially in the implementation phase (Herren and Neuenschwander 1991)—those against invasive weeds, invariably, are under-sold to stakeholders and donors alike. Almost certainly this is because there is less political leverage, since invasive pests can have an immediate and devastating impact on food security and farmer livelihoods, compared to cryptic yet insidious exotic weeds—which, of course, are not directly life threatening. Needless to say, the investment in CBC is but a fraction of that needed to develop and market chemical herbicides. For *M. micrantha*, the research component was funded from UK aid, whilst the Indian Government partly funded the implementation phase. There was little leeway for substantial supplementary funding to pursue additional research when the need arose as, inevitably, it did. Thus, it became clear at an early stage that, despite the relatively narrow genetic base of the plant in India, the selected rust strain from Trinidad was not compatible with all the weed biotypes in Assam (Fig. 6.2c), and that rust pathotypes from Latin America held more promise (Ellison et al. 2004). Indeed, preliminary testing of the new species from Mexico, *Dietelia mesoamericana*, showed this to be a more aggressive species against a much wider range of weed biotypes throughout Asia and the Pacific (Evans and Ellison 2005). However, keeping multiple pathotypes or different species of fungal CBC agents in quarantine is an immense logistical challenge, and can only be achieved with substantial investment. The clear lesson here is that more funding should have been sought in order to expand the surveys and increase the collections of both plant biotypes and rust pathotypes, in order to identify the source(s) of weed introductions and then match the best CBC agent. Because of under-investment in this research phase, as well as in the implementation phase, will this CBC programme be deemed a failure in India? Ironically, it appears that it may well be a success in other countries, such as PNG. For such low stakes—compared to any other weed management strategy—the financial benefits, not to mention the ecological ones, are disproportionally high, as shown by an analysis of Australian CBC programmes (Page and Lacey 2006; Palmer et al. 2010), and elsewhere (Culliney 2005; McFadyen 2008). However, the mikania weed programme has exposed a potentially even greater threat to CBC than shortfalls in funding: namely, strict enforcement of the **Convention on Biological Diversity** (CBD 2011), in all its many guises and interpretations since its inception in 1991. Permission to use additional strains of *P. spegazzinii* from Argentina, Ecuador and Peru—better-suited to the range of biotypes in the India sub-continent and other Asian regions—has been blocked as a result of these countries imposing a blanket ban on the export of their biological diversity (Vurro and Evans 2008). Despite diplomatic approaches over a number of years, there has been no change of stance, centred on an illogical suspicion of the aims of the CBC strategy, especially of its non-commercial philosophy. This threat to CBC—from an over-zealous interpretation of the CBD, essentially drafted to combat bio-piracy—has been highlighted recently (Cock 2010).

b) Japanese Knotweed: *Fallopia japonica (Polygonaceae)*

Europe has fallen behind other continents in embracing the CBC approach for the management of invasive alien weeds. In fact, CBC has never even appeared on the European horizon until recently, despite many of the practitioners operating from here (Shaw 2008). As highlighted by Sheppard et al. (2006), the **European Union** (EU) has been slow to assess and to quantify the impact of invasive alien plants, and even slower to appreciate the potential of CBC as a management tool. This is shown in the

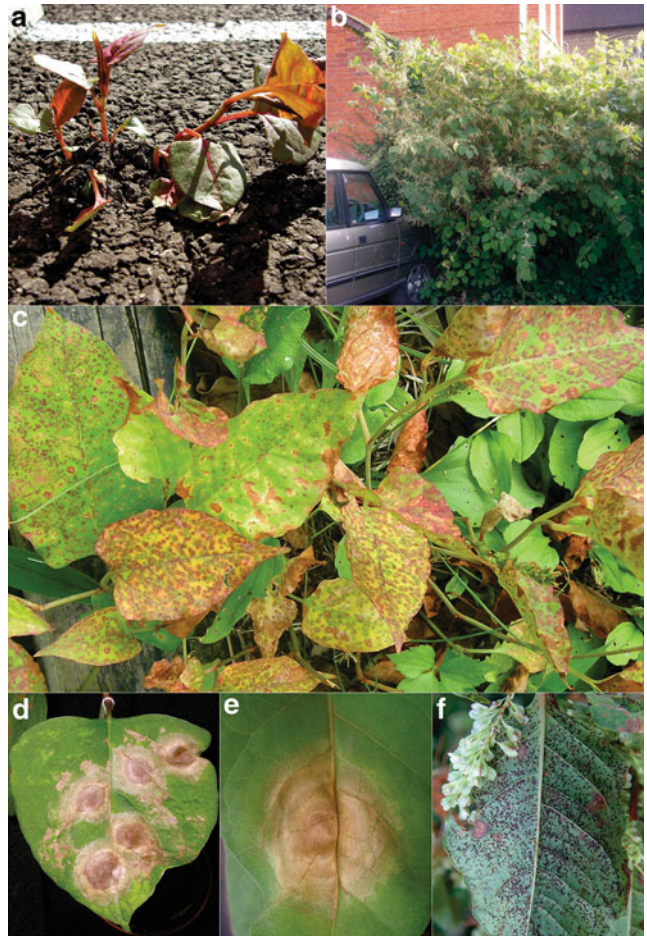

Fig. 6.3. (**a**) Japanese knotweed, *Fallopia japonica*, pushing through tarmac from overwintering rhizome, in early spring (Surrey, South-east England). (**b**) Same site in late summer, in full flower—effectively, the plant is clonal in the UK, since the flowers never set viable seed in the absence of male plants. (**c**) Japanese knotweed heavily attacked by *Mycosphaerella polygoni-cuspidati* (Kyushu Island, Southern Japan), showing leaf chlorosis and necrosis. (**d**) Plant in quarantine (CABI, UK) following inoculation with mycelium of *M. polygoni-cuspidati*. (**e**) Detail of lesion showing central rings of developing spermogonia—ascomata develop later, and the fungus cycles only through ascospores in the field. (**f**) Detail of infection by a *Puccinia* rust (Kyushu Island), that was rejected as a classical biological control agent because it proved to be heteroecious

ambiguous legislative and regulatory framework. For example, **the use of fungal pathogens for biological control comes under the EU directive for chemical pesticide regulation**, prompting Seier (2005) to argue that release of fungal pathogens as CBC agents "should be dealt with under a different non-pesticide legislation enforced by appropriate national bodies". Thus, when funding was approved by a consortium of UK environmental agencies and local stakeholders for a biological control programme against Japanese knotweed—following estimates of the damage it was inflicting on both the economy and biodiversity; costs to control the weed being put at $1.56 billion (Sheppard et al. 2006; Fig. 6.3a, b)—particular emphasis was placed on surveys in Japan for arthropod agents, as well as in the UK for indigenous, opportunistic pathogens that could be exploited as mycoherbicides (Evans 2003). This has become the flagship programme "to launch classical biological control into European waters" (Vurro and Evans 2008). The

legislative and licensing hurdles to be overcome—before the final ministerial approval for release of an insect CBC agent was granted—have been well-documented, as has the supporting science (Shaw et al. 2009, 2011). Some initially promising fungal agents have now been consigned to history: the two rust species from Japan were confirmed to be heteroecious with alternative hosts in the life cycle and, therefore, non-starters as CBC agents (Kurose et al. 2006; Fig. 6.3f), whilst no suitable pathogens were recorded in the UK. However, an unusual hemibiotrophic *Mycosphaerella* species—highly damaging and widespread in Japan (Kurose et al. 2009; Fig. 6.3c–e)—had been put on the 'back-burner', until recently, when funding was released by the UK Government to revive the studies on this pathogen, in the expectation that it may complement and enhance the impact of the insect agent on this particularly intractable weed (Kurose et al. 2006). Spectacular control of the highly invasive bridal creeper weed (*Asparagus asparagoides*) has been achieved in Australia by deploying this strategy, whereby different CBC agents can partition the weed resources in both space and time (Palmer et al. 2010; Turner et al. 2010).

c) Himalayan Balsam: *Impatiens glandulifera (Balsaminaceae)*

This alien riparian weed is now listed as **the tallest annual plant in the British Isles**, enabling it to out-compete the native flora for resources and thus reduce biodiversity (Chittka and Schürkens 2001; Tanner et al. 2008). It also ranks amongst the top 20 environmental weeds in Europe (Sheppard et al. 2006). Once again, a UK consortium of local government agencies and stakeholders has invested in a CBC programme. Interestingly, even before the field surveys began in the Himalayas, a new rust record on this host was found on a botanical specimen in Kew Herbarium, collected from India in the mid-nineteenth century, together with notes on where the seeds were distributed in the UK. Thus, prior knowledge of the natural enemies and where to search for them, as well as the possible source of origin of the UK invasions, was gathered beforehand. DNA profiling of UK and Himalayan plants has now confirmed that northern India is the target area for field surveys and that the rust, *Puccinia* cf *komarovii*, is widespread and damaging in that region. The use of both molecular techniques and pathogenicity studies has shown that the spermogonia and aecia appearing on swollen seedlings early in the season represent spore stages of the same rust species producing uredinia and telia on foliage later in the season. In order to break the dormancy of the teliospores for the life cycle studies, lessons learned from earlier programmes relating to the use of bleaching agents and experimenting with temperature protocols—in this case, freezing the teliospores and then germinating at low temperatures (4 °C)—were essential (Evans 1987b, Tanner, 2012, personal communication). Host-range studies are at an advanced stage, and it is probable that this rust could be the first pathogen to be released for CBC of an invasive alien weed in Europe, and not, as predicted, the *Mycosphaerella* pathogen targeted at Japanese knotweed (Kurose et al. 2006, 2009). There are minor concerns about some degree of infection of the ornamental plant *Impatiens balsamina*, but this is almost certainly the well-documented result of **host-range extension** in artificial greenhouse conditions (Evans and Tomley 1996; Evans 2000). Circumstantial evidence from the field supports this conclusion, since the same rust species is common and damaging on *Impatiens parviflora* in mainland Europe, whilst nearby Himalayan balsam remains uninfected (Bacigalova et al. 1998; Piskorz and Klimko 2006).

Similarly, *I. balsamina* is recorded as an invasive alien weed in northern India, where there are at least 50 native *Impatiens* species (Khuroo et al. 2012), indicating that it is highly competitive and hence not attacked by natural enemies, such as the rust, from related endemics. DNA profiling shows minor differences in sequences between isolates of the rust from different *Impatiens* species, and this, together with the field data, is indicative of species-specific pathotypes or *formae speciales* within the *P. komarovii* species complex. Final proof of rigid host specificity is awaited following the deployment of 'sentinel' plants—also referred to as a 'trap garden' (Morin et al. 2011)—of UK biotypes of *I. glandulifera* amongst rust-infested plants of *I. parviflora* in mainland Europe. The UK agency Defra has already stipulated that if release of the rust is undertaken by transfer of infected plants from quarantine

into monitored field sites—as has been the normal methodology in Australia, for example—then the introduction would not come under the **UK Pesticide Regulation Act** (Tanner, 2012, personal communication).

III. Inundative Biological Control (IBC)

A. Introduction

In the IBC approach, otherwise known as the mycoherbicide strategy, the selected fungal pathogen—invariably an **indigenous necrotroph**—is mass-produced, formulated, marketed, and applied in basically the same way as a chemical herbicide. Thus, in essence, it is conceived and marketed as a replacement for traditional herbicides in agriculture by offering a potentially safer or 'greener' and theoretically cheaper option. Unfortunately, IBC has failed to deliver as a viable and sustainable alternative solution to chemical control of weeds, at least in the wider agricultural context. Here, the reasons for this perceived failure (Weaver et al. 2007; Vurro and Evans 2008)—after the initial optimism (Templeton et al. 1979; Templeton 1982)—are explored in relation to past programmes, whilst the potential role of mycoherbicides in the future is evaluated.

B. Early History

As noted previously (Evans 2002a), Wilson (1969) in a pre-emptive review of the subject stressed that there was a need for "microbial herbicides" not only because of environmental concerns but also because of the increasing trend towards multiple methods of pest control (**Integrated Pest Management**). He considered that: "Plant pathogens as weed control agents fit nicely into such a scheme"; and listed their advantages over chemical herbicides, notably that they were more specific and safer. Ironically, these are the very virtues that have now effectively relegated them to niche-market products. To be a commercial success, herbicides need to have a wide application against a range of weeds in a variety of crops: the first mycoherbicides, although environmentally benign and seemingly successful, were targeted at a single weed in one crop system (TeBeest and Templeton 1985). Moreover, the significant investment in both time and money required to overcome the many technical and regulatory hurdles meant that there were considerable delays between the highly promising research phase and commercialisation (Daniel et al. 1973; Woodhead 1981; Bowers 1986; Kenney 1986; Ridings 1986), tempering the initial optimism and, perhaps, dissuading the multinational agrochemical companies from investing in this alternative technology.

For a general overview of the history and development of IBC from these beginnings up to 2002, as well as lists of products ostensibly on the market, consult Boyette (2000), Charudattan (2001) and Evans et al. (2002a, b).

C. Recent Developments

Have there been any developments of significance in the field of IBC in the last decade? In reality, little has changed and agriculture is still reliant for weed management on chemical herbicides, albeit a diminishing range of products, whilst **mycoherbicides have effectively been relegated to or remained at the cottage-industry level**, targeting niche-market amenity weeds rather than crops. The constraints to the development of mycoherbicides are the same today as those identified nearly 20 years ago (Auld and Morin 1995), and these have been revisited in the interim (El-Sayed 2005; Hallett 2005; Weaver et al. 2007; Ash 2010). Suffice it to say that the problem areas are the usual suspects, as detailed in Evans (2002a): production technology, formulation, application technology, compatibility with other management practices, regulatory issues, and marketing strategy. A closer analysis of several IBC programmes, the complete history of which has only recently been documented, is included here to demonstrate these failings.

1. The BioMal® Story

This began in the early 1980s in Canada with the discovery of a serious anthracnose disease, caused by a pathotype of *Colletotrichum gloeosporioides*,

attacking round-leaved mallow, *Malva pusilla* (Mortensen 1988), a troublesome urban weed and also problematic in a number of annual crops (Mortensen and Bailey 2002). There followed a lengthy period of greenhouse screening to determine specificity within the Malvaceae, and then, for registration purposes, field trials were conducted in nine crop ecosystems. In addition, over 20 field experiments were conducted to determine efficacy under different climatic parameters. As with previous mycoherbicide programmes (TeBeest and Templeton 1985), these early stages of product development were funded publicly; typically this is at the university or agricultural research station level. However, for **commercialisation**—which involves investment to develop a manufacturing process and to generate the data necessary to register a product with the relevant pesticide directorate—an industry partner was sought. There were considerable technical hurdles in mass production, as well as in formulation, to overcome before the product could be registered. In this case, with no real guidelines for microbial products, the same toxicology and environmental persistence data as required for a chemical pesticide had to be provided (Bailey et al. 2010). Finally, BioMal® was registered in 1992 as a **post-emergence mycoherbicide**—a decade after the initial research. Nevertheless, the product was withdrawn from the market within a few years by the industry partner, because the investment needed to manufacture it and expand commercialisation was not covered by projected sales: in effect, the initial market research was poor and it misjudged totally the demand for a product that was aimed at a single target weed. Further licensing agreements were made with other industry partners designed to expand the market to the USA, as well as to target closely related agricultural weeds also susceptible to the fungal pathotype—notably, velvet leaf (*Abutilon theophrasti*). However, despite the reported optimism (Evans 2002a), the technical difficulties were such—especially in relation to consistent field efficacy—that the economics of trying to overcome these meant that the mycoherbicide could not compete with chemical herbicides (Boyetchko et al. 2007).

2. The *Cirsium arvense* Story

Creeping thistle is a plant of Eurasian origin which is weedy in its European range (Tiley 2010) and highly invasive in its extensive exotic range, where it is more commonly known as Canada or California thistle (Moore 1975). *Cirsium arvense* is recognised as one of the world's worst weeds (Holm et al. 1977), and it has been the target of numerous attempts to develop mycoherbicides as an alternative to chemicals, since control can be problematic because of the deep perennial root system. Müller and Nentwig (2011) list the fungal candidates—in the genera *Alternaria*, *Fusarium*, *Phoma*, *Phomopsis*, *Phyllosticta*, *Sclerotinia*, *Septoria*, and *Stagonospora*—that have been screened against this weed, with two patents being taken out concerning the herbicidal potential of the active components from *Septoria cirsii* and *Phomopsis cirsii*. The authors conclude "that the potential of pathogens for biocontrol of *C. arvense* has largely been overestimated". Interestingly, the potential of the systemic rust *Puccinia punctiformis* (see Fig. 6.4c), either as a mycoherbicide or in an IPM strategy, has been investigated both in North America and Europe (French and Lightfield 1990; Frantzen 1994; Völker and Boyle 1994; Guske et al. 2004; Demers et al. 2006), with mixed results but no product. The rust appears to have moved together with its host throughout the weed's invasive range. In fact, mass production of rust inoculum for application against this weed was even attempted in New Zealand long before the concept of mycoherbicides had been conceived (Cockayne 1915; Cunningham 1927; Wilson 1969). "The water containing the (uredinio)spores should then be sprayed on young healthy thistles with a spray-pump provided with a Bordeaux nozzle" (Cockayne 1915). Ironically, after nearly a century, interest in the management potential of this rust is still on-going: "its usefulness for biological control in New Zealand has not been fully explored" (Cripps et al. 2008).

The practicality of using a biotrophic fungus as a mycoherbicide had also been evaluated previously against yellow nutsedge, *Cyperus esculentus*, in the USA, when the rust *Puccinia*

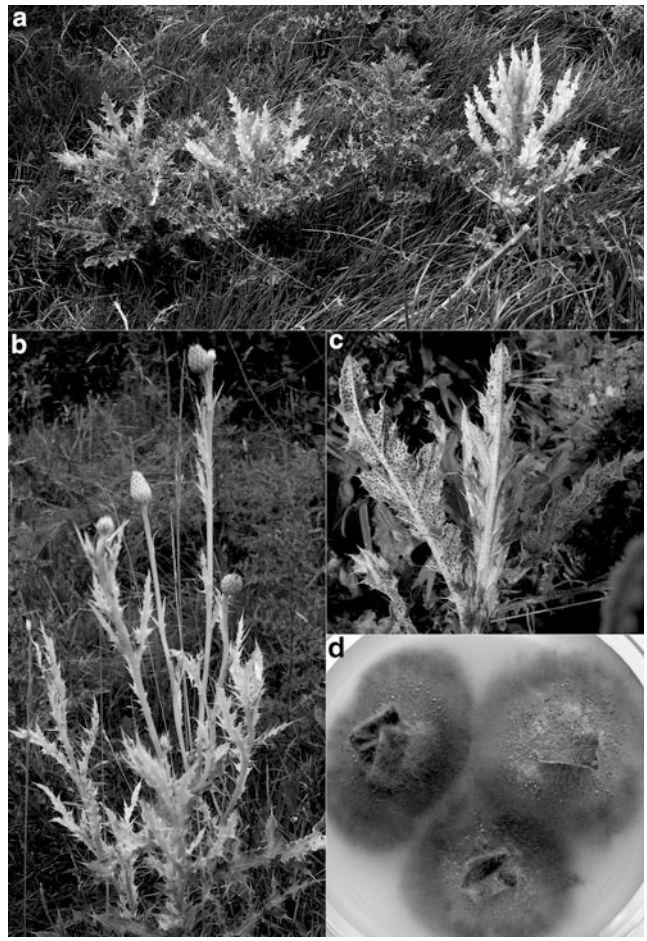

Fig. 6.4. White tip disease of *Cirsium arvense* (creeping or Canada thistle) in southern England, caused by a strain of *Phoma macrostoma* that produces novel plant toxins (macrocidins). (**a**) Early stage with characteristic bleaching of the vegetative shoots. (**b**) Later stage with bleaching of the inflorescence. (**c**) Bleached leaves also infected by the systemic rust, *Puccinia punctiformis*. (**d**) Surface-sterilised, white-tip leaf pieces on agar plate, showing consistent isolation of *Phoma macrostoma*, typified by a red pigment in the mycelium and pycnidia with prominent ostioles

canaliculata was shown to impact severely on plant vigour and long-term survival. The rust was mass-produced, formulated with inert carriers, and applied artificially early in the season (Beste et al. 1992). The application for a patent and the registration of a product name, Dr Biosedge (Phatak 1992), seemed to herald the arrival of a new approach in IBC, but, for unspecified reasons—presumed to be related to technical and logistical problems of producing and marketing a biotrophic fungus—the initiative foundered. It could be argued that the IBC strategy can work with rust fungi, but not at a commercial level. For example, because of the urgency of the situation and vast area to be treated, the rubber-vine rust in Australia was mass-produced, harvested, formulated (in water with a spore dispersant), and applied by conventional sprayers and also from light aircraft to control the invasive front of the weed (Tomley and Evans 2004). Because this was a publicly-funded programme, undertaken and implemented by scientists rather than farmers, there was no necessity to develop a registered, saleable product and hence no need to compete with chemical herbicides in the marketplace.

In effect, it was never interpreted as a pesticide *sensu lato*, and so escaped the potentially prohibitive regulatory costs that have dogged more conventional mycoherbicide initiatives or prevented their registration entirely, especially in Europe (Vurro and Evans 2008).

However, there still could be a happy, if somewhat serendipitous and fortuitous ending to the *C. arvense* story, rather than yet another perceived failure (Müller and Nentwig 2011); although this somewhat simplistic interpretation is now being questioned (Cripps et al. 2012). During a government-funded programme to assess the mycoherbicidal potential of indigenous pathogens found on *C. arvense* in Canada, a disease causing spectacular bleaching of the shoots was encountered (Fig. 6.4a–c). The causal agent was discovered to be the coelomycete fungus *Phoma macrostoma* (Fig. 6.4d)—a minor plurivorous pathogen of woody plants (Holliday 1989). Further investigation revealed that the Canadian isolates of the fungus, from the so-called white tip disease of *C. arvense*, are genetically distinct from the type strain and, moreover, all were found to produce unique **phytotoxins**, the **macrocidins** (Graupner et al. 2003; Bailey et al. 2011). Additional research on host range, environmental safety and product formulation, in conjunction with an industrial partner, has resulted in the registration in North America of a product targeted not specifically at Canada thistle, but as a pre-emergence mycoherbicide against broad-leaved weeds in turf grass and grass-seed production systems, as well as in cereal crops (Bailey and Falk 2011; PMRA 2011). Since then, the disease has been reported from southern England—and the causal agent has been shown to have a similar DNA profile to the Canadian fungus—leading to the conclusion that the pathogen was taken to North America together with its coevolved host (Evans et al. 2013).

3. The *Chondrostereum purpureum* Story

This story, more than any other, encapsulates the history and evolution of mycoherbicides, beginning with the original premise and central tenet of IBC, especially using fungal pathogens of plants: that the agent should exhibit a high degree of specificity and, thus, have a narrow host range. Many of the original IBC initiatives were based on the concept of *formae speciales* of indigenous pathogens—notably in the genus *Colletotrichum*—that, purportedly, were restricted to the target weed. Some of these proto- and established mycoherbicides proved to be not as specific as claimed (Weidemann 1991). This is not necessarily a 'death sentence' for IBC products, which are based on indigenous pathogens, and, as shown above for BioMal®, it can even be advantageous. In fact, as intimated previously, the rigid host specificity of a mycoherbicide is not, an attractive selling point to investors, since the market size is limited. Hence, the use of an infamous, previously notifiable plant pathogen, such as *C. purpureum*, was something of a seismic shift in IBC policy, and involved a different approach to the risk assessment. Here, the **PRA was based on epidemiological data and simulation models** which demonstrated that minimal risks were posed to crops, mainly stone fruits, and that background inoculum (basidiospores) of the pathogen was not perceptibly different 0.5 km from release sites (de Jong et al. 1990; de Jong 2000). The target weed of this research was the American black cherry, *Prunus serotina*, a highly invasive tree in the Netherlands, and mycelial preparations were applied to cut stumps to prevent re-sprouting, the only safety stipulation being that the mycoherbicide is not applied within 500 m of a susceptible tree crop. The resultant product, BioChon®, has been commercially available for over a decade in the Netherlands where it is marketed as a **wood-rot promoter** rather than a mycoherbicide; although the most recent reports indicate that it is not currently on the market because of on-going problems with registration and quality control (Ehlers 2008, 2011). Nevertheless, this approach has been adopted in other countries against woody invasive weeds. Most advanced is Canada, where alternative solutions are needed as a matter of urgency for control of woody invasives because of legislation restricting the use of chemical herbicides, especially in forestry and utility rights-of-way. Reportedly, two stump-treatment products have now been registered with the Canadian regulatory agency (Bailey 2010). The story behind the development of one of these mycoherbicides has been

detailed recently (de la Bastide and Hintz 2007; Hintz 2007), and the product is now registered and available under the trade name Chontrol (MycoLogic 2011). Testing of local strains and an assessment of the mycoherbicide potential is on-going against European gorse and other woody exotics in New Zealand (Bourdôt et al. 2006), rhododendron (*Rhododendron ponticum*) in the UK (Evans 2003; Seier, 2012, personal communication) and invasive hardwoods in Nordic countries (Vartiamäki et al. 2008).

IV. Conclusions

A. CBC: Problems

As pointed out previously (Evans 2002a), the concept of employing fungal plant pathogens for the management of weeds—especially the CBC approach involving the transcontinental movement and the release of alien pathogenic microbes—has always met with suspicion and alarm by legislative authorities and environmental scientists alike. This so-called '**pathophobia**' (Freeman and Charudattan 1985) continues to be expressed to the present day (Warner 2012) and, to some extent, has slowed or even hampered the general acceptance and use of this technology as a viable or front-line weed-management strategy, particularly of invasive alien plant species. Almost certainly, it has delayed the process in the USA (Miller and Aplet 2005) and in Europe (Sheppard et al. 2006; Vurro and Evans 2008).

Lively debates have ensued between ecologists, conservationists and biological control practitioners, with provocative statements such as "Mechanical and localized chemical control methods exist and, although they take time and resources, may represent less harmful containment strategies in the long run than introducing self-replicating, self-dispersing, irretrievable biological 'natural enemies' with unexpected side-effects" (Louda and Stiling 2004), to counter those weed scientists advocating CBC as the strategy of choice to control invasive alien species (Hoddle 2004a, b; Carruthers 2004). Meanwhile, others adopt a more paternal, overarching stance, pointing out the possible knock-on effects, potential risks, unpredictable scenarios, and pleas for ecologists to become more engaged with CBC (Pearson and Callaway 2003; Müller-Schärer et al. 2004; Fowler et al. 2012; Simberloff 2012)—best encapsulated, perhaps, by the even more cryptic and somewhat sinister implications of CBC—"Assuming it could be done, scientific introduction of target-specific control species would threaten the entrenched interests of a wide variety of dominant actors in the U.S. agricultural–industrial field" (Proffitt 2004). And these are the views of scientists on the perceived 'safer' option of using insect CBC agents. The views of the 'amateur' scientists and layman can be even more worrying and damning, as a magazine editorial – entitled "Defra's alien creepy-crawly that threatens our landscapes" – demonstrates, following the release of a psyllid CBC agent in the UK (see above, II.C.2b), that included the statement: "The Japanese knotweed is bad, but not as bad as the Pandora's box that is the wilful releasing of alien insects" (Anon 2009). It does not bear thinking about what the editor's response will be if the fungal pathogen (*Mycosphaerella polygoni-cuspidati*), currently under assessment in UK quarantine (see II.C.2b), is approved for release against Japanese knotweed.

In defence, CBC pathologists can point to the high-level science and environmental concern underpinning this approach (Barratt and Moeed 2005; Berner and Bruckart 2005; Martin and Paynter 2010), the unblemished safety record (Barton 2004, 2012), and the high success rate (Culliney 2005; Page and Lacey 2006; McFadyen 2008; Palmer et al. 2010). However, the final assurance that no unexpected, **non-target impacts** will occur from a CBC introduction—categorised as 'Risks not Foreseen' (Berry 2006)—cannot be given because nature is never entirely predictable, and "attempts to do so would be stultifying" (Berry 2006). Of course, this can be used against CBC, but, as has been highlighted recently, if the **precautionary principle** were to be followed and "if everything we did had to be absolutely safe, risk-free, proven to have no adverse outcomes for anyone or anything, we'd never get anywhere. . . . science would be stifled" (Berry C in O'Neill 2004). The basis of the PRA currently employed in CBC programmes is to assuage any

fears of environmental mishaps. However, some have considered that current demands can be too strict, citing successful introductions of CBC insect agents in the past which would fail to be sanctioned today (Groenteman et al. 2011).

Perhaps even more problematic are the mainly non-scientific issues that have come to the fore recently. First, the re-interpretation of the CBD legislation—with the recent **International Regime on Access and Benefit Sharing—designed primarily to combat bio-piracy can effectively prevent the transfer of CBC agents between countries and continents** (Cock 2010). This problem was touched upon in the case of the mikania weed programme (see II.C.2a), in which the screening of alternative rust pathotypes—potentially better-adapted to the range of weed biotypes occurring in its Asian invasive range—has effectively reached an impasse, because most Latin American countries do not have the legislative mechanism to allow for the interchange of material for CBC purposes, nor the necessary experience to understand and react to the needs of CBC. This was dramatically illustrated during the early phase of the mikania programme, when Indian quarantine authorities initially refused to sanction the transfer of weed biotypes to the UK for host-range studies, on their understanding that the plant constituted part of the biodiversity of the Western Ghats. Second, the concept of 'embracing invasives' (Vince 2011) is potentially more intractable if it becomes more than just a mantra amongst conservationists and ecologists. Fortunately, there was an immediate knee-jerk reaction by both ecologists and CBC practitioners alike (Simberloff et al. 2011). Certainly, there should be no place for complacency about and acceptance of highly invasive alien plant species, especially so in World Heritage ecosystems such as the Galápagos Archipelago where this furore has centred recently. The stated objective of the authorities tasked with preserving the biological integrity of these emblematic islands is to restrict the invasiveness of alien weeds, and thereby enable the native flora to compete on a more equal footing: that is precisely what CBC aims to deliver.

B. CBC: Prospects

Until recently, CBC of invasive weeds has been played out almost exclusively on the continental stages of Australasia, Africa, and Latin America, but new initiatives in Asia and Europe give cause for optimism. Latin America was the scene of some pioneering CBC activity—and the history and prospects for this region have been reviewed (Ellison and Barreto 2004)—but little has been achieved since, and efforts thus far to generate interest in this approach in Brazil, Costa Rica, and Ecuador (Galápagos) continue to be disappointing, and have virtually been put on hold (CABI unpublished reports). The experience in Costa Rica has been particularly frustrating since a CBC programme involving the use of a systemic head smut (*Sporisorium ophiuri*) against a major invasive grassy weed of agricultural ecosystems, *Rottboellia cochichinensis*, was stalled at the eleventh hour—the implementation and release phase—as a result of relatively minor funding and legislative issues (Evans 2002a; Ellison and Barreto 2004), which remain unresolved to the present day. Nevertheless, the tide may be turning in favour of CBC as an acceptable, if not entirely mainstream weed management strategy for the control of alien plant species: increasing environmental awareness and decreasing choice of 'safe' chemical herbicides, coupled with increasing costs of developing new products, imply that the prospects are favourable. This low-tech approach is especially appropriate for small islands where the risks from and environmental consequences of exotic plant invasions are accentuated because of the fragile nature of the ecosystems, and where CBC can be at its most effective; offering sustainable, environmentally benign, and low-cost control.

However, it cannot be overstated that the public, in general, and stakeholders and policy makers, in particular, remain ill-informed about the science, as well as the socio-economic and environmental benefits of CBC for the management of invasive alien weeds (Warner et al. 2008; Warner 2012). Unfortunately, CBC practitioners make poor lobbyists. Until these issues are addressed, CBC will not realise its full potential and it will continue to be an 'underachiever'.

C. IBC: Problems and Prospects

These are mutually intertwined, and the prospects for IBC will continue to be linked inevitably with the seemingly insurmountable problems of marketing mycoherbicides. The very strength of CBC—the rigid host specificity of the pathogen—has been the weakness of IBC, severely limiting the weed targets and thus the size of the market, and, of course, the big investment to solve the technical problems with developing a biological pesticide: mass production, formulation to confer stability, shelf-life, and consistent efficacy under varying field conditions, as well as the application strategy. In effect, the initial concept of host specificity has been turned on its head and fungal agents for IBC are now selected on the basis of their pathogenicity to a range of weed hosts—the more the merrier for commercial development—in order to increase market size, with the PRA being based on evidence from epidemiological rather than pathogenicity studies. The BioMal® story (III.C. 1.), perhaps best encapsulates the technical and commercial problems involved in developing a mycoherbicide. Seemingly gone are the prospects of replacing the multi-billion dollar agricultural herbicides with 'green' IBC products, and mycoherbicides are not even included in the actual and perceived rapidly-expanding world market for biopesticides (Anon 2012; Glare et al. 2012). The current trend appears to be for **niche markets**, specifically for specialist products based on indigenous, plurivorous, wood-rotting fungal pathogens active against shrubs or trees—indigenous, naturalised or exotic—which are difficult or environmentally unsafe to control with chemicals. At present, this market is small, but the prospects could improve, since there are increasing incidences of invasive woody plants throughout the world, where CBC or more conventional weed management strategies may not supply the answer: quinine in the Galápagos, rhododendron in Europe, paper mulberry in Asia and Africa, for example. Whether funding for product development will come from public (government, aid agencies) or private investment is difficult to predict.

The encouraging development would seem to be selecting pathogens producing novel phytotoxins—such as *Phoma macrostoma*—which can compete with traditional herbicides in efficacy and speed of kill. This is provided, of course, that the mycoherbicides are not subject to the same legislative and registration protocols as for chemical pesticides (Vurro and Evans 2008), otherwise the costs would be prohibitive. In addition, other initiatives have been suggested or are already in the pipeline, such as synergistic mixtures of low-dose chemicals with a fungal pathogen (Duke et al. 2007). However, it is felt that research involving **genetic engineering** to enhance the virulence of mycoherbicides (Amsellem et al. 2002; Gressel et al. 2007) is a step too far and would not sit well with the public, certainly in Europe, if the selling point of a microbial pesticide is for a 'greener', or natural product.

References

Agrios GN (2005) Plant pathology, 5th edn. Elsevier, Amsterdam

Aime MC (2006) Toward resolving family-level relationships in rust fungi (Uredinales). Mycoscience 47:112–122

Alves JL, Pereira OL, Barreto RW (2008) *Pseudocercospora cryptostegiae-madagascariensis* sp. nov. on *Cryptostegia madagascariensis*, an exotic vine involved in major biological invasions in northeast Brazil. Mycopathologia 165:364–367

Amsellem Z, Cohen BA, Gressel J (2002) Engineering hypervirulence in a mycoherbicidal fungus for efficient weed control. Nat Biotechnol 20:1035–1039

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends Ecol Evol 19:535–544

Anon (2009) Defra's alien creepy-crawly that threatens our landscapes. Country Life August 12:2

Anon (2012) Biopesticides: a global strategic business report (RPT 927963). Global Industry Analysts, http://www.companiesandmarkets.com. Accessed 25 May 2012

Ash GJ (2010) The science and art of successful bioherbicides. Biol Control 52:230–240

Auld BA, Morin L (1995) Constraints in the development of bioherbicides. Weed Technol 9:638–652

Ayres P (2005) Harry Marshall Ward and the fungal thread of death. APS Press, St. Paul

Bacigalova K, Elias P, Srobarova A (1998) *Puccinia komarovii* a rust fungus on *Impatiens parviflora* in Slovakia. Biologia 53:7–13

Bailey KL (2010) Canadian innovations in microbial biopesticides. Can J Plant Pathol 32:113–121
Bailey KL, Falk S (2011) Turning research on microbial bioherbicides into commercial products – a *Phoma* story. Pest Technol 5:73–79
Bailey KL, Chandler D, Grant WP, Greaves J, Prince G, Tatchell M (2010) The regulation of biopesticides: an international analysis. In: Bailey A, Chandler D, Grant WP, Greaves J, Prince G (eds) Biopesticides: pest management and regulation. CAB International, Wallingford, pp 148–176
Bailey KL, Pitt WM, Leggett F, Sheedy C, Derby J (2011) Determining the infection process of *Phoma macrostoma* that leads to bioherbicidal activity on broadleaved weeds. Biol Control 59:268–276
Barneby RC (1989) Reflections on typification and application of the names *Mimosa pigra* L. and *M. asperata* L. (Mimosaceae). Notes R Bot Gard Edinburgh 45:129–134
Barratt BIP, Moeed A (2005) Environmental safety of biological control: policy and practice in New Zealand. Biol Control 35:247–252
Barreto RW, Evans HC (1988) Taxonomy of a fungus introduced into Hawaii for biological control of *Ageratina riparia* (Eupatorieae; Compositae), with observations on related weed pathogens. Trans Br Mycol Soc 91:81–97
Barreto RW, Evans HC (1995) The mycobiota of the weed *Mikania micrantha* in southern Brazil with particular reference to fungal pathogens for biological control. Mycol Res 99:343–352
Barreto RW, Evans HC, Ellison CA (1995) The mycobiota of the weed *Lantana camara* in Brazil, with particular reference to biological control. Mycol Res 99:769–782
Barton J (2004) How good are we at predicting the field host-range of fungal pathogens used for classical biological control of weeds? Biol Control 31:99–122
Barton J (2012) Predictability of pathogen host range in classical biological control of weeds: an update. BioControl 57:289–305
Barton J, Fowler SV (2008) Benefits to New Zealand's native flora from the successful biological control of mistflower (*Ageratina riparia*). In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, p 361
Barton J, Fowler SV, Gianotti AF, Winks CJ, de Beurs M, Arnold GC, Forrester G (2007) Successful biological control of mist flower (*Ageratina riparia*) in New Zealand: agent establishment, impact and benefits to the native flora. Biol Control 40:370–385
Baskin Y (2002) A plague of rats and rubbervines. Island Press, Washington, DC
Berner DK, Bruckart WL (2005) A decision tree for evaluation of exotic plant pathogens for classical biological control of introduced invasive weeds. Biol Control 34:222–232
Berry C (2006) The precautionary principle – more sorry than safe. Sci Parliament 63:18–19
Beste CE, Frank JR, Bruckart WL, Johnson DR, Potts WE (1992) Yellow nutsedge (*Cyperus esculentus*) control in tomato with *Puccinia canaliculata* and pebulate. Weed Technol 6:980–984
Blumenthal DM (2006) Interactions between resource availability and enemy release in plant invasions. Ecol Letters 9:887–895
Bourdôt G, Barton J, Hurrell G, Gianotti A, Saville D (2006) *Chondrostereum purpureum* and *Fusarium tumidum*, independently reduce regrowth in gorse (*Ulex europaeus*). Biocontrol Sci Technol 16:307–327
Bowers RC (1986) Commercialization of Collego – an industrialist's view. Weed Sci 34:24–25
Boyetchko SM, Bailey KL, Hynes RK, Peng G (2007) Development of the mycoherbicide, BioMal®. In: Vincent C, Goettel MS, Lazarovits G (eds) Biological control: a global perspective. CAB International, Wallingford, pp 274–283
Boyette CD (2000) The bioherbicide approach: using plant pathogens to control weeds. In: Cobb AH, Kirkwood RC (eds) Herbicides and their mechanism of action. Sheffield University Academic Press, Sheffield, pp 134–152
Bruzzese E, Hasan S (1983) A whole leaf clearing and staining technique for host specificity studies of rust fungi. Plant Pathol 32:335–338
Burdon JJ, Groves RH, Cullen JM (1981) The impact of biological control on the distribution and abundance of *Chondrilla juncea* in south-eastern Australia. J Appl Ecol 8:857–866
Burrows NJ, Lukitsch BV, Liberato JR (2012) Rediscovery of the rust *Diabole cubensis*, released as a biological control agent against the invasive weed *Mimosa pigra* in Australia. Australas Plant Pathol Notes 7:171–175
Cannon PF (2007) *Diabole cubensis*. IMI Descr Fungi Bact 1725:1–3
Carpenter JE, Gianessi L (2010) Economic impacts of glyphosate-resistant weeds. In: Nandula VK (ed) Glyphosate resistance in crops and weeds: history, development, and management. Wiley, Hoboken, pp 297–312
Carruthers RI (2004) Biological control of invasive species, a personal perspective. Conserv Biol 18:54–57
Carvalho CR, Fernandes RC, Carvalho GMA, Barreto RW, Evans HC (2011) Cryptosexuality and the genetic diversity paradox in coffee rust, *Hemileia vastatrix*. PLoS ONE 6:e26387, 7 pp
CBD (2011) Text of the convention on biological diversity. htpp://www.cbd.int/convention/text. Accessed 29 Nov 2011
Charudattan R (2001) Biological control of weeds by means of plant pathogens: significance for

integrated weed management in modern agro-ecology. BioControl 46:229–260
Chew MK (2009) The monstering of Tamarisk: how scientists made a plant into a problem. J Hist Biol 42:231–266
Chittka L, Schürkens S (2001) Successful invasion of a floral market. Nature 411:653
Cock MJW (2010) Biopiracy rules should not block biological control. Nature 467:369
Cock MJW, Ellison CA, Evans HC, Ooi PAC (2000) Can failure be turned into success for biological control of mile-a-minute weed (*Mikania micrantha*)? In: Spencer NR (ed) Proceedings of the X international symposium biological control weeds. Montana State University Press, Bozeman, pp 155–167
Cockayne AH (1915) California thistle rust. N Z J Agric 11:300–302
Cox GW (1999) Alien species in North America and Hawaii: impacts on natural ecosystems. Island Press, Washington, DC
Cripps MG, Edwards GR, Waipara NW, Fowler SV, Bourdôt GW (2008) The degree of polymorphism in *Puccinia punctiformis* virulence and *Cirsium arvense* resistance: implications for biological control. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, p 246
Cripps MG, Bourdôt GW, Bailey KL (2012) Plant pathogens as biocontrol agents for *Cirsium arvense* – an answer to Műller and Nentwig. Neobiota 13:31–39
Cronk QCB, Fuller JA (1995) Invasive plants: the threat to natural ecosystems worldwide. Chapman and Hall, London
Cullen JM, Kable PF, Catt M (1973) Epidemic spread of a rust imported for biological control. Nature 244:262–264
Culliney TW (2005) Benefits of classical biological control for managing invasive plants. Critical Rev Plant Sci 24:131–150
Cunningham GH (1927) 'Natural control' of weeds and insects by fungi. Bull N Z Dept Agric 132:244–251
Daniel JT, Templeton GE, Smith RJ, Fox WT (1973) Biological control of northern joinvetch in rice with an endemic fungal disease. Weed Sci 21:303–307
Darwin C (1859) On the origin of species. Murray, London
Davis MA, Chew MK, Hobbs RJ, Lugo AE, Ewel JJ, Vermeij GJ et al (2011) Don't judge species on their origins. Nature 474:153–154
Day MD, Kawi A, Tunabuna A, Fidelis J, Swamy B, Ratutuni J, Saul-Maura J, Dewhurst CF, Orapa W (2011) The distribution and socio-economic impacts of *Mikania micrantha* (Asteraceae) in Papua New Guinea and Fiji and prospects for its biocontrol. In: Proceedings of 23rd Asian-Pacific weed science society conference, Cairns, pp 146–153
De Jong MD (2000) The BioChon story: deployment of *Chondrostereum purpureum* to suppress stump sprouting in hardwoods. Mycologist 14:58–62
De Jong MD, Scheepens PC, Zadoks JC (1990) Risk analysis for biological control: a Dutch case studying biocontrol of *Prunus serotina* by the fungus *Chondrostereum purpureum*. Plant Dis 74:189–194
de la Bastide PY, Hintz WE (2007) Developing the production system for *Chondrostereum purpureum*. In: Vincent C, Goettel MS, Lazarovits G (eds) Biological control: a global perspective. CAB International, Wallingford, pp 291–299
Demers AM, Berner DK, Backman PA (2006) Enhancing incidence of *Puccinia punctiformis*, through mowing, to improve management of Canada thistle (*Cirsium arvense*). Biol Control 39:481–488
Dennill GB, Donnelly D, Stewart K, Impson FAC (1999) Insect agents used for the biological control of Australian *Acacia* species and *Paraserianthes lophantha* (Willd.) Nielsen (Fabaceae) in South Africa. Afr Entomol Mem 1:45–54
Duke SO, Wedge DE, Cerdeira AL, Matallo MB (2007) Interactions of synthetic herbicides with plant disease and microbial herbicides. In: Vurro M, Gressel J (eds) Novel biotechnologies for biocontrol agent enhancement and management. Springer, Dordrecht, pp 277–296
Ehlers R-U (2008) Regulation of biological weed biocontrol agents in Europe: results of the EU Policy Support Action REBECA. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 369–375
Ehlers R-U (2011) Regulation of biological control agents and the EU Policy Support Action REBECA. In: Ehlers R-U (ed) Regulation of biological control agents. Springer, Dordrecht, pp 3–24
Ellison CA (2001) Classical biological control of *Mikania micrantha*. In: Sankaran KV, Murphy ST, Evans HC (eds) Alien weeds in moist tropical zones: banes and benefits. Kerala Forestry Research Institute, Peechi, pp 131–138
Ellison CA (2004) Biological control of weeds using fungal natural enemies: a new technology for weed management in tea? Int J Tea Sci 3:4–20
Ellison CA, Barreto RW (2004) Prospects for the management of invasive alien weeds using co-evolved fungal pathogens: a Latin American perspective. Biol Invasions 6:23–45
Ellison CA, Day M (2011) Current status of release of *Puccinia spegazzinii* for *Mikania micrantha* control. Biocontrol News Info 32:1N–2N
Ellison CA, Evans HC, Ineson J (2004) The significance of intraspecies pathogenicity in the selection of a rust biotype for the classical biological control of *Mikania micrantha* (mile-a-minute weed) in Southeast Asia. In: Cullen JM, Briese DT, Kriticos

DJ, Lonsdale WM, Morin L, Scott JK (eds) Proceedings of the XI international symposium biological control weeds. CSIRO Entomology, Canberra, pp 102–107

Ellison CA, Pereira JM, Thomas SE, Barreto RW, Evans HC (2006) Studies on the rust *Prospodium tuberculatum*, a new classical biological control agent released against the invasive alien weed *Lantana camara* in Australia. I: life-cycle and infection parameters. Australas Plant Pathol 35:306–319

Ellison CA, Evans HC, Djeddour DH, Thomas SE (2008) Biology and host range of the rust fungus *Puccinia spegazzinii*: a new classical biological control agent for the invasive, alien weed *Mikania micrantha* in India. Biol Control 45:133–145

El-Sayed W (2005) Biological control of weeds with pathogens: current status and trends. J Plant Dis Prot 112:209–221

Elton CS (1958) The ecology of invasions. Chapman and Hall, London

Evans HC (1987a) Fungal pathogens of some tropical and subtropical weeds and the possibilities for biological control. Biocontrol News Info 8:7–30

Evans HC (1987b) Life-cycle of *Puccinia abrupta* var. *partheniicola*, a potential biological control agent of *Parthenium hysterophorus*. Trans Br Mycol Soc 88:105–111

Evans HC (1993) Studies on the rust *Maravalia cryptostegiae*, a potential biological control agent of rubber-vine weed *Cryptostegia grandiflora* (Asclepiadaceae: Periplocoideae), in Australia. I: Life cycle. Mycopathologia 124:163–174

Evans HC (2000) Evaluating plant pathogens for biological control: an alternative view of pest risk assessment. Australas Plant Pathol 29:1–14

Evans HC (2002a) Biological control of weeds. In: Kempken F (ed) The Mycota XI, agricultural applications. Springer, Berlin, pp 135–152

Evans HC (2002b) Invasive neotropical pathogens of tree crops. In: Watling R, Frankland JC, Ainsworth AM, Isaac S, Robinson CH (eds) Tropical mycology 2, Micromycetes. CAB International, Wallingford, pp 83–112

Evans HC (2003) Biological control of invasive alien weeds using fungi, with particular reference to *Rhododendron ponticum* in the British Isles. In: Argent G, McFarlane M (eds) Rhododendrons in horticulture and science. Royal Botanic Garden Edinburgh, Edinburgh, pp 8–19

Evans HC (2008) The endophyte-enemy release hypothesis: implications for classical biological control. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 20–25

Evans HC, Ellison CA (2005) The biology and taxonomy of rust fungi associated with the neotropical vine *Mikania micrantha*, a major invasive weed in Asia. Mycologia 97:935–947

Evans HC, Tomley AJ (1996) Greenhouse and field evaluations of the rubber-vine rust, *Maravalia cryptostegiae*, on Madagascan and Australian Asclepiadaceae. In: Moran VC, Hoffmann JH (eds) Proceedings of the international symposium biological control weeds. Cape Town University Press, Cape Town, pp 165–169

Evans HC, Waller JH (2010) Globalisation and the threat to biosecurity. In: Strange RN, Gullino ML (eds) The role of plant pathology in food safety and food security. Springer, Dordrecht, pp 53–71

Evans HC, Carrión G, Guzman G (1993) A new species of *Sphaerulina* and its *Phloeospora* anamorph, with potential for biological control of *Mimosa pigra*. Mycol Res 97:59–67

Evans HC, Carrión G, Ruiz-Belin F (1995) Mycobiota of the giant sensitive plant, *Mimosa pigra sensu lato* in the Neotropics. Mycol Res 99:420–428

Evans HC, Frölich J, Shamoun SF (2002a) Biological control of weeds. In: Pointing SB, Hyde KD (eds) Bio-exploitation of filamentous fungi. Fungal Diversity Press, University of Hong Kong, Hong Kong, pp 349–401

Evans HC, Greaves MP, Watson AK (2002b) Fungal biocontrol agents of weeds. In: Butt TM, Jackson C, Magan N (eds) Fungi as biocontrol agents. CAB International, Wallingford, pp 169–192

Evans HC, Seier MK, Derby J-A, Falk S, Bailey KL (2013) Tracing the origins of White Tip disease of *Cirsium arvense* and its causal agent, *Phoma macrostoma*. Weed Res 53:42–52

Fowler SV, Paynter Q, Dodd S, Groenteman R (2012) How can ecologists help practitioners minimize non-target effects in weed biocontrol? J Appl Ecol. doi:10.1111/j.1365-2664.2011.02106.x

Frantzen J (1994) The role of clonal growth in the pathosystem *Cirsium arvense*: *Puccinia punctiformis*. Can J Bot 72:832–836

Freeman TE, Charudattan R (1985) Conflicts in the use of plant pathogens as biocontrol agents of weeds. In: Delfosse ES (ed) Proceedings of the VI international symposium biological control weeds. Agriculture Canada, Ottawa, pp 351–357

French RC, Lightfield AR (1990) Induction of systemic aecial infection in Canada thistle (*Cirsium arvense*) by teliospores of *Puccinia punctiformis*. Phytopathology 80:872–877

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J, Marrone P, Morin L, Stewart A (2012) Have biopesticides come of age? Trends Biotechnol. doi:10.1016/j.tibtech.2012.01.003

Graupner PR, Carr A, Clancy E, Gilbert J, Bailey KL, Derby J, Gerwick BC (2003) The macrocidins: novel cyclic tetramic acids with herbicidal activity produced by *Phoma macrostoma*. J Nat Prod 66:1558–1561

Gressel J, Meir S, Herschkovitz Y, Al-Ahmad H, Green-spoon I, Babalola O, Amsellem Z (2007) Approaches to and success in developing transgenetically enhanced mycoherbicides. In: Vurro M, Gressel J (eds) Novel biotechnologies for biocontrol agent enhancement and management. Springer, Dordrecht, pp 297–305

Groenteman R, Fowler SV, Sullivan JJ (2011) St. John's wort beetles would not have been introduced to New Zealand: a retrospective host range test of New Zealand's most successful weed biocontrol agents. Biol Control 57:50–58

Guske S, Schulz B, Boyle C (2004) Biocontrol options for *Cirsium arvense* with indigenous fungal pathogens. Weed Res 44:107–116

Hallett SG (2005) Where are the bioherbicides? Weed Sci 53:404–415

Hart JA (1988) Rust fungi and host plant coevolution: do primitive hosts harbor primitive parasites? Cladistics 4:339–366

Heard TA, Paynter Q (2009) *Mimosa pigra* L. (Leguminosae). In: Muniappan R, Reddy DVR, Raman A (eds) Biological control of tropical weeds using arthropods. Cambridge University Press, Cambridge, UK, pp 256–273

Hennecke B (2006) Failure of *Diabole cubensis*, a promising biological control agent, to establish in Australia. Biol Control 39:121–127

Herren HR, Neuenschwander P (1991) Biological control of cassava pests in Africa. Annu Rev Entomol 36:257–283

Herrera O, Major I (2006) Visitantes perigosos no Nordeste – Brasil Tropical. Ciência Hoje 38:42–44

Heystek F, Wood AR, Neser S, Kistensamy Y (2011) Biological control of two *Ageratina* species (Asteraceae: Eupatorieae) in South Africa. Afr Entomol 19:208–216

Hintz W (2007) Development of *Chondrostereum purpureum* as a mycoherbicide for deciduous bush control. In: Vincent C, Goettel MS, Lazarovits G (eds) Biological control: a global perspective. CAB International, Wallingford, pp 284–290

Hoddle MS (2004a) Restoring the balance: using exotic species to control invasive exotic species. Conserv Biol 18:38–49

Hoddle MS (2004b) The strength of biological control in the battle against invasive pests: a reply. Conserv Biol 18:61–64

Hoffmann JH, Moran VC (2008) Assigning success in biological weed control: what do we really mean? In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 687–692

Holden ANG, Mahlberg PG (1992) Application of chemotaxonomy of leafy spurges (*Euphorbia* spp.) in biological control. Can J Bot 70:1529–1536

Holliday P (1989) A dictionary of plant pathology. Cambridge University Press, Cambridge, UK

Holm LG, Plucknett DL, Pancho JV, Herberger JP (1977) The world's worst weeds, distribution and biology. University Press of Hawaii, Honolulu

Keane RM, Crawley MJ (2002) Exotic plant invasions and the enemy release hypothesis. Trends Ecol Evol 17:164–170

Kenney DS (1986) DeVine – the way it was developed. Weed Sci 34:15–16

Khuroo AA, Reshi ZA, Malik AH, Weber E, Rashid I, Dar GH (2012) Alien flora of India: taxonomic composition, invasion status and biogeographic affiliations. Biol Invasions 14:99–113

Kumar PS, Rabindra RJ, Ellison CA (2008) Expanding classical biological control of weeds with pathogens in India: the way forward. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 163–172

Kurose D, Renals T, Shaw R, Furuya N, Takagi M, Evans H (2006) *Fallopia japonica*, an increasingly intractable weed problem in the UK: can fungi help cut through this Gordian knot? Mycologist 20:126–129

Kurose D, Evans HC, Djeddour DH, Cannon PF, Furuya N, Tsuchiya K (2009) Systematics of *Mycosphaerella* species associated with the invasive weed *Fallopia japonica*, including the potential biological control agent *M. polygoni-cuspidati*. Mycoscience 50:179–189

Lambetini M, Leape J, Marton-Lefeuvre J, Mittermeier RA, Rose M, Robinson JG et al (2011) Invasives: a major conservation threat. Science 333:404–405

Large EC (1940) The advance of the fungi. Jonathan Cape, London

Le Roux J, Wieczorek AM (2009) Molecular systematic and population genetics of biological invasions: towards a better understanding of invasive species management. Ann Appl Biol 154:1–17

Leather RI (1967) A catalogue of some plant diseases and fungi in Jamaica. Bull Min Ag Lands Jamaica 61:1–92

Louda SM, Stiling P (2004) The double-edged sword of biological control in conservation and restoration. Conserv Biol 18:50–53

Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M, Bazzaz FA (2000) Biotic invasions: causes, epidemiology, global consequences, and control. Ecol Appl 10:689–710

Maier W, Wingfield BD, Menniken M, Wingfield MJ (2007) Polyphyly and two emerging lineages in the rust genera *Puccinia* and *Uromyces*. Mycol Res 111:176–185

Marohasy J, Forster PI (1991) A taxonomic revision of *Cryptostegia* R. Br. (Asclepiadaceae: Periplocoideae). Australas Syst Bot 4:571–577

Martin N, Paynter Q (2010) Assessing the biosecurity risk from pathogens and herbivores to indigenous plants: lessons from biological control. Biol Invasions 12:3237–3248

McCook S (2006) Global rust belt: *Hemileia vastatrix* and the ecological integration of world coffee production since 1850. J Global Hist 1:177–195

McFadyen REC (1998) Biological control of weeds. Annu Rev Entomol 43:369–393

McFadyen REC (2008) Return on investment: determining the economic impact of biological control programmes. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 67–74

McFadyen REC, Harvey GT (1990) Distribution and control of rubber-vine, *Cryptostegia grandiflora*, a major weed in northern Queensland. Plant Protect Q 5:152–155

Meyer S (2006) The end of the wild. MIT Press, Cambridge, MA

Miller ML, Aplet GH (2005) Applying legal sunshine to the hidden regulations of biological control. Biol Control 35:358–365

Mitchell CE, Power AG (2003) Release of invasive plants from fungal and viral pathogens. Nature 421:625–627

Moore RJ (1975) The biology of Canadian weeds. 13. *Cirsium arvense* (L.) Scop. Can J Plant Sci 55:1033–1048

Morin L, Hill RL, Matayoshi S (1997) Hawaii's successful biological control strategy for mist flower (*Ageratina riparia*) – can it be transferred to New Zealand? Biocontrol News Info 18:77N–88N

Morin L, Evans KJ, Sheppard AW (2006) Selection of pathogen agents in weed biological control: critical issues and peculiarities in relation to arthropod agents. Australas J Entomol 45:349–365

Morin L, Reid AM, Sims-Chilton NM, Buckley YM, Dhileepan K, Hastwell GT, Nordblom TL, Raghu S (2009) Review of approaches to evaluate the effectiveness of weed biological control agents. Biol Control 51:1–15

Morin L, Evans KJ, Jourdan M, Gomez DR, Scott JK (2011) Use of a trap garden to find additional genetically distinct isolates of the rust fungus *Phragmidium violaceum* to enhance biological control of European blackberry in Australia. Eur J Plant Pathol 131:289–303

Mortensen K (1988) The potential of an endemic fungus, *Colletotrichum gloeosporioides*, for biological control of round-leaved mallow (*Malva pusilla*) and velvetleaf (*Abutilon theophasti*). Weed Sci 36:473–478

Mortensen K, Bailey KL (2002) *Malva pusilla* Smith, round-leaved mallow (Malvaceae). In: Mason PG, Huber JT (eds) Biological control programmes in Canada, 1981–2000. CAB International, Wallingford, pp 391–395

Müller E, Nentwig W (2011) Plant pathogens as biocontrol agents of *Cirsium arvense* – an overestimated approach? Neobiota 11:1–24

Müller-Schärer H, Schaffner U, Steinger T (2004) Evolution in invasive plants: implications for biological control. Trends Ecol Evol 19:417–422

Muniappan R, Viraktamath CA (1993) Invasive alien weeds in the Western Ghats. Curr Sci 64:555–557

MycoLogic (2011) Products: Chontrol peat paste. http://www.mycologic.ca/products/. Accessed 12 Dec 2011

Newcombe G, Dugan FM (2010) Fungal pathogens of plants in the Homogocene. In: Gherbawy Y, Voigt K (eds) Molecular identification of fungi. Springer, Berlin, pp 3–34

O'Neill B (2004) More sorry than safe. http://www.spiked-online.com/Articles/0000000CA592htm. Accessed 29 Jun 2004

Olden JD, Poff NL, Douglas MR, Douglas ME, Fausch KD (2004) Ecological and evolutionary consequences of biotic homogenization. Trends Ecol Evol 19:18–24

Paganelli A, Gnazzo V, Acosta H, López SL, Carrasco AE (2010) Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signalling. Chem Res Toxicol 23:1586–1595

Page AR, Lacey KL (2006) Economic impact assessment of Australian weed biological control. Technical series 10, CRC for Australian Weed Management, Adelaide

Palmer WA, Heard TA, Sheppard AW (2010) A review of Australian classical biological control of weeds programs and research activities over the past 12 years. Biol Control 52:271–287

Pearson DE, Callaway RM (2003) Indirect effects of host specific biological control agents. Trends Ecol Evol 18:456–461

Phatak SC (1992) Development and commercialization of the rust (*Puccinia canaliculata*) for biological control of yellow nutsedge. In: Richardson RG (ed) Proceedings of the 1st international weed control congress. Weed Science Society of Victoria, Frankston, pp 388–391

Piskorz R, Klimko M (2006) The effect of *Puccinia komarovii* Tranzsch. infection on characters of *Impatiens parviflora* DC. Acta Soc Botanica Pol 75:51–59

PMRA (2011) Pest Management Regulatory Agency, Evaluation report ERC2011-09, *Phoma macrostoma* strain 94-44B. Catalogue number H113-26/201109E-PDF. Online at http://www.hc-sc.gc.ca/cps-spc/pubs/pest/_decisions/erc2011-09/index-eng.php. Accessed 12 Apr 2012

Powles S, Duke SO (2008) Evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Manag Sci 64:360–365

Proffitt WT (2004) Legitimacy and adoption of a scientific biological control program: an institutional analysis of Hoddle. Conserv Biol 18:58–60

Quimby PC (1982) Impact of plant diseases on plant populations. In: Charudattan R, Walker HL (eds) Biological control of weeds with plant pathogens. Wiley, New York, pp 47–60

Relyea RA (2012) New effects of Roundup on amphibians: predators reduce herbicide mortality; herbicides induce antipredator morphology. Ecol Appl 22:634–647

Ridings WH (1986) Biological control of stranglervine (*Morrenia odorata* Lindl.) in citrus – a researcher's view. Weed Sci 34:31–32

Ritschel A (2005) Monograph of the genus *Hemileia*. Bibl Mycol 200:1–132

Rodriguez-Estrella R, Navarro JJP, Granados B, Rivera L (2010) The distribution of an invasive plant in a fragile ecosystem: rubber vine (*Cryptostegia grandiflora*) in oases of the Baja California peninsula. Biol Invasions 12:3389–3393

Rosenzweig ML (2001) The four questions: what does the introduction of exotic species do to diversity? Evol Ecol Res 3:361–367

Rudgers JA, Koskow JM, Clay K (2004) Endophytic fungi alter relationships between diversity and ecosystem properties. Ecol Lett 7:42–51

Ryan MJ, Ellison CA (2002) Development of a cryopreservation protocol for the microcyclic rust fungus *Puccinia spegazzinii*. CryoLetters 24: 43–48

Sandel B, Dangremond EM (2011) Climate change and the invasion of California by grasses. Global Change Biol 18:277–289

Sankaran KV, Puzari KC, Ellison CA, Kumar PS, Dev U (2008) Field release of the rust fungus *Puccinia spegazzinii* to control *Mikania micrantha* in India: protocols and raising awareness. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 384–389

Savile DBO (1976) Evolution of the rust fungi (Uredinales) as reflected by their ecological problems. Evol Biol 9:137–207

Schulz B, Boyle C (2005) The endophyte continuum. Mycol Res 109:661–686

Seier MK (2005) Fungal pathogens as classical biological control agents for invasive alien weeds – are they a viable concept for Europe? Neobiota 6:165–175

Seier MK, Evans HC (1996) Two fungal pathogens of *Mimosa pigra* var. *pigra* from Mexico: the finishing touch for biological control of this weed in Australia? In: Moran VC, Hoffmann JH (eds) Proceedings of the IX international symposium biological control weeds. Cape Town University Press, Cape Town, pp 87–92

Seier MK, Morin L, van der Merwe M, Evans HC, Romero A (2009) Are the microcyclic rust species *Puccinia melampodii* and *Puccinia xanthii* conspecific? Mycol Res 113:1271–1282

Service RF (2007) A growing threat down on the farm. Science 316:114–117

Shaw D (2008) What chance classical biocontrol of weeds in Europe? Int Pest Control 50:165–168

Shaw RH, Bryner S, Tanner R (2009) The life history and host range of the Japanese knotweed psyllid, *Aphalara itadori* Shinji: potentially the first classical biological weed control agent for the European Union. Biol Control 49:105–113

Shaw RH, Tanner R, Djeddour D, Cortat G (2011) Classical biological control of *Fallopia japonica* in the United Kingdom – lessons for Europe. Weed Res 51:552–558

Sheppard AW, Hill R, DeClerck-Floate RA, McClay A, Olckers T, Quimby PC, Zimmermann HG (2003) A global review of risk-benefit-cost analysis for the introduction of classical biological control agents against weeds: a crisis in the making? Biocontrol News Info 24:91N–108N

Sheppard AW, Shaw RH, Sforza R (2006) Top 20 environmental weeds for classical biological control in Europe: a review of opportunities, regulations and other barriers to adoption. Weed Res 46:93–117

Simberloff D (2004) A rising tide of species and literature: a review of some recent books on biological invasions. Bioscience 54:247–254

Simberloff D (2012) Risk of biological control for conservation purposes. BioControl 57:263–276

Simberloff D, Genovesi P, Pyšek P, Campbell K (2011) Recognizing conservation success. Science 332:419

Steffen W, Grinevald J, Crutzen P, McNeill J (2011) The Anthropocene: conceptual and historical perspectives. Phil Trans R Soc A 369:842–867

Tanner R, Ellison C, Shaw R, Evans H, Gange A (2008) Losing patience with *Impatiens*: are natural enemies the solution? Outlook Pest Manage 19:86–91

TeBeest DO, Templeton GE (1985) Mycoherbicides: progress in the biological control of weeds. Plant Dis 69:6–10

Templeton GE (1982) Biological herbicides: discovery, development, deployment. Weed Sci 30:430–433

Templeton GE, TeBeest DO, Smith RJ (1979) Biological weed control with mycoherbicides. Annu Rev Phytopathol 17:301–310

Tiley GED (2010) Biological flora of the British Isles: *Cirsium arvense* (L.) Scop. J Ecol 98:938–983

Tomley AJ, Evans HC (2004) Establishment of, and preliminary impact studies on, the rust *Maravalia cryptostegiae* of the invasive alien weed, *Cryptostegia grandiflora* in Queensland. Australas Plant Pathol 53:475–484

Trueman M, Atkinson R, Guézou A, Wurm P (2010) Residence time and human-mediated propagule pressure at work in the alien flora of the Galápagos. Biol Invasions 12:3949–3960

Trujillo EE (1985) Biological control of Hamaku pamakani with *Cercosporella* sp. in Hawaii. In: Delfosse ES (ed) Proceedings of the VI international symposium biological control weeds. Agriculture Canada, Ottawa, pp 661–671

Trujillo EE (2005) History and success of plant pathogens for biological control of introduced weeds in Hawaii. Biol Control 33:113–122

Turner PJ, Morin L, Williams DG, Kriticos DJ (2010) Interactions between a leafhopper and rust fungus on the invasive plant *Asparagus asparagoides* in Australia: a case of two agents being better than one for biological control. Biol Control 54:322–330

Van Wilgen BW, de Wit MP, Andersen HJ, Le Maitre DC, Kotze IM, Ndala S, Brown B, Rapholo MB (2004) Costs and benefits of biological control of invasive alien plants: case studies from South Africa. S Afr J Sci 100:113–122

Vartiamäki H, Uotila A, Vasaitis R, Hantula J (2008) Genetic diversity in Nordic and Baltic populations of *Chondrostereum purpureum*: a potential herbicide biocontrol agent. For Pathol 38:381–393

Vince G (2011) Embracing invasives. Science 331:1383–1384

Vogler W, Lindsay A (2002) The impact of the rust fungus *Maravalia cryptostegiae* on three rubbervine (*Cryptostegia grandiflora*) populations in tropical Queensland. In: Jacobs HS, Dodd J, Moore JH (eds) Proceedings of the 13th Australian weeds conference. Western Australian Plant Protection Society, Perth, pp 180–183

Völker K, Boyle C (1994) Bean rust as a model system to evaluate efficiency of teliospore induction, especially in the potential mycoherbicide *Puccinia punctiformis*. Weed Res 34:275–281

Vurro M, Evans HC (2008) Opportunities and constraints for the biological control of weeds in Europe. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 455–462

Waipara NW, Barton J, Smith LA, Harman HM, Winks CJ, Massey B, Wilkie JP, Gianotti AF, Cripps MG (2009) Safety in New Zealand weed biocontrol: a nationwide pathogen survey for impacts on non-target plants. N Z Plant Prot 62:41–49

Wapshere AJ (1974a) A strategy for evaluating the safety of organisms for biological weed control. Ann Appl Biol 77:201–211

Wapshere AJ (1974b) Host specificity of phytophagous organisms and the evolutionary centres of plant genera and subgenera. Entomophaga 19:301–309

Wapshere AJ (1989) A testing sequence for reducing rejection of potential biological control agents for weeds. Ann Appl Biol 114:515–526

Warner KD (2012) Fighting pathophobia: how to construct constructive public engagement with biocontrol for nature without augmenting public fears. BioControl 57:307–317

Warner KD, McNeill J, Getz C (2008) What every biocontrol researcher should know about the public. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the XII international symposium biological control weeds. CAB International, Wallingford, pp 390–394

Weaver MA, Lyn ME, Boyette CD, Hoagland RE (2007) Bioherbicides for weed control. In: Upadhyaya MK, Blackshaw RE (eds) Non-chemical weed management. CAB International, Wallingford, pp 93–110

Weidemann GJ (1991) Host-range testing: safety and science. In: TeBeest DO (ed) Microbial control of weeds. Chapman and Hall, New York, pp 83–96

Wilson CL (1969) Use of plant pathogens in weed control. Annu Rev Phytopathol 7:422–434

Wingfield BD, Ericson L, Szaro T, Burdon JJ (2004) Phylogenetic patterns in the Uredinales. Australas Plant Pathol 33:327–335

Wood AR, Morris MJ (2007) Impact of the gall-forming fungus *Uromycladium tepperianum* on the invasive tree *Acacia saligna* in South Africa: 15 years of monitoring. Biol Control 41:68–77

Woodhead SH (1981) Field efficacy of *Phytophthora palmivora* for control of milkweed vine. Phytopathology 71:913

7 Disease Management of *Phoma* Infections

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CONTENTS

I. Introduction

The imperfect genus *Phoma*, with more than 2,000 species, is traditionally placed into the Sphaeropsidaceae family (Sphaeropsidales). The family is far from being monophyletic, and is characterized by dark-pigmented pycnidia that normally contain an ostiolum. **The genus itself is also not monophyletic** and is, in addition to family features, essentially defined by hyaline conidia that are formed by conidiogenous cells in distinct, dark and thin-walled pycnidia, not grouped on a common stroma. Earlier publications classified these fungi, mainly according to host specificity, into the genera *Phoma*, *Phyllosticta*, and *Ascochyta*. Modern approaches are more consistent, and are based on the morphology of conidiogenesis and on cultural characteristics (van der Aa et al. 1990). Today, the genus *Phoma* is subdivided into the sections Phyllostictoides, Peyronellaea, and Paraphoma (van der Aa et al. 1990; Boerema 1997). Most of the species are saprotrophs, but many species are phytopathogens on a huge variety of plants.

Commercially the most important species is ***Ph. lingam***, being identical with its perfect form ***Leptosphaeria maculans***. This important plant pathogen will be the main topic of this review. It will generally be addressed by its teleomorphic name.

Infections of crucifers, especially of **rapeseed**, with this pathogen are widespread and may cause tremendous losses. The disease caused in *Brassica napus* (**rapeseed** or today increasingly *canola* for *Can*adian *o*il *l*ow *a*cid) is normally called '**blackleg disease**' (*Umfallkrankheit* in Germany). In Germany, crop loss in **rapeseed** cultivation resulting from ***L. maculans*** may amount to 10 dt/ha, with expected yields from healthy fields around 30 dt/ha. In 2009, **worldwide economic loss due to *L. maculans* was estimated at 1,000 million $** (Eckert et al. 2009).

Blackleg disease as a major problem worldwide is increasingly important because of considerable increase in **rapeseed** cultivation. Between the 1950s with approximately 3.5 million tons and today with nearly 60 million tons worldwide, production has increased 17-fold. Virtually all

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B. napus cultivars belong to 00-varieties with low erucic acid and low glucosinolate content, with the exception of a few varieties that have, for industrial purposes, been optimized for high erucic acid. Presently, **rapeseed is on the second rank in oil plants**, making up 13 % of these crops, preceded by soybean with 58 %. In the future, it is assumed that cultivation of *Brassica* will gain even more importance due to increasing demands for fuel oils, especially bio-diesel. The European Community is, with 35 % of worldwide production, the leading region worldwide in **rapeseed** growth, followed by China with 22 % and Canada with 19 %. Australia and the US are countries with increasing efforts in canola cultivation.

As a result of the high agricultural impact of infections by ***L. maculans***, much effort has been invested in understanding the physiology and genetics of the fungus. This review deals predominantly with those biological aspects that are, today or in the future, important for disease management in the field.

II. Relevant *Phoma* Species

Apart from ***Leptosphaeria maculans*** (anamorph: ***Phoma lingam***) there are many *Phoma* species with agricultural relevance, although most of them are believed normally to grow saprotrophically on dead plant material in soil.

Ph. exigua is a species with isolates differing considerably in cultural properties and host specificity, which has been found on more than 200 host species. In addition, these fungi occur on litter and in soil in nearly all parts of the world.

The fungus is a species of the section Phyllostictoides (van der Aa et al. 2000). Despite pronounced differences in cultural characteristics and also of ***a***mplified ***f***ragment ***l***ength ***p***olymorphisms (**AFLPs**) that allowed clear correlation with cultural varieties, **ITS** sequences are nearly identical throughout *Ph. exigua* and do not allow definition of subgroups (Abeln et al. 2002). Even the two distinct species ***Ph. foveata*** and ***Didymella lycopersici*** could not be separated from the ***Ph. exigua*** complex by **ITS** analysis.

Ph. sorghina is a cosmopolitan facultative phytopathogen, included in the Peyronellae section (Aveskamp et al. 2008) with a broad host range, but with special relevance in *sorghum* and pearl millet cultivation.

Didymella holci is most probably the corresponding teleomorph (Pažoutová 2009). Similarly to ***Ph. exigua***, the fungus has a broad host range and shows little genetic variation at the **ITS** sequence level, whereas PCR-based **fingerprinting** allowed differentiation of individual isolates (Pažoutová 2009). Interestingly, in this pathogen, **fingerprinting** has been done with primers derived from bacterial sequences that are meant to target repetitive sequences, **rep-PCR** with the M13 core sequence (Lieckfeldt et al. 1993) and **ERIC**-primers (Versalovic et al. 1991). Sometimes the fungus is also found as an endophyte (Feldman et al. 2008).

Ph. tracheiphila occurs in the Mediterranean region, and is a major threat for all citrus species. The fungus enters its hosts via wounds and presumably stomata, grows systemically, and finally invades xylem vessels, thus interfering with water transport in the plant (Perrotta and Graniti 1988). Some of the symptoms in shoots or leaves are caused also in vitro by injecting a purified glycoprotein isolated from the fungus (Fogliano et al. 1998).

Italian populations of ***Ph. tracheiphila*** isolates are highly identical at the genetic level, behaving essentially identically at the levels of ITS sequences (>98 % identity), **RAPD**- with 12, and **microsatellite fingerprinting** with 7 different primers. (Balmas et al. 2005)

Ph. medicaginis is normally found in soil, but has a tendency to associate with the rhizosphere of legumes and sometimes of herbs and potatoes (Boerema et al. 1965a, b). It is a weak root pathogen that, under normal conditions, does not give rise to severe symptoms or considerable losses.

This species, too, seems to be genetically homogeneous, at least if analyzed at the level of rDNA-**ITS**, although sampling of isolates was geographically more restricted than for the other *Phoma* species mentioned. (Castell-Miller et al. 2008)

Ph. destructiva (Morgan-Jones and Burch 1988a) is found worldwide, causing **tomato black spot disease** on *Lycopersicon esculentum*. ***Ph. lycopersici*** (teleomorph: ***Didymella lycopersici***) is morphologically related to ***Ph. destructiva.*** In phytopathological studies, these two species are often not distinguishable, although some

morphological features, symptoms, and host range can be distinguished. ***Ph. lycopersici*** is considered as a causative agent of tomato **stem canker** and fruit rot; it seems to be restricted to tomatoes (Morgan-Jones and Burch 1988b).

Ph. betae, teleomorph ***Pleospora betae*** or ***P. bjoerlingii***, grows on various *Beta* species and causes losses during storage especially of sugar beets. Germ plasms of sugar beet, resistant to the root rot fungus *Rhizoctonia solani*, also exhibit some resistance against ***Ph. betae*** (Bugbee 1990).

Ph. glomerata isolates are highly variable in culture, and are found frequently and ubiquitously on plant litter and in soil. The fungus causes disease on many host plants, and is also known as a rare opportunistic human pathogen (Boerema et al. 1965a, b). Under wet conditions, it infects wheat and causes severe leaf spots (Hosford 1975).

Pyrethrum (*Tanacetum cinerariifolium*) is grown for producing the pyrethrins, a naturally occurring broad-range insecticide. Besides others, these plants are infected by ***Ph. ligulicola*** (teleomorph: ***Didymella ligulicola***), the typical symptoms being, in particular, necroses in ray florets as well as in stems and leaves (Pethybridge and Hay 2001). Resultant crop loss can be tremendously high, reaching nearly 100 % in some reported cases, and the fungus occurs in the Australian state Tasmania as well as in Kenya, the two main pyrethrum-producing countries (Pethybridge et al. 2004).

With respect to **ITS** sequences, the species is genetically essentially homogeneous, with sequence identities above 99.3 %, thus providing the opportunity to develop species-specific primers for **PCR** diagnosis (Pethybridge et al 2004). The two variants of this species exhibit a somewhat different host range: var. *ligulicola* causes disease especially on chrysanthemums, var. *inoxydablis* is the main pyrethrum pathogen. (Pethybridge et al. 2008)

III. The Causative Agent of Blackleg Disease in *Brassica*

The most important pathogen of rapeseed cultivation is ***Ph. Lingam***, with its teleomorphs ***Leptosphaeria maculans*** and ***L. biglobosa***. The fungus infects *Brassica* crops, and in addition many more cruciferous plants. Early observations claimed infection of plants other than crucifers, but these reports should be regarded with care, since today the species have been delineated with higher stringency. Here we will deal exclusively with the ***maculans/biglobosa* species complex**, two species that are traditionally referred to as isolates, designated virulent or avirulent (McGee and Petrie 1978), aggressive and non-aggressive (Hassan et al. 1991; Koch et al. 1989), tox^+ and tox^0 (Balesdent et al. 1992), or group A and B (Mengistu et al. 1991). These two groups differ at the morphological level by a characteristically formed, clearly visible beak on pseudothecia of non-aggressive isolates (Shoemaker and Brun 2001), the ability to produce the toxin **sirodesmin PL** (Koch et al. 1989), which is exclusively produced by the non-aggressive fungi, and also by various **fingerprinting** techniques (Eckert et al. 2005; Goodwin and Annis 1991; Meyer et al. 1992; Purwantara et al. 2000; Schäfer and Wöstemeyer 1992; Voigt et al. 1998). The differences between aggressive and non-aggressive pathotypes of ***L. maculans*** became evident early during molecular strain typing of fungi (Voigt et al. 2001), together with establishing a new species, ***L. biglobosa***, for the non-aggressive pathotype group (Shoemaker and Brun 2001). The *maculans*/*biglobosa* classification for the two major blackleg fungi was consistently confirmed by **ITS** sequence analysis (Mendes-Pereira et al. 2003). In most of these analyses, ***L. biglobosa*** was revealed as genetically more heterogeneous than ***L. maculans*** (Koch et al. 1989). These observations match with those earlier studies that classify the non-aggressive isolates into different groups, NA1 to NA3, with distinguishable symptoms on plants (Gall et al. 1995; Koch et al. 1989). Clear separation of the *maculans* and *biglobosa* clades, as well as the more pronounced genetic diversity in ***L. biglobosa***, is confirmed at the level of the mating type locus and the protein coding genes for actin and β-tubulin (Voigt et al. 2005). Thorough taxonomic revision of the genus *Phoma* and related genera at morphological and sequence levels revealed that *Didymella* must be regarded exclusively as teleomorph of *Phoma* species *sensu stricto*, whereas the attribution of *Phoma*

lingam alone to either the species *biglobosa* or *maculans* is confirmed (Aveskamp et al. 2010).

IV. Diagnosis

The *Phoma* group is taxonomically complex, with more than 3,000 different fungi at the subgenus level (Monte et al. 1991), approximately half of them being relevant phytopathogens (Aveskamp et al. 2008). In practice, identification of species is not always straightforward because of lack of enough and sufficiently clear morphological characteristics, and the high degree of intraspecific colony variation between isolates in culture, even within species.

A. Conventional Diagnosis

In phytopathology, identification of *Phoma* species often relies more on symptoms on host plants rather than on defined taxonomic characteristics. For understanding functional relationships of the interaction between host and pathogen, and for understanding distribution of pathogens in nature, this practice tends to produce misleading results. For these aspects and for efficient disease management, this approach is far from being sufficient. Only thorough and reliable diagnosis provides a reasonable platform for this purpose. In any case, classical morphological identification in *Phoma* and related genera requires profound expertise and long experience.

In the field, diagnosis normally starts by inspecting the plants for symptoms at early stages of development based on assumptions of the expected pathogen. Special attention is given to the most important rapeseed pathogen, ***Phoma lingam***. Characteristic leaf spotting can often be seen in autumn for winter crops. The lesions are initially small and brown; sometimes they are accompanied by surrounding chlorosis. With some experience, ***L. maculans***, the aggressive *Phoma*-type, and the less aggressive ***L. biglobosa*** can sometimes be differentiated by the development of primary leaf lesions. ***L. maculans*** tends to produce somewhat larger spots that develop pycnidia, whereas ***L. biglobosa*** infections produce smaller and darker spots, with few if any pycnidia (Thürwächter et al. 1999; West et al. 1999). These observations may be accompanied by testing fungal isolates in culture. In most instances, ***L. biglobosa*** isolates germinate with longer germ tubes, grow faster on potato-dextrose agar, and produce yellow to brown pigments on Czapek Dox agar (Kuswinanti et al. 1999; Williams and Fitt 1999).

Good results for identifying isolates of the ***L. maculans*** and **L. biglobosa** groups have been obtained by artificial inoculation of wounded cotyledons of defined, differentiating cultivars. This approach even allows identification of pathotypes within these major pathogenicity groups (Koch et al. 1991; Kutcher et al. 1993). These experiments need, however, several weeks and considerable expertise for interpreting the results.

B. Chemotaxonomy and Enzyme Analysis

Secondary metabolites are useful for differentiating isolates. Sirodesmin PL and related toxins in particular are very helpful for distinguishing aggressive ***L. maculans*** from non-aggressive ***L. biglobosa*** isolates. These compounds can easily be extracted from culture supernatants (Sjödin et al. 1988) and even from infected plants (Pedras and Séguin-Swartz 1990). Identification of the toxins can be done by simple thin-layer chromatography procedures (Sjödin et al. 1988) or by HPLC (Pedras and Biesenthal 2000a), the latter also allowing analysis of additional toxins. These techniques allow very clear and unequivocal discrimination between toxin-producing *maculans* and non-producing *biglobosa* isolates.

A different strain-typing approach relies on measuring isozyme patterns either from cell extracts of the fungi grown in vitro or from culture supernatants. Compared with ***L. biglobosa*, *L. maculans*** shows elevated levels of the extracellular cell-wall-degrading enzymes, cellulase, α- and β-glucanase, and polygalacturonase (Hassan et al. 1991). A similar approach was followed by Annis and Goodwin (1996), who reported on elevated levels of cell-wall-

degrading enzymes in non-aggressive *Phoma* isolates. No evidence was found for correlations between aggressivity and enzyme secretion. The differences between these analyses are probably due to different cultivation conditions. Characteristic isozyme pattern on native gels, characteristic for species or pathogenicity groups, can be generated for several enzymes. In addition, these approaches differentiate between aggressive and non-aggressive isolates. Examples are the intracellular metabolic enzymes glucose phosphate isomerase (Sippell and Hall 1995) or malate dehydrogenase (Hill et al. 1984). On this basis, more detailed results were obtained for the tox^0 ***L. biglobosa*** group, which can be differentiated into pathotypes based on glutamate oxaloacetate transaminase, glucose-6-phosphate-dehdrogenase, and shikimate dehydrogenase (Gall et al. 1995).

C. DNA-Based Diagnosis

It is extremely helpful to make use of the numerous molecular approaches that have been developed over the years. These techniques are typically fast, less time-consuming than enzyme assays or inoculation experiments, and can be performed with reasonable equipment and by personnel with average laboratory expertise. Although we are far from being able to classify any given *Phoma* species by simple and reliable **fingerprinting** techniques, the major pathogens can readily be identified with comparably low effort and reasonable technical expertise. For ***Ph. Lingam*** in particular, these approaches have been driven to a level that can be used for differentiating the aggressive pathotypes, now ***L. maculans*** *sensu strict*, and the considerably less non-aggressive pathotypes, now ***L. biglobosa***. Even differentiation of individual pathotypes within these groups is possible by **fingerprinting**; the technique used decides on analytical depth between the level of genera, species, pathotypes, or individual isolates. Fungi, at all levels of genomic organization between karyotype and **microsatellite** distribution, are generally highly variable; all analytical intentions can normally be fulfilled.

Especially attractive are procedures working independently of sequence information. Most of these target repetitive DNA in the fungal genomes. Positions and genetic context of such elements in genomes are highly diverse even between isolates, but the repeated sequences themselves are sufficiently conserved to be found by simple **PCR**- or hybridization assays.

1. Electrophoretic Karyotype

In ***L. maculans***, karyotype patterns obtained by **pulsed-field gel electrophoresis** (Howlett 1997) discriminate clearly between the *maculans* and the *globosa* group, and reveal minor size polymorphisms also within these groups, especially between non-aggressive isolates (Taylor et al 1991; Voigt et al. 2001). Different strains are sufficiently heterogeneous to establish genetic maps after going through meiosis (Kuhn et al. 2006). This technique can be used for identifying isolates, but the advantages reside more in the potential to ascribe genes to individual chromosomes by hybridization than in identifying field isolates.

In principle, any **fingerprinting** technique can be used for diagnosis of *Phoma* species, and nearly all of them have been employed for differentiation of the *L. maculans/biglobosa* complex, with different aims and for different levels of resolution.

2. Hybridization Techniques

Restriction fragment length polymorphism (**RFLP**) is the oldest technique for studying genetic diversity at the molecular level. It relies on specific detection of defined genetic elements by hybridization with a labelled complementary probe in Southern-type experiments. Differences between isolates are revealed by size polymorphisms of these targets due to differential occurrence and distribution of recognition sites for restriction endonucleases. This technique works nicely for differentiation of the *L. maculans*/*L. biglobosa* groups. It can be performed with many individual clones from genomic libraries that show polymorphism;

these hybridization probes must not necessarily be identified at the functional or sequence levels (Koch et al. 1991). The clear separation by **RFLP** patterns between the two groups, and the absence of intermediate patterns, provided indications for compatibility between these organisms and led to early assumptions of a two-species concept for the major pathogenicity groups of ***Ph. lingam*** (Johnson and Lewis 1990).

A different approach uses generalized hybridization probes that aim at universal repetitive targets. This has the advantage that polymorphism studies can be started without cloning and with standard hybridization probes. Distinction between ***L. maculans*** and ***L. biglobosa*** was achieved by using synthetic **microsatellites** as probes (Meyer et al. 1992).

3. PCR Techniques

Whereas the reliability of **RFLP** approaches is high, it still requires considerable experimental effort for DNA preparation, Southern blotting, and hybridization that can be minimized by **PCR**-based approaches. The **microsatellite**-dependent fingerprinting approach can easily be converted into reliable **PCR**-assays by applying di-, tri- or tetranucleotide repeats of the microsatellite. This convenient technique has, to my knowledge, not been established for ***L. maculans***, but has proven its value for strain typing in ***Ascochyta rabiei*** (teleomorph: *Didymella rabiei*; Bayraktar et al. 2007), a fungus phylogenetically related to the *Phoma* group.

A combination of **RFLP** and **PCR**, amplified fragment length polymorphism (**AFLP**) combines the advantages of restriction analysis and the polymerase chain reaction (**PCR**). Several variations of this approach are published. For strain typing of ***L. maculans***, restriction fragments were linked to adapters, the sequence of which served as binding site for the **PCR**. This technique has a high resolution and has been used for epidemiological studies; the band patterns were also suitable for recognizing pathotypes (Pongam et al. 1999). An **AFLP** approach for strain typing has been used in the ***Ph. exigua*** vicinity, too. Resolution proved to be much higher than by sequencing **ITS** regions (Abeln et al. 2002).

A conceptually different high-resolution fingerprint technique is **RAPD**-PCR (random-primed polymorphism of DNA). The basis is to use very low annealing temperatures around 37 °C, which renders the reaction essentially independent of primer length. Normally, oligonucleotides between 9 and 12 nucleotides are employed, but in principle any primer will do, regardless of length and sequence. This approach offers the advantage that no sequence information about the target DNA is required. It has, however, the intrinsic disadvantage of high requirements for maintaining salt concentration and exact annealing temperature in order to obtain highly reproducible patterns. **RAPD**-PCR has successfully been used to unequivocally distinguish ***L. maculans*** from ***L. biglobosa*** isolates (Goodwin and Annis 1991; Schäfer and Wöstemeyer 1992).

Very often, in 16 out of 20 cases, diagnostic **RAPD** bands proved to be specific for the taxon analyzed, also at the sequence level. It is thus possible to construct hybridization probes or longer and, thus, target-specific **PCR**-primers based on sequenced **RAPD** fragments (Voigt and Wöstemeyer 1995). This technique has been employed for identifying *Brassica* pathogens other than ***Ph. lingam***, especially those that sometimes are found in mixed infections. (Schleier et al. 1997)

Very similar molecular approaches have been adapted to recognition, strain typing, and population studies in ***Ph. tracheiphila***, the causal agent of ***mal secco*** **disease** and a very important pathogen in *Citrus* cultivation (Balmas et al. 2005; Licciardello 2006). **PCR** analysis in this pathogen has been extended to direct diagnosis with DNA from infected plants (Ezra et al. 2007) and can also be performed in a quantitative way by adapting real-time **PCR** to this problem (Demontis et al. 2008).

V. Infection Pathway

Winter oilseed rape (*B. napus*) stands in the field for the major part of the year, thus offering many opportunities for pathogens to invade the

plant. Because ***L. maculans*** shows different symptom complexes, primarily leaf lesions in autumn and winter, and **stem canker** in spring and summer, it was especially attractive and necessary to understand the infection pathway and spreading of mycelium in the plant. Both symptoms go normally back to the infection of the leaves in autumn, and are not due to independent events.

The typical infection pathway has been elucidated by microscopy of artificially infected plants under controlled conditions (Hammond et al. 1985). Leaves can be inoculated either by pycnidiospores or by ascospores. After germination, hyphae grow on the leaves' surface, and invade the plants via stomata or lesions. The mycelium spreads between the epidermis and palisade layer without causing cell or tissue damage. After entering the spongy mesophyll first, necrotic effects are seen behind the hyphal front. These are limited in size, and do not follow the spread of mycelium in the plant. ***L. maculans*** grows systemically into the plant by following intracellular spaces around veins and by entering xylem vessels. During this phase, where infection becomes systemical, essentially no symptoms are seen and the fungus behaves as a biotroph. **Only after reaching the stem after following the petiole, the lifestyle of the fungus changes to necrotrophy.** Along the stem, and later on, especially in the hypocotyl region, hyphae leave the xylem vessel and colonize the surrounding tissue. This stage is highly necrotic and corresponds to the **stem canker** observed in the field (Hammond and Lewis 1986). At this developmental stage, the heterothallic ***L. maculans*** enters into sexual reproduction, in cases where the plant is infected by complementary mating types. This process is normally completed on the stubble, where the fungus grows saprotrophically anyhow. The ascospores, differentiated in bitunicate asci within typical pseudothecia, are the major inoculum for subsequent infection cycles.

In leaves of *B. napus* var. *oleifera*, the establishment of systemic infections depends on the age of the leaves, which tend to become more resistant with age (Hammond and Lewis 1987). The infection pathway is confirmed by more sophisticated studies that make use of genetically manipulated ***L. maculans***, expressing green fluorescent protein (GFP) under control of the promoter for the putative pathogenicity gene *Cht* encoding cyanide hydratase (Sexton and Howlett 2001). This approach conveniently makes it possible to follow hyphal growth at very early stages of infection and in xylem vessels containing only limited amounts of fungal biomass, and especially in necrotic areas of the stem, where the tissue becomes brown.

L. maculans is known to invade plant roots. Its appearance in roots coincides with flowering of host plants (Sprague et al. 2007). By using a genetically labelled fungus that expresses a translational fusion of **GFP** and a gene with strong similarity to an alcohol dehydrogenase, it became possible to follow fungal growth in roots also. At the onset of flowering ***L. maculans*** may grow through the hypocotyl into roots. It has also been shown that intact roots can be infected from outside, without formation of infection structures, by growing through an appropriate layer of inoculum (Sprague et al. 2007). A field study in Australia that included fields without **rapeseed** cultivation in the past, confirmed the hypothesis that most root rot infestations are the consequence of primary infections on leaves. The situation may be different in fields with remnant stubbles harboring ascospores (Sprague et al. 2009).

Infection pathways in *Phoma* species other than ***Ph. lingam*** are less clear. Many species share penetration without the formation of appressoria, examples being ***Ph. macdonaldii*** (Roustaee et al. 2000), a pathogen of *Helianthus annuus*, or ***Ph. narcissi***, a leaf pathogen of *Hippeastrum* plants (Saniewska and Dyki 1997). ***Ph. exigua***, however, infects via specialized appressoria, and ***Ph. clematidina*** exhibits several penetration styles between direct penetration and cushion-shaped appressoria. (Van de Graaf et al. 2002)

VI. Disease and Virulence Factors

Virulence factors are discussed in phytopathology and medical microbiology, since we have tried to understand functional relationships between disease and individual properties of pathogens, be they structural elements, enzymes, or other proteins, or metabolites such as toxins or signalling molecules. Sometimes functional relations seem obvious. However, if studied in

detail, it can be seen that many assumptions on the importance of putative virulence factors for disease manifestation have not passed stringent experimental scrutiny. Many functions are redundant in pathogens, and their inactivation by mutagenesis does not necessarily lead to the expected phenotype. It thus remains difficult to describe the contribution of single factors to initiation and development of disease. In addition, if studied in detail, **most of the properties normally ascribed to pathogenicity by intuition, can also be found in fungi growing as saprotrophs**. It is nevertheless worthwhile to study the contribution of individual putative virulence factors to growth on plants, and to consider their suitability as targets for pathogen control approaches.

A. Sirodesmins

Sirodesmins are the most important toxins synthesized and secreted by *L. maculans*. Their contribution to disease symptoms is not totally clear, but it seems to be generally accepted that they contribute considerably to later, necrotic stages of the fungal lifestyle in the plant. The control of their synthesis could be one possible target to influence disease development and severity. It is therefore necessary to consider synthesis, genetics, and biological consequences of this putative virulence factor in more depth.

1. Biosynthesis

Already in early studies on the reasons for pathogenicity of ***Ph. lingam***, a toxin with low specificity with respect to the organisms affected was recognized and described as a substance belonging to the group of epipolythiodioxopiperazines, **sirodesmin PL** (Férézou et al. 1977). The name goes back to structurally very similar toxins, isolated **from *Sirodesmium diversum*** (Curtis et al. 1977), a fungus belonging to the Dothideomycetes like ***L. maculans***, but to a different order. The toxin can easily be extracted by chloroform extraction and methanol precipitation from culture supernatants of *L. maculans sensu stricto*. Detailed analysis of culture supernatants by thin-layer chromatography provides evidence for at least eight chemically similar substances that differ in the number of acetyl groups and the number of sulphur atoms in the epipolythio chain (Badawy and Hoppe 1989a).

Chemically related substances are also known as secondary metabolites in other fungi. A well-known compound studied in depth is gliotoxin, secreted by the opportunistic human pathogen ***Aspergillus fumigatus*** and other fungi (Scharf et al. 2012). Gliotoxin exerts apoptotic as well as necrotic effects and inhibits NF-κB activation.

Labelling in culture with putative ^{13}C- and ^{14}C-precursors led to the conclusion that **sirodesmin** synthesis depends on acetate metabolism. The tetrahydrofuranone ring of **sirodesmin PL** comes from mevalonic acid, whereas the cyclopentenylpyrrolidine part of the molecule depends on the amino acids serine and tyrosine (Férézou et al. 1980b). In analogy to the gliotoxin situation (Bose et al. 1968), introducing the two amino acids involved was assumed to be the consequence of a **non-ribosomal peptide synthetase** (Gardiner et al. 2004a).

2. The Sirodesmin Gene Cluster

Identifying and cloning the genes for biosynthesis of the epidithiodioxopiperazine **sirodesmin PL**, and indeed for the first member of this group ever, was based on establishing a reasonable but hypothetical biosynthetic pathway, together with the assumption that the genes involved should be clustered in the genome, analogous to some other prominent secondary metabolites (Rosewich and Kistler 2000). By this approach, a cluster of open reading frames containing 18 genes assumed to be involved in **sirodesmin** synthesis was identified on merely 68 kb of the genome (Gardiner et al 2004a). All postulated activities, e.g., the **non-ribosomal peptide synthetase** (*sirP*), a prenyl transferase (*sirD*), a thioredoxin reductase (*sirT*), and an acetyltransferase (*sirH*) were identified based on sequence similarities. The identity of the cluster is independently proven by constructing a gene disruption mutant in the peptide

synthetase gene. These mutants fail to secrete **sirodesmin**. In addition, all of these genes show the same regulation pattern, which parallels **sirodesmin** formation.

One of the genes (*sirA*) in the **sirodesmin** cluster encodes an ABC type transporter (Gardiner et al. 2004b). Mutations in this gene increase transcription of the **non-ribosomal peptide–synthetase** and, consequently, the amount of sirodesmin in the medium. Thus, this transporter is not responsible for secreting the toxin. On the other hand, the mutant is considerably more sensitive to its own toxin. As the transporter gene is transcribed at high levels even after down-regulation of the biosynthetic genes, the most probable role of the transporter is getting along with sirodesmin re-entering the cell from outside (Gardiner et al. 2004b).

A very early step, immediately preceding the peptide synthetase reaction in **sirodesmin** biosynthesis, is the *O*-prenylation of tyrosine with dimethylallyldiphosphate to dimethylallyl–tyrosine (Gardiner et al. 2004a; Kremer and Li 2010). This step is catalyzed by the *sirD* gene product that has been analysed in detail after over-expressing the gene in *Escherichia coli* and purifying the protein. The enzyme acts as a homodimer and is able, in the reaction with tryptophan, to catalyze formation of C–C bonds in addition to C–O prenylation necessary for producing **sirodesmin**. The K_m-value is, however, lower for the natural substrate tyrosine (Kremer and Li 2010). If compared with other prenyltransferases, the sirD protein shows unexpectedly high substrate flexibility (Zou et al 2011). It has not been elucidated whether this prenylase has additional targets other than the sirodesmins in ***L. maculans***.

The **sirodesmin** biosynthesis gene cluster is largely regulated by a transcription factor gene (*sirZ*) that is part of the cluster itself (Fox et al. 2008). Down-regulation of the gene product by RNA-mediated silencing reduces **sirodesmin** levels considerably, and directly affects transcription of the investigated genes directly involved in biosynthesis. Other phenotypes are not affected by these genetic manipulations at the expression level.

Apart from the *sirZ* transcription factor, three additional genes controlling **sirodesmin** synthesis were identified by screening 200 independent T-DNA insertion mutants, two of them being affected in transcription factor genes by sequence similarity. The most interesting **sirodesmin** control gene turned out to be a homologue of the cross-pathway control system, *cpcA*, from ***Aspergillus fumigatus***. In *A. fumigatus*, and probably in other fungi, this transcription factor is responsible for reacting to amino acid availability in the environment. Artificially induced amino acid starvation does not affect the **sirodesmin** pathway in the wild type, whereas RNA silencing of *cpcA* under starvation conditions stimulates transcription of the *sirZ* regulator gene and also of the peptide synthetase gene *sirP*, and leads to increased **sirodesmin** secretion (Elliott et al. 2011).

3. Biological Effects

If **sirodesmin PL** is applied to *Brassica* leaves or cotyledons, the plant reacts with chlorosis and collapsing lesions of the tissue. Apart from this phenotypic effect, application of the toxin at micromolar concentrations inhibits RNA synthesis (Rouxel et al. 1988). Most of the toxicity is due to the disulphate bridge spanning the pyrrolidine ring. The argument is based on Zn^{2+}, Hg^{2+}, and Cd^{2+} ions being able to titrate toxicity in vitro (Rouxel et al. 1988) and partially in vivo too (Rouxel et al. 1990). This points towards a toxicity mechanism via formation of disulphides between proteins and **sirodesmin**, and is probably the basis for antiviral activity and inhibition of RNA synthesis.

The major and very basic method of action of epidithiodioxopiperazine derivatives is most probably due to the property of thiols to undergo redox cycling initiated by glutathione and other reductive substances in the cell. The resulting disulphate is rapidly oxidized again, while producing considerable amounts of reactive oxygen molecules, especially superoxide ions and hydroxyl radicals. (Munday 1989)

Sirodesmin PL is also found in infected hypocotyls, and leads to various toxic effects

in various plants and other organisms, but its causative relationship with the aggressivity of ***Ph. lingam*** has been questioned from the beginning. Already shortly after discovery, nitrosoguanidine-induced toxin-deficient mutants with highly reduced amounts of toxin were analyzed with respect to their ability to induce necroses. No correlation between aggressivity and the amount of secreted toxin was established (Boudart 1978). In an independent study with many more UV-induced mutants, derived from a defined single-spore isolate of an aggressive **sirodesmin** producer, these early observations have been substantiated and refined (Sock and Hoppe 1999). Such mutants are found in amazingly high frequency around 10^{-3}, and reduce toxin production between 100- and 1,000-fold. These mutants behave like the wild type in cotyledon inoculation tests, lead to identical tissue collapses in leaves, and are not affected in pycnidium and ascus formation. However, symptoms at the stem base are considerably reduced, providing **strong evidence for the sirodesmins as important virulence factors during the necrotic growth stage**. These early results are supported by the phenotype of a defined mutant that had been disrupted in the *sirP* gene for the peptide synthetase (Elliott et al. 2007). In infection experiments, **sirodesmin** is clearly recognized as an agent contributing to aggressivity in the stem.

Other studies with more and different fungal isolates led to contradictory results, stating a direct correlation between phytotoxicity of culture filtrates and aggressivity of the corresponding isolates in cotyledon and leaf-infection experiments (De March et al. 1986). Some of the difficulties in evaluating these primary studies results from defining A- and NA-type fungal isolates as belonging to a single species. Thus, the results are often interpreted in the light of regarding the non-aggressive ***L. biglobosa*** strains more or less as naturally occurring toxin-deficient varieties of the same species.

While essentially all cells are sensitive towards **sirodesmin PL**, some specificity has been observed in experiments with regenerating protoplasts and cells of susceptible plants and non-hosts. Protoplasts seem generally to be highly sensitive, regardless of the plant species. With increasing age of cells and tissues regenerated from protoplasts, *Brassica* host cells retain sensitivity, whereas comparable assays with cells from *Solanum tuberosum* and *Nicotiona tabacum* acquire some resistance (Sjödin et al. 1988; Sjödin and Glimelius 1989a).

Toxicity of sirodesmins is not restricted to plants. Toxic effects have been found for all organismic groups. Even at the time of detailed chemical description, their antiviral properties were recognized (Curtis et al. 1977). They also inhibit bacterial growth, an observation that offers the possibility for convenient screening of non-producing mutants, simply by screening for the lack of inhibition zones around such mutants. Like in plants, the toxic character of the compound depends on the presence of the epi-disulphide bridge (Boudart 1989). Sirodesmin also affects fungi (Poiret et al. 1985) as well as rats and mice (Bousquet et al. 1977).

The low degree of selectivity of the **sirodesmins** leads to the assumption that secretion of **sirodesmins** by ***L. maculans*** in planta is regulated. During the biotrophic stage of the pathogen in the leaves, toxin secretion should be low, whereas later on, during the necrotrophic stage, especially during canker formation, **sirodesmin** is secreted in high amounts and is one of the virulence factors contributing to tissue damage in the plants. Measurements of **sirodesmin** concentrations in infected leaves and in **stem cankers** confirm this hypothesis. Cotyledons contain around 5 $\mu g\ ml^{-1}\ g^{-1}$ fresh weight, whereas the concentration is 10 times higher in **stem cankers** (Sock and Hoppe 1999), a concentration sufficient to exhibit phytotoxicity, which has been observed at concentrations above 10–20 $\mu g\ ml^{-1}$ (Badawy and Hoppe 1989b). A later study, based on HPLC analysis of leaf and stem extracts confirms the presence of **sirodesmin** in infected plants (Elliott et al. 2007). It is, however, not trivial to measure the amount of toxin produced by a defined amount of mycelium. Until now, this has not been done. The genetic basis for regulating **sirodesmin** production, if there is any, is also not known. In any case, the different **sirodesmin** levels in the plant are related to light. Sirodesmins are absent from plants kept with normal light/dark cycles, and need 4 days in the dark in order to

be found (Sock et al. 1995; Sock and Hoppe 1999). It is not clear if this difference is because of mere physical factors or based on genetic regulation. It will be rewarding to analyze gene expression and **sirodesmin** formation directly in planta during the infection cycle. Already to date, the described regulatory factors (Fox et al. 2008; Elliott et al. 2011), identified in axenic fungal cultures under in vitro conditions, open this possibility. More regulatory principles will most certainly be detected.

B. Additional Toxins

In addition to **sirodesmins**, ***Ph. lingam*** strains synthesize and secrete a bunch of additional secondary metabolites with, and sometimes without, approved toxicity. In any case, these substances may play roles in communication between plant and pathogen, and therefore are interesting with respect to understanding interaction between partners and, as a consequence, for breeding plants that are not recognized as potential hosts by these pathogens.

Shortly after describing **sirodesmin** and deacetylsirodesmin, **phomamide**, a related piperazine-2,5-dione was reported (Férézou et al. 1980a). This substance, assumed to be a **sirodesmin** precursor, is not phytotoxic. Biologically more interesting is the branched, unsaturated, and polyfunctional long-chain carboxylic acid phomenoic acid and the corresponding intracellular ester phomenoic acid δ-lactone (Devys et al. 1984, 1986).

These substances are synthesized late during growth in liquid culture, and can be extracted with organic solvents from the mycelium. Antifungal and antibacterial activities are reported. Published data on biological activity are, however, scarce, and the relevance of the substance during infection or maybe for competing with other microorganisms in planta has not been investigated.

Phomalirazine, an untypical epidithiopiperazine (Pedras et al. 1989), was also isolated from ***Ph. lingam*** cultures. Toxicity in cotyledon tests was shown to be approximately 20 times higher than for **sirodesmin**, and pollen of *Brassica juncea* in particular is cited to be highly sensitive to **phomalirazine** (Howlett et al. 2001).

A biologically very interesting toxin, produced by ***L. maculans***, is the depsipeptide **phomalide**. The substance is down-regulated by **sirodesmin**, and among the other toxins it is the only one that exhibits species or cultivar selectivity. A cultivar of brown mustard, *Brassica juncea*, resistant to ***L. maculans***, is much less affected by the toxin in vitro than a susceptible *B. napus* cultivar (Pedras and Biesenthal 2000b). This specificity may open reasonable possibilities for direct resistance screening breeding programs.

Occasionally, however, relationships between fungi, their toxins, and host plants are unexpected. **Maculansin A**, an unusual branched mannitol derivative from a highly virulent ***L. maculans*** isolate, behaves more toxically towards a resistant *B. juncea* plant than to a susceptible *B. napus*. (Pedras and Yu 2008)

While the epidithiopiperazine substances referred to are characteristic for aggressive strains of ***Ph. lingam***, the non-aggressive isolates, now ***L. biglobosa***, are chemically characterized by the completely different polyketides **phomaligol** and **phomaligadione**. These compounds supported the view of distinct species for both isolate groups (Pedras et al. 1993a). The biological activity of these compounds in communication between the fungus and hosts plants is not known. This also holds true for the substances **phomaligin** (Pedras et al. 1995) and the **phomapyrones** (Pedras et al. 1993b). No substances toxic for *Brassica* leaves have been found in these non-aggressive isolates; they should however be analyzed with regard to signal character in the communication between plant and fungus.

C. Exoenzymes

Nearly all fungi live at least partially on substances that they produce extracellularly by degradation of polymers with exoenzymes. In this respect, **there are no obvious differences between phytopathogens and fungi living saprotrophically on plant material**. It is, however,

less clear to which degree exoenzymes, in particular cell-wall-degrading enzymes, contribute to the infection process and to colonisation of the host plant, and thus have to be seen as pathogenicity or virulence factors. Probably these questions have to be answered individually for host/pathogen associations and, of course, for individual enzyme specificities.

For the *Phoma* species, very little has been done in this respect, although ideas about such enzymes as important factors involved in disease establishment were published early for the peppermint (*Mentha piperita*) pathogen *Ph. strasseri* (Melouk and Horner 1972). The authors found pectinolytic activity and an enzymatic complex leading to maceration either of potato or of peppermint rhizome tissue in culture filtrates, as well as in infected peppermint rhizomes.

L. maculans secretes many different cell wall degrading enzymes in liquid culture, several of which could also be measured in **stem canker** lesions. Polygalacturonase and pectate lyase are induced by cultivation on *Brassica* cell wall. Polygalacturonase and α-arabinosidase were found in **stem cankers**, whereas pectate lyase, a major activity in culture, was not measured in vivo. These results were interpreted as hints towards involvement of these enzymes in early stages of canker lesions, whereas secretion of sufficiently high activities during the biotrophic phase is regarded as improbable (Easton and Rossall 1985).

In addition, α- and β-glucanases and cellulases were found in liquid cultures of *L. maculans* (Hassan et al. 1991), as well as xylanase, β-galactosidase, and xylosidase (Annis and Goodwin 1996). In these studies that compare ***L. maculans*** with ***L. biglobosa*** isolates, **aggressivity does not necessarily correlate with the amount of enzyme activity secreted**. Interestingly, infected plants seem to regulate the activity of secreted cell-wall lytic enzymes. Substances inhibiting fungal polygalacturonases were extracted from *Brassica* stem regions. The inhibitory activity correlates with resistance of the tested cultivars, measured as the ability to increase the size of the lesions (Annis and Goodwin 1997), a phenomenon strongly suggesting participation of polygalacturonases in disease development. A similar observation has been made in sugar beets infected with ***Ph. betae*** (Bugbee 1993). In this interaction, the plant reacts by secreting a protein inhibiting fungal pectin lyase. The amount of inhibitor correlates with the degree of resistance also in this system.

More details at the expression level have been obtained for an endopolygalacturonase (*pg1*) and two cellobiohydrolase (*cel1*, *cel2*) genes from ***L. maculans*** (Sexton et al. 2000). *pg1* and *cel1* are induced by their substrates in culture; *cel1* but not *pg1* is catabolite-repressed by glucose. *cel2* is the only gene of which transcripts have been detected by **RT-PCR** even in infected cotyledons of *B. napus* and *B. juncea*. Transcription in stem canker regions was not measured, and nothing is known with respect to protein expression and activity in planta.

Another class of extracellular enzymes possibly affecting the integrity of plant cells are lipases. Many fungi secrete such enzymes, including ***Ph. glomerata***, a saprotrophic and parasitic species with low host specificity. Lipolytic activity has been characterized in some detail at the biochemical level (Pollero et al. 1997, 2001), but the relevance for pathogenicity has not been investigated in any plant pathogen.

D. Other Putative Virulence Factors

It is far from trivial to define the terms pathogenicity or virulence factor or to identify the corresponding genes. Mostly, these terms are used for functions that are necessary for causing disease and for improving severity of infection and symptoms. Sometimes it is helpful to exclude those functions that are important only in planta and not for growth in axenic culture, but in the end it will probably never be possible to discriminate strictly between these only seemingly unrelated lifestyles. The fuzzy border between the lifestyles becomes immediately evident when shotgun approaches are used to detect novel prerequisites for pathogenicity or virulence.

Convenient approaches make use of random insertion techniques for mutagenesis. Random integration of a cassette conferring hygromycin resistance to transformants revealed that more than 2 % of all ***L. maculans***

mutants are affected in pathogenicity in cotyledon infection tests. A very interesting mutant depicts a primarily **unexpected relationship between fatty acid metabolism and pathogenicity**. Deleting the gene for isocitrate lyase destroys the ability to use fatty acids as carbon source, shows different growth and branching patterns already on cotyledon surfaces, and produces less severe lesions (Idnurm and Howlett 2002). Further analysis of the mutant led to the assumption that the peroxisome-localized glyoxalate pathway is essential for infection. In this respect, the *Leptosphaeria* situation resembles those of *Candida albicans* and other human pathogens (Brock 2009).

A similar experimental approach, based on random T-DNA insertion, allowed the identification of a gene from ***L. maculans*** responsible for the biosynthesis of the glycosylphosphatidylinositol membrane anchor (Remy et al. 2008b). The product of this gene, *Lmgpi15*, is normally an integrated constituent of the endoplasmatic reticulum. While primary infection of cotyledons is not impaired in the mutant, invasive growth is severely affected, and colonization and stem lesions are very rare events. In culture, growth rate is reduced, and hyphal appearance differs from the wild type. A second pathogenicity gene, *Lmpma1*, identified by the same approach, codes for a membrane-bound ATPase. The corresponding mutant carries the T-DNA insertion in the promoter region of the gene, and is down-regulated two-fold in transcription. At the phenotype level, it is unable to germinate on cotyledons, and thus completely unable to infect. Viability of the spores is not affected, even after exposure to the plant surface. The reduced expression level of the H^+-ATPase protein is believed to interfere with the ability to grow under the severely hypotonic and primarily nutrient-limited conditions on the leaf surface (Remy et al. 2008b).

The intimate connection between primary metabolism and pathogenicity is emphasized by the analysis of a ***L. maculans*** mutant affected in the gene for UDP-glucose-4-epimerase (Remy et al. 2009). The enzyme mediates the connection between galactose and glucose metabolism. Whereas cotyledon infection is still possible, the mutant will not grow systemically in the plant, and cannot switch to necrotrophic growth. The mutant is also not able to degrade host cell walls substantially, but in contrast to the inability to grow on the polysaccharide xylan, pectin degradation and growth on this substance in culture is still possible.

VII. *Phoma* Genetics

Traditionally, very little has been done at the genetic level in the imperfect genus *Phoma*. Even after recognition of ascospores as the natural major inoculum (Venn 1979) of the most important sexual fungus in this group, the heterothallic species ***L. maculans***, research projects were aimed mainly at immediate phytopathological problems rather than at introducing the fungus into fundamental genetic research in order to understand the interaction between host and fungus. Although it was shown early that, despite high intraspecies variability, many isolates from distant geographical locations can be crossed and form pseudothecia and fertile ascospores deliberately in culture (Pertrie and Lewis 1985), genetic analysis had to wait for later molecular approaches. Today, the sexual system of the fungus is studied in some detail. At the genomic level ***L. maculans*** matches essentially the idiomorph type of mating type locus, typical for other ascomycetes (Cozijnsen and Howlett 2003; Voigt et al. 2005). We see today that detailed genetic and genomic studies provide opportunities for the development of novel strategies in disease management.

L. maculans **has a genome size of approximately 45 Mbp** (Rouxel et al. 2011), distributed over 15–16 chromosomes (Howlett 2004) with sizes between 0.7 and 3.5 Mbp, which is a normal picture for filamentous ascomycetes. Some strains have been shown to contain additional linear plasmids (Hassan et al. 1991; Lim and Howlett 1994). Even before the era of sequencing complete genomes, repetitive elements were cloned and characterized. A long element of more than 5 kbp was isolated from ***L. maculans***, and found to reside on every chromosome with a total copy number around 80 (Taylor and Borgmann 1994). This element, *LmR1*, is unusual, because despite its length no transcripts and indeed no open reading frames

were found. The element was found in all isolates, independent of geographic origin, pathogenicity group or mating type, and is thus suitable to serve as a species-specific marker. Species from the ***L. biglobosa*** complex do not contain this DNA. The single exception is interpreted as a **rare transfer event from the aggressive *L. maculans* to the non-aggressive *L. biglobosa* group**. Independent proof for this interesting interpretation, based on additional sequences, is however not provided.

Another **minisatellite** but single-locus type of element was found to occur in six alleles differing by the number of tandem repeats. It is found in a region rich in various **microsatellites**, and carries 6 bp direct repeats at the ends (Attard et al. 2001; Wöstemeyer and Kreibich 2002). Species-specific elements of this multi-allelic type may be valuable for epidemiological screening.

Analysis and annotation of the complete genome sequence revealed several unexpected features of ***L. maculans*** (Rouxel et al. 2011). Compared with other ascomycetes, ***L. maculans*** has an unusually high content of repeated sequence, around 30 %. Their sequence characterizes them as transposon-like elements, although they are inactivated by deletions, and only very few seem to be transcribed. Comparing the sequences within repeat families reveals frequent C-to-T and G-to-A changes, indicative of repeat-induced point mutations (RIP). At the genetic level, ***L. maculans*** has been shown before to undergo RIP events (Idnurm and Howlett 2003). Interestingly, and in contrast to the general expectation, the repeated rDNA units also appear as ripped. A novelty for fungal genomes is the pronounced isochore character of the genome, with clear separation of GC-and AT-enriched blocks, the latter harboring genes interpreted as effectors for communication between parasite and host, including the known **avirulence genes**.

As protoplast preparation is feasible and chromosomes can easily be separated, many genes and other useful genetic markers have been physically mapped by blotting techniques (Kuhn et al. 2006; Plummer and Howlett 1995). Today, physical mapping on gels, genetic crosses, and tetrad analysis are complementary techniques that permitted mapping of several genes including **avirulence genes** (Attard et al. 2002; Ghanbarnia et al. 2012). Genetic transformation of ***L. maculans*** by plasmids conferring hygromycin or phleomycin resistance was established early (Farman and Oliver 1988, 1992), and form the experimental basis for providing conclusive evidence for putative pathogenicity genes (Remy et al. 2008a, b). These basic techniques were supplemented by constructing convenient vectors, including genes for fluorescent proteins that made it possible to follow mycelia even during growth in planta (Sexton and Howlett 2001).

VIII. Avirulence and Effector Genes

Those determinants modulating the aggressivity of pathogens on their hosts are normally addressed as virulence factors. These play a role once the interaction between host and pathogen has been started at the level of recognition. This very important level that decides on infection or resistance is mediated by highly specific gene products, normally referred to as ***avirulence genes*** in the fungal partner. Ideally, recognition and consequently defence response follows the classical **gene-for-gene model** between fungal avirulence and cognate plant resistance genes.

Specificity at the level of recognition was recognized between different *Brassica* cultivars and distinguishable ***L. maculans*** genotypes, characterized by **RAPD**-PCR polymorphisms. Using these molecular markers in crosses made it possible to identify and map **the first avirulence gene for *L. maculans*, *AvrLm1***, controlling host-range specificity for a defined *Brassica* cultivar (Ansan-Melayah et al. 1995). Additional *Avr* genes followed, including identification of corresponding specific resistance or *Rlm* genes in individual *Brassica* cultivars (Ansan-Melayah et al. 1998; Balesdent et al. 2001, 2002, 2005). Taken together, nine genetically identified *Avr* genes were identified and mapped to four separate loci (Gout et al. 2006), many of them being polymorphic between isolates (Balesdent et al. 2006). *AvrLm7* is recognized by plants containing either *Rlm4* or *Rlm7*, and consequently was renamed as *Avr4-7* (Parlange et al. 2009). The gene encodes

a small secreted protein, rich in cysteine, and according to expectation for a recognition molecule, is expressed early during infection. Apart from acting as effector molecule, the *AvrLm4-7* product behaves additionally as a virulence factor. This interpretation is based on the observation that strains containing the wild-type allele are more aggressive than variants without (Parlange et al. 2009).

Other *Avr*-products look similar: Cloning and sequencing of *AvrLm1*, primarily by a map-based approach (Attard et al. 2002), revealed the gene product as a small protein that is expressed early during infection and secreted into the apoplast (Rouxel et al. 2011). The corresponding resistance gene, *Rlm1*, is assumed to be associated with increasing physical barriers in the host plant. Lignification is stimulated and vascular plugs, inhibiting fungal spread in the xylem, were formed after infection of the model plant *Arabidopsis thaliana*, as a consequence of the interaction between *AvrLm1*- and *Rlm1*-gene products (Persson et al. 2009).

As a general rule, effector proteins or peptides are secreted to the apoplast. Putative effectors with Avr-functions can thus be identified biochemically by analyzing fungal secretomes grown under appropriate conditions. Ideally, but experimentally far from being trivial, fungi are grown in planta for this purpose. This approach was successful for the first fungal *Avr*-product ever, the Avr9-protein from the tomato pathogen ***Cladosporium fulvum*** (van Kan et al. 1991). Recently, an initiative for fungal and oophyte secretome research and for maintaining the corresponding database was started (Choi et al. 2010; htttp://fsd.snu.ac.kr), in order to understand, on a broader scale, the nature of putative effector proteins.

IX. Disease Management

B. napus, at least in winter oilseed rape cultivation, stands in the field for 10–11 months. **This very long growth period alone requires reasonable management for warranting economic success.** Several fungicides and, generally even more important, herbicides are helpful tools, reasonable crop rotations are mandatory, and probably the most rewarding preventive measure is the choice of suitable cultivars under the given climate and soil conditions.

A. Breeding for Resistance

Already towards the middle of the nineteenth century, botanists perceived the ability of *Brassica* and related genera for interspecies fertilization. In particular, the origin of rapeseed, *B. napus* L., was recognized as a spontaneous hybridisation event between *B. rapa* L. (Rübsen) and *B. oleracea* L. (Kohl). This hypothesis, based on molecular traits, was corroborated by cytogenetic analysis (Morinaga 1934), and finally led to establishing the *B. napus* amphidiploid karyotype as AACC (2n = 38) with the AA-parent *B. rapa* (2n = 20) and the CC-parent *B. oleracea* (2n = 18). In order to increase the narrow genetic basis of *B. napus*, and thus to introduce additional *Phoma* resistance genes, resynthesis from different genotypes of the parental species was suggested and followed actively already during early systemic oilseed rape breeding (Hoffmann and Peters 1958; Olsson 1960). Breeding of 0- without low erucic acid and 00-cultivars with low erucic acid and glucosinolate content followed later. During this breeding for quality, the resynthesis technique lost acceptation, but successful introduction of *Phoma* resistance genes is reported (Crouch et al. 1994; Diederichsen and Sacristán 1996).

Many oilseed rape cultivars are genetically more complex, and as parts of the *Brassica* B-genome have been added in breeding programs. B-genome species are the non-*Sinapis* mustards *B. nigra* (German: *Schwarzer Senf*) with a diploid BB genome, *B. carinata* (German: Abbessinischer Senf) containing a BBCC-genome, and *B. juncea* (German: Brauner or Indischer Senf) with an AABB karyotype. In interspecific crosses between several oilseed rape varieties and the resistant species *B. carinata* and *B. juncea*, **B-genomes have been shown to be a good source of *Ph. lingam* resistance genes** (Sacristán and Gerdemann 1986). Whereas both hybrids were equally

resistant as their resistant parents, backcrossing with *B. napus* led to loss of resistance already in the F_1 backcross generation for the *B. carinata* hybrid. However, the *B. juncea* hybrid proved to be permanently resistant after several backcross generations. B-genome **resistance genes** are monogenically dominant in the parental lines, as well as in hybrids with *B. napus* (Plieske et al. 1998). A more detailed genetic study of similar hybrids revealed that *B. juncea* transfers a single resistance gene to the *B. napojuncea* line, whereas *B. nigra* introduces two such genes in the *B. naponigra* line (Dixelius (1999). By applying **RFLP**-mapping to the DNA from hybrid genotypes, the stability of introduced B-type markers in *B. napus* could be followed. Generally, *B. nigra* as well as *B. carinata* and *B. juncea* genes exhibit instability in subsequent backcrosses, although to different degrees (Dixelius and Wahlberg 1999). In summary, and although neither complete chromosomes nor recognizable chromosomal arms were detected in backcross material, four markers leading to cotyledon and leaf resistance were identified. Comparison of the three B-genome donors revealed a single triplicate region harboring the resistance loci in all three species.

A complementary approach to recruit ***Phoma* resistance genes** makes use of *Sinapis arvensis*, a species that is resistant against several ***L. maculans*** pathotypes. This novel approach became even more interesting, since the B-genome resistance was reported to be broken by several ***L. maculans*** genotypes (Purwantara et al. 1998). After repeated backcrossing, interspecies crosses with *B. napus* led to fertile progeny with high resistance for cotyledons and adult plants. Genomic in situ hybridization (**GISH**) permitted identification of the intergeneric hybrids as aneuploid monosomic or double chromosome addition events (Snowdon et al. 2000). Similar results with respect to acquisition of *Phoma* **resistance genes** from *Sinapis arvensis* were obtained by protoplast fusion of mesophyll cells (Hu et al. 2002), an approach that had already proven its value for constructing *Phoma* resistant *B. naponigra* somatic hybrids (Sjödin and Glimelius 1989b).

Somatic hybridization may also allow introducing **resistance genes** from more distantly related species into *B. napus*. By fusing protoplasts of *Phoma*-resistant ecotypes of *Arabidopsis thaliana* with *B. napus*, cotyledon and leaf resistance traits were transferred. However, the cotyledon resistance was lost in subsequent generations (Bohman et al. 2002). These experiments demonstrate clearly that crucifers other than Brassicas can be used as novel resistance sources for breeding. In the long run, however, we will probably need to circumvent the imponderability of interspecies crosses or somatic hybridization by introducing genes that are clearly defined at the sequence level into the desired genetic background. It will also be helpful to understand details of the biochemistry determined by **resistance genes**. Analyzing expression patterns in intergeneric recombinants between *B. napus* and resistant plants offers a convenient experimental approach in this direction (Subramanian et al. 2005).

Recently, intergeneric and interspecific transfer of resistances following protoplast fusion was tested on a broader scale for applicability in *Brassica* vegetables, with more pathogens in addition to ***Ph. lingam***. Among other valuable observations, *B. nigra* and *B. juncea* as well as *S. arvensis* were found to be good sources for **resistance genes** also in interactions with pathogens of the genus *Alternaria* (Scholze et al. 2010).

Breeding for resistance has been facilitated by using haploid cell suspension cultures and embryonic cultures (Thomas et al. 1976; Sacristán 1982). Growth of cultures after mutagenic treatment on sirodesmin-containing medium permitted the isolation of toxin-resistant plants. Very often, however, the progeny of these regenerants is genetically unstable, and resistance is only partially inherited (Sacristán 1985). A similar approach was followed based on doubled haploid lines. Haploid plantlets were grown from microspores followed by selection for resistance by exposing them to pycnidiospores of ***L. maculans*** (Bansal et al. 1998). After diploidisation with colchicines, the plants were found to be enriched for

resistant phenotype in infection tests on cotyledons.

Combining genetic results about genes conferring *Phoma* resistance with molecular mapping approaches makes it possible to identify these genes at the molecular and sequence level, especially since the genome sequence of ***L. maculans*** has been established (Dusabenyagasani and Fernando 2008; Mayerhofer et al. 1997). These map-based approaches are promising but difficult for the complex *Brassica* genome, and have not been driven to a point where they can immediately be introduced in breeding programs. However, **resistance genes** are successfully identified and mapped with high resolution (van de Wouw et al. 2009; Long et al. 2011).

Besides qualitative resistance, determined by single, race-specific genes, linked to the biology of the pathogen via gene-for-gene relationships, quantitative resistance traits play a major role also in the interaction between *B. napus* and ***L. maculans***. A good cultivar needs both **specific qualitative resistance genes**, which are normally highly efficient, but unfortunately subject to rapid breakdown in the field, and **durable quantitative resistance traits**, which have a broad action spectrum and cannot easily be circumvented by the pathogen, but are less effective. Quantitative resistance is generally regarded as more valuable in the field, but because of its multilocus character is also hard to handle in breeding programs. Characterization of such quantitative resistance loci at the molecular level would be extremely useful for future breeding strategies. First experiments in this direction have been started. For mapping of quantitative trait loci (QTL), molecular markers were derived from expressed sequence tags (ESTs), and mapping identified several interesting candidate genes (Kaur et al. 2009). A broad screening for validation of QTL was performed in many different genetic backgrounds by marker-assisted selection. This promising study reveals 61 marker alleles to be associated with the development of **stem canker**, thus providing excellent analytical tools for monitoring promising alleles in introgression breeding programs (Jestin et al. 2011).

In order to improve resistance management in the field, especially with respect to durable **stem canker** control, a regime concept was proposed that integrates conventional, cultural, and chemical control into an intelligent avirulence management. Basic ideas are to reduce population sizes of *Leptosphaeria*, to limit the selection pressure on pathogens, thus slowing down resistance breaking in the field, and combining various monogenic or polygenic resistances for intelligent crop management (Aubertot et al. 2006).

B. Phoma Forecasting

Early infection of winter oilseed rape at the cotyledon stage causes the highest losses. Knowledge about factors influencing infection rates and forecasting risks of **stem canker** helps to choose adequate prevention measures and to avoid unnecessary losses. **Stem canker** epidemics are related to weather conditions, especially rainfall immediately before planting in autumn. Pseudothecia development depends on temperature and moisture; dry climate in August and September leads to later maturation of ascospores, whereas rainfall during this time increases development and the number of mature spores (West et al. 1999). Detailed relationships between climate and development of ascospores as the main source of infection have been reviewed (West et al. 2001, 2002). **A reasonable way to evaluate the degree of *Phoma* infection is to estimate number and size of early leaf spots in the field**. This analysis helps to determine reasonable regimes for fungicide spraying. **Stem canker** risk is increased for early infections. Later infections provide more time for reaching the stem region, and the timespan for effective spraying is extended under these conditions (West et al. 1999).

As ascospores are the major infective agents, it is also regarded as helpful to measure the amount of airborne ascospores around fields with rapeseed stubble in late summer and autumn. For this purpose, continuously working spore samplers are used that fix spores from defined air volumes to an adhesive tape. After staining, the spores are inspected and counted microscopically. It is very important to differentiate the blackleg complex into ***L. maculans*** and ***L. biglobosa***. This has been achieved by DNA extraction directly from

spores and identifying the species by taxon-specific PCR (Kaczmarek et al. 2009).

C. Fungicides

Fungicides diminish infection pressure if applied early during the cultivation period. They can reduce germination of ascospores on leaves, and are useful to suppress growth in primarily infected leaves. They are not able to control systemic growth in the stem. It is thus **highly important to apply fungicides at the right growth stage, after the first incidence of *Phoma* leaf spots.** In a field experiment, the first leaf spots were detected 2–3 weeks after measuring the increase of airborne ascospores in autumn. New leaf spots were suppressed for 1–2 months by a fungicide mixture of carbendazim and difenoconazole, depending on the time of treatment. Spraying also reduced incidences and severity of **stem cankers** (West et al. 2002). Carbendazim is a benzimide imidazole, binding to tubulin and thus inhibiting processes depending on the cytoskeleton; difenoconazole is a chlorinated triazole derivative, interfering with ergosterol metabolism.

Similar results were obtained for the combination of carbendazim and flusilazole that also inhibited novel *Phoma* leaf spots for several weeks, and increased yield by decreasing **stem canker** development (Steed et al. 2007). Spraying was found to have the best effect with plants already having 7–11 leaves. Flusilazole is a fluorinated triazole, with silicium instead of a carbon atom in the centre of the molecule. Like other triazoles, the substance inhibits steroid biosynthesis.

A detailed study in Australia on application of the chlorinated triazole fluquiconazole in the field led to valuable recommendations about effectiveness of such treatment (Marcroft and Potter 2008). Treatment of seed prior to sowing reduces blackleg losses considerably, especially under extreme conditions of high disease pressure; experimentally, *Brassica* seed was sown into infected **rapeseed** stubble. Under normal conditions, the economic effect at the level of grain yield was much lower and is not recommended. Treatment is also not recommended for cultivars with low *Leptosphaeria* susceptibility.

Recently, the first systematic investigation on differences in sensitivity between ***L. maculans*** and the less aggressive ***L. biglobosa*** were published (Eckert et al. 2009). The measurements were performed in culture. Ascospore germination was not affected by fungicides; they germinated at rates above 94 % in both species. Pycnidiospores behaved differentially, with ***L. maculans*** being more sensitive than ***L. biglobosa***. The triazoles tebuconazole and flusilazole completely prevented ***L. maculans*** spores from germinating. Measurements on the sensitivity of mycelial growth led to similar differences. If these observations apply to field conditions, fungicide treatment could shift mixed populations of the fungi towards the less aggressive ***L. biglobosa***. The authors assume that subsequently ***L. maculans*** could be suppressed by ***L. biglobosa*** during later growth stages. Field experiments support this idea. Pre-treatment of seed with ***L. biglobosa*** spore suspensions had indeed similar protective effects as fungicide treatment. The number of leaf spots as well as canker development was decreased by this biological fungicide (Liu et al. 2006). The differential behavior of ***L. biglobosa*** and ***L. maculans*** towards fungicide treatment was also affirmed by assessing leaf-spot development after treatment with a combination of flusilazole and carbendazim (Huang et al. 2011). Fungicide application reduced lesion size and also the amount of fungal DNA in affected leaves considerably for ***L. maculans***, but did not affect ***L. biglobosa*** for either parameter. Lesion sizes were also not affected in mixed infections with both fungi, indicating that interactions between these species in planta have an effect on the efficacy of fungicide treatment.

D. Cultivation Regime

The choice of appropriate plant cultivars and chemical treatment should be accompanied by good cultivation practice in order to obtain optimal results (Gabrielson 1983). The general rules are easy to follow, and often part of intuitional knowledge in **rapeseed** cultivation. As

the major source of infection are ascospores that mature on stubble and plant debris, it is helpful to remove this material immediately after harvest. The highest ascospore discharge from stubble of the preceding year on European fields is observed during the first 2 months after planting (Alabouvette and Brunin 1970). **Burning of stubble and deep ploughing help considerably** in removing infectious inoculum from the field. As ascospores survive on stubble and in dry soils for at least 3 years, a balanced crop rotation is mandatory; **4-year intervals** have been proposed **for rapeseed cultivation** (Aubertot et al. 2006). Chemical treatment of stubble in order to reduce ascospore load has been proposed, but causes additional expenses (Humpherson-Jones and Burchill 1982). All these measures, together with optimized sowing time for minimizing contact with airborne ascospores, reduce exposure of plants to the inoculum. Distances between **rapeseed** fields should also be considered. Most airborne ascospores sediment within the first 100 m, but can be found at distances around 500 m. Larger distances have been reported, but are rare (Marcroft et al. 2004; West et al. 2001). A good discussion of recommended cultivation procedures together with choice of cultivars and fungicide treatment has recently been published, aiming at integrative disease management concepts (Aubertot et al. 2006).

X. Conclusions

Phoma species are widespread pathogens of many different plants. Economically most important and encountered worldwide the *Phoma* complex is causing **blackleg disease** in oilseed rape (*B. napus*). Blackleg is a symptom complex, originating from infection by isolates of the aggressive ascomycete ***L. maculans*** or the much less aggressive ***L. biglobosa***. Simultaneous infections with both fungi occur in some countries where both fungi co-exist, and mixed infections with other fungi and even bacteria are found in the field. The two species, formerly designated as aggressive or A- and non-aggressive or B- isolates of *Ph. lingam*, differ in many respects.

The most obvious physiological difference between ***L. maculans*** and ***L. biglobosa*** is the secretion of the general epidithiopiperazine toxin **sirodesmin**, including its derivatives, by ***L. maculans***, which is regarded as a major virulence factor by most authors. The importance of other putative virulence factors, exoenzymes, or differing toxins, is less clear.

The interaction between ***L. maculans*** with its host plants is determined by several **avirulence genes** and the cognate **resistance genes** in the plant. These have already been introduced in breeding programs, technically often based in interspecies crosses or on protoplast fusion with other *Brassica* or *Sinapis* species. Mapping of quantitative trait loci has been started, and introducing those into breeding strategies is to be expected. In the future, **breeding for resistance should be considerably facilitated by using the recently established complete genome sequence of *L. maculans***.

Disease management in the field depends largely but not exclusively on choosing appropriate *Brassica* cultivars with resistance traits that match local *Phoma* populations. In addition, economic oilseed rape cultivation requires adequate cultural precautions and, in most years, a reasonable fungicide-spraying regime. *Phoma* forecasting strategies help to make spraying decisions.

Most important are plant protection approaches avoiding high selection pressure on monogenic, race-specific resistances. Such conditions will select for *Phoma* populations with changed *Avr*-complement and thus lead to breaking of resistance in the field. Integrative approaches that also consider these important long-term aspects have been proposed. They need continuous improvement and adaption to the highly different requirements in *Brassica* growth regions.

References

Abeln ECA, Stax AM, De Gruyter J, van der Aa HA (2002) Genetic differentiation of *Phoma exigua* varieties by means of AFLP fingerprints. Mycol Res 106:419–427

Alabouvette C, Brunin B (1970) Recherches sur la maladie de colza due a *Leptosphaeria maculans* (Desm.): Ces. et de Not. I. Rôle des restes de culture dans la conservation et la dissemination du parasite. Ann Phytopathol 6:463–475

Annis SL, Goodwin PH (1996) Comparison of cell wall-degrading enzymes produced by highly and weakly virulent isolates of *Leptosphaeria maculans* in culture. Microbiol Res 151:401–406

Annis SL, Goodwin PH (1997) Inhibition of polygalacturonase activity produced by *Leptosphaeria maculans* with stem extracts of canola, and the relationship of inhibition to resistance. J Phytopathol 145:217–223

Ansan-Melayah D, Balesdent MH, Buée M, Rouxel T (1995) Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. Phytopathol 85:1525–1529

Ansan-Melayah D, Balesdent MH, Delourme R, Pilet ML, Tanguy X, Renard M, Rouxel T (1998) Genes for race-specific resistance against blackleg disease in *Brassica napus* L. Plant Breed 117: 373–378

Attard A, Gourges M, Gout L, Schmit J, Roux J, Narcy JP, Balesdent MH, Rouxel T (2001) Molecular characterisation and polymorphism of *MinLm1*, a minisatellite from the phytopathogenic ascomycete *Leptosphaeria maculans*. Curr Genet 40:54–64

Attard A, Gout L, Gourgues M, Kühn ML, Schmit J, Laroche S, Ansan-Melayah D, Billault A, Cattolico L, Balesdent MH, Rouxel T (2002) Analysis of molecular markers genetically linked to the *Leptosphaeria maculans* avirulence gene *AvrLm1* in field populations indicates a highly conserved event leading to virulence on *Rlm1* genotypes. Mol Plant Microbe Interact 15:672–682

Aubertot JN, West JS, Bousset-Vaslin L, Salam MU, Barbetti MJ, Diggle AJ (2006) Improved resistance management for durable disease control: a case study of *Phoma* stem canker of oilseed rape (*Brassica napus*). Eur J Plant Pathol 114:91–106

Aveskamp MM, De Gruyter J, Crous PW (2008) Biology and recent development in the systematics of *Phoma*, a complex genus of major quarantine significance. Fungal Divers 31:1–18

Aveskamp MM, De Gruyter J, Woudenberg JHC, Verkley GJM, Crous PW (2010) Highlights of the Didymellaceae: a polyphasic approach to characterize *Phoma* and related pleosporalean genera. Stud Mycol 65: 1–60

Badawy HMA, Hoppe HH (1989a) Production of phytotoxic sirodesmins by aggressive strains of *Leptosphaeria maculans* differing in interactions with oil seed rape genotypes. J Phytopathol 127:146–157

Badawy HMA, Hoppe HH (1989b) Nonspecific phytotoxic effects of sirodesmins on host and nonhost plants of *Leptosphaeria maculans*. J Phytopathol 127:137–145

Balesdent MH, Gall C, Robin P, Rouxel T (1992) Intraspecific variation in soluble mycelial protein and esterase patterns of *Leptosphaeria maculans* French isolates. Mycol Res 96:677–684

Balesdent MH, Attard A, Ansan-Melayah D, Delourme R, Renard M, Rouxel T (2001) Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. Phytopathology 91:70–76

Balesdent MH, Attard A, Kühn ML, Rouxel T (2002) New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. Phytopathology 92: 1122–1133

Balesdent MH, Barbetti MJ, Li H, Sivasithamparam K, Gout L, Rouxel T (2005) Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. Phytopathology 95:1061–1071

Balesdent MH, Louvard K, Pinochet X, Rouxel T (2006) A large-scale survey of races of *Leptosphaeria maculans* occurring on oilseed rape in France. Eur J Plant Pathol 114:53–65

Balmas V, Scherm B, Ghignone S, Salem AOM, Cacciola AO, Migheli Q (2005) Characterisation of *Phoma tracheiphila* by RAPD-PCR, microsatellite-primed PCR and ITS rDNA sequencing and development of specific primers for in planta PCR detection. Eur J Plant Pathol 111:235–247

Bansal VK, Thiagarajah MR, Stringam GR, Hardin RT (1998) Haploid plantlet screening in the development of blackleg resistant DH lines of *Brassica napus*. Plant Breed 117:103–106

Bayraktar H, Dolar FS, Tor M (2007) Determination of genetic diversity within *Ascochyta rabiei* (Pass.) Labr., the cause of *Ascochyta* blight of chickpea in Turkey. J Plant Pathol 89:341–347

Boerema GH (1997) Contributions towards a monograph of *Phoma* (coelomycetes). 5. Subdivision of the genus in sections. Mycotaxon 64:321–333

Boerema GH, Dorenbosch MMJ, Leffring L (1965a) A comparative study of the black stem fungi on lucerne and red clover and the footrot fungus on pea. Neth J Plant Pathol 71:79–89

Boerema GH, Dorenbosch MMJ, van Kesteren HA (1965b) Remarks on species of *Phoma* referred to Peyronellaea. Persoonia 4:47–68

Bohman S, Wang M, Dixelius C (2002) *Arabidopsis thaliana*-derived resistance against *Leptosphaeria maculans* in a *Brassica napus* genomic background. Theor Appl Genet 105:498–504

Bose AK, Khanchan KS, Tavares R, Funke PT (1968) Biochemical studies II. The mode of incorporation of phenylalanine into gliotoxin. J Am Chem Soc 90: 3593–3594

Boudart G (1978) Phytotoxine et nécrose des hypocotyles de crucifères infectées par *Leptosphaeria maculans* (Ces. et de Not.) et sa forme imparfaite *Phoma lingam*. Phytopathology 92:76–82

Boudart G (1989) Antibacterial activity of sirodesmin PL phytotoxin: application to the selection of phytotoxin-deficient mutants. Appl Environ Microbiol 55:1555–1559

Bousquet JF, Férézou JP, Devys M, Barbier M (1977) Sur une toxine produite par le champignon *Phoma lingam* Tode, parasite du colza: isolement et propriétés. C R Acad Sci Paris, Ser D 284:927–928

Brock M (2009) Fungal metabolism in host niches. Curr Opin Microbiol 12:371–376

Bugbee WM (1990) Combined resistance in sugar beet to *Rhizoctonia solani*, *Phoma betae*, and *Botrytis cinerera*. Plant Dis 74:353–355

Bugbee WM (1993) A pectin lyase inhibitor protein from cell walls of sugar beet. Phytopathology 83: 63–68

Castell-Miller CV, Szabo LJ, Gale LR, O'Neill NR, Samac DA (2008) Molecular variability of a Minnesota population of *Phoma medicaginis* var. *medicaginis*, the causal agent of spring black stem and leaf spot of alfalfa. Can J Plant Pathol 30:85–96

Cozijnsen AJ, Howlett BJ (2003) Characterisation of the mating-type locus of the plant pathogenic ascomycete *Leptosphaeria maculans*. Curr Genet 43:351–357

Crouch JH, Lewis BG, Mithen RF (1994) The effect of a genome substitution on the resistance of *Brassica napus* to infection by *Leptosphaeria maculans*. Plant Breed 112:265–278

Curtis PJ, Greatbanks D, Hesp B, Forbes Cameron A, Freer AA (1977) Sirodesmins A, B, C, and G, antiviral epipolythiopiperazine-2,5-diones of fungal origin: X-ray analysis of sirodesmin A diacetate. J Chem Soc Perkin I:180–189

De March G, Séguin-Swartz G, Petrie GA (1986) Virulence and culture filtrate phytotoxicity in *Leptosphaeria maculans*: perspectives for in vitro selection. Can J Plant Pathol 8:422–428

Demontis MA, Cacciola SO, Orrù M, Balmas V, Chessa V, Maserti BE, Mascia L, Raudino F, di San Lio GM, Migheli Q (2008) Development of real-time PCR systems based on SYBR Green I and TaqMan technologies for specific quantitative detection of *Phoma tracheiphila* in infected *Citrus*. Eur J Plant Pathol 120:339–351

Devys M, Férézou JP, Topgi RS, Barbier M, Bousquet JF, Kollmann A (1984) Structure and biosynthesis of phomenoic acid, an antifungal compound isolated from *Phoma lingam* Tode. J Chem Soc Perkin Trans I:2133–2137

Devys M, Topgi RS, Férézou JP, Quaino L, Bousquet JF, Kollman A, Barbier M (1986) Phomenolactone, an antifungal substance from *Phoma lingam*. Phytochemistry 25:531–532

Diederichsen E, Sacristán MD (1996) Disease response of resynthesized *Brassica napus* L. lines carrying different combinations of resistance to *Plasmodiophora brassicae* Wor. Plant Breed 115:5–10

Dixelius C (1999) Inheritance of the resistance to *Leptosphaeria maculans* of *Brassica nigra* and *B. juncea* in near-isogenic lines of *B. napus*. Plant Breed 118: 151–156

Dixelius C, Wahlberg S (1999) Resistance to *Leptosphaeria maculans* is conserved in a specific region of the *Brassica* B genome. Theor Appl Genet 99: 368–372

Dusabenyagasani M, Fernando WGD (2008) Development of a SCAR marker to track canola resistance against blackleg caused by *Leptosphaeria maculans* pathogenicity group 3. Plant Dis 92:903–908

Easton CJ, Rossall S (1985) The production of certain cell wall-degrading enzymes by *Leptosphaeria maculans* in culture and in stem canker lesions of oilseed rape. Physiol Plant Pathol 26:185–197

Eckert M, Gout L, Rouxel T, Blaise F, Jedryczka M, Fitt B, Balesdent MH (2005) Identification and characterization of polymorphic minisatellites in the phytopathogenic ascomycete *Leptosphaeria maculans*. Curr Genet 47:37–48

Eckert MR, Rossall S, Selley A, Fitt BDL (2009) Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (*Phoma* stem canker of oilseed rape). Pest Manag Sci 66:396–405

Elliott CE, Gardiner DM, Thomas GD, Cozijnsen A, van de Wouw A, Howlett BJ (2007) Production of the toxin sirodesmin PL by *Leptosphaeria maculans* during infection of *Brassica napus*. Mol Plant Pathol 8:791–802

Elliott CA, Fox EM, Jarvis RS, Howlett BJ (2011) The cross-pathway control system regulates production of the secondary metabolite toxin, sirodesmin PL, in the ascomycete, *Leptosphaeria maculans*. BMC Microbiol 11:169

Ezra D, Kroitor T, Sadowsky A (2007) Molecular characterization of *Phoma tracheiphila*, causal agent of *mal secco* disease in *Citrus*, in Israel. Eur J Plant Pathol 118:183–191

Farman ML, Oliver RP (1988) The transformation of protoplasts of *Leptosphaeria maculans* to hygromycin B resistance. Curr Genet 13:327–330

Farman ML, Oliver RP (1992) Transformation frequencies are enhanced and vector DNA is targeted during retransformation of *Leptosphaeria maculans*, a fungal plant pathogen. Mol Gen Genet 231: 243–247

Feldman TS, O'Brien HE, Arnold AE (2008) Moths that vector a plant pathogen also transport endophytic fungi and mycoparasitic antagonists. Microb Ecol 56:742–750

Férézou JP, Riche C, Quesneau-Thierry A, Pascard-Billy C, Barbier M, Bousquet JF, Boudart G (1977) Structures de deux toxins isolées des cultures du champignon *Phoma lingam* Tode: La sirodesmine PL et la désacétylsirodesmine PL. Nouv J Chim 1: 327–333

Férézou JP, Quesneau-Thierry A, Barbier M, Kollmann A, Bousquet JF (1980a) Structure and synthesis of phomamide, a new piperazine-2,5-dione related to the sirodesmins, isolated from the culture medium of *Phoma lingam* Tode. J Chem Soc Perkin I:113–115

Férézou JP, Quesneau-Thierry A, Servy C, Zissmann E, Barbier M (1980b) Sirodesmin PL biosynthesis in

Phoma lingam Tode. J Chem Soc Perkin I: 1739–1746

Fogliano V, Marchese A, Scaloni A, Ritieni A, Visconti A, Randazzo G, Graniti A (1998) Characterization of a 60 kDa phytotoxic glycoprotein produced by *Phoma tracheiphila* and its relation to malseccin. Physiol Mol Plant Pathol 53:149–161

Fox EM, Gardiner DM, Keller NP, Howlett BJ (2008) A $Zn(II)_2Cys_6$ DNA binding protein regulates the sirodesmin PL biosynthetic gene cluster in *Leptosphaeria maculans*. Fung Genet Biol 45: 671–682

Gabrielson RL (1983) Blackleg disease of crucifers caused by *Leptosphaeria maculans* (*Phoma lingam*) and its control. Seed Sci Technol 11:749–780

Gall C, Balesdent MH, Desthieux I, Robin P, Rouxel T (1995) Polymorphism of Tox^0 *Leptosphaeria maculans* isolates as revealed by soluble protein and isozyme electrophoresis. Mycol Res 99: 221–229

Gardiner DM, Cozijnsen AJ, Wilson LM, Pedras MSC, Howlett BJ (2004a) The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Mol Microbiol 53:1307–1318

Gardiner DM, Jarvis RS, Howlett BJ (2004b) The ABC transporter gene in the sirodesmin biosynthetic gene cluster of Leptosphaeria maculans is not essential for sirodesmin production but facilitates self-protection. Fungal Genet Biol 42:257–263

Ghanbarnia K, Lydiate DJ, Rimmer SR, Li G, Kutcher HR, Larkan NJ, McVetty PBE, Fernando WGD (2012) Genetic mapping of the *Leptosphaeria maculans* avirulence gene corresponding to the *LepR1* resistance gene of *Brassica napus*. Theor Appl Genet 124: 505–513

Goodwin PH, Annis SL (1991) Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl Environ Microbiol 57:2482–2486

Gout L, Fudal I, Kuhn ML, Blaise F, Eckert M, Cattolico L, Balesdent M-H, Rouxel T (2006) Lost in the middle of nowhere: the *AvrLm1* avirulence gene of the dothideomycete *Leptosphaeria maculans*. Mol Microbiol 60:67–80

Hammond KE, Lewis BG (1986) The timing and sequence of events leading to stem canker disease in populations of *Brassica napus* var. *oleifera* in the field. Plant Pathol 35:551–564

Hammond KE, Lewis BG (1987) The establishment of systemic infection in leaves of oilseed rape by *Leptosphaeria maculans*. Plant Pathol 36:135–147

Hammond KE, Lewis BG, Musa TM (1985) A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. Plant Pathol 34: 557–565

Hassan AK, Schulz C, Sacristan MD, Wöstemeyer J (1991) Biochemical and molecular tools for the differentiation of aggressive and non-aggressive isolates of the oilseed rape pathogen, *Phoma lingam*. J Phytopathol 131:120–136

Hill CB, Xu XH, Williams PH (1984) Correlations of virulence, growth rate, pigment production and allozyme banding patterns which differentiate virulent and avirulent isolates of *Leptosphaeria maculans*. Cruciferae Newslett 9:79

Hoffmann W, Peters R (1958) Versuche zur Herstellung synthetischer und semisynthetischer Rapsformen. Der Züchter 28:40–51

Hosford RM (1975) *Phoma glomerata*, a new pathogen of wheat and Triticales, cultivar resistance related to wet period. Phytopathology 65:1236–1239

Howlett BJ (1997) Genome analysis of the fungal plant pathogen *Leptosphaeria maculans* using pulsed field gel electrophoresis. Electrophoresis 18: 1544–1547

Howlett BJ (2004) Current knowledge of the *Brassica napus* – *Leptosphaeria maculans* interaction: a review. Can J Plant Pathol 26:245–252

Howlett BJ, Idnurm A, Pedras MSC (2001) *Leptosphaeria maculans*, the causal agent of blackleg disease of Brassicas. Fung Genet Biol 33:1–14

Hu Q, Andersen SB, Dixelius C, Hansen LN (2002) Production of fertile intergeneric somatic hybrids between *Brassica napus* and *Sinapis arvensis* for the enrichment of the rapeseed gene pool. Plant Cell Rep 21:147–152

Huang YJ, Hood JR, Eckert MR, Stonard JF, Cools HJ, King GJ, Rossall S, Ashworth M, Fitt BDL (2011) Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of *Phoma* stem canker on oilseed rape (*Brassica napus*). Plant Pathol 60:607–620

Humpherson-Jones FM, Burchill RT (1982) Chemical suppression of the sexual stage of *Leptosphaeria maculans* on oil-seed rape and turnip seed crop straw. Ann Appl Biol 100:281–288

Idnurm A, Howlett BJ (2002) Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). Eukaryot Cell 1:719–724

Idnurm A, Howlett BJ (2003) Analysis of loss of pathogenicity mutants reveals that repeat-induced point mutations can occur in the Dothideomycete *Leptosphaeria maculans*. Fungal Genet Biol 39:31–37

Jestin C, Lodé M, Vallée P, Domin C, Falentin C, Horvais R, Coedel S, Manzanares-Dauleux MJ, Delourme R (2011) Association mapping of quantitative resistance for *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). Mol Breed 27:271–287

Johnson RD, Lewis BG (1990) DNA polymorphism in *Leptosphaeria maculans*. Physiol Mol Plant Pathol 37:417–424

Kaczmarek J, Jedryczka M, Fitt BDL, Lucas JA, Latunde-Dada AO (2009) Analyses of air samples for ascospores of *Leptosphaeria maculans* and *L. biglobosa* by light microscopy and molecular techniques. J Appl Genet 50:411–419

Kaur S, Cogan NOI, Ye G, Baillie RC, Hand ML, Ling AE, Mcgeary AK, Kaur J, Hopkins CJ, Todorovic

M, Mountford H, Edwards D, Batley J, Burton W, Salisbury P, Gororo N, Marcroft S, Kearney G, Smith KF, Forster JW, Spangenberg GC (2009) Genetic map construction and QTL mapping of resistance to blackleg (*Leptosphaeria maculans*) disease in Australian canola (*Brassica napus* L.) cultivars. Theor Appl Genet 120:71–83

Koch E, Badawy HMA, Hoppe HH (1989) Differences between aggressive and non-aggressive single spore lines of *Leptosphaeria maculans* in cultural characteristics and phytotoxin production. J Phytopathol 124:52–62

Koch E, Song K, Osborn TC, Williams PH (1991) Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Mol Plant-Microbe Interact 4:341–349

Kremer A, Li SM (2010) A tyrosine O-prenyltransferase catalyses the first pathway-specific step in the biosynthesis of sirodesmin PL. Microbiology 156: 278–286

Kuhn ML, Gout L, Howlett BJ, Melayah D, Meyer M, Balesdent MH, Rouxel T (2006) Genetic linkage maps and genomic organization in *Leptosphaeria maculans*. Eur J Plant Pathol 114:17–31

Kuswinanti T, Koopmann B, Hoppe HH (1999) Virulence pattern of aggressive isolates of *Leptosphaeria maculans* on an extended set of *Brassica* differentials. J Plant Dis Protect 106:12–20

Kutcher HR, van den Berg CGJ, Rimmer SR (1993) Variation in pathogenicity of *Leptosphaeria maculans* on *Brassica spp*. Based on cotyledon and stem reactions. Can J Plant Pathol 15:253–258

Licciardello G (2006) Identification and detection of *Phoma tracheiphila*, causal agent of *Citrus* mal secco disease, by real-time polymerase chain reaction. Plant Dis 90:1523–1530

Lieckfeldt E, Meyer W, Börner T (1993) Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. J Basic Microbiol 33:413–426

Lim L, Howlett BJ (1994) Linear plasmids, pLm9 and pLM10, can be isolated from the phytopathogenic ascomycete *Leptosphaeria maculans* by pulsed-field gel electrophoresis. Curr Genet 26:276–280

Liu SY, Liu Z, Fitt BDL, Evans N, Foster SJ, Huang YJ, Latunde-Dada AO, Lucas JA (2006) Resistance to *Leptosphaeria maculans* (*Phoma* stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. Plant Pathol 55:401–412

Long Y, Wang Z, Sun Z, Fernando DWG, McVetty PBE, Li G (2011) Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar 'Surpass 400'. Theor Appl Genet 122:1223–1231

Marcroft SJ, Potter TD (2008) The fungicide fluquinconazole applied as a seed dressing to canola reduces *Leptosphaeria maculans* (blackleg) severity in south-eastern Australia. Australas Plant Pathol 37:396–401

Marcroft SJ, Sprague SJ, Pymer SJ, Salisbury PA, Howlett BJ (2004) Crop isolation, not extended rotation length, reduces blackleg (*Leptosphaeria maculans*) severity of canola (*Brassica napus*) in south-eastern Australia. Aust J Exp Agric 44:601–606

Mayerhofer R, Bansal VK, Thiagarajah MR, Stringam GR, Good AG (1997) Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of *Brassica napus*. Genome 40:294–301

McGee GC, Petrie GA (1978) Variability of *Leptosphaeria maculans* in relation to blackleg of oilseed rape. Phytopathology 68:625–630

Melouk HA, Horner CE (1972) Production of pectolytic and macerating enzyme by *Phoma strasseri*. Can J Microbiol 18:1065–1072

Mendes-Pereira E, Balesdent MH, Brun H, Rouxel T (2003) Molecular phylogeny of the *Leptosphaeria maculans – L. biglobosa* species complex. Mycol Res 107:1287–1304

Mengistu A, Rimmer SR, Koch E, Williams PH (1991) Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling brassicas. Plant Dis 75:1279–1282

Meyer W, Lieckfeldt E, Wöstemeyer J, Börner T (1992) DNA fingerprinting for differentiating aggressiveness groups of the rapeseed pathogen *Leptosphaeria maculans*. Mycol Res 96:651–657

Monte E, Bridge PD, Sutton BC (1991) An integrated approach to *Phoma* systematics. Mycopathologia 115:89–103

Morgan-Jones G, Burch KB (1988a) Studies in the genus *Phoma* XII. Concerning *Phoma destructiva*, a second species implicated as a pathogen of tomato. Mycotaxon 32:253–265

Morgan-Jones G, Burch KB (1988b) Studies in the genus *Phoma* XI. Concerning *Phoma lycopersici*, the anamorph of *Didymella lycopersici*, causal organism of stem canker and fruit rot of tomato. Mycotaxon 32:133–142

Morinaga T (1934) Interspecific hybridisation in *Brassica*. VI. The cytology of F1 hybrids of *B. juncea* and *B. nigra*. Cytologia 6:62–67

Munday R (1989) Toxicity of thiols and disulphides: involvement of free radical species. Free Radical Biol Med 7:659–673

Olsson G (1960) Species crosses within the genus *Brassica*. II. Artificial *Brassica napus* L. Hereditas 46: 351–386

Parlange F, Daverdin G, Fudal I, Kuhn ML, Balesdent MH, Blaise F, Grezes-Besset B, Rouxel T (2009) *Leptosphaeria maculans* avirulence gene *AvrLm4-7* confers a dual recognition specificity by the *Rlm4* and *Rlm7* resistance genes of oilseed rape, and circumvents *Rlm4*-mediated recognition through a single amino acid change. Mol Microbiol 71:851–863

Pažoutová S (2009) Genetic variation of *Phoma sorghina* isolates from Southern Africa and Texas. Folia Microbiol 54:217–229

Pedras MSC, Biesenthal CJ (2000a) HPLC analyses of cultures of *Phoma* spp.: differentiation among groups and species through secondary metabolite profiles. Can J Microbiol 46:685–691

Pedras MSC, Biesenthal CJ (2000b) Vital staining of plant cell suspension cultures: evaluation of the phytotoxic activity of the phytotoxins phomalide and destruxin B. Plant Cell Rep 19:1135–1138

Pedras MSC, Séguin-Swartz G (1990) Rapid high-performance liquid chromatographic analysis of phytotoxins from *Phoma lingam*. J Chromatogr 519:383–386

Pedras MSC, Yu Y (2008) Structure and biological activity of maculansin A, a phytotoxin from the phytopathogenic fungus *Leptosphaeria maculans*. Phytochemistry 69:2966–2971

Pedras MSC, Abrams SR, Séguin-Swartz G, Quail JW, Jia Z (1989) Phomalirazine, a novel toxin from the phytopathogenic fungus *Phoma lingam*. J Am Chem Soc 111:1904–1905

Pedras MSC, Morales VM, Taylor JL (1993a) Phomaligols and phomaligadiones: new metabolites from the blackleg fungus. Tetrahedron 49:8317–8322

Pedras MSC, Morales VM, Taylor JL (1993b) Phomapyrones: three metabolites from the blackleg fungus. Phytochemistry 36:1315–1318

Pedras MSC, Taylor JL, Morales VM (1995) Phomaligin A and other yellow pigments in *Phoma lingam* and *P. wasabiae*. Phytochemistry 38:1215–1222

Perrotta G, Graniti A (1988) *Phoma tracheiphila* (Petri) Kanchaveli and Gikashvili. In: Smith IM, Dunez J, Lelliott RA, Phillips DH, Archer SA (eds) European handbook of plant diseases. Blackwell Scientific, Oxford, pp 396–398

Persson M, Staal J, Oide S, Dixelius C (2009) Layers of defense responses to *Leptosphaeria maculans* below the *RLM1*- and camalexin-dependent resistances. New Phytol 182:470–482

Pertrie GA, Lewis PA (1985) Sexual compatibility of isolates of the rapeseed blackleg fungus *Leptosphaeria maculans* from Canada, Australia, and England. Can J Plant Pathol 7:253–255

Pethybridge SJ, Hay FS (2001) Influence of *Phoma ligulicola* on yield, and site factors on disease development, in Tasmanian pyrethrum crops. Australas Plant Pathol 30:17–20

Pethybridge SJ, Scott JB, Hay FS (2004) Genetic relationships among isolates of *Phoma ligulicola* from pyrethrum and chrysanthemum based on ITS sequences and its detection by PCR. Australas Plant Pathol 33:173–181

Pethybridge SJ, Hay FS, Clarkson RA, Groom T, Wilson CR (2008) Host range of Australian *Phoma ligulicola* var. *inoxydablis* isolates from pyrethrum. J Phytopathol 156:506–508

Plieske J, Struss D, Röbbelen G (1998) Inheritance of resistance derived from the B-genome of *Brassica* against *Phoma lingam* in rapeseed and the development of molecular markers. Theor Appl Genet 97: 929–936

Plummer KM, Howlett BJ (1995) Inheritance of chromosomal length polymorphism in the ascomycete *Leptosphaeria maculans*. Mol Gen Genet 247: 416–422

Poiret B, Kollmann A, Bousquet JF (1985) Activités antifongiques de la sirodesmine PL et de deux analogues naturels isolés de *Phoma lingam* (Tode) Desm. Action antagoniste du zinc. Agronomie 5:533–538

Pollero R, Gaspar ML, Cabello M (1997) Lipolytic activity in free and immobilized cells of *Phoma glomerata*. JAOCS 74:451–454

Pollero R, Gaspar ML, Cabello M (2001) Extracellular lipolytic activity in *Phoma glomerata*. World J Microbiol Biotechnol 17:805–809

Pongam P, Osborn TC, Williams PH (1999) Assessment of genetic variation among *Leptosphaeria maculans* isolates using pathogenicity data and AFLP analysis. Plant Dis 83:149–154

Purwantara A, Salisbury PA, Burton WA, Howlett BJ (1998) Reaction of *Brassica juncea* (Indian mustard) lines to Australian isolates of *Leptosphaeria maculans* under glasshouse and field conditions. Eur J Plant Pathol 104:895–902

Purwantara A, Barrins JM, Cozijnsen AJ, Ades PK, Howlett BJ (2000) Genetic diversity of isolates of the *Leptosphaeria maculans* species complex from Australia, Europe and North America using amplified fragment length polymorphism analysis. Mycol Res 104:772–781

Remy E, Meyer M, Blaise F, Chabirand M, Wolff N, Balesdent MH, Rouxel T (2008a) The *Lmpma1* gene of *Leptosphaeria maculans* encodes a plasma membrane H^+-ATPase isoform essential for pathogenicity towards oilseed rape. Fung Genet Biol 45: 1122–1134

Remy E, Meyer M, Blaise F, Simon UK, Kuhn D, Chabirand M, Riquelme M, Balesdent MH, Rouxel T (2008b) The *Lmgpi15* gene, encoding a component of the glycosylphosphatidylinositol anchor biosynthesis pathway, is required for morphogenesis and pathogenicity in *Leptosphaeria maculans*. New Phytol 179:1105–1120

Remy E, Meyer M, Blaise F, Simon UK, Kuhn D, Balesdent MH, Rouxel T (2009) A key enzyme of the Leloir pathway is involved in pathogenicity of *Leptosphaeria maculans* towards oilseed rape. Mol Plant Microbe Interact 22:725–736

Rosewich UL, Kistler HC (2000) Role of horizontal gene transfer in the evolution of fungi. Annu Rev Phytopathol 38:325–363

Roustaee A, Dechamp-Guillaume G, Gelie B, Savy C, Dargent R, Barrault G (2000) Ultrastructural studies of the mode of penetration by *Phoma*

macdonaldii in sunflower seedlings. Phytopathology 90:915–920

Rouxel T, Chupeau Y, Fritz R, Kollmann A, Bousquet JF (1988) Biological effects of sirodesmin PL, a phytotoxin produced by *Leptosphaeria maculans*. Plant Sci 57:45–53

Rouxel T, Kollmann A, Bousquet JF (1990) Zinc suppresses sirodesmin PL toxicity and protects *Brassica napus* plants against the blackleg disease caused by *Leptosphaeria maculans*. Plant Sci 68:77–86

Rouxel T, Grandaubert J, Hane JK, Hoede C, van de Wouw AP, Couloux A, Dominguez V, Anthouard V, Bally P, Bourras S, Cozijnsen AJ, Ciufetti LM, Degrave A, Dilmaghani A, Duret L, Fudal I, Goodwin SB, Gout L, Glaser N, Linglin J, Kema GHJ, Lapalu N, Lawrence CB, May K, Meyer M, Ollivier B, Poulain J, Schoch CL, Simon A, Spatafora JW, Stachowiak A, Turgeon BG, Tyler BM, Vincent D, Weissenbach J, Amselem J, Quesneville H, Oliver RP, Wincker P, Balesdent MH, Howlett BJ (2011) Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by repeat-induced point mutations. Nat Commun 2:202. doi:10.1038/ncomms1189

Sacristán MD (1982) Resistance responses to *Phoma lingam* of plants regenerated from selected cell and embryogenic cultures of haploid *Brassica napus*. Theor Appl Genet 61:193–200

Sacristán MD (1985) Selection for disease resistance in *Brassica* cultures. Hereditas 3(Suppl):57–63

Sacristán MD, Gerdemann M (1986) Different behaviour of *Brassica juncea* and *B. carinata* as sources of *Phoma lingam* resistance in experiments of interspecific transfer to *B. napus*. Plant Breed 97:304–314

Saniewska A, Dyki B (1997) Development of *Phoma narcissi* within tissues of *Hippeastrum* leaves. Phytopathol Polonica 14:55–60

Schäfer C, Wöstemeyer J (1992) Random primer dependent PCR differentiates aggressive from non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (*Leptosphaeria maculans*). J Phytopathol 136:124–136

Scharf DH, Heinekamp T, Remme N, Hortschansky P, Brakhage AA, Hertweck C (2012) Biosynthesis and function of gliotoxin in *Aspergillus fumigatus*. Appl Microbiol Biotechnol 93:467–472

Schleier S, Voigt K, Wöstemeyer J (1997) RAPD-based molecular diagnosis of mixed fungal infections on oilseed rape (*Brassica napus*): Evidence for genus- and species-specific sequences in the fungal genomes. J Phytopathol 145:81–87

Scholze P, Krämer R, Ryschka U, Klocke E, Schumann G (2010) Somatic hybrids of vegetable Brassicas as source for new resistances to fungal and virus diseases. Euphytica 176:1–14

Sexton AC, Howlett BJ (2001) Green fluorescent protein as a reporter in the *Brassica-Leptosphaeria maculans* interaction. Physiol Mol Plant Pathol 58:13–21

Sexton AC, Paulsen M, Wöstemeyer J, Howlett BJ (2000) Cloning, characterization and chromosomal location of three genes encoding host-cell-wall-degrading enzymes in *Leptosphaeria maculans*, a fungal pathogen of *Brassica* spp. Gene 248:89–97

Shoemaker RA, Brun H (2001) The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. Can J Bot 79:412–419

Sippell DW, Hall R (1995) Glucose phosphate isomerase polymorphisms distinguish weakly from highly virulent strains of *Leptosphaeria maculans*. Can J Plant Pathol 17:1–6

Sjödin C, Glimelius K (1989a) Differences in response to the toxin sirodesmin PL produced by *Phoma lingam* (Tode ex Fr.) Desm. on protoplasts, cell aggregates and intact plants of resistant and susceptible *Brassica* accessions. Theor Appl Genet 77:76–80

Sjödin C, Glimelius K (1989b) *Brassica naponigra*, a somatic hybrid resistant to *Phoma lingam*. Theor Appl Genet 77:651–656

Sjödin C, Nyhammar T, Glimelius K (1988) Effects of toxic metabolites produced by *Phoma lingam* (Tode ex Fr.) Desm. on protoplasts, cells and plants of hosts and non-hosts of the pathogen. Physiol Mol Plant Pathol 32:301–312

Snowdon RJ, Winter H, Diestel A, Sacristán MD (2000) Development and characterisation of *Brassica napus-Sinapis arvensis* addition lines exhibiting resistance to *Leptosphaeria maculans*. Theor Appl Genet 101:1008–1014

Sock J, Hoppe HH (1999) Pathogenicity of sirodesmin-deficient mutants of *Phoma lingam*. J Phytopathol 147:169–173

Sock J, Mennen H, Hoppe HH (1995) Pathogenicity of sirodesmin-deficient mutants of *Leptosphaeria maculans*. In: Proceedings of the 9th international rapeseed congress, Cambridge, pp 613–615

Sprague SJ, Watt M, Kirkegaard JA, Howlett BJ (2007) Pathways of infection of *Brassica napus* roots by *Leptosphaeria maculans*. New Phytol 176:211–222

Sprague SJ, Howlett BJ, Kirkegaard JA (2009) Epidemiology of root rot caused by *Leptosphaeria maculans* in *Brassica napus* crops. Eur J Plant Pathol 125:189–202

Steed JM, Baierl A, Fitt BDL (2007) Relating plant and pathogen development to optimise fungicide control of *Phoma* stem canker (*Leptosphaeria maculans*) on winter oilseed rape (*Brassica napus*). Eur J Plant Pathol 118:359–373

Subramanian B, Bansal VK, Kav NNV (2005) Proteome-level investigation of *Brassica carinata*-derived resistance to *Leptosphaeria maculans*. J Agric Food Chem 53:313–324

Taylor JL, Borgmann IE (1994) An unusual repetitive element from highly virulent isolates of *Leptosphaeria maculans* and evidence of its transfer to a weakly virulent isolate. Mol Plant Microbe Interact 7:181–188

Taylor JL, Borgmann I, Séguin-Swartz G (1991) Electrophoretic karyotyping of *Leptosphaeria maculans* differentiates highly virulent from weakly virulent isolates. Curr Genet 19:273–277

Thomas E, Hoffmann F, Potrykus I, Wenzel G (1976) Protoplast regeneration and stem embryogenesis of haploid androgenetic rape. Mol Gen Genet 145:245–247

Thürwächter F, Garbe V, Hoppe HH (1999) Ascospore discharge, leaf infestation and variation in pathogenicity as criteria to predict impact of *Leptosphaeria maculans* on oilseed rape. J Phytopathol 147:215–222

van de Graaf P, Joseph ME, Chartier-Hollis JM, O'Neill TM (2002) Prepenetration stages in infection of *Clematis* by *Phoma clematidina*. Plant Pathol 51:331–337

van de Wouw AP, Marcroft SJ, Barbetti MJ, Hua L, Salisbury PA, Gout L, Rouxel T, Howlett BJ, Balesdent MH (2009) Dual control of avirulence in *Leptosphaeria maculans* towards a *Brassica napus* cultivar with 'sylvestris-derived' resistance suggests involvement of two resistance genes. Plant Pathol 58:503–513

van der Aa HA, Noordeloos ME, De Gruyter J (1990) Species concepts in some larger genera of the coelomycetes. Stud Mycol 32:3–19

van der Aa HA, Boerema GH, de Gruyter J (2000) Contributions towards a monograph of *Phoma* (coelomycetes). 6–1 – section phyllostictoides: characteristics and nomenclature of its type species *Phoma exigua*. Persoonia 17:435–456

van Kan JAL, van den Ackerweken GFJM, de Wit PJGM (1991) Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen *Cladosporium fulvu*m, causal agent of tomato leaf mold. Mol Plant Microbe Interact 4:52–59

Venn L (1979) The genetic control of sexual compatibility in *Leptosphaeria maculans*. Australas Plant Pathol 8:5–6

Versalovic J, Koeuth T, Lupski R (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823–6831

Voigt K, Wöstemeyer J (1995) The combination of Gilbert/Maxam chemical sequencing and the dideoxynucleotide chain termination approach facilitates the construction of species specific PCR-primers based on diagnostic RAPD bands. Microbiol Res 150:373–377

Voigt K, Schleier S, Wöstemeyer J (1998) RAPD-based molecular probes for the blackleg fungus *Leptosphaeria maculans* (*Phoma lingam*): evidence for pathogenicity group-specific sequences in the fungal genomes. J Phytopathol 146:567–576

Voigt K, Jedryczka M, Wöstemeyer J (2001) Strain typing of Polish *Leptosphaeria maculans* isolates supports at the genomic level the multi-species concept of aggressive and non-aggressive strains. Microbiol Res 156:169–177

Voigt K, Cozijnsen AJ, Kroymann J, Pöggeler S, Howlett BJ (2005) Phylogenetic relationships between members of the crucifer pathogenic *Leptosphaeria maculans* species complex as shown by mating type (*MAT1-2*), actin, and β-tubulin sequences. Mol Phylogenet Evol 37:541–557

West JS, Biddulph JE, Fitt BD, Gladders P (1999) Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. Ann Appl Biol 135:535–546

West JS, Kharbanda PD, Barbetti MJ, Fitt BDL (2001) Epidemiology and management of *Leptosphaeria maculans* (*Phoma* stem canker) on oilseed rape in Australia, Canada and Europe. Plant Pathol 50:10–27

West JS, Fitt BDL, Leech PK, Biddulph JE, Huang YJ (2002) Effects of timing of *Leptosphaeria maculans* ascospore release and fungicide regime on *Phoma* leaf spot and *Phoma* stem canker development on winter oilseed rape (*Brassica napus*) in southern England. Plant Pathol 51:454–463

Williams RH, Fitt BDL (1999) Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. Plant Pathol 48:161–175

Wöstemeyer J, Kreibich A (2002) Repetitive DNA elements in fungi (Mycota): impact on genomic architecture and evolution. Curr Genet 41:189–198

Zou HX, Xie X, Zheng XD, Li SM (2011) The tyrosine *O*-prenyltransferase SirD catalyzes *O*-, *N*-, and *C*-prenylations. Appl Microbiol Biotechnol 89:1443–1451

8 Biology, Diversity, and Management of FHB-Causing *Fusarium* Species in Small-Grain Cereals

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CONTENTS

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I. Introduction

One of the greatest challenges of humankind is to secure the food supply for a growing world population under changing climate conditions in the future. Therefore, knowledge of important crop diseases and their effective management are most essential. ***Fusarium* head blight (FHB**; synonyms: **ear blight, scab)** is one of the most destructive diseases of small-grain cereals. The disease was initially described in the UK at the end of the nineteenth century, as reviewed before (Parry et al. 1995). Throughout the twentieth century, FHB epidemics have been repeatedly reported from many wheat-growing regions of the world. Consequently, it has been concluded that FHB is a chronic problem in North America, Europe, and China (Shaner 2003). Under favourable conditions, this monocyclic flower disease may cause

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tremendous economic damage by reducing crop yield and kernel quality. The accumulation of mycotoxins generally occurring with FHB infections poses potential threats to human and animal health, and may heavily limit the use of crops for food and feed production. Several soil- and residue-borne species of the genus *Fusarium* may cause FHB. Among those, ***F. graminearum*** [teleomorph ***Gibberella zeae*** (Schwein) Petch] is the most prominent in North America and many other parts of the world (Goswami and Kistler 2004). Dependent on the region, additional species may appear as major FHB pathogens. In Europe, this includes ***F. culmorum, F. avenaceum*** (teleomorph *G. avenacea*) and ***F. poae*** (Parry et al. 1995; Xu and Nicholson 2009). In Australia, ***F. pseudograminearum*** (teleomorph *G. coronicola*) may associate with FHB (Miedaner et al. 2008). In Asia, *F. graminearum* and ***F. asiaticum*** are frequent causes of FHB, as reported from China (Qu et al. 2008), Japan (Suga et al. 2008) and Korea (Lee et al. 2009). The latter are two of at least 15 phylogenetically defined species that were recently distinguished within the so-called ***F. graminearum*** **species complex (FGSC)** also referred to as *F. graminearum* sensu lato (s. l.), which was formerly assumed to represent a single panmictic species (see II.B). Thus, FHB is triggered by a complex of *Fusarium* species.

In addition to these, less prominent species causing FHB are *F. acuminatum* (teleomorph *G. accuminata*), *F. arthrosporioides*, *F. cerealis* (syn. *F. crookwellense*), *F. equiseti* (teleomorph *G. intricans*), *F. langsethiae*, *F. oxysporum*, *F. sambucinum* (*G. pulicaris* var. *pulicaris*), *F. sporotrichioides*, *F. tricinctum* (teleomorph *G. tricincta*), and *F. verticillioides* (teleomorph *G. moniliformis*) (Parry et al. 1995; Xu and Nicholson 2009). Most of these species are found frequently only in certain regions and/or under certain environmental conditions. Beside these *Fusarium* species, two *Microdochium* species, i.e., *M. nivale* and *M. majus* are also considered FHB pathogens. Although they may have regional importance, they have received much less research interest, and thus we do not consider them further in this review.

Increased awareness of food safety and the (re-)emergence of FHB epidemics in the last decades in the USA, China, and elsewhere in the world, which resulted in huge economic losses (see III.D), have intensified research on FHB-causing pathogens, predominantly *F. graminearum* s. l. and *F. culmorum*. This has resulted in the accumulation of a huge body of epidemiological and population-genetic data. Numerous laboratory and field experiments have made it possible to determine disease-favouring environmental conditions, disease initiation and pathogens' progression *in plants*, as well as sources and ways of inoculum dispersal and resistance sources. This knowledge has promoted the development of reliable field management and plant-breeding strategies, from which farmers, the grain-processing industry, and consumers profit.

II. Systematics of FHB Causing *Fusarium* Pathogens

A. Species Definitions in Mycology

The definition of species is not only important for studies in fungal systematics and evolution, but also has far-reaching practical implications. Acknowledged species names are needed for quarantine and biosecurity policies, admission of fungicides, monitoring of resident and invading pathogens, and forecasting and treatment of emerging epidemics, just to give a few examples.

Because of the nature of most fungi, their taxonomy is arguably more problematic than, for instance, that of animals. Difficulties arise among others from the scarceness, the small size, and the variability of useful morphological characters. Sterility in sexual crosses and homothallism pose additional problems. Fungal taxonomy has applied several species concepts, as discussed in previous reviews (Cai et al. 2011; Giraud et al. 2008; Taylor et al. 2000). These comprise the **biological species concept (BSC)** that focuses on reproductive barriers, the **morphological species concept (MSC)** relying on morphological characters, the **ecological species concept (ESC)** employing adaptation to a certain ecological niche, for example a host species, and the **phylogenetic species concept (PSC)** using nucleotide and amino acid sequences, to distinguish fungal species.

Traditionally, the MSC was most widely used in fungal taxonomy, while today the PSC is frequently exploited to delineate species borders and to link anamorphs with teleomorphs. In early studies applying the PSC, phylogenies were constructed for single genes, implying that their evolution would exactly reflect that of the examined organisms, which is not necessarily true. Robust phylogenies that overcome this drawback and provide higher resolution result when the evolution of several unlinked genes is analysed in parallel, as has been done with the **genealogical concordance phylogenetic species recognition (GCPSR)** concept (Taylor et al. 2000). The GCPSR concept, which can be considered an extension of the PSC, merges different isolates to the same species when the branching patterns seen in the phylogenies of several genes are highly similar. Isolates belonging to the same branch have thus not exchanged genetic material with isolates from a neighbouring branch after the corresponding furcation took place. Therefore, such independent evolutionary lineages are regarded as species under GCPSR. For additional reading on speciation processes in fungi, we recommend previous reviews (Giraud et al. 2008; Kohn 2005).

B. *Fusarium* Systematics and Speciation in the FHB Species Complex

The taxonomy of the anamorph genus *Fusarium*, which connects with teleomorphs in the genera *Gibberella* and *Nectria* (Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Nectriaceae), has experienced considerable changes in the application of species concepts. The initial descriptions of *Fusarium* by Heinrich Friedrich Link in 1809 and Elias Magnus Fries in 1821 applied the MSC. Fusoid macroconidia including a foot cell commonly defined the genus. Typical characteristics to differentiate species were the morphology (i.e., shapes and sizes) of macroconidia, microconidia, and chlamydospores, but also growth rates, colony morphology, and pigmentation (Leslie and Summerell 2006). The number of species varied considerably over the decades, depending on the morphological characteristics included and their weighting. In several cases, species defined by the MSC comprised several mating populations or species according to the BSC. Application of the PSC uncovered in *Fusarium* numerous cryptic species often forming groups of more or less closely related species, e.g., the *F. chlamydosporum*, *F. dimerum*, *F. graminearum*, *F. incarnatum-equiseti*, *F. oxysporum*, *F. solani*, *F. sporotrichioides*, *F. tricinctum,* and *Gibberella fujikuroi* species complexes. The latter is particularly extended, with about 50 phylogenetic lineages, 34 of which are also distinguishable by morphological characters (Kvas et al. 2009). Currently, 1374 species names are listed for *Fusarium* in the MycoBank database (http://www.mycobank.org/).

F. graminearum is worldwide the most important species causing FHB. The perception of this species has, however, changed substantially over time. Even 40 years ago, two distinct groups of fungi were found to exist within *F. graminearum* that were differentiated among others by mating behaviour and aggressiveness in causing crown rot, but not by morphological characteristics (Purss 1971; Scott and Chakraborty 2006). Isolates belonging to group I are heterothallic, cause heavier crown rot symptoms, and are today known as *F. pseudograminearum* (*Gibberella coronicola*). Isolates belonging to group II kept their original name. During recent years, the application of GCPSR yielded remarkable insights into the evolution of fungi within this group. Phylogenetic analysis of six single-copy nuclear genes amplified from DNAs of a worldwide collection of isolates showed concordance of the six trees (O'Donnell et al. 2000). Seven distinct lineages were resolved within *F. graminearum* which were also clearly separated from other species causing FHB such as *F. culmorum* and *F. cerealis*. Follow-up studies that extended these phylogenetic analyses by using up to 13 genes and numerous isolates from many regions around the globe identified a further ten lineages (Desjardins and Proctor 2011; O'Donnell et al. 2004, 2008; Sarver et al. 2011; Starkey et al. 2007; Ward et al. 2002; Yli-Mattila et al. 2009) (Fig. 8.1). The origins of the analysed isolates indicated that several of the lineages are linked to distinct geographic regions, which suggested that they had evolved allopatrically. Fertile crosses between members of different lineages were established in the laboratory (Bowden and Leslie 1999; Cumagun et al. 2004), which would contradict their status as distinct species under the BSC. However, recombination events between lineages were

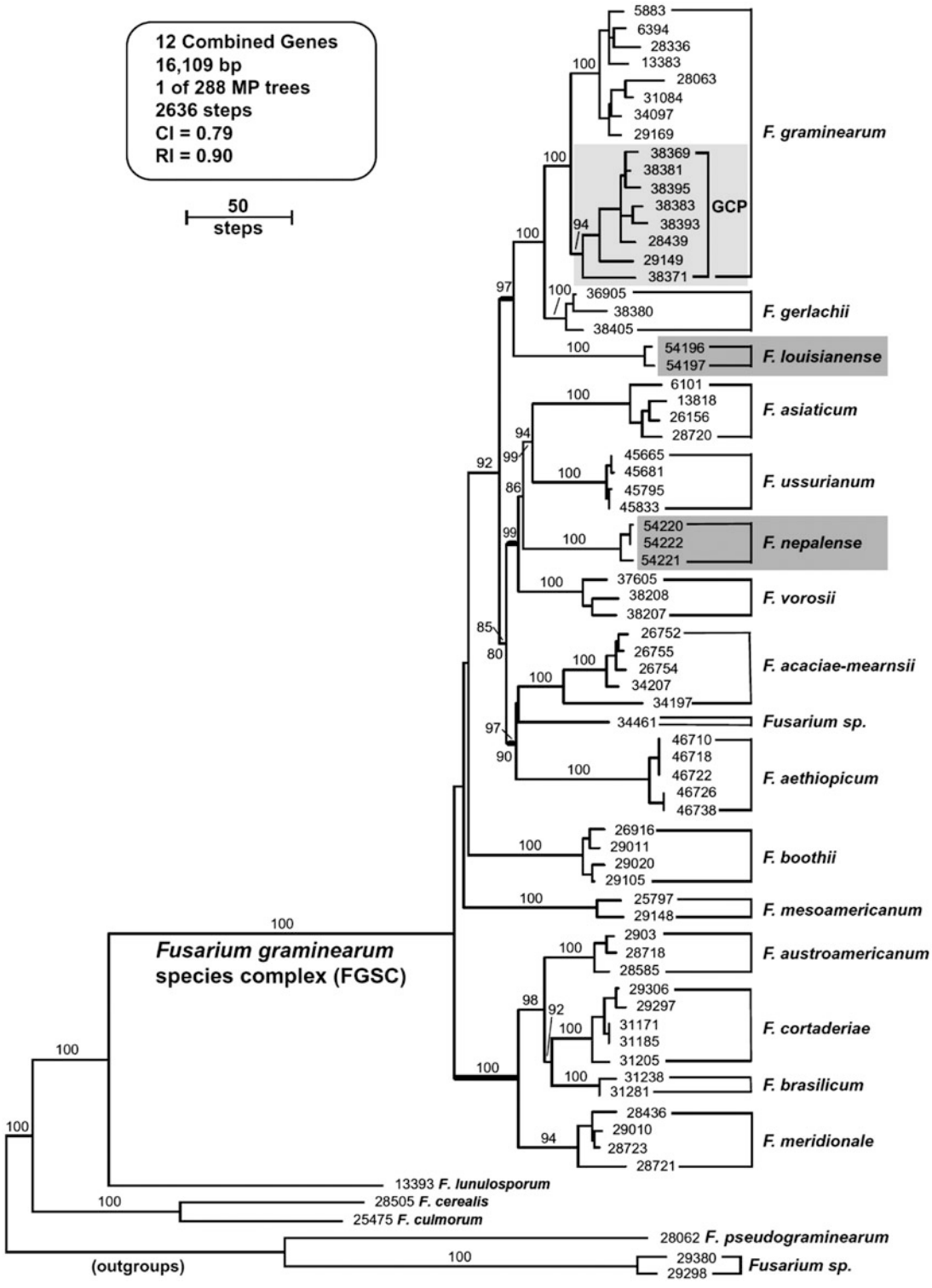

Fig. 8.1. Molecular phylogeny of B-type trichothecene toxin-producing fusaria inferred from sections of 12 genes. *F. pseudograminearum* and two *Fusarium* sp. were used to root the tree. Maximum likelihood (*ML*)

extremely rarely observed in field isolates, although different lineages were found to co-occur at sometimes small distances, suggesting the existence of as yet unknown reproductive barriers counteracting their hybridisation in nature. Therefore, the lineages resolved among isolates, which would have all been ascribed previously to *F. graminearum*, appear to have been reproductively separated for a while. Thus, many researchers consider the phylogenetic lineages nowadays as distinct species. Nonetheless, some researchers question the species status of the described *F. graminearum* lineages (Leslie et al. 2007; Leslie and Bowden 2008).

The *F. graminearum* species complex (FGSC) is treated here as synonymous with *F. graminearum* sensu lato (s. l.) (Fig. 8.1). **Lineage 7, which is not linked to a particular continent but rather occurs pandemically, kept the original name, i.e., *F. graminearum***, and is also referred to as *F. graminearum* sensu stricto (s. str.).

The other species and their initially presumed origins are: *F. austroamericanum* (lineage 1, South America), *F. meridionale* (lineage 2, Africa), *F. boothii* (lineage 3, Africa), *F. mesoamericanum* (lineage 4, Central America), *F. acaciae-mearnsii* (lineage 5, Africa), *F. asiaticum* (lineage 6, Asia), *F. cortaderiae* (lineage 8, South America), *F. brasilicum* (South America), *F. vorosii* (Asia), *F. gerlachii* (upper Midwest of the USA), *F. aethiopicum* (East Africa), *F. ussurianum* (Russian Far East), *F. louisianense* (Louisiana, USA), *F. nepalense* (Nepal) and two as yet unnamed lineages/species.

It was suggested that the phylogenetically basal species were endemic in the southern hemisphere whereas the more derived species, in particular *F. graminearum* s. str., evolved in the northern hemisphere (O'Donnell et al. 2000; Starkey et al. 2007). Several of these lineages/species also occur in other regions or even continents, which might be the result of human activities. The identification of distinct lineages/species within *F. graminearum* s. l. under the GCPSR concept made it possible to evaluate the discriminatory power of some of the morphological characters that had been used previously to define species in the FHB complex using the MSC (O'Donnell et al. 2004; Sarver et al. 2011; Starkey et al. 2007) (Fig. 8.2). *F. culmorum* was distinct from all other species analysed. However, within the FGSC most species were not discernible by morphological characters.

Phylogenetic studies were also conducted for additional species causing FHB. Isolates of *F. culmorum* originating from four continents were analysed for portions of three genes which separated four lineages (Obanor et al. 2010). Three lineages comprised isolates from more than one continent. In addition, there were no strong signals of linkage disequilibrium. Therefore, it was suggested that in contrast to *F. graminearum* s. l., *F. culmorum* is a single species showing little biogeographic structure. Sequencing parts of four genes in isolates of *F. pseudograminearum* from three continents resulted in a multilocus tree separating six lineages (Scott and Chakraborty 2006). It was also suggested that *F. pseudograminearum* is a single species exhibiting little biogeographic structure. The same conclusion resulted for *F. poae* in a study analysing two genes in isolates from two continents (Stenglein et al. 2010). Therefore, it appears that the distinct biogeographical separations seen in *F. graminearum* s. l. are not a feature typical for all species causing FHB.

In addition to geographical separation, also diverging preferences for temperature and host species were discussed to contribute to the divisions within the FGSC. In China, *F. graminearum* was the most frequent FHB species on wheat in regions with average temperatures of at most 15 °C, while *F. asiaticum* dominated warmer regions in the south (Zhang et al. 2007). However, a more recent large study suggested that not temperature per se but rather the cropping system, which in turn depends on the climate, is more important in

Fig. 8.1. (continued) bootstrap values are indicated above nodes, whereas maximum parsimony (*MP*) bootstrap values are only indicated if they differed by ≥5 % from the ML bootstrap value. Thick internal nodes are used to identify four strongly supported, biogeographically structured subclades within the FGSC. *GCP*, genetically divergent Gulf Coast population of *F. graminearum* (Figure is reprinted from Sarver et al., © 2011, with permissions from Elsevier and the author)

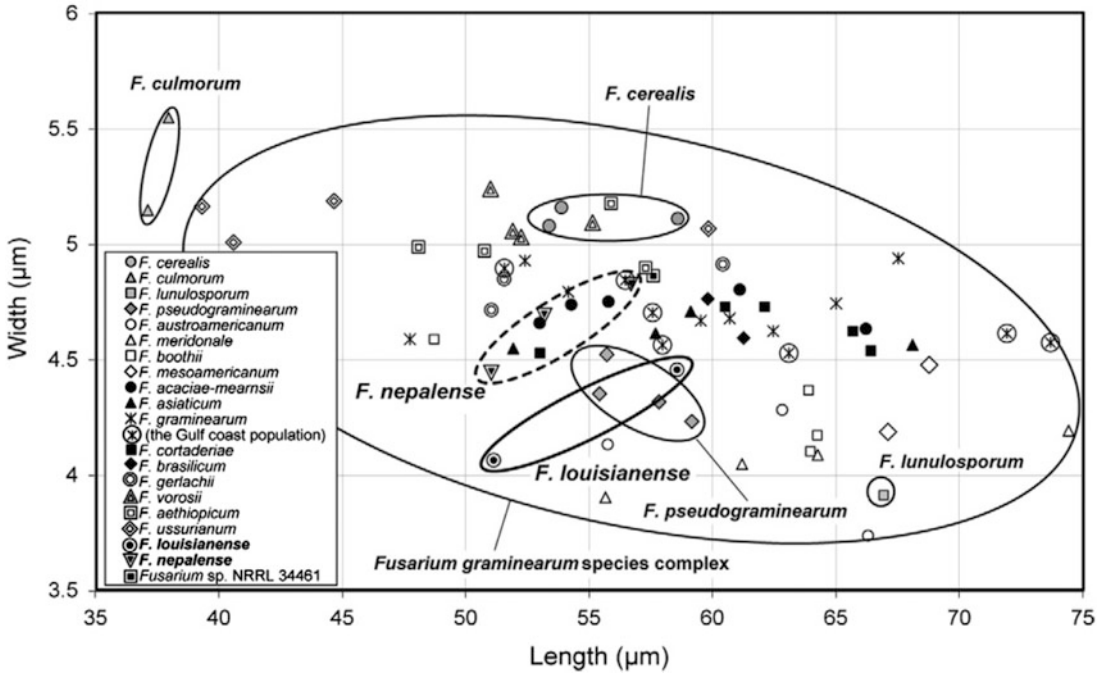

Fig. 8.2. Length and width of 5-septate conidia of B-clade species. The *oval* circumscribing the two isolates of *F. louisianense* and three isolates of *F. nepalense* graphically illustrates that conidial length and width cannot be used to identify these species (Figure is reprinted from Sarver et al., © 2011, with permissions from Elsevier and the author)

determining regional prevalence of the two species (Zhang et al. 2012). A previous study from Korea also indicated that *F. graminearum* associates more frequently with maize–wheat rotation, whereas *F. asiaticum* with rice–wheat rotation (Lee et al. 2009).

C. Trichothecene Chemotypes

Members of the genus *Fusarium* produce a variety of different mycotoxins. These include **zearalenone**, **fumonisins**, and **trichothecenes** that have been intensively investigated with respect to biosynthesis, toxicity, and genetics. The trichothecenes are tricyclic sesquiterpenes, and are divided into four types A to D. **For the species discussed here in detail, the type B-trichothecenes are most important.** They are characterized by the presence of a keto group at the C-8 site of the ring system, which is missing in the A-trichothecenes. Many of the species causing FHB produce compounds belonging to the B-trichothecenes. Some FHB species produce A-trichothecenes, such as T-2 toxin derivatives (*F. acuminatum*, *F. sporotrichioides*, *F. poae*, *F. langsethiae*), or diacetoxyscirpenol (*F. equiseti*, *F. poae*) (Desjardins 2006). A few species make both A- and B-trichothecenes (*F. equiseti*, *F. poae*). It should also be mentioned that a few species do not produce trichothecenes at all, but other mycotoxins (see III.E).

Depending on the strain analysed, in the B-trichothecene producers, nivalenol (NIV), deoxynivalenol (DON), and variants of them that are acetylated at the C-3, C-4 and the C-15 positions (4-ANIV; 4,15-diANIV; 3-ADON; 15-ADON; 3,15-diADON) were identified from in vitro cultures (Alexander et al. 2011). Depending on the medium used for cultivation, individual isolates produced essentially single compounds (in a particular liquid medium) or mixtures of two or three compounds (on rice grains). Three different chemotypes (NIV, 3ADON, 15ADON) that had been proposed more than 20 years ago, although by using other names (Miller et al. 1991), were substantiated by many other studies. Isolates representing the **NIV chemotype** produced on rice

grains a mix of NIV, 4-ANIV, and 4,15-diANIV, those of the **3ADON chemotype** 3-ADON and DON, and those of the **15ADON chemotype** 15-ADON, DON, and 3,15-diADON (Alexander et al. 2011). The biosynthesis of these compounds is accomplished by proteins encoded by genes mostly contained in the *Tri* gene cluster, a typical finding for genes involved in the synthesis of secondary metabolites in fungi. Here, we will not go into the details of the biosynthesis of B-trichothecenes, but do refer to recent literature (Alexander et al. 2009; Kimura et al. 2007; Proctor et al. 2009). However, we need to mention that variations in certain *Tri* genes determine the chemotype of a given isolate. Mutations in the *Tri13* and *Tri7* genes of isolates representing the 3ADON and 15ADON chemotypes distinguish them from the NIV chemotype, a finding that has been confirmed by functional molecular genetics (Lee et al. 2002). Sequence variations in the *Tri8* gene leading to differential enzymatic activities discriminate the 3ADON and 15ADON chemotypes (Alexander et al. 2011). **In populations of *F. graminearum* and *F. asiaticum* all three chemotypes were found, but only the NIV and 3ADON chemotypes seem to exist in *F. culmorum*** (Miller et al. 1991; Ward et al. 2002; Zhang et al. 2012). In other species, which, however, have been less intensively sampled, only one chemotype may occur, e.g., NIV in *F. cerealis* and *F. meridionale*.

When the biogeographical separations within the FGSC were recognized, it became important to assess whether they would correspond to chemotypes. A study comparing combined phylogenies of eight genes in the *Tri* cluster with six unrelated genes in a collection of strains representing eight of the *F. graminearum* s. l. species plus four additional *Fusarium* spp. found no congruence of the trees (Ward et al. 2002). It was suggested that the chemotypes originated in an ancestral species and that they were then passed on through several speciation events. The B-trichothecene chemotypes are maintained in extant populations by balancing selection, indicating that variable selective forces resulting from variations in spatial and/or temporal factors may allow for their co-existence.

III. Fusarium Head Blight

A. Hosts and Symptoms

FHB was originally described for wheat, but the causative fungi were subsequently found to infect a broad range of small-grain cereals including rye, barley, spelt, and less commonly oat (Atanasoff 1920; Stack 2003). In addition, some FHB pathogens and their mycotoxins were also found in rice (Desjardins et al. 2000; Nyvall et al. 1999) and maize (Logrieco et al. 2002).

The typical FHB symptoms were described about 100 years ago, and were more or less confirmed by later authors (Brown et al. 2010; Bushnell et al. 2003; Stack 2003). The first symptoms of a *Fusarium* head infection are small (2–3 mm), slightly brown, water-soaked spots on the glumes (Fig. 8.3). Under favourable conditions, these lesions occur 3–4 days after infection, and increase more or less rapidly. They differ somewhat between the different cereal species, being for example less uniform in barley than in wheat and rye (Atanasoff 1920). Awns often become deformed, twisted, and curved downward (Goswami and Kistler 2004). With progression of the infection, the colonized spikelets may finally die, dry up, and bleach comparable to ripe spikelets. This **premature bleaching of cereal spikelets** rendering ears partly white and partly green is the typical symptom of the disease being manifested in its designation as head blight. Whereas in barley those bleaching symptoms appear generally as discrete spikelets sometimes scattered throughout the head, in wheat multiple adjoining spikelets are often affected. Entire apical parts of the wheat ear may dry up completely, as fungal infection can progress through the rachis, cutting off the water and nutrient supplies of the affected spikelets. In barley, the spread of the fungus into the rachis is inhibited at the rachis node and rachilla, thus preventing further infection of adjacent florets (Jansen et al. 2005). However, depending on the weather conditions, FHB infection can also become visible by cottony salmon-pink to red fungal mycelia spreading outside the glumes

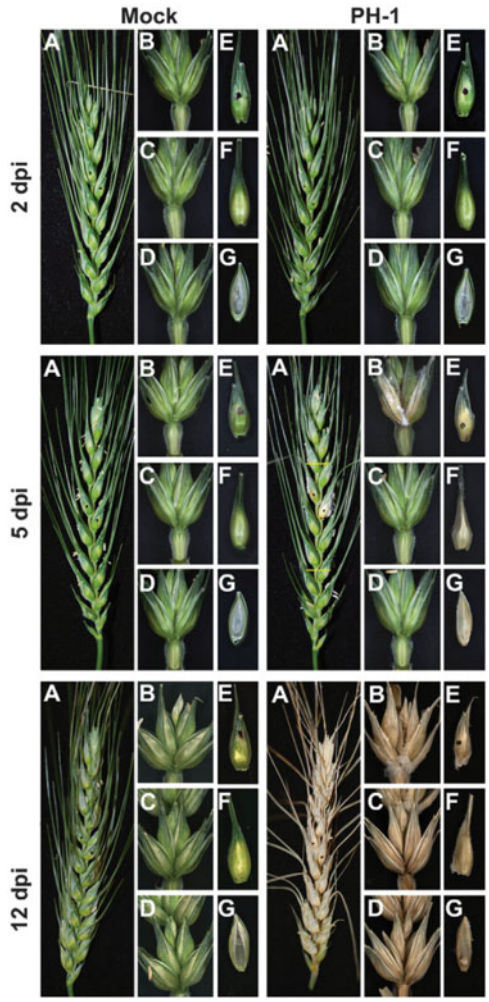

Fig. 8.3. Symptoms of *F. graminearum* infection at 2, 5 and 12 dpi following the addition of conidia into two adjacent spikelets in the middle of the ear. *Left and right panels* compare water-only inoculated controls (*Mock*) with infections using the PH-1 wild type reference. (*A*) Entire ear with a *black dot* marking each of the two inoculated spikelets. The two *yellow horizontal lines* superimposed on the inoculated PH-1 ear at 5 dpi indicate the extent of symptomless colonisation identified by microscopy. (*B*) The inoculated spikelet and adjoining rachis node and rachis segment. (*C* and *D*) Successive spikelets below the inoculated spikelet. The individually excised tissues of the inoculated spikelet, glume (*E*), lemma (*F*), and palea (*G*). In the PH-1 infected ear, the grain remained in the floral cavity but had not developed post inoculation and appeared shrivelled at 12 dpi (Figure is reprinted from Brown et al., © 2010, with permissions from Elsevier and the author)

(Atanasoff 1920; Parry et al. 1995). In advanced infection stages, reddish fungal spore masses (sporodochia) or black-coloured, ascospore-containing perithecia may become visible (Bushnell et al. 2003).

B. Disease Cycle

Detailed knowledge of a pathogen's life cycle and infection process is essential for effective disease management (see V). An illustration of the life cycle of *F. graminearum* is given in Fig. 8.4. A wealth of literature covering these topics has accumulated since the discovery of the causal agents of FHB. We will focus here on important and recent findings.

1. Source and Spreading of Inoculum

Fusarium species causing head blight are facultative pathogens, since they survive saprophytically on residues of cereal hosts and many species of gramineous and broad-leaf weeds (Pereyra and Dill-Macky 2008). Such weeds may have been already colonized as living hosts, often without visible symptoms (Inch and Gilbert 2003; Jenkinson and Parry 1994a). Endophytic colonisation of annual weeds is discussed as enhancing survival of the fungus (Summerell et al. 2011). The authors suggested that with the onset of senescence, the fungus might derive advantage from rapid growth in dying tissues, thereby outcompeting saprophytic competitors. ***F. graminearum* may survive up to 3 years on plant debris**, depending on the plant species and whether the residues remain on top of the soil or are buried (Pereyra and Dill-Macky 2008; Pereyra et al. 2004). Buried substrate is degraded faster by soil microbes, which could affect survival of FHB fungi. However, in contrast to *F. graminearum*, other FHB species attain higher prevalence on residues over time, as they possess greater saprophytic competitiveness (Pereyra and Dill-Macky 2008).

Colonized plant residue is the principal source of inoculum, as it may give rise to asexual (conidia) and/or sexual spores

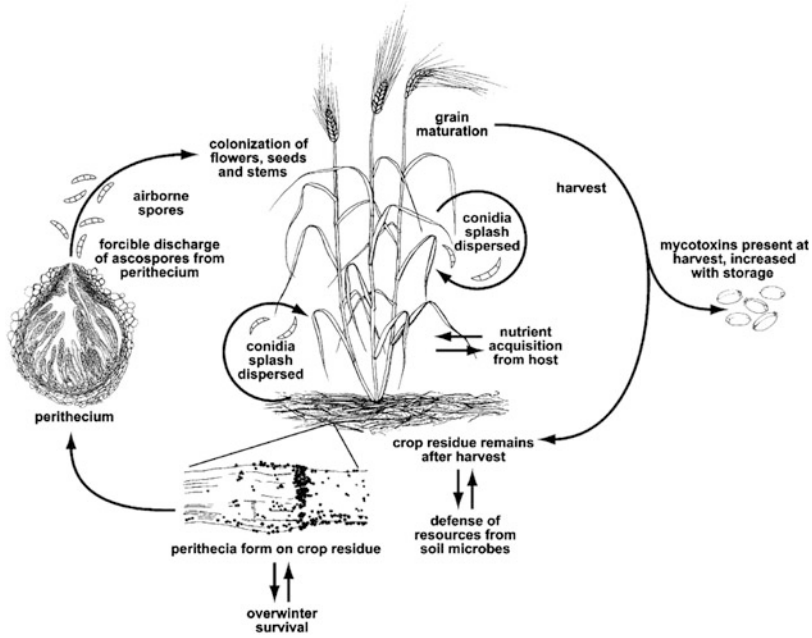

Fig. 8.4. Life cycle of *Gibberella zeae* (anamorph *Fusarium graminearum*) (Figure is reprinted from Trail et al., © 2009, with permissions from American Society of Plant Biologists and the author)

(ascospores) both of which may initiate cereal head infection. Sexual development is only known from some species of the FHB complex, including *F. graminearum*/*G. zeae*. Ascospores are produced in and forcibly discharged from purplish-black, flask-shaped perithecia (Trail and Common 2000; Trail et al. 2005). Maturation of perithecia is favoured by temperatures of 20–24 °C and residue moisture levels greater than −1.35 MPa (Dufault et al. 2006). Another study indicated an even higher temperature optimum for ascospore production (Sutton 1982). In contrast, ascospore release rates peaked at 16–17 °C (Tschanz et al. 1976). In addition to temperature, relative **humidity also influences ascospore expulsion, which has been found to be highest 1–4 days after a rainfall**, but was low during heavy rain or continuous relative humidity of more than 80 % (Doohan et al. 2003; Fernando et al. 2000; Inch et al. 2005; Paulitz 1996). Furthermore, ascospore discharge appears to be influenced by diurnal rhythms, with a maximum at night-time usually before midnight. It has been suggested that the stimulus for the release of ascospores is a sharp increase in humidity during early evening (Paulitz 1996; Trail et al. 2002).

An additional factor influencing perithecial density, and thus the number of ascospores produced, is the plant species on whose residues perithecia are formed in the field. **Maize debris allows for the production of the highest numbers of ascospores of *G. zeae*** (see also V.A.1), but wheat and barley residues may also promote considerable inoculum production (Dill-Macky and Jones 2000; Pereyra and Dill-Macky 2008). Because laboratory experiments indicated very short shooting distances of less than 1 cm in still air (Trail et al. 2005), **wind needs to be taken into account as a factor promoting ascospore drift.** This was supported by field experiments suggesting that **most ascospores travel in the meter range**. Ascospore densities declined by 50 % at a distance of 18 m from the inoculums, and by 90 % within 60 m (de Luna et al. 2002). A release–recapture study using AFLP markers to identify strains found that at distances of ≥24 m from the source, only 5 % of the recaptured genotypes corresponded to those of the released genotypes, and they caused only 1 % of the FHB

infections at that distance (Keller et al. 2010). However, **long-distance transport of some ascospores also seems to occur**, since *G. zeae* was isolated from wheat heads at distances of tens of kilometres away from any known source of inoculum (Francl et al. 1999). In addition, during the time of anthesis, large numbers of viable *G. zeae* spores were regularly detectable in the lower atmosphere (Maldonado-Ramirez et al. 2005). AFLP haplotypes of airborne spores were highly diverse at a single location in New York State, and resembled those found in populations in seven other states (Schmale et al. 2006). This suggested that spores originating from multiple locations over large geographic distances may serve as inoculum, in addition to local populations.

Although *F. graminearum*/*G. zeae* ascospores have been considered the primary source of inoculum, in other species of the FHB complex, they are rarely formed or not at all, e.g., *F. culmorum* and *F. poae*. For such species, asexual spores are the primary inoculum for cereal flower infection. The generally fusiformed, three- to seven-septated, straight to banana-like curved macroconidia of *Fusarium* spec. are often produced in slimy, orange-coloured sporodochia. Similar to ascospores, the production of conidia is also influenced by temperature and moisture (Doohan et al. 2003; Xu 2003). Optimal temperatures for conidiation of FHB-causing *Fusarium* species range between 28 °C and 32 °C, whereas temperatures below 16 °C and above 36 °C inhibit their production. Aerial abundance of **macroconidia peaked during and shortly after rainfall, suggesting that their dispersal mainly depended on rain splashing** (Rossi et al. 2002). Thus, **conidia may travel rather short distances, typically less than 1 m in vertical and horizontal directions** (Horberg 2002; Jenkinson and Parry 1994b). In contrast to ascospores, release of macroconidia does not exhibit diurnal periodicity (Fernando et al. 2000).

2. Infection Process and Colonisation of the Host

Depending on the species involved, both ascospores and macroconidia can effectively initiate FHB (Stack 1989). In addition to these primary inocula, hyphal fragments and chlamydospores, which have thick cell walls permitting their prolonged survival in soil, may also serve as propagules, potentially contributing to cereal-head infections (Sutton 1982). **Flower infections primarily occur during anthesis**, as open florets provide access to pathogens' primary penetration sites, i.e., the developing caryopses as well as the adaxial surfaces of lemma and palea. Studies on the penetration, subsequent ingress into host tissues, and molecular host–pathogen interactions have been a matter of extensive research, which has been reviewed recently (Kazan et al. 2012; Trail 2009; Walter et al. 2010) and is thus rather concisely treated here. Germination of ascospores requires a relative humidity (R.H.) of at least 30 %, whereas optimal conditions are 90 % R.H. and 15 °C (Gilbert et al. 2008). Macroconidia have been shown to need more than 80 % R.H. to germinate (Beyer et al. 2004). In contrast to R.H., germination was shown to be much less affected by light, temperature, and pH conditions (Beyer et al. 2004).

Subsequent to germination, hyphae initially colonize the exterior floret surface without immediate penetration. The latter occurs when hyphae arrive at stomata or more susceptible interior floret tissues (Bushnell et al. 2003). Extruding anthers also offer an opportunity for floret invasion. Inside the florets, direct penetration of epidermal cells by infection hyphae, infection cushions, and lobate appressoria has been described (Boenisch and Schäfer 2011; Kang and Buchenauer 2000). After penetration, two distinct phases of infection have been distinguished (Brown et al. 2010). Hyphae advance intercellularly through living host tissue without causing visible symptoms initially (Fig. 8.3). After 2–3 days, older parts of the mycelium invade host cells leading to necrosis, whereas the hyphal front continues with biotrophic growth in the apoplast. Thus, it has been suggested that FHB pathogens such as *F. graminearum* should be considered as hemibiotrophs instead of necrotrophs (Kazan et al. 2012).

Interestingly, fungal spreading inside the plant from the initially infected spikelets into

adjacent spikelets differs with the cereal host species. In barley, *F. graminearum* is blocked at the rachis node, whereas in wheat it invades neighbouring florets (Fig. 8.3), which depends on trichothecenes such as DON (Jansen et al. 2005). Thus, **DON is not a pathogenicity factor, but rather a virulence factor promoting the proliferation of the fungus within the head of wheat.** The innate type II resistance (see V.B.1) of barley seems to be mediated by several defence mechanisms, including the detoxification of DON by conversion to DON-3-O-glucoside (Gardiner et al. 2010; Schweiger et al. 2010). Apparently, in wheat this activity is not sufficiently available at the right time and place.

This host-specific effect of DON was demonstrated by GFP-tagged mutants of *F. graminearum* carrying deletions in the *Tri5* gene, which is essential for trichothecene production. In contrast to the wild type, *tri5* mutants remained restricted to the inoculated spikelet of wheat (Jansen et al. 2005). Correspondingly, similar results were obtained for triticale, durum wheat, and in an alleviated manner for rye (Langevin et al. 2004). Similarly to DON, NIV is also important for spreading in wheat but not in barley, as shown by a *tri5* mutant of a NIV producer. Interestingly, this mutant was less virulent on maize, in contrast to the corresponding DON mutant, underlining the fact that the impact of trichothecenes may vary with the host (Maier et al. 2006; Proctor et al. 1995).

The production of trichothecenes is induced by, among others, reactive oxygen species (ROS), polyamines, and low pH (Kazan et al. 2012; Walter et al. 2010). Generally, DON and related compounds are non-specific toxins that bind to protein L3 of eukaryotic ribosomes, leading to the inhibition of protein synthesis. This may have several consequences including programmed cell death and hydrogen peroxide production (Desmond et al. 2008).

In the case of wheat, FHB pathogens may colonize plants systemically from the heads downwards through the culms. *F. graminearum* initiates this growth during seed development of wheat, and slows down with the onset of senescence (Guenther and Trail 2005). Hyphae penetrate culms through xylem vessels and pith cavities. Subsequently, radial colonisation is initiated and extended through the parenchyma and, finally, the chlorenchyma. About 16–18 days after infection, hyphae fill substomatal cavities, but do not grow through stomata. Instead, association of *F. graminearum* with stomata and silica cells specifically initiates sexual development. Perithecial initials serve as overwintering structures, and in the following spring maturation of perithecia completes on crop debris.

C. Related *Fusarium* Diseases in Small-Grain Cereals: Seedling Blight, Foot Rot and Crown Rot

FHB-causing pathogens are not exclusively flower-infecting pathogens, but may also cause seedling, foot, crown, and root diseases. **Seedling blight disease** can be caused by *F. graminearum*, *F. culmorum*, and other FHB species (Yang et al. 2011). Seedling blight can result either from root or subcrown infections of the emerging seedling or from infected seeds, allowing for vertical transmission of the pathogen (Jones 1999; Wang et al. 2006). The respective symptoms range from pre- and post-emergence death, lesions, and low plant vigour (Imathiu et al. 2010).

Foot and crown rot caused by *Fusarium* spp. are two distinct cereal diseases (Cook 1981). **Foot rot** occurs worldwide under humid conditions, and is caused by, among others, *F. culmorum* and *F. graminearum*. In Central Europe, mixed infections together with *Oculimacula yallundae* (anamorph names *Helgardia herpotrichoides*, *Pseudocercosporella herpotrichoides*), *Thanatephorus cucumeris* (anamorph *Rhizoctonia solani*), and/or *Microdochium* spp. occur often (Miedaner et al. 1995). Symptoms are brown lesions at the stem base with visible fungal mycelium in the centre of the culm. Later on, the whole culm gets soft in the necrotic regions, which might lead to early lodging and, as a consequence, reduction in grain weight. Foot rot occurs mainly in dense stands with high nitrogen input, when weather conditions are moist at jointing stage (EC 31) or later (Cook 1981). In contrast, ***Fusarium* crown rot (FCR)** is mainly caused by *F. pseudograminearum*, and leads to

necrosis and dry rot of the crown, basal stem, and root tissue (Chakraborty et al. 2006). Further symptoms include brown, necrotic lesions, stand reduction, root rotting, tiller abortion, and the formation of so-called whiteheads (Hogg et al. 2007; Scherm et al. 2011). FCR was first reported from Australia, but occurs in other wheat-growing areas also, and is promoted by hot and dry weather conditions (Burgess et al. 1981). Therefore, a synonymous disease name is "dryland foot rot" or "dryland root rot". Especially late-season drought during flowering and grain-fill periods result in FCR symptoms and the occurrence of whiteheads that contain no or only shrivelled grain (Chakraborty et al. 2006). Moreover, grain from plants infected by FCR may also become contaminated by mycotoxins as a result of their potential vascular translocation (Covarelli et al. 2012). Some species of the FHB complex can in addition to FCR also cause root rots of wheat (Beccari et al. 2011). Therefore, it needs to be considered that FHB belongs to a complex of several cereal *Fusarium* diseases with epidemiological relationships among each other (Covarelli et al. 2012; Parry et al. 1995). Beyond infections in small-grain cereals, FHB fungi may also cause diseases in maize, rice, and some wild grasses. In maize, red ear rot is mostly caused by *F. graminearum*, *F. culmorum*, and *F. cerealis*, whereas pink ear rot is typically caused by *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*, thus resulting in different mycotoxin profiles in contaminated grains (Logrieco et al. 2002).

D. Economic Impact

FHB damage to grain production may be multifold. This disease can result in tremendous **yield losses due to reduction of number, size, and weight of the grains** produced by infected spikes. In addition, FHB seriously affects grain quality, impeding its marketing, export, and processing (McMullen et al. 1997). **Fusarium-damaged kernels (FDK)** may appear shrivelled and discoloured, and often have an altered composition of starch and storage proteins, which may result in problems for the production and processing of flour and flour-based products (Boyacioglu and Hettiarachchy 1995; Nightingale et al. 1999; Wang et al. 2005). Furthermore, high levels of **FDK impede the production of healthy seeds**, which holds the risk for seedling blight (Snijders 1990a). This would result in reduced numbers of tillers per acreage, which could thereby affect also the following harvest (Paul et al. 2006). However, the most prominent problem with grain from FHB-infected plants is **contamination with mycotoxins being harmful to humans and animals** (see III.E). Depending on toxin levels, harvested grains might be completely unsuitable for consumption. In the European Union, the USA, and some other countries, mycotoxin contamination of grain is of increasing importance to farmers, because legally enforceable limits for mycotoxin contents in food have been applied (see III.E). With regard to the above-mentioned aspects, FHB is a unique plant disease, as it results in negative economic effects for the whole grain production and processing industry (Gilbert and Tekauz 2000).

Although FHB epidemics occurred rather sporadically in both space and time in the past, **disease incidence and severity has apparently increased worldwide**, and thus resulted in massive economic losses in recent decades. In the USA, FHB has been ranked as the worst plant disease since the stem-rust epidemics of the 1950s (Windels 2000).

In the 1990s, several states were repeatedly hit by FHB epidemics. It has been estimated that during 1991 to 1997 wheat producers in affected regions experienced yield and price reductions accumulating to $1.3 billion (Johnson et al. 2003). Barley growers in the upper Midwestern states lost about $200 million during the same period (Nganje and Johnson 2003). Losses from FHB to the US wheat and barley producers were estimated at 871 million dollars for the period of 1998–2000 (Nganje et al. 2004). In 2003, FHB caused yield losses of about 30–50 % in Southeastern states, resulting in pre-milling losses of about $14 million and further losses of several millions to the mills as a result of increased costs for shipping, testing, and handling (Cowger and Sutton 2005). However, the total economic impact is probably about two to four times higher than the direct losses if secondary effects impacting other sectors of rural and state economies are also considered (Johnson et al. 2003; Nganje et al. 2002, 2004). Similarly, FHB epidemics also caused

huge losses to the Canadian cereal industry that were estimated at more than $1 billion for 1993–2000 (Pandeya and Graf 2006). An outbreak in 1996 in Ontario resulted in yield losses of 8.4 million bushels, and in addition because of mycotoxin contamination a loss of quality, and thus sales profits of about 27.5 million bushels (Schaafsma 2002). In China, FHB is potentially threatening more than 7 million hectares, which represents about one-fourth of the world's wheat acreage (Bai et al. 2003). Between 1950 and 1990, moderate to severe FHB epidemics were recorded in 21 years, resulting in yield losses of up to 40 % (Wang 1997). In the 1990s, FHB occurred at higher frequency and severity. Total annual yield losses resulting from FHB are estimated for China at about 1 million metric tons (Bai and Shaner 2004), and up to 2.5 million tons of grains may be lost in epidemic years (Dubin and Ruckenbauer 1997). Because of the devastating impact of FHB, regional agricultural markets have changed notably as many farmers abandoned wheat production, as evidenced by the southern province Hubei where the wheat acreage decreased from 1.2 million to less than 0.6 million hectares (Yang et al. 2008).

E. Health Aspects

FHB-causing *Fusarium* species are well-known producers of a broad range of biologically active secondary metabolites, including several that are hazardous to human and animal health and thus referred to as mycotoxins. There exist several documented cases of severe outbreaks of human and animal toxicoses that were traced back to the consumption of grain-based food or feed contaminated with *Fusarium* mycotoxins, as reviewed elsewhere (Desjardins 2006).

Because of their acute toxicity and their prevalence in *Fusarium*-infected grain, trichothecenes have been most strongly associated with chronic and fatal toxicoses of humans and animals (Desjardins and Proctor 2007). At the molecular level, by binding to protein L3 of the 60S subunit of eukaryotic ribosomes **trichothecenes inhibit peptidyltransferase activity, leading to the cessation of protein biosynthesis** (Feinberg and McLaughlin 1989). Ingestion of grain contaminated with trichothecenes can cause intestinal irritations including nausea, emesis, diarrhea, and/or anorexia, potentially followed by aleukia and anaemia, which may even lead to death (Foroud and Eudes 2009; Li et al. 2011; Pestka and Smolinski 2005). Toxicity of trichothecenes differs between farm animals, and depends on age and gender. In general, pigs are most susceptible, followed by poultry and then ruminants. Prolonged dietary uptake may adversely affect the haematopoietic, the immune, the nervous, and possibly also the female reproductive systems (Bennett and Klich 2003; Sugita-Konishi et al. 2008). Noticeably, the toxicity of the trichothecenes differs. T-2 toxin is much more toxic than DON in experiments testing lethal oral doses in mice and cytotoxicity in human cells (Desjardins 2006). However, in Europe DON (including its acetylated derivatives) is the most prevalent toxin associated with FHB (Logrieco et al. 2002).

As pointed out above, species in the FHB complex produce additional classes of mycotoxins in addition to the trichothecenes. In terms of food security, the most important is the resorcyclic acid lactone compound zearalenone (ZEA), which has a relatively low acute toxicity but a high biological potency (Bennett and Klich 2003). **The oestrogen-like structure of ZEA leads to a high affinity to oestrogen receptors** (Ueno 1985), which in turn causes malfunctions of female reproductive organs (Kuiper-Goodman et al. 1987). In pigs and cattle, low ZEA doses may lead to infertility, and higher doses may affect the nidation and development of the foetus as well as the viability of the new-born (Conkova et al. 2003). Metabolic transformations in animals and man result in ZEA derivatives with increased oestrogenic activity, e.g., α-zearalenol (Ueno 1985). In addition, ZEA has hepatotoxic, haematotoxic, immunotoxic, and genotoxic properties (Zinedine et al. 2007). Furthermore, ZEA has been indicated as a potential promoter of cancer tumorigenesis (Ahamed et al. 2001).

Some of the species in the FHB complex contaminate grains with additional mycotoxins including moniliformin, beauvericin, and enniatins. This is especially true for Europe, where *F. avenaceum* and *F. poae* are among the major FHB pathogens (see IV.A). Acute toxication with the hydroxycyclobutenedione compound **moniliformin** (MON), which is the major mycotoxin of *F. avenaceum* but also of some other, less frequently occurring FHB species such as

F. poae and *F. tricinctum*, can cause muscular weakness, respiratory stress, and myocardial degeneration (Jestoi 2008) resulting from the inhibition of several mitochondrial enzymes. The resulting damage to cellular respiration is responsible for the observed respiratory distress and muscle weakness (Desjardins 2006). The cardiotoxicity of MON may result from failing to remove free radicals in myocardium cells due to the inhibition of glutathione–peroxidase and glutathione–reductase (Chen et al. 1990). Several FHB species including *F. acuminatum*, *F. avenaceum*, *F. equiseti*, *F. poae*, *F. sporotrichioides*, and *F. tricinctum*, produce nonribosomal cyclic hexadepsipeptides, such as **beauvericin** (BEA) and **enniatins** (ENNs). Formerly, these secondary metabolites were not associated with known animal or human diseases (Desjardins 2006). However, recent studies highlighted their potential health risks, since these compounds had cytotoxic effects (Jestoi 2008). BEA and ENNs possess ionophoric activity, probably by forming cation-selective membrane channels (Kamyar et al. 2004; Kouri et al. 2003) which results in mitochondrial dysfunctions (Tonshin et al. 2010).

These naturally occurring *Fusarium* mycotoxins cannot be completely eliminated from the food supply chain (Desjardins 2006). Moreover, most mycotoxins withstand thermal processing to some degree (Kabak 2009). Therefore, knowledge of the infection biology of the causative *Fusarium* species should be applied to restrict FHB in the first instance, thus keeping mycotoxin contents in the grain at minimal levels. Governmental agencies provide advisory levels or even strict legal regulations for maximum tolerable mycotoxins levels in raw and/or processed cereal products for human consumption, as well as animal feed. In the European Union, the limits in unprocessed soft wheat grain intended for use as food are 1.25 mg kg^{-1} for DON and 0.25 mg kg^{-1} for ZEA (Verstraete 2008). For animal grain feed, guidance values are 0.9–12 mg kg^{-1} for DON and 0.1–3 mg kg^{-1} for ZEA depending on the animal species and the age.

IV. Population Biology

A. Biodiversity of *Fusarium* Species Causing FHB in Wheat

Up to 17 *Fusarium* species can be isolated from infected wheat heads (Parry et al. 1995), of which *F. graminearum* s. l. is thought to be the most important pathogen. **In Europe, four species are regularly found in FHB-infected heads; *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae*** (Table 8.1). These four species occur in varying frequencies depending on the weather conditions and regions, but by analysing 20 comprehensive surveys across several years, it becomes clear that *F. avenaceum* and especially *F. poae* play a much larger role nowadays than previously believed (Table 8.1). In eight surveys, *F. avenaceum* was the most frequently occurring species. In Norway, Denmark, and Ireland, this pathogen reached >80 % incidence. *F. avenaceum* was frequently isolated in Germany, France, Hungary, Slovakia, and Poland also. In eight out of 20 surveys, *F. poae* was the most frequently occurring species. Incidences of >80 % were observed in UK, Bavaria, and Slovakia. This species was prevalent noticeably in most other countries/regions analysed, even in Southern Europe (Italy). *F. graminearum* was the most frequent species in three surveys (Netherlands, Luxembourg, Poland) and came in second in nine other studies from all over Europe. *F. culmorum* was the most frequent species in only one study (Flanders) (Table 8.1). This species has declined in the last decade, while *F. graminearum* and *F. poae* have become more prominent (Waalwijk et al. 2003; Xu and Nicholson 2009). *F. culmorum* is still frequent in Norway, Ireland, and Denmark (≥60 % incidence). The high incidences of *F. poae* and *F. avenaceum* seemed somewhat surprising, because *F. graminearum* and *F. culmorum* were more aggressive in artificial inoculations (Xu et al. 2007).

In addition to these four species, others may reach high incidences only in certain regions. For example, *F. langsethiae* was first found in Norway (Torp and Nirenberg 2004), and seems to play a major role in Northern

Table 8.1. Incidences of *Fusarium* species on wheat heads in Europe

Country[a]	Years	M[b]	Fg[c]	Fc	Fa	Fp	Reference
Norway	1994–1996	A	26	60	99	78	Uhlig et al. (2007)
	2001–2002	A	35	85	100	51	
Finland	2001–2002	–[d]	1	2	11	0.2	Uhlig et al. (2007)
United Kingdom	2001–2002	A	43	18	18	95	Xu et al. (2005)
Ireland	2001–2002	A	56	68	79	32	Xu et al. (2005)
Denmark	2003–2007	A	87	81	93	71	Nielsen et al. (2011)
Netherlands	2000–2001	S	59	25	1	1	Waalwijk et al. (2003)
Belgium (Wallonia)	2003–2009	A	53	25	59	61	Chandelier et al. (2011)
Belgium (Flanders)	2002–2005	S	27	30	7	8	Isebaert et al. (2009)
Belgium (Flanders)	2007–2008	S	37	9	22	49	Audenaert et al. (2009)
Germany (Rhineland)	1997–1998	S	17	19	41	18	Birzele et al. (2002)
Germany (Rhineland)	1998–2000	A	11	18	41	10	Lienemann (2002)
Germany (Bavaria)	2003–2004	A	63	27	60	95	Büttner (2006)
France	2000–2002	A	67	20	65	75	Ioos et al. (2004)
Luxembourg	2007–2008	S	39	10	19	18	Giraud et al. (2010)
Poland	1998–1999	S	28	13	42	–[d]	Stepien and Chelkowski (2010)
	2005–2009	S	43	31	18	–[d]	
Slovakia	1999–2003	A	8	21	33	100	Rohacik and Hudec (2005)
Hungary	2001–2002	A	34	7	34	74	Xu et al. (2005)
Italy	2001–2002	A	37	0	3	61	Xu et al. (2005)

[a]When no region is indicated, samples originated from several regions
[b]Method (M) of scoring: Numbers refer to percentages of all samples (A) or percentages of samples with FHB symptoms (S)
[c]Fg *F. graminearum*, Fc *F. culmorum,* Fa *F. avenaceum*, Fp *F. poae*
[d]Not recorded

Europe. In Denmark, it was isolated over a period of 5 years with an average incidence of 59 % (Nielsen et al. 2011), whereas in Norway it was found in 72 % of the samples in 1994 (Kosiak et al. 2003). *F. langsethiae* has also been observed in Finland and Russia (Yli-Mattila 2010).

In most surveys, several species occurred in the same sample. In Denmark, for example, the four major species were found in nearly all samples. Consequently, co-occurrence of several mycotoxins is reported frequently. Co-inoculations of wheat heads with two or three FHB species under controlled environmental conditions resulted in lower biomasses of the individual fungi compared to single inoculations, but highly increased mycotoxin contents (Xu et al. 2007). Because *F. avenaceum* and *F. poae* produce toxins different from *F. graminearum* and *F. culmorum* (see III.E), the occurrence and quantity of toxins cannot be predicted from naturally infected field samples, and the detection of DON can merely be used as an indicator for the presence of additional toxins. Especially the high percentage at which *F. poae* occurs is of toxicological significance, because it produces a large array of mycotoxins (Stenglein 2009).

B. Structure and Dynamics of FHB-Pathogen Populations

The amount of genetic variance within and among populations, and of migration between populations, are essential parameters of population ecology (Hartl and Clark 1997). They are affected by all evolutionary processes that have influenced a population. Recombination, gene flow, and mutation increase genetic variation, whereas selection and genetic drift decrease it. Knowledge of the extent and nature of spatial and temporal distribution of genotypic diversity within populations, the level of population subdivision, and its links to phenotypic traits such as aggressiveness and mycotoxin production, will aid in predicting the evolutionary potential of FHB pathogens, with implications for the deployment of resistance.

1. Spatial and Temporal Variation

Several hierarchical levels of space are commonly used to analyse genetic diversity within and between populations: (a) different parts of the world (e.g., continents), (b) geographically defined large regions (e.g., countries), (c) individual fields and (d) individual host plants. Molecular markers are an ideal tool for estimating genetic diversity, because some marker types are highly polymorphic, selection-neutral, and can be analysed in high-throughput systems.

Large genetic diversity is found in worldwide collections of *F. graminearum* isolates as well as in collections from geographically distinct, but large regions (states, provinces, and countries). High to maximal percentages of unique haplotypes have been found by analysing, for instance, isolates from Canada (Dusabenyagasani et al. 1999), North Carolina (Walker et al. 2001), and Germany (Miedaner et al. 2001).

Analyses of variation of individual field populations that have been systematically sampled are, however, more adequate for population analyses, because they allow direct conclusions on evolutionary forces that have shaped the populations. In strict terms, population genetics works with allele frequencies (Hartl and Clark 1997). Because Pezizomycotina including the *Fusarium* species are haploid throughout most parts of their life cycle, DNA markers allow the direct monitoring of allelic frequencies that are also phenotypically relevant. Such population analyses have been performed during the last decade for *F. graminearum* in North America (Gale et al. 2007, 2011; Guo et al. 2008; Schmale et al. 2006; Zeller et al. 2003, 2004), Europe (Naef and Defago 2006; Talas et al. 2011b), and Asia (Gale et al. 2002; Karugia et al. 2009a; Lee et al. 2009; Suga et al. 2008). These studies identified the species by PCR assays using species-specific primers, including numerous isolates ($N > 250$, $N_{max} = 1{,}200$) and are, therefore, highly compelling. Similar large population analyses have been accomplished for *F. asiaticum* (Karugia et al. 2009b; Suga et al. 2008; Zhang et al. 2010a, b, 2012). However, they are still missing for *F. culmorum* and other FHB pathogens.

All large-scale studies revealed a **high level of genetic diversity in *F. graminearum* within individual field populations** or populations sampled across a definite geographic scale. Haplotype diversity within individual populations was maximal, mostly >90 %, i.e., most isolates sampled from one population had unique haplotypes. Accordingly, in all studies, Nei's gene diversity or number of alleles per locus had high values, and the low level of linkage disequilibrium was consistent with the hypothesis of randomly mating populations (Miedaner et al. 2008). There is some debate about population subdivision and gene flow, two parameters reflecting the exchange of alleles between different populations (Lowe et al. 2004). In earlier studies (Schmale et al. 2006; Zeller et al. 2003, 2004), the US population of *F. graminearum* was found to be relatively homogeneous with a low population subdivision and a high gene flow, even between geographically distinct populations. In contrast, Gale et al. (2007, 2011) reported a significant population subdivision in the USA associated with molecular markers and trichothecene chemotype differences. They defined several subpopulations (such as Midwestern 15ADON, Upper Midwestern 15ADON, Upper Midwestern 3ADON, Gulf Coast or Louisiana populations) with restricted gene flow and rather high genetic distances. It has been suggested that some of these US populations are distinct species, e.g., the Midwestern population has been named *F. gerlachii* and the Louisiana population *F. louisianense* (see II. B). Interestingly, **at all sampling sites one subpopulation was dominant, but some isolates of other subpopulations were also found.** Furthermore, at each sampling site some isolates (7–46 %) could not be assigned to one of the subpopulations, illustrating either previously undescribed subpopulations or recombination between subpopulations (Gale et al. 2011). In addition, among *F. asiaticum* isolates of China and Japan, distinct subpopulations determined by trichothecene production and geographic origin were found (Karugia et al. 2009b; Zhang et al. 2010a, 2012). The genotypic variation within (sub)populations was similarly large as in *F. graminearum*.

Subjecting multi-population data of *F. graminearum* to analysis of molecular variance (AMOVA) indicated that most of the variance resulted from differences within populations (Gale et al. 2011; Talas et al. 2011b). Spatial structuring within one field plays no major role, because diversity occurs on a very small spatial scale. Among the isolates sampled from just 0.25 m^2, a high level of diversity for vegetative compatible groups (VCGs) existed (Bowden and Leslie 1994). Analysing the same isolates by molecular markers, on average 67 % unique haplotypes were found (Zeller et al. 2003). Most haplotypes were recovered only once, but on rare occasions, more than one isolate of the same haplotype was found on the same head. Often adjacent heads were colonized by different haplotypes. This suggests that more than one genotype initiates infection, but some haplotypes may colonize more than one spikelet by subsequent growth within the head. This is consistent with initial infection by genetically diverse ascospores. The role of secondary infection by conidia is probably not very prominent because in this case neighboring heads would have been infected more often by the same haplotypes. Such conclusions are also supported by reports from Germany (Miedaner et al. 2001) and Canada (Fernando et al. 2006).

Worldwide collections of isolates from *F. culmorum* also display a high level of genetic diversity, as determined by haplotype frequency and/or diversity (Miedaner et al. 2001; Mishra et al. 2003; Obanor et al. 2010; Toth et al. 2004). If these isolates were to be considered as one (global) population, a recombining structure of this species would be most likely. Alternative explanations could be the widespread distribution of genotypes (clones) by international trade, for example by infected seeds, or by long-distance aerial dispersal of spores or the existence of a cryptic teleomorph (Toth et al. 2004). Interestingly, mating type genes, homologous to *MAT-1* and *MAT-2* of *G. zeae*, have been found in *F. culmorum* (Toth et al. 2004), and both genes were evenly distributed among 100 isolates originating from all continents (Obanor et al. 2010). The fact that isolates from different continents cluster frequently points to large diverse populations (Obanor et al. 2010).

In conclusion, genetic diversity within *F. graminearum*, *F. asiaticum*, and *F. culmorum* populations is tremendously high, even on very small spatial scales. For *F. graminearum*, this might be mainly caused by sexual recombination with some percentage of outcrossing, but other evolutionary factors may also play a role (Miedaner et al. 2008). Sexual recombination of *F. graminearum* was confirmed for field populations with outcrossing rates of 6–21 % (Chen and Zhou 2009b).

2. Variation in Chemotypes

Historically, in *F. graminearum* the 15ADON chemotype was dominant in North America (Guo et al. 2008; Ward et al. 2008). Of 998 isolates from the Eastern USA, 92 % were still of the 15ADON, 7 % of the 3ADON and 1 % of the NIV chemotype (Schmale et al. 2011). Subpopulations as defined by molecular markers were found to be associated with specific chemotypes (Gale et al. 2011). In the Midwestern population also, the 15ADON chemotype prevailed (>90 %), whereas the majority of isolates from Southern Louisiana (94 %) were of the NIV chemotype. In contrast, some isolates from the Upper Midwest population and most isolates from the Gulf coast population were of the 3ADON chemotype (Gale et al. 2007, 2011). Very recently, isolates with increased 15ADON production have been found in the USA that might result from a recombination event with 3ADON isolates (Foroud et al. 2012).

The distribution of chemotypes is different in Asia, where both *F. graminearum* and *F. asiaticum* occur, sometimes at the same sampling sites (Zhang et al. 2012). Out of 50 isolates of *F. graminearum* sampled across Japan, 70 % produced 3ADON and the remainder 15ADON, whereas the NIV chemotype was not detected (Suga et al. 2008). In contrast, 70 % out of 246 isolates of *F. asiaticum* were NIV producers, whereas the other isolates produced 3ADON and only one isolate 15ADON. In isolates from China, the NIV chemotype occurred frequently in *F. asiaticum* (42 %); all

other isolates were 3ADON producers (Karugia et al. 2009b). An even more comprehensive recent study detected that in China 35.3 % of the *F. asiaticum* isolates were of the NIV, 62.2 % of the 3ADON and 2.5 % of the 15ADON chemotype, whereas the *F. graminearum* isolates represented only the 15ADON chemotype (Zhang et al. 2012). The authors concluded from population-genetic data that within *F. asiaticum* hybridization between 3ADON and NIV chemotypes is common, but not between *F. asiaticum* and *F. graminearum*.

In Europe, diversity in trichothecenes seems to be high in *F. graminearum*. All three chemotypes have even been found in the same field (Talas et al. 2011b), but the 15ADON chemotype prevailed (Jennings et al. 2004; Talas et al. 2011b). Whereas NIV producers were rare in Germany (1.2 %), (Talas et al. 2011b), they occurred in the UK in 25 % of all *F. graminearum* isolates (Jennings et al. 2004). The frequency of the NIV chemotype was even higher (43 %) among the *F. culmorum* isolates sampled at the same locations and years (Jennings et al. 2004).

3. Recent Shifts in *Fusarium* Populations

Several studies have demonstrated **dynamic changes in populations of *Fusarium* spp.** which may have been caused by changing environmental conditions, and/or agronomical practices such as the increased planting of maize and of resistant wheat genotypes, changed tillage practice, or fungicide application. This is illustrated by the shift from *F. culmorum* to *F. graminearum* as the most important *Fusarium* species in the Netherlands (Waalwijk et al. 2003), Great Britain (Jennings et al. 2004), and Northern Europe (Yli-Mattila 2010). This shift seems to be accompanied by co-occurrence with *F. avenaceum* and *F. poae* in astonishingly high frequencies (see IV.A). Other examples of changes are the movement of *F. graminearum* in Canada from eastern to western Prairie Provinces (Mishra et al. 2004), and the recent colonisation of Western Canada by *F. pseudograminearum* (Mishra et al. 2006). Previously, the latter species seemed to be restricted to Australia (Miedaner et al. 2008). Meanwhile, *F. pseudograminearum* has been also connected with FHB in North Africa (Fakhfakh et al. 2011). The shift in *F. graminearum* from the former, resident 15-ADON to the 3-ADON producing isolates resulted in a west-to-east trend in Canada, with about 94 % of the isolates in Alberta still being 15ADON while 100 % of the isolates from Prince Edward Island were 3ADON (Ward et al. 2008). Interestingly, the proportion of the 3ADON chemotype has also expanded in populations of *F. asiaticum* in China, from east to west (Zhang et al. 2010a, 2012). In the USA, (sub)populations of *F. graminearum*, seem to have evolved in a rather dynamic manner, where the 3ADON chemotype expanded in some regions (Gale et al. 2007, 2011; Puri and Zhong 2010). The 3ADON isolates were found to produce higher levels of trichothecenes and more macroconidia. In South Korea, this species appears to have been relatively recently introduced and connects to regions where maize is a major crop (Lee et al. 2012). In Northern Europe, NIV-producers of *F. graminearum* are on the rise in maize-dominated crop rotations (Carter et al. 2002; Waalwijk et al. 2003). Additionally, benzimidazole, tebuconazole, and carbendazim fungicide resistances in China and Europe (see V.A.3) have been shown to develop in *F. graminearum* populations. These examples give unambiguous hints that species and populations underlie shifts by evolutionary selection, although the driving forces are not always clear yet.

C. Variation and Inheritance of Parasitic Fitness-Related Traits

1. Aggressiveness

Aggressiveness is defined as the quantitative ability of an isolate to cause disease on a compatible host in a non-race-specific pathosystem (Vanderplank 1984), which is set apart from the term ‘virulence’ used in race-specific pathosystems implying highly specific effector–receptor interactions. High aggressiveness of an isolate subsequently enhances its chance to contribute to the next generation, i.e., improves its fitness

(Hartl and Clark 1997). Aggressiveness is generally measured by symptom development and host colonization. Clearly, isolates of both, *F. graminearum* and *F. culmorum,* display a large variation of aggressiveness (Gang et al. 1998; Miedaner et al. 2000; Muthomi et al. 2000). This held true when aggressiveness of isolates sampled from the same field plot was tested on seedlings in the greenhouse (Miedaner and Schilling 1996) as well as on adult plants in the field (Talas et al. 2012a). Interestingly, partitioning of genotypic variation into variance within and between populations resulted in aggressiveness in about the same numbers as for variation monitored by molecular markers (72 % vs. 28 % respectively).

In an interspecific cross between a NIV-producing Japanese barley isolate of *F. asiaticum* and a DON-producing US wheat isolate of *F. graminearum*, pathogenicity, and aggressiveness were inherited by different loci (Cumagun et al. 2004). A gene for pathogenicity (*PATH1*) explaining 60 % of phenotypic variance was mapped in close vicinity to major genes for toxin content (*TOX1*), female fertility (*PERI1*), and colony pigmentation (*PIG1*). Two linked major QTLs for aggressiveness explaining 51 % and 29 % of phenotypic variance, respectively, were found in a different genomic region near the trichothecene cluster (Cumagun et al. 2004). Significant ($P<0.01$) quantitative-genetic variation for aggressiveness, host colonization, and DON content was also recognized in a companion study with 153 progeny of two European isolates of *F. graminearum* with low and moderate aggressiveness, respectively (Cumagun and Miedaner 2004). Several transgressive segregants towards higher aggressiveness and higher DON production occurred in this population, implying that both parental isolates had different alleles for these traits that recombined in the progeny. Even when crossing two highly aggressive isolates, a few transgressions towards even more aggressive isolates arose by recombination (Voss et al. 2010).

Such studies illustrate the **potential of the pathogen to reach higher levels of aggressiveness through sexual recombination** within a narrow geographic region or a field. This is further supported by the rather simple inheritance of pathogenicity and aggressiveness in the mapping population (Cumagun et al. 2004). The molecular or physiological causes of the widely varying aggressiveness in *Fusarium* populations are still unknown. In a first candidate gene association mapping approach, SNPs in three genes (*Tri1*, *MetAP1*, *Erf2*) significantly associated with aggressiveness (Talas et al. 2012b).

2. Mycotoxin Production

Close association between aggressiveness in wheat and DON production has been demonstrated in several field studies (e.g., Gang et al. 1998; Mesterhazy 2002; Miedaner et al. 2000). However, it is unclear whether isolates are more aggressive because of high DON production, or vice versa, produce more DON because they are colonizing the host tissue faster due to other factors. No correlation or only low correlation remains if DON production is calculated relative to fungal biomass within the tissue, e.g., by measuring ergosterol or fungal proteins (Gang et al. 1998; Miedaner et al. 2000; Voss et al. 2010; Wanyoike et al. 2002). One explanation for these findings might be that DON is required only for the inhibition of host resistance reactions and promotion of fungal spread within the wheat spike until a threshold level has been reached, and a further increase of the DON level would not necessarily cause more disease. DON production is, similar to aggressiveness, inherited as a quantitative trait in a mainly additive manner (Cumagun et al. 2004).

D. Implications for Resistance Breeding

The high polymorphism for selection-neutral markers and the large genotypic variance for fitness-related traits within individual field populations imply that the analysed ***Fusarium* species are able to adjust to widely differing conditions**. They should be considered as "high-risk pathogens" in terms of their evolutionary potential (see Box 8.1) (McDonald and Linde 2002). For a durable control of FHB by cultivar resistance, it is of utmost importance to know whether *Fusarium* populations will shift to higher aggressiveness and mycotoxin concentrations if they are confronted with resistant cultivars on large acreages. *F. graminearum* populations have already shown their ability

for temporary and regional changes concerning their chemotype and fungicide sensitivity (see V.A.3). Current wheat breeding efforts (see V.B.3) focus on identifying and incorporating multiple loci for FHB resistance into a single cultivar. Selection of *Fusarium* populations by host genotype should be weak, because no specific isolate–cultivar interaction has been observed, and the fungi spend the longest part of their life cycle saprophytically on plant debris or in the soil. Continuously improved resistance of cultivars grown on large acreages, however, might represent a constant unspecific selection force on *Fusarium* populations for enhanced aggressiveness. Inoculation with binary mixtures of *F. graminearum* and *F. culmorum* displaying various aggressiveness levels showed significant competition between these species on host genotypes possessing no, one or two potent resistance QTLs (von der Ohe and Miedaner 2011). However, this competition was mainly associated with weather conditions and disease severity in a given year, and not with the level of host resistance. Neither the type of mycotoxin (DON/NIV) nor the DON concentration at harvest determined the outcome of the competition. It is thus unlikely that genetic variation present in *Fusarium* populations will negatively affect host resistance. Nevertheless, multiple resistance genes of different origins should be employed in wheat breeding programs to obtain a high, long-lasting FHB resistance.

Box 8.1: Population Dynamics in *Fusarium*

- Large population sizes during epidemics
- High genetic variation in terms of markers, chemotypes, and phenotypic traits
- Genetic drift should play minor role
- Frequent sexual recombination with outcrossing in *F. graminearum*; no known teleomorph in *F. culmorum*
- Migration by splash-dispersed conidia, airborne ascospores, and seed transport
- Selection by host genotype weak because of saprophytic survival and no race specificity

V. Disease Management

A. Agronomic Practices

Epidemiology, infection process, and the diversity of FHB pathogens require multifaceted defence measures integrating various agricultural practices to effectively prevent disease outbreak and thus to limit mycotoxin contamination of cereal grains. Several studies have indicated that the susceptibility of the planted cereal cultivar has a great influence on FHB severity and mycotoxin levels (Koch et al. 2006; Schaafsma et al. 2001). Therefore, and because of recent scientific progress, we will discuss resistance breeding in more detail in a separate section (see V.B). In this section, we will review additional measures that are available to the farmer to confine the risk of FHB epidemics. These include appropriate application of crop rotation, tilling, and agrochemicals, as well as chemical and biological control measures. **In practice, the integration of several control measures promises to achieve best results** (Blandino et al. 2012; Willyerd et al. 2012).

1. Crop Rotation

Among the agronomic factors affecting the occurrence of the disease, crop rotation is critical. The previous crop significantly influences FHB severity in the following year, since it is the substrate for the production of the inoculum. In this regard, two aspects need to be considered (Beyer et al. 2006): (1) the pre-crop itself may serve as a host for FHB fungi, thus resulting in increased inoculum amount compared to non-host plant cultivation. (2) certain pre-crops may support saprophytic fungal growth and inoculum production by producing large amounts of crop debris. **FHB severity and mycotoxin contamination of wheat were highest when the previous crop was maize** and lowest when this was soybean, whereas wheat after wheat was in between (Dill-Macky and Jones 2000). Although soybean residues were saprophytically colonized, the lower infection pressure was explained by the lower amount

of debris remaining in the field after harvest. The average mycotoxin levels were twofold higher when wheat was planted into maize debris compared to soybean or wheat debris (Schaafsma et al. 2001). Maize residues are particularly suited for the survival and ascospore formation of *F. graminearum* in the next spring (Sutton 1982). In this context, it has to be considered that current maize harvest technologies result in a significant amount of crop debris (Beyer et al. 2006). Grain maize as the preceding crop causes more infections in wheat than silage maize, because more residues remain on the soil surface (Obst et al. 1997). Thus, maize or other cereals that are susceptible to FHB should be omitted as the pre-crop for wheat. In addition to this clear effect of maize, FHB was more severe when the preceding crop was durum wheat or oats rather than soft wheat or barley (Champeil et al. 2004). When wheat was grown after sugar beet, which has been considered rather a non-host for FHB pathogens, mycotoxin concentrations remained substantially lower compared to grains harvested from wheat-after-wheat trials (Koch et al. 2006). However, pathogen populations seem to adjust to rotational cropping systems, since *F. graminearum* increasingly emerges as a pathogen of potato and sugar beet (Burlakoti et al. 2008; Christ et al. 2011). This may reduce the benefits of these crops in rotation systems attempting to confine FHB to cereals in the future. Nevertheless, reduced risks for FHB infection will result when planting legumes, sunflower, rape, flax, and pea as pre-crops for wheat (Chandelier et al. 2011; Pereyra and Dill-Macky 2008).

2. Tilling

Of similar importance to crop rotation is adequate field preparation to restrict the saprophytic survival of FHB species on crop debris, thus minimizing the production of inoculum. Conventional tillage practice (mouldboard ploughing) buries crop residues, which accelerates their degradation by microbes. The survival of *F. graminearum* is inversely correlated to the rate at which degradation occurs, since residues at the soil surface provide substrate to the pathogen for a longer period than buried residues (Pereyra et al. 2004). However, conservation tillage practice, i.e., reduced or no tilling, is increasingly applied worldwide, as it saves fuel, increases yields, reduces erosion, and increases soil moisture (Lori et al. 2009). This move towards tillage reduction has probably contributed to U.S. FHB epidemics in the 1990s mentioned earlier (Dill-Macky and Jones 2000). Consistently, **several studies have indicated conventional tillage practice reduces risk of FHB infection or mycotoxin contaminations** (Blandino et al. 2010; Koch et al. 2006; Obst et al. 1997; Yi et al. 2001). The influence of tilling practice on FHB is rather difficult to evaluate, because it may be masked by infection pressure (Gilbert and Tekauz 2000), which in turn depends on the pre-crop used (Edwards 2004). Therefore, the pros and cons of conserving tillage practice need to be evaluated for a given location and climate in the context of the utilized crop rotation. When maize is a pre-crop to wheat, conventional tillage always leads to lower disease incidence. In cases where reduced tillage is necessary because of erosion constraints, maize residues in particular should be crushed as fine as possible to promote their microbial degradation (Pereyra and Dill-Macky 2008).

3. Chemical Control

In addition to the practices mentioned above, fungicides are a supplemental option to reduce both FHB incidence and mycotoxin accumulation (Gilbert and Tekauz 2000). However, as indicated by the inconsistent success of treatment, **the proper choice and rate of the fungicide applied, as well as the timing of its application, are critical** (Jones 2000; Pirgozliev et al. 2003). Although triazole fungicides have been proven to be effective agents for control of FHB and mycotoxin contamination, efficacies of individual triazoles may considerably differ (Mesterhazy 2003). A previous summary of several field studies concluded that tebuconazole is the best performing triazole fungicide (Mesterhazy 2003). Later on, a meta-analysis

covering 100 U.S. fungicide studies conducted over several years and in several states has indicated that **the combination of prothioconazole plus tebuconazole was most effective (52 %) in confining disease severity, whereas metconazole performed best in reducing DON levels (45 %)** (Paul et al. 2008). It was further concluded that these fungicides could increase yields by 13.8–15 % (Paul et al. 2010). In addition to triazoles, benzimidazole fungicides have also been proven to be effective against FHB, thus playing regionally an important role in disease management, i.e., in several countries in South America, East Asia, and Europe (Jones 2000). Especially in China, benzimidazoles have been extensively used since the 1970s (Yin et al. 2009). The success of triazole and benzimidazole fungicides may differ with respect to the geographic region, because of the different etiological components of FHB (Jones 2000). Furthermore, decreases in fungal susceptibility to those fungicides have been observed in field populations (Chen and Zhou 2009a; Klix et al. 2007), potentially threatening their efficacy in the future. Since decreased susceptibility of *F. graminearum* to triazoles may be associated with increased mycotoxin production (Becher et al. 2010), resistance development in *Fusarium* populations should be monitored. Other fungicide classes such as strobilurines or amine fungicides (including morpholines, piperidines and spiroketalmines), being highly active against other ascomycete plant pathogens, have been often found inconsistently effective in controlling FHB-causing *Fusarium* species. Interestingly, *F. graminearum* exhibits intrinsic resistance to these fungicides (Dubos et al. 2011; Liu et al. 2011). Even worse, application of strobilurines that are often used against other fungi infecting leaves of cereals may increase DON levels under conditions conducive for FHB fungi (Eiblmeier and von Gleissenthall 2007; Magan et al. 2002; Simpson et al. 2001). This might result from the elimination of competitors such as *Microdochium* spp., which are sensitive to strobilurines and are not known to produce mycotoxins, thereby facilitating the establishment of FHB (Pirgozliev et al. 2003). Occasionally, azoles might also stimulate DON production (Gareis and Ceynowa 1994; Ramirez et al. 2004).

Even for well-performing fungicides, practical efficacy is strongly dependent on the time-point of application. Although they are systemic fungicides, triazoles do not sufficiently translocate from leaves to the wheat head, and should therefore be directly applied to flowering heads (Mesterhazy 2003). Fungicide treatments are most effective 2–3 days before or after head infection, and much less effective earlier or later (Pirgozliev et al. 2003). Since the susceptibility of wheat for FHB fungi is highest when the anthers extrude from open flowers, **azole application around mid-anthesis (GS65) is considered optimal for field practice. In barley cultivars of the closed-flowering type (cleistogamous), a somewhat later application is more appropriate**, as anthers not extruded at anthesis are rather pushed out several days later by the developing kernel (Yoshida et al. 2008). Interestingly, recent field experiments with the rather novel triazole prothioconazole have indicated that FHB severity and mycotoxin contamination of grains were also significantly reduced when the fungicide was sprayed onto leaves at earlier growth stages, i.e., GS31 and GS39 (Edwards and Godley 2010).

The farmer should not rely solely on fungicide treatment to control for FHB, because successful application needs exact timing and because approaches combining fungicide treatment with additional measures provide better results. A study representing the outcomes of 40 field experiments in 12 U.S. states assessed three levels of susceptibility of wheat (susceptible, moderately susceptible, moderately resistant) combined with a single application of tebuconazole plus prothioconazole at anthesis in comparison to the corresponding untreated controls (Willyerd et al. 2012). Among all possible combinations, the lowest levels of FHB symptoms and DON resulted when combining a moderately resistant variety with the fungicide treatment. Another study assessed combined effects of tillage practice, susceptibility of the planted wheat variety, and triazole treatment on three sites in Northern Italy (Blandino et al. 2012). The combination of ploughing, a moderately resistant variety,

and triazole application at heading performed best among all possible combinations.

4. Biological Control

For reasons of food safety, as also environmental aspects and cost efficiency, efforts have been made to develop biological protection measures against FHB. Accordingly, several bacterial and fungal strains have been identified as potential biocontrol agents against FHB-causing *Fusarium* species. In most cases, such organisms were isolated from cereal hosts such as wheat and maize, but also from other cultivated plants such as soybeans or pea. Promising bacterial antagonists include strains in the genera *Bacillus* (Khan et al. 2001), *Brevibacillus* (Palazzini et al. 2007), *Lysobacter* (Jochum et al. 2006), *Paenibacillus* (He et al. 2009), *Pseudomonas* (Schisler et al. 2006), and *Streptomyces* (Palazzini et al. 2007). Effective fungal antagonists were strains of *Cryptococcus* ssp. (Khan et al. 2001), *Trichoderma* ssp. (Hasan et al. 2012; Matarese et al. 2012), and *Clonostachys* ssp. (Xue et al. 2009). Most of the antagonists which showed good results under laboratory and green house conditions performed much less efficiently in the field. Complex environmental interactions such as climate, competition with the resident microflora, and the composition of the local FHB population, could contribute to these discrepancies (Schisler et al. 2006). In this context, it has been suggested that an improved physiological quality of the applied propagules of the antagonists would enhance their survival under field conditions, and thus improve biocontrol efficacy (Palazzini et al. 2009). Nevertheless, some biocontrol agents, such as *Pseudomonas* ssp. strain AS 64.4, have achieved efficacies (>60 %) under field conditions which were almost as good as obtained with tebuconazole (Schisler et al. 2006). Field experiments applying other *Pseudomonas* strains resulted in less reduction of disease symptoms, but showed significant reduction of trichothecene contamination (74–78 %) (Khan and Doohan 2009). Although it was possible to confine mycotoxin contents to almost undetectable levels in the greenhouse through *Bacillus* spp. and *Streptomyces* spp., successful transfer of such results to the field remains challenging, since application (including timing, sequence, and technology), together with the concentrations and formulations of the antagonists, need optimisation to assure their survival and efficacy under harsh field conditions (Palazzini et al. 2007). Results from greenhouse experiments have indicated that bacterial antagonists were more effective when applied prior to infection of *Fusarium* spp. than afterwards (Khan and Doohan 2009). The earlier application may allow the biocontrol agents to (i) out-compete *Fusarium* for space and/or nutrients, (ii) inhibit the germination of *Fusarium* spores, and/or (iii) induce defence reactions in cereal heads. For the *Clonostachys rosea* strain ACM941, it was shown that it not only reduced disease severity but also reduced the production of perithecia, leading to a putative attenuation of infection pressure in the subsequent vegetation period (Xue et al. 2009).

However, although research has underscored the potential of biocontrol organisms to contribute to FHB management strategies, to our knowledge there is currently no biocontrol product approved for cereal head application against FHB as. However, as indicated by information retrievable from the internet, e.g., from the AAFC (Agriculture and Agri-Food Canada; www.agr.gc.ca), there are likely commercial biocontrol products underway.

5. Fertilizers, Plant Growth Regulators, and Herbicides

Because of discrepancies in the literature, it seems uncertain whether the amount and type of the applied nitrogen fertilizer has a specific impact on FHB and mycotoxin contamination. Studies on wheat report on increases in FHB severity and DON levels in the grain when increasing the N input (Heier et al. 2005; Lemmens et al. 2004). In contrast, a recent study on FHB in barley has indicated that the disease was more severe when plants were grown with low N input (Yang et al. 2010). Trichothecene levels were significantly higher in lodged wheat, barley, and rice plants than in controls (Nakajima et al. 2008).

Therefore, appropriate application of fertilizers and other agrochemicals is needed to restrict lodging.

The application of plant growth regulators leading to shorter stems of wheat increased FHB incidence, probably because of a reduced distance between the heads and the debris on the ground providing the inoculum (Fauzi and Paulitz 1994).

There are also indications that application of herbicides, especially glyphosate, may lead to higher FHB incidence in wheat and barley (Fernandez et al. 2009). It has been argued that this might be due to the effects of herbicides on the soil microflora, which may benefit the survival of FHB pathogens (Levesque and Rahe 1992). In contrast, a current study was unable to confirm any significant effect of glyphosate on disease incidence and severity, as well as trichothecene production (Berube et al. 2012).

B. Host-Plant Resistance

Host-plant resistance is the most effective and environmentally sound means of managing *Fusarium* diseases, although it is not easy to achieve (see Box 8.2). Resistance to FHB is quantitatively inherited, with a continuous distribution of FHB ratings among progenies in wheat (e.g., (Buerstmayr et al. 1996; Gosman et al. 2007; Mesterhazy 1995; Snijders 1990b), triticale (Oettler et al. 2004), and rye (Miedaner and Geiger 1996)). This type of resistance is considered as more durable than monogenic, hypersensitivity-based resistance, but also prone to non-genetic interactions, in particular genotype x environment interaction (Miedaner and Korzun 2012). Since the resistance ranking of genotypes varies with the tested environments (locations and/or years), **several environments should be tested to identify durable genotypes.** Interestingly, highly resistant lines are generally more stable than susceptible and moderately resistant lines (Buerstmayr et al. 2008). Because FHB does not necessarily occur at test plots each year, artificial infection is recommended. Three methods are available: **single-floret inoculation** (see V.B.1), **spray inoculation** of whole ears or plots, and **inoculation of the soil surface** using *Fusarium*-colonized kernels or maize stubbles. Inoculation can additionally be supported by irrigating the plots to enhance infection frequency. These methods for inoculation and the respective rating scales have been described elsewhere (Miedaner et al. 2003a; Yang et al. 1999). It should be considered that there is neither a correlation between *Fusarium* resistances in seedling and head blight in wheat (Arseniuk et al. 1993; Tamburic-Ilincic et al. 2009) nor between foot rot and head blight in rye (Miedaner et al. 1997). A non-significant correlation implies that there might be genotypes that are resistant during both growth stages and against both diseases, but not as a rule.

Once FHB-resistant cultivars are available, they can be widely used, because resistance acts similarly against several *Fusarium* species, e.g., *F. culmorum* and *F. graminearum* (Mesterhazy et al. 1999; Van Eeuwijk et al. 1995), against at least eight species of the *F. graminearum* species complex (Toth et al. 2008), and against different chemotypes, e.g., DON- and NIV-producing isolates (Miedaner and Reinbrecht 2001) or 3ADON- and 15ADON-producing isolates (von der Ohe et al. 2010b). Although genotype x isolate interactions can be found in some experiments caused by scaling effects (Voss et al. 2010), no races or isolate-specific resistances have been detected. Accordingly, resistance is believed to persist even after inoculation with highly aggressive isolates of *F. graminearum* and *F. culmorum* (Voss et al. 2010).

Box 8.2: General Features of FHB Resistance

- Quantitative, i.e., polygenic inheritance
- High impact of genotype x environment interaction
- Specific for host growth stage (young vs. adult plant) and organ (seedling, stem, head)
- Not specific for *Fusarium* species or chemotypes
- Not race-/isolate-specific

Table 8.2. Types of FHB resistance in cereals

Type	Description
I	Resistance to initial infection
II	Resistance to disease spread within the head
III	Resistance to kernel infection
IV	Resistance to toxin accumulation
V	Tolerance mechanisms

1. Types of Resistance

Fusarium resistance is mediated by many factors including passive factors (e.g., morphological or developmental features influencing infection and/or disease development) and active physiological mechanisms, i.e., gene products of defence/resistance genes. Several types of resistance have been described (Table 8.2). Whereas type I and type II resistance have been known for a long time (Schroeder and Christensen 1963), types III to V were defined later (Mesterhazy et al. 1999). **Type I resistance** describes a reduced infection efficiency affected by plant morphology, such as awning, head density, peduncle length. **Type II resistance**, which reduces the spreading of the disease within the head, is tested by injecting spores directly into individual florets (= point or single-floret inoculation) followed by monitoring symptom development. This can be easily assessed in the greenhouse with high accuracy, while in the field usually a spray inoculation is performed affecting both type I and type II resistance. Therefore, type I resistance can be determined only indirectly when the results from spray and single floret inoculations differ (Miedaner et al. 2003a). Resistant European cultivars have both types of resistance to some extent, with type I resistance being more important. This is not surprising, because a full type II resistance would not be sufficient for disease control when inoculum density is so high that most single florets become infected. Indeed, even the highly resistant wheat strain CM 82036 has different quantitative trait loci (QTLs) for both type I and type II resistance (Buerstmayr et al. 2002, 2003a). Some authors assess the number of infected heads in a first rating shortly after inoculation (= disease incidence) as type I and the number of infected spikelets per head (= disease severity) in a later rating as type II resistance. A FHB index is then calculated by multiplying both ratings. In Europe, mostly the percentage of affected spikelets per plot is rated; thus, this FHB rating includes both types of resistance (Buerstmayr et al. 2008; Mesterhazy 1995).

Type III resistance exists if kernel infection is less severe than expected from FHB rating. **Type IV resistance** leads to toxin contents in grains that are lower than expected from FHB rating. **Type V resistance** exists if significant differences in grain yield occur but disease symptoms show no difference, or vice versa. Alternatively, type III and type IV resistances can also be regarded as special features of type II resistance, because they are also caused by restricted fungal growth/mycotoxin spread within the spike. The trait of *Fusarium*-damaged kernels (FDK) is often employed to test for type III resistance (Mesterhazy et al. 1999). With regard to type IV resistance, a degradation or modification of DON has been reported in at least two instances, Frontana and *Fhb1* from Sumai 3 (Lemmens et al. 2005; Miller and Arnison 1986), but it is unclear how widespread this mechanism is in commercial cultivars. Validation of resistance types III to V is laborious because significant differences have to be confirmed for each trait in several environments (locations, years).

2. Resistance Sources and Meta-QTL

Three pools of resistance sources have been uncovered in the past few decades by testing tens of thousands of genotypes worldwide (Bai and Shaner 2004; Snijders 1990a; Wan et al. 1997): (1) several old Chinese and Japanese spring wheat landraces, (2) a few old South-American spring wheat cultivars and (3) Central European winter wheat cultivars including a few East European entries. **The most effective loci for FHB resistance were found in stocks from China, especially Sumai 3** and its derivatives such as CM82036, Ning7840 (Anderson et al. 2001; Buerstmayr et al. 2002, 2003a). Other Chinese sources with some resistance loci being different from Sumai 3 are Wangshuibai and Wuhan 1 (Wan et al.

Table 8.3. Meta-QTL with a mean explained phenotypic variance (R^2) >10 %

Chromosome	Position on consensus map [cM]	Flanking SSR markers	Number of initial QTL	Mean R^2 [%]	Resistance source(s)
1B_1	33.2	*barc137 – wmc320*	6	12.1	Arina, Cansas, Dream, F201-R, Wangshuibai
1B_2	75.2	*gwm124 – cfa2219*	2	13.8	Arina, Frontana
2D	91.6	*gwm608 – wmc261*	4	12.7	DH181,Wangshuibai, Wuhan1
3B (*Fhb1*)	16.1	*barc133 – gwm161/ gwm493*	13	17.7	CM82036, Ning7840, Sumai3, Wangshuibai, Wuhan1, W14-2
4D	32.9	*wmc473/Rht-D1b – wmc457a/gwm133*	6	19.3	*Rht-D1* locus
5A	62.8	*barc040/barc56 – barc180/gwm304*	6	13.5	CM82036, DH181, Frontana, F201-R, Wangshuibai
6A	50.0	*barc23 – wmc182*	2	13.3	Dream, ND2603
6B *(Fhb2)*	41.3	*gwm88 – wmc179*	8	12.4	Arina, DH181, Sumai3, Wangshuibai

Data are taken from Löffler et al. (2009)

1997). From South America, the Brazilian wheats Encruzilhada and Frontana have been identified as resistance sources (Snijders 1990a). The European winter wheats are a genetically highly heterogeneous group, with no known common ancestors that could be identified as resistance donors. Their resistance is inferior to Sumai 3, and is considered moderate. Various species of wild grasses, such as *Aegilops squarrosa*, *Roegneria* and *Elymus* species, have been reported to possess FHB resistance (Wan et al. 1997). Tetraploid wheat species, including durum wheat, are generally highly susceptible, but some moderately resistant cultivars were recently reported from Tunisia and Syria (Huhn et al. 2012; Talas et al. 2011a) and from their progenitor species. Eight promising entries were found among 151 accessions of *T. turgidum* L. var. *dicoccoides* from Israel and Turkey (Buerstmayr et al. 2003b). Furthermore, testing of 376 accessions of five wheat subspecies uncovered 16 moderately resistant lines in *T. turgidum* subsp. *carthlicum* and four in *T. turgidum* subsp. *dicoccum* (Oliver et al. 2008).

Knowledge of the genetic control of FHB resistance has continually increased over recent years as a result of a substantial number of published quantitative trait loci (QTL) studies (Buerstmayr et al. 2009). Up to 2009, 176 QTLs had been reported, which are distributed over all chromosomes of wheat (Buerstmayr et al. 2009; Löffler et al. 2009). A recent meta-analysis based on 101 published QTLs for FHB resistance from 30 mapping populations suggested the presence of 19 independent meta-QTLs, with the majority of meta-QTLs derived exclusively from adapted germplasm (Löffler et al. 2009). Out of these, **eight meta-QTLs on seven chromosomes have a major impact on FHB resistance** (Table 8.3).

Fhb1 on chromosome 3B is among the QTLs with the strongest effects and is only known from Asian resistance sources; it seems to be common in this region. It provides mainly a type II resistance (Buerstmayr et al. 2002) that can be tested easily in the greenhouse. Obviously, the Asian resistance sources have in addition to *Fhb1* several common QTLs on chromosomes 2D, 5A, and 6B. The QTL *Qfhs.ifa-5A* has been detected in all gene pools and has been associated with type I resistance (Buerstmayr et al. 2003a).

Certain European and Asian resistance sources comprise additional meta-QTLs. A QTL on chromosome 6B, named *Fhb2*, was detected in at least eight studies (Cuthbert et al. 2007). Its contribution to resistance was confirmed in the greenhouse by single-floret inoculation, and by spray inoculation in the field as well. A QTL on chromosome 1BL (*Qfhs.lfl-1BL*) has also been defined as a meta-QTL (1B_3), but with a low R^2 (5.5 %) (Löffler et al. 2009). This QTL is a

major FHB-resistance locus in the European winter wheat cultivars Biscay, Cansas, History, and Pirat (Häberle et al. 2009). It was also found in a genome-wide association study using 455 European winter wheat entries (Miedaner et al. 2011) that detected nine significant associations on seven chromosomes: 1B, 1D, 2B, 2D, 3A, 4D, and 7A.

The QTL on chromosome 4D co-localizes with the dwarfing locus *Rht-D1* (syn. *Rht2*) in many European cultivars such as Arina, Biscay, Pirat, and Rubens (Draeger et al. 2007; Holzapfel et al. 2008). In a meta-analysis (Löffler et al. 2009) (Table 8.3), this QTL had by far the largest effect and the most narrow confidence interval (0.1 cM). Further genetic and molecular studies confirmed that the dwarfing *Rht-D1b* allele is associated with increased FHB severity (Lu et al. 2011; Mao et al. 2010; Srinivasachary et al. 2008; Voss et al. 2008). Up to now, it is not known whether this is due to linkage or pleiotropy. Because this dwarfing allele is widely distributed in European wheat cultivars, this finding has a high impact on resistance breeding (see V.B.3.a).

Four major QTLs for FHB in tetraploid wheat have been identified so far on chromosomes 3A (*Qfhs.ndsu-3AS*,) (Otto et al. 2002), 7A (*Qfhs.fcu-7AL*) (Kumar et al. 2007), 6BS, and 2BL (Somers et al. 2006), with the three former QTLs derived from wild relatives of durum wheat or emmer. QTLs for FHB resistance from wild relatives can be successfully transferred to adapted durum wheat (Somers et al. 2006). Despite the identification of some major QTLs, the genetic basis of FHB resistance is complex, with many different, independent resistance loci having only small to medium effects. Even the most potent resistance donor, Sumai 3, has at least three QTLs and presumably more.

3. Achieving Durable Resistance

Resistance breeding is challenging given the complex inheritance of the resistance, the need to select plants plotwise at near maturity, and the large environmental effects on disease resistance. To enhance and accelerate progress of selection in developing FHB-resistant wheat germplasm, three strategies are applicable: (a) Phenotypic selection within adapted germplasm, (b) marker-assisted selection (MAS) and pyramidisation of QTLs from adapted resistance sources, and (c) marker-assisted backcross (MAB) breeding of QTLs from non-adapted ('exotic') resistance sources.

a) Phenotypic Selection Within Adapted Germplasm

Phenotypic selection for FHB resistance in the field has been successfully applied by breeding companies. Fairly resistant varieties have been established in Germany (e.g., Dream, Petrus, Solitaer, Toras), Switzerland (e.g., Arina) and France (e.g., Apache, Arche). Phenotypic selection, however, is laborious, costly, and highly dependent on inoculation experiments. Single-plant assays are possible only for type II resistance loci, like *Fhb1* (Buerstmayr et al. 2002). Type I resistance has to be tested plotwise in the field, because selection among single plants of e.g. F_2 generation does not result in reliable repeatability (Oettler et al. 2004; Snijders 1990b). Estimates of expected selection gain based on genetic variances and trait heritabilities are encouraging for further improvement of germplasm (Miedaner et al. 2006a).

Midparent values generally resembled the means of their progeny, illustrating a predominance of additive inheritance (Miedaner et al. 2006a). Significant ($P<$ 0.01) genotypic variance was detected in each of five wheat crosses, but genotype x environment interaction and error variances were high. Medium to high entry-mean heritabilities (0.6–0.8), however, underline the feasibility of selecting F_2-derived bulks in a later generation on a plot basis across environments (locations, years). In a parallel study, correlation between FHB severity and DON content was high in wheat and rye ($r=0.8$, $P=0.01$) (Miedaner et al. 2003b).

Selection for reduced DON contents in grains and for FHB resistance can thus be effectively started in early generations when bulked seed is used and entries are tested plotwise across environments. Lines with low DON content can be obtained indirectly by selecting for reduced head blight severity across environments (Wilde and Miedaner 2006). A major drawback is the **negative correlation between FHB resistance and plant height** in many European materials caused by the widespread use of the dwarfing alleles *Rht-B1b* or *Rht-D1b*, both of which increase FHB susceptibility considerably (Miedaner and Voss 2008). In the UK, most varieties, and in Germany about half of the registered varieties, contain the dwarfing *Rht-D1b* allele. Because it increases FHB, it must be counterbalanced by several potent

resistance QTLs on other chromosomal regions (Lu et al. 2011). Even in populations without a *Rht* gene, QTLs for FHB resistance are often localized near QTLs for tallness or lateness (Häberle et al. 2007; Mao et al. 2010; Schmolke et al. 2005). These associations have to be taken into account when breeding for early, short-strawed, FHB-resistant varieties by phenotypic selection. In this respect, the use of large population sizes with strict culling levels for selection of the wanted traits is essential.

b) Marker-Assisted Selection and Pyramidisation of QTLs Within Adapted Germplasm

The use of molecular markers is feasible, since QTLs for FHB resistance have been increasingly detected in diverse mapping populations and marker costs are being reduced with the introduction of SNP (single-nucleotide polymorphism) markers.

For genome-wide analyses, high-throughput marker systems based on microarrays allow for a fast and cost-effective screening of large plant populations with a high number of marker data points. For single-marker assays, the Competitive Allele-Specific PCR SNP genotyping system (KASPar) assay was recently introduced (http://www.kbioscience.co.uk/reagents/KASP/KASP.html).

Marker-assisted selection (MAS) can subsequently be performed in the first segregating generation or in the D1 generation after doubled-haploid (DH) production, to enrich the population for beneficial QTL alleles.

It is clear from recent studies that **several QTLs should be pyramided to gain a high level of FHB resistance**. Even the most potent QTL (*Fhb1*) alone was able to reduce disease severity only by two rating points on a 1–9 scale, when the parent is highly susceptible (von der Ohe et al. 2010a). Published FHB-resistance QTLs should be validated in different elite breeding material prior to a broad adaptation of MAS. For example, no congruent QTL was found in three mapping populations using the Swiss variety Arina as a source of resistance (Draeger et al. 2007; Paillard et al. 2004; Semagn et al. 2007).

A crucial question for the breeder is whether phenotypic or marker-based selection will achieve a higher selection success. We analysed a double cross where three FHB-resistance QTL alleles were introgressed in an elite winter wheat background. The QTLs derived from 'Dream' (*Qfhs.lfl-6AL, Qfhs.lfl-7BS)* and 'G16-92' (Chromosome 2BL) were selected in a population of 600 lines, applying one SSR marker per QTL on the one hand and pure phenotypic selection on the other (Miedaner et al. 2009). The mean realized response from selection per year was 2.1 % versus 2.5 % for the phenotypic versus the marker variant respectively. The higher selection gain per year of the marker variant resulted from the shorter procedure, because no field test for resistance was necessary. However, no selection for other agronomic traits is possible by MAS unless verified QTLs for those traits are available. This is especially crucial for plant height and heading date, because several QTLs for FHB resistance are linked with QTLs for both traits. Accordingly, the marker variant in this study resulted in significantly taller progeny than the phenotypic variant (Miedaner et al. 2009). **After marker selection, phenotypic selection in the field should follow** to exploit the full range of quantitative variation for resistance caused by genes that have so far remained undetected in QTL-mapping studies, and to select for other agronomic traits. In this case, population size should be large, to allow after selection for marker alleles also a selection for other traits. For this reason, genotyping following phenotyping has also been discussed (Agostinelli et al. 2012).

c) Marker-Assisted Backcrossing (MABC) of QTLs from Non-adapted Sources

The QTLs *Fhb1* and *Qfhs.ifa-5A* are to date the only disease resistance QTLs that are routinely used in international wheat breeding (Miedaner and Korzun 2012). Marker-based introgression of both QTLs in elite spring wheat reduced FHB rating by 10 % for each QTL individually and by 15 % when combined into a single line (Miedaner et al. 2006b). These values are considerably lower than those estimated in the original mapping populations, which is however quite common for QTL studies. Performing one cycle of recurrent selection with a spring wheat

population where both QTLs have been introgressed resulted in a mean realised response from selection of 3.2 % versus 4.4 % per year for the phenotypic versus the marker variant respectively (Wilde et al. 2007). Again, it was suggested that marker selection should be followed by phenotypic selection in the field. The best progeny of this study exhibited resistance and DON levels that were similar to those of the resistant donor, but now introgressed into the elite background. Both QTLs acted additively, independent of the genetic background, which was confirmed later on (Salameh et al. 2011). This underlines the interest in combining major QTLs to increase FHB resistance levels. MAS allowed for introgressing six validated FHB resistance QTLs, orange blossom midge resistance (*Sm1*) and leaf rust resistance (*Lr21*) into elite spring wheat during two backcrosses and two selfings within 25 months (Somers et al. 2005). However, it is not clear whether new varieties arose from this program, or whether selected lines were used in prebreeding.

Marker-based backcrossing of the two QTLs *Fhb1* and *Qfhs.ifa-5A* into European elite winter wheat also resulted in significantly improved FHB resistance (von der Ohe et al. 2010a). Because of the high yield levels of winter wheat in Germany, Northern France, and the UK, breeders are cautious in using non-adapted germplasm. In the marker class containing both QTLs, grain yields of progeny were significantly reduced by 1.6 % in one of the two backcross populations; the other population was not affected (von der Ohe et al. 2010a). Because no fine mapping of these QTLs is available, it cannot be concluded whether the small yield penalty in one backcross population is due to pleiotropy or linkage drag. Entries with only one of either QTL showed no significantly different grain yield compared to the class without any QTL in both backcross populations. Because of the significant phenotypic variation for FHB resistance and grain yield within each marker class, **selection of lines with improved resistance and a high yield level** similar to that in the recurrent parent **should be feasible**. Although other agronomic and quality traits often varied significantly, the absolute differences between classes with and without non-adapted QTL, however, were small. Similar results underline the effect of the recurrent parent on the final FHB resistance level after MABC (Salameh et al. 2011). In conclusion, both *Fhb1* and *Qfhs.ifa-5A* are effective and durable in elite spring and winter wheat backgrounds, and can be used for MAS without any known agronomical penalties.

4. Transgenic Approaches

Several strategies relying on transgenic approaches have been proposed to control FHB. Among the first attempts was the **overexpression of plant defence genes** under control of constitutive promoters in transgenic wheat. These encoded, for example, wheat alpha-thionin, barley beta-1,3-glucanase, barley class II chitinase or barley thaumatin-like-protein, maize b-32-RIP (ribosome inactivating protein), and bean polygalacturonase-inhibiting proteins (PGIPs) (Anand et al. 2003; Balconi et al. 2007; Dahleen et al. 2001; Ferrari et al. 2012; Mackintosh et al. 2007; Shin et al. 2008). Greenhouse tests of transgenic lines showed that type II resistance was improved by 20–50 % compared with their non-transformed parents. When transgenic plants were inoculated with *F. graminearum* in field experiments (Anand et al. 2003; Mackintosh et al. 2007; Shin et al. 2008), lines carrying the glucanase or class II chitinase transgenes showed simultaneous reduction of DON accumulation, percentage of *Fusarium*-damaged kernels, and disease severity. Constitutive expression of the *Arabidopsis thaliana NPR1* (*AtNPR1*) gene in the highly susceptible wheat cultivar Bobwhite also resulted in improved type II resistance in greenhouse tests (Makandar et al. 2006). *NPR1* [nonexpressor of pathogenesis-related (PR) genes] is a transcription coactivator controlling the salicylic acid signalling pathway regulating systemic acquired resistance, which is an inducible plant defence response against a broad range of pathogens. Recently, expression of a fusion protein comprising a recombinant *Fusarium*-specific antibody and an antifungal peptide from *Aspergillus giganteus* conferred type I and type II resistance to transgenic wheat (Li et al. 2008).

Other strategies aim to reduce or even eliminate the toxicity of mycotoxins. Heterologous expression in the wheat cultivar Bobwhite of a trichothecene 3-O-acetyltransferase from *F. sporotrichioides* (TRI101) reduced symptom spreading after inoculation with *F. graminearum* (Okubara et al. 2002). Expression of this gene in transgenic wheat was pursued and tested in the field by Syngenta (Karlovsky 2011). In addition, glucosylation of DON contributes to type II resistance, and a UDP-glycosyltransferase was isolated from *A. thaliana* (Poppenberger et al. 2003). Another UDP-glycosyltransferase was found in barley, and a candidate gene, *HvUGT13248*, has been identified (Schweiger et al. 2010). When overexpressed in *A. thaliana*, this gene caused a high level of DON resistance (Shin et al. 2012). The manipulation of the molecular target of trichothecenes, ribosomal protein L3, could also be used to reduce their toxicity. Transgenic wheat expressing an N-terminal fragment of L3 from yeast exhibited decreased levels of symptoms and DON in the greenhouse and the field (Di et al. 2010).

Recently, constitutive overexpression of a wheat transcription factor (*TaWRKY45*), which is upregulated during infection with *F. graminearum*, conferred partial resistance to wheat against FHB in greenhouse experiments (Bahrini et al. 2011). In another approach, the constitutive expression of bovine lactoferrin in the cultivar Bobwhite mediated partial resistance in a greenhouse experiment spray-inoculating *F. graminearum* conidia at flowering (Han et al. 2012). Lactoferrin, which is found in mucous secretions of mammals, is an iron-binding protein of the transferrin family that has broad antimicrobial activity. However, it remains uncertain whether the levels of free iron were reduced and whether this might have negative effects for the host.

Up to now, transgenic lines have fallen short of reaching levels of FHB resistance comparable to those of highly resistant cultivars. Moreover, effects on important agronomic traits such as yield and several quality parameters in transgenic lines await rigorous field tests in the future.

VI. Conclusions and Future Prospects

A. Population Structure and Evolution of FHB Fungi

FHB in small-grain cereals results from infections of the heads with lineages/species of the *F. graminearum* species complex, and in addition by *F. acuminatum*, *F. arthrosporioides*, *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. langsethiae*, *F. oxysporum*, *F. poae*, *F. pseudograminearum*, *F. sambucinum*, *F. sporotrichioides*, *F. tricinctum*, and *F. verticillioides*, as well as *Microdochium nivale* and *M. majus*. The frequency at which these species occur in diseased fields is affected by several factors, such as the planted crop, the previous crop, the weather during flowering, the geographical region, and probably others not yet determined. Furthermore, since most studies have used methods based on the traditional microbiological isolation of FHB fungi from diseased heads, the observed frequencies may not always reflect exactly the situation in the field. Therefore, it will be interesting to compare previous results with those from future studies based on sequence analyses of DNA that is directly extracted from environmental samples, as performed in fungal ecology (Neubert et al. 2006). Results of such studies will not be influenced by conditions of microbial cultivation, competition in vitro, dormancy and so forth.

Knowledge of the population structure of FHB fungi is important for several reasons. First, the success of a particular management practice may vary by species. Generally, an attempt should be made to control the entire FHB species complex. If this is unfeasible, control measures should be directed against the most prevalent species. Second, the types and levels of mycotoxins produced vary with the FHB species and the chemotype of the infecting fungal strains. Thus, shifts in *Fusarium* populations, which may arise, among other factors, also from climate changes, may have a quantitative and qualitative impact on the toxication of grains. Furthermore, sound knowledge of population structures of FHB species in various environments will help

to find answers for several questions that have emerged from previous studies:

- Why do some FHB-causing species exhibit strong biogeographical signals whereas others do not?
- Which biotic and abiotic factors have promoted the separation of lineages/species in *F. graminearum* s. l.?
- Were the lineages observed in *F. graminearum* s. l. in the process of forming new species, or were these processes completed before the onset of agriculture about 10,000 years ago?
- In case that their splitting is not yet completed, do human activities effectively counteract further separations of formerly isolated lineages/species?
- Which factors influence the balancing selection for chemotypes, and which lead to dynamic changes recently observed in some regions?
- Do some mycotoxins provide competitive advantages for the producer during its saprophytic growth on plant debris in the soil? Are other mycotoxins necessary for pathogenesis?

B. Management of FHB

Essential for effectively preventing epidemics of FHB are approaches integrating all cultural practices that reduce the disease. Most important is the use of cultivars providing high levels of resistance, and the avoidance of maize as the crop preceding wheat. If this is not possible, finely chopped maize residues should be ploughed. Fungicides can be used to secure seed production or to prevent the most severe consequences of infection when other strategies fail. In the future, biological plant protection schemes may become available for regular field application. Breeding for highly resistant wheat cultivars should take into account the fact that FHB populations are dynamic. The expansion of more aggressive strains such as the 3ADON chemotype of *F. graminearum* in several regional populations may partly result from selection induced by increased levels of FHB resistance in currently planted host cultivars. Therefore, breeding programs should use a broad range of FHB fungi that include different chemotypes when testing for resistance. Most promising are approaches that combine highly effective QTLs originating from independent sources. In addition, monitoring of FHB fungi in the field is needed to detect shifts in population structures early on. The implementation of all available knowledge about FHB in agronomical practice should help to avoid heavy epidemics in the future, and to support the production of healthy food and feed.

References

Agostinelli AM, Clark AJ, Brown-Guedira G, Van Sanford DA (2012) Optimizing phenotypic and genotypic selection for *Fusarium* head blight resistance in wheat. Euphytica 186:115–126

Ahamed S, Foster JS, Bukovsky A, Wimalasena J (2001) Signal transduction through the Ras/Erk pathway is essential for the mycoestrogen zearalenone-induced cell-cycle progression in MCF-7 cells. Mol Carcinog 30:88–98

Alexander NJ, Proctor RH, McCormick SP (2009) Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*. Toxin Rev 28:198–215

Alexander NJ, McCormick SP, Waalwijk C, van der Lee T, Proctor RH (2011) The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in *Fusarium*. Fungal Genet Biol 48:485–495

Anand A, Zhou T, Trick HN, Gill BS, Bockus WW, Muthukrishnan S (2003) Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*. J Exp Bot 54:1101–1111

Anderson JA, Stack RW, Liu S, Waldron BL, Fjeld AD, Coyne C, Moreno-Sevilla B, Fetch JM, Song QJ, Cregan PB, Frohberg RC (2001) DNA markers for *Fusarium* head blight resistance QTLs in two wheat populations. Theor Appl Genet 102:1164–1168

Arseniuk E, Goral T, Czembor HJ (1993) Reaction of triticale, wheat and rye accessions to gramineous *Fusarium* spp. infection at the seedling and adult plant growth stages. Euphytica 70:175–183

Atanasoff D (1920) The *Fusarium* blight (scab) of wheat and other cereals. Dissertation, Plant Pathology, University of Wisconsin, Madison

Audenaert K, Van Broeck R, Bekaert B, De Witte F, Heremans B, Messens K, Hofte M, Haesaert G (2009) *Fusarium* head blight (FHB) in Flanders: population diversity, inter-species associations and DON contamination in commercial winter wheat varieties. Eur J Plant Pathol 125:445–458

Bahrini I, Sugisawa M, Kikuchi R, Ogawa T, Kawahigashi H, Ban T, Handa H (2011) Characterization of a wheat transcription factor, TaWRKY45, and its effect on *Fusarium* head blight resistance in transgenic wheat plants. Breed Sci 61:121–129

Bai GH, Shaner G (2004) Management and resistance in wheat and barley to *Fusarium* head blight. Annu Rev Phytopathol 42:135–161

Bai GH, Chen LF, Shaner G (2003) Breeding for resistance to *Fusarium* head blight of wheat in China. In: Leonard KJ, Bushnell WR (eds) *Fusarium* head blight of wheat and barley. APS Press, St. Paul, pp 296–317

Balconi C, Lanzanova C, Conti E, Triulzi T, Forlani F, Cattaneo M, Lupotto E (2007) *Fusarium* head blight evaluation in wheat transgenic plants expressing the maize *b-32* antifungal gene. Eur J Plant Pathol 117:129–140

Beccari G, Covarelli L, Nicholson P (2011) Infection processes and soft wheat response to root rot and crown rot caused by *Fusarium culmorum*. Plant Pathol 60:671–684

Becher R, Hettwer U, Karlovsky P, Deising HB, Wirsel SGR (2010) Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. Phytopathology 100: 444–453

Bennett JW, Klich M (2003) Mycotoxins. Clin Microbiol Rev 16:497–516

Berube ME, Vanasse A, Rioux S, Bourget N, Dion Y, Tremblay G (2012) Effect of glyphosate on *Fusarium* head blight in wheat and barley under different soil tillages. Plant Dis 96:338–344

Beyer M, Roding S, Ludewig A, Verreet JA (2004) Germination and survival of *Fusarium graminearum* macroconidia as affected by environmental factors. J Phytopathol 152:92–97

Beyer M, Klix MB, Klink H, Verreet JA (2006) Quantifying the effects of previous crop, tillage, cultivar and triazole fungicides on the deoxynivalenol content of wheat grain – a review. J Plant Dis Prot 113:241–246

Birzele B, Meier A, Hindorf H, Kramer J, Dehne HW (2002) Epidemiology of *Fusarium* infection and deoxynivalenol content in winter wheat in the Rhineland, Germany. Eur J Plant Pathol 108: 667–673

Blandino M, Pilati A, Reyneri A, Scudellari D (2010) Effect of maize crop residue density on *Fusarium* head blight and on deoxynivalenol contamination of common wheat grains. Cereal Res Commun 38:550–559

Blandino M, Haidukowski M, Pascale M, Plizzari L, Scudellari D, Reyneri A (2012) Integrated strategies for the control of *Fusarium* head blight and deoxynivalenol contamination in winter wheat. Field Crop Res 133:139–149

Boenisch MJ, Schäfer W (2011) *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. BMC Plant Biol 11:110

Bowden RL, Leslie JF (1994) Diversity of *Gibberella zeae* at small spatial scales. Phytopathology 84:1140

Bowden RL, Leslie JF (1999) Sexual recombination in *Gibberella zeae*. Phytopathology 89:182–188

Boyacioglu D, Hettiarachchy NS (1995) Changes in some biochemical components of wheat grain that was infected with *Fusarium graminearum*. J Cereal Sci 21:57–62

Brown NA, Urban M, van de Meene AM, Hammond-Kosack KE (2010) The infection biology of *Fusarium graminearum*: defining the pathways of spikelet to spikelet colonisation in wheat ears. Fungal Biol 114:555–571

Buerstmayr H, Lemmens M, Grausgruber H, Ruckenbauer P (1996) Scab resistance of international wheat germplasm. Cereal Res Commun 24:195–202

Buerstmayr H, Lemmens M, Hartl L, Doldi L, Steiner B, Stierschneider M, Ruckenbauer P (2002) Molecular mapping of QTLs for *Fusarium* head blight resistance in spring wheat. I. Resistance to fungal spread (type II resistance). Theor Appl Genet 104:84–91

Buerstmayr H, Steiner B, Hartl L, Griesser M, Angerer N, Lengauer D, Miedaner T, Schneider B, Lemmens M (2003a) Molecular mapping of QTLs for *Fusarium* head blight resistance in spring wheat. II. Resistance to fungal penetration and spread. Theor Appl Genet 107:503–508

Buerstmayr H, Stierschneider M, Steiner B, Lemmens M, Griesser M, Nevo E, Fahima T (2003b) Variation for resistance to head blight caused by *Fusarium graminearum* in wild emmer (*Triticum dicoccoides*) originating from Israel. Euphytica 130:17–23

Buerstmayr H, Lemmens M, Schmolke M, Zimmermann G, Hartl L, Mascher F, Trottet M, Gosman NE, Nicholson P (2008) Multi-environment evaluation of level and stability of FHB resistance among parental lines and selected offspring derived from several European winter wheat mapping populations. Plant Breed 127:325–332

Buerstmayr H, Ban T, Anderson JA (2009) QTL mapping and marker-assisted selection for *Fusarium* head blight resistance in wheat: a review. Plant Breed 128:1–26

Burgess LW, Dodman RL, Pont W, Mayers P (1981) *Fusarium* diseases of wheat, maize, and grain sorghum in Eastern Australia. In: Nelson PE, Toussoun TA, Cook RJ (eds) *Fusarium* – diseases, biology, and taxonomy. Pennsylvania State University Press, University Park, pp 64–76

Burlakoti RR, Ali S, Secor GA, Neate SM, McMullen MP, Adhikari TB (2008) Genetic relationships among populations of *Gibberella zeae* from barley, wheat, potato, and sugar beet in the upper Midwest of the United States. Phytopathology 98:969–976

Bushnell WR, Hazen BE, Pritsch C (2003) Histology and physiology of *Fusarium* head blight. In: Leonard KJ, Bushnell WR (eds) *Fusarium* head blight of wheat and barley. APS Press, St. Paul, pp 44–83

Büttner P (2006) Das Artenspektrum der Gattung *Fusarium* an Weizen und Roggen in Bayern in den Jahren 2003–2004. Gesunde Pflanzen 58:28–33

Cai L, Giraud T, Zhang N, Begerow D, Cai GH, Shivas RG (2011) The evolution of species concepts and species recognition criteria in plant pathogenic fungi. Fungal Divers 50:121–133

Carter JP, Rezanoor HN, Holden D, Desjardins AE, Plattner RD, Nicholson P (2002) Variation in pathogenicity associated with the genetic diversity of *Fusarium graminearum*. Eur J Plant Pathol 108:573–583

Chakraborty S, Liu CJ, Mitter V, Scott JB, Akinsanmi OA, Ali S, Dill-Macky R, Nicol J, Backhouse D, Simpfendorfer S (2006) Pathogen population structure and epidemiology are keys to wheat crown rot and *Fusarium* head blight management. Australas Plant Pathol 35:643–655

Champeil A, Dore T, Fourbet JF (2004) *Fusarium* head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by *Fusarium* in wheat grains. Plant Sci 166:1389–1415

Chandelier A, Nimal C, Andre F, Planchon V, Oger R (2011) *Fusarium* species and DON contamination associated with head blight in winter wheat over a 7-year period (2003–2009) in Belgium. Eur J Plant Pathol 130:403–414

Chen Y, Zhou MG (2009a) Characterization of *Fusarium graminearum* isolates resistant to both carbendazim and a new Fungicide JS399-19. Phytopathology 99:441–446

Chen Y, Zhou MG (2009b) Sexual recombination of carbendazim resistance in *Fusarium graminearum* under field conditions. Pest Manag Sci 65: 398–403

Chen LY, Tian XL, Yang B (1990) A study on the inhibition of rat myocardium glutathione-peroxidase and glutathione-reductase by moniliformin. Mycopathologia 110:119–124

Christ DS, Marlander B, Varrelmann M (2011) Characterization and mycotoxigenic potential of *Fusarium* species in freshly harvested and stored sugar beet in Europe. Phytopathology 101: 1330–1337

Conkova E, Laciakova A, Kovac G, Seidel H (2003) Fusarial toxins and their role in animal diseases. Vet J 165:214–220

Cook RJ (1981) *Fusarium* diseases of wheat and other small grains in North America. In: Nelson PE, Toussoun TA, Cook RJ (eds) *Fusarium* – diseases, biology, and taxonomy. Pennsylvania State University Press, University Park, pp 39–52

Covarelli L, Beccari G, Steed A, Nicholson P (2012) Colonization of soft wheat following infection of the stem base by *Fusarium culmorum* and translocation of deoxynivalenol to the head. Plant Pathol. 61:1121–1129

Cowger C, Sutton AL (2005) The Southeastern U.S. *Fusarium* head blight epidemic of 2003. Plant Health Prog. doi:10.1094/PHP-2005-1026-1001-RS

Cumagun CJR, Miedaner T (2004) Segregation for aggressiveness and deoxynivalenol production of a population of *Gibberella zeae* causing head blight of wheat. Eur J Plant Pathol 110:789–799

Cumagun CJR, Bowden RL, Jurgenson JE, Leslie JF, Miedaner T (2004) Genetic mapping of pathogenicity and aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. Phytopathology 94:520–526

Cuthbert PA, Somers DJ, Brule-Babel A (2007) Mapping of *Fhb2* on chromosome 6BS: a gene controlling *Fusarium* head blight field resistance in bread wheat (*Triticum aestivum* L.). Theor Appl Genet 114:429–437

Dahleen LS, Okubara PA, Blechl AE (2001) Transgenic approaches to combat *Fusarium* head blight in wheat and barley. Crop Sci 41:628–637

de Luna L, Bujold I, Carisse O, Paulitz TC (2002) Ascospore gradients of *Gibberella zeae* from overwintered inoculum in wheat fields. Can J Plant Pathol 24:457–464

Desjardins AE (2006) *Fusarium* mycotoxins: chemistry, genetics, and biology. The American Phytopathological Society, St. Paul

Desjardins AE, Proctor RH (2007) Molecular biology of *Fusarium* mycotoxins. Int J Food Microbiol 119:47–50

Desjardins AE, Proctor RH (2011) Genetic diversity and trichothecene chemotypes of the *Fusarium graminearum* clade isolated from maize in Nepal and identification of a putative new lineage. Fungal Biol 115:38–48

Desjardins AE, Manandhar HK, Plattner RD, Manandhar GG, Poling SM, Maragos CM (2000) *Fusarium* species from nepalese rice and production of mycotoxins and gibberellic acid by selected species. Appl Environ Microbiol 66:1020–1025

Desmond OJ, Manners JM, Stephens AE, MaClean DJ, Schenk PM, Gardiner DM, Munn AL, Kazan K (2008) The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat. Mol Plant Pathol 9:435–445

Di R, Blechl A, Dill-Macky R, Tortora A, Tumer NE (2010) Expression of a truncated form of yeast ribosomal protein L3 in transgenic wheat improves resistance to *Fusarium* head blight. Plant Sci 178:374–380

Dill-Macky R, Jones RK (2000) The effect of previous crop residues and tillage on *Fusarium* head blight of wheat. Plant Dis 84:71–76

Doohan FM, Brennan J, Cooke BM (2003) Influence of climatic factors on *Fusarium* species pathogenic to cereals. Eur J Plant Pathol 109:755–768

Draeger R, Gosman N, Steed A, Chandler E, Thomsett M, Srinivasachary, Schondelmaier J, Buerstmayr H,

Lemmens M, Schmolke M, Mesterhazy A, Nicholson P (2007) Identification of QTLs for resistance to *Fusarium* head blight, DON accumulation and associated traits in the winter wheat variety Arina. Theor Appl Genet 115:617–625

Dubin HJ, Ruckenbauer P (1997) Foreword. In: Dubin HJ, Gilchrist L, Reeves J, McNab A (eds) Fusarium head scab: global status and future prospects. CIMMYT, El Batan, Mexico, pp V–VI

Dubos T, Pasquali M, Pogoda F, Hoffmann L, Beyer M (2011) Evidence for natural resistance towards trifloxystrobin in *Fusarium graminearum*. Eur J Plant Pathol 130:239–248

Dufault NS, De Wolf ED, Lipps PE, Madden LV (2006) Role of temperature and moisture in the production and maturation of *Gibberella zeae* perithecia. Plant Dis 90:637–644

Dusabenyagasani M, Dostaler D, Hamelin RC (1999) Genetic diversity among *Fusarium graminearum* strains from Ontario and Quebec. Can J Plant Pathol 21:308–314

Edwards SG (2004) Influence of agricultural practices on Fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. Toxicol Lett 153:29–35

Edwards SG, Godley NP (2010) Reduction of *Fusarium* head blight and deoxynivalenol in wheat with early fungicide applications of prothioconazole. Food Addit Contam A 27:629–635

Eiblmeier P, von Gleissenthall JL (2007) Risk evaluation of deoxynivalenol levels in Bavarian wheat from survey data. J Plant Dis Prot 114:69–75

Fakhfakh MM, Yahyaoui A, Rezgui S, Elias EM, Daaloul A (2011) Identification and pathogenicity assessment of *Fusarium* spp. sampled from durum wheat fields in Tunisia. Afr J Biotechnol 10:6529–6539

Fauzi MT, Paulitz TC (1994) The effect of plant-growth regulators and nitrogen on *Fusarium* head blight of the spring wheat cultivar max. Plant Dis 78:289–292

Feinberg B, McLaughlin CS (1989) Biochemical mechanism of action of trichocenene mycotoxins. CRC Press, Boca Raton

Fernandez MR, Zentner RP, Basnyat P, Gehl D, Selles F, Huber D (2009) Glyphosate associations with cereal diseases caused by *Fusarium* spp. in the Canadian prairies. Eur J Agron 31:133–143

Fernando WGD, Miller JD, Seaman WL, Seifert K, Paulitz TC (2000) Daily and seasonal dynamics of airborne spores of *Fusarium graminearum* and other *Fusarium* species sampled over wheat plots. Can J Bot 78:497–505

Fernando WGD, Zhang JX, Dusabenyagasani M, Guo XW, Ahmed H, McCallum B (2006) Genetic diversity of *Gibberella zeae* isolates from Manitoba. Plant Dis 90:1337–1342

Ferrari S, Sella L, Janni M, De Lorenzo G, Favaron F, D'Ovidio R (2012) Transgenic expression of polygalacturonase-inhibiting proteins in *Arabidopsis* and wheat increases resistance to the flower pathogen *Fusarium graminearum*. Plant Biol 14 (Suppl 1):31–38

Foroud NA, Eudes F (2009) Trichothecenes in cereal grains. Int J Mol Sci 10:147–173

Foroud NA, McCormick S, MacMillan T, Badea A, Kendra DF, Ellis BE, Eudes F (2012) Greenhouse studies reveal increased aggressiveness of emergent Canadian *Fusarium graminearum* chemotypes in wheat. Plant Dis 96:1271–1279

Francl L, Shaner G, Bergstrom G, Gilbert J, Pedersen W, Dill-Macky R, Sweets L, Corwin B, Jin Y, Gallenberg D, Wiersma J (1999) Daily inoculum levels of *Gibberella zeae* on wheat spikes. Plant Dis 83:662–666

Gale LR, Chen LF, Hernick CA, Takamura K, Kistler HC (2002) Population analysis of *Fusarium graminearum* from wheat fields in eastern China. Phytopathology 92:1315–1322

Gale LR, Ward TJ, Balmas V, Kistler HC (2007) Population subdivision of *Fusarium graminearum* sensu stricto in the Upper Midwestern United States. Phytopathology 97:1434–1439

Gale LR, Harrison SA, Ward TJ, O'Donnell K, Milus EA, Gale SW, Kistler HC (2011) Nivalenol-type populations of *Fusarium graminearum* and *F. asiaticum* are prevalent on wheat in southern Louisiana. Phytopathology 101:124–134

Gang G, Miedaner T, Schuhmacher U, Schollenberger M, Geiger HH (1998) Deoxynivalenol and nivalenol production by *Fusarium culmorum* isolates differing in aggressiveness toward winter rye. Phytopathology 88:879–884

Gardiner SA, Boddu J, Berthiller F, Hametner C, Stupar RM, Adam G, Muehlbauer GJ (2010) Transcriptome analysis of the barley-deoxynivalenol interaction: evidence for a role of glutathione in deoxynivalenol detoxification. Mol Plant Microbe Interact 23:962–976

Gareis M, Ceynowa J (1994) Effect of the fungicide matador (tebuconazole/triadimenol) on mycotoxin production by *Fusarium culmorum*. Z Lebensm Unters Forsch 198:244–248

Gilbert J, Tekauz A (2000) Review: recent developments in research on *Fusarium* head blight of wheat in Canada. Can J Plant Pathol 22:1–8

Gilbert J, Woods SM, Kromer U (2008) Germination of ascospores of *Gibberella zeae* after exposure to various levels of relative humidity and temperature. Phytopathology 98:504–508

Giraud T, Refregier G, Le Gac M, de Vienne DM, Hood ME (2008) Speciation in fungi. Fungal Genet Biol 45:791–802

Giraud F, Pasquali M, El Jarroudi M, Vrancken C, Brochot C, Cocco E, Hoffmann L, Delfosse P, Bohn T (2010) *Fusarium* head blight and associated mycotoxin occurrence on winter wheat in Luxembourg in 2007/2008. Food Addit Contam A 27:825–835

Gosman N, Bayles R, Jennings P, Kirby J, Nicholson P (2007) Evaluation and characterization of resistance to *Fusarium* head blight caused by *Fusarium culmorum* in UK winter wheat cultivars. Plant Pathol 56:264–276

Goswami RS, Kistler HC (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. Mol Plant Pathol 5:515–525

Guenther JC, Trail F (2005) The development and differentiation of *Gibberella zeae* (anamorph: *Fusarium graminearum*) during colonization of wheat. Mycologia 97:229–237

Guo XW, Fernando WGD, Seow-Brock HY (2008) Population structure, chemotype diversity, and potential chemotype shifting of *Fusarium graminearum* in wheat fields of Manitoba. Plant Dis 92:756–762

Häberle J, Schmolke M, Schweizer G, Korzun V, Ebmeyer E, Zimmermann G, Hartl L (2007) Effects of two major *Fusarium* head blight resistance QTL verified in a winter wheat backcross population. Crop Sci 47:1823–1831

Häberle J, Holzapfel J, Schweizer G, Hartl L (2009) A major QTL for resistance against *Fusarium* head blight in European winter wheat. Theor Appl Genet 119:325–332

Han J, Lakshman DK, Galvez LC, Mitra S, Baenziger PS, Mitra A (2012) Transgenic expression of lactoferrin imparts enhanced resistance to head blight of wheat caused by *Fusarium graminearum*. BMC Plant Biol 12:33

Hartl DL, Clark AG (1997) Principles of population genetics. Sinauer Associates, Sunderland

Hasan MM, Rahman SM, Kim GH, Abdallah E, Oh DH (2012) Antagonistic potentiality of *Trichoderma harzianum* towards seed-borne fungal pathogens of winter wheat cv. protiva in vitro and in vivo. J Microbiol Biotechnol 22:585–591

He J, Boland GJ, Zhou T (2009) Concurrent selection for microbial suppression of *Fusarium graminearum*, *Fusarium* head blight and deoxynivalenol in wheat. J Appl Microbiol 106:1805–1817

Heier T, Jain SK, Kogel KH, Pons-Kuhnemann J (2005) Influence of N-fertilization and fungicide strategies on *Fusarium* head blight severity and mycotoxin content in winter wheat. J Phytopathol 153:551–557

Hogg AC, Johnston RH, Dyer AT (2007) Applying real-time quantitative PCR to *Fusarium* crown rot of wheat. Plant Dis 91:1021–1028

Holzapfel J, Voss HH, Miedaner T, Korzun V, Haberle J, Schweizer G, Mohler V, Zimmermann G, Hartl L (2008) Inheritance of resistance to *Fusarium* head blight in three European winter wheat populations. Theor Appl Genet 117:1119–1128

Horberg HM (2002) Patterns of splash dispersed conidia of *Fusarium poae* and *Fusarium culmorum*. Eur J Plant Pathol 108:73–80

Huhn MR, Elias EM, Ghavami F, Kianian SF, Chao SM, Zhong SB, Alamri MS, Yahyaoui A, Mergoum M (2012) Tetraploid Tunisian wheat germplasm as a new source of *Fusarium* head blight resistance. Crop Sci 52:136–145

Imathiu SM, Hare MC, Ray RV, Back M, Edwards SG (2010) Evaluation of pathogenicity and aggressiveness of *F. langsethiae* on oat and wheat seedlings relative to known seedling blight pathogens. Eur J Plant Pathol 126:203–216

Inch S, Gilbert J (2003) The incidence of *Fusarium* species recovered from inflorescences of wild grasses in southern Manitoba. Can J Plant Pathol 25:379–383

Inch S, Fernando WGD, Gilbert J (2005) Seasonal and daily variation in the airborne concentration of *Gibberella zeae* (Schw.) Petch spores in Manitoba. Can J Plant Pathol 27:357–363

Ioos R, Belhadj A, Menez M (2004) Occurrence and distribution of *Microdochium nivale* and *Fusarium* species isolated from barley, durum and soft wheat grains in France from 2000 to 2002. Mycopathologia 158:351–362

Isebaert S, De Saeger S, Devreese R, Verhoeven R, Maene P, Heremans B, Haesaert G (2009) Mycotoxin-producing *Fusarium* species occurring in winter wheat in Belgium (Flanders) during 2002–2005. J Phytopathol 157:108–116

Jansen C, von Wettstein D, Schäfer W, Kogel KH, Felk A, Maier FJ (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proc Natl Acad Sci USA 102:16892–16897

Jenkinson P, Parry DW (1994a) Isolation of *Fusarium* species from common broad-leaved weeds and their pathogenicity to winter wheat. Mycol Res 98:776–780

Jenkinson P, Parry DW (1994b) Splash dispersal of conidia of *Fusarium culmorum* and *Fusarium avenaceum*. Mycol Res 98:506–510

Jennings P, Coates ME, Walsh K, Turner JA, Nicholson P (2004) Determination of deoxynivalenol- and nivalenol-producing chemotypes of *Fusarium graminearum* isolated from wheat crops in England and Wales. Plant Pathol 53:643–652

Jestoi M (2008) Emerging *Fusarium* mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin – a review. Crit Rev Food Sci 48:21–49

Jochum CC, Osborne LE, Yuen GY (2006) *Fusarium* head blight biological control with *Lysobacter enzymogenes*. Biol Control 39:336–344

Johnson DD, Flaskerud GK, Taylor RD, Satyanarayana V (2003) Quantifying economic impacts of *Fusarium* head blight in wheat. In: Leonard KJ, Bushnell WR (eds) *Fusarium* head blight of wheat and barley. APS Press, St. Paul, pp 461–483

Jones RK (1999) Seedling blight development and control in spring wheat damaged by *Fusarium graminearum* group 2. Plant Dis 83:1013–1018

Jones RK (2000) Assessments of *Fusarium* head blight of wheat and barley in response to fungicide treatment. Plant Dis 84:1021–1030

Kabak B (2009) The fate of mycotoxins during thermal food processing. J Sci Food Agric 89:549–554

Kamyar M, Rawnduzi P, Studenik CR, Kouri K, Lemmens-Gruber R (2004) Investigation of the electrophysiological properties of enniatins. Arch Biochem Biophys 429:215–223

Kang ZS, Buchenauer H (2000) Cytology and ultrastructure of the infection of wheat spikes by *Fusarium culmorum*. Mycol Res 104:1083–1093

Karlovsky P (2011) Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. Appl Microbiol Biotechnol 91:491–504

Karugia GW, Suga H, Gale LR, Nakajima T, Tomimura K, Hyakumachi M (2009a) Population structure of the *Fusarium graminearum* species complex from a single Japanese wheat field sampled in two consecutive years. Plant Dis 93:170–174

Karugia GW, Suga H, Gale LR, Nakajima T, Ueda A, Hyakumachi M (2009b) Population structure of *Fusarium asiaticum* from two Japanese regions and eastern China. J Gen Plant Pathol 75:110–118

Kazan K, Gardiner DM, Manners JM (2012) On the trail of a cereal killer: recent advances in *Fusarium graminearum* pathogenomics and host resistance. Mol Plant Pathol 13:399–413

Keller MD, Waxman KD, Bergstrom GC, Schmale DG (2010) Local distance of wheat spike infection by released clones of *Gibberella zeae* disseminated from infested corn residue. Plant Dis 94:1151–1155

Khan MR, Doohan FM (2009) Bacterium-mediated control of *Fusarium* head blight disease of wheat and barley and associated mycotoxin contamination of grain. Biol Control 48:42–47

Khan NI, Schisler DA, Boehm MJ, Slininger PJ, Bothast RJ (2001) Selection and evaluation of microorganisms for biocontrol of *Fusarium* head blight of wheat incited by *Gibberella zeae*. Plant Dis 85:1253–1258

Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M (2007) Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. Biosci Biotechnol Biochem 71:2105–2123

Klix MB, Verreet JA, Beyer M (2007) Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. Crop Prot 26:683–690

Koch HJ, Pringas C, Maerlaender B (2006) Evaluation of environmental and management effects on *Fusarium* head blight infection and deoxynivalenol concentration in the grain of winter wheat. Eur J Agron 24:357–366

Kohn LM (2005) Mechanisms of fungal speciation. Annu Rev Phytopathol 43:279–308

Kosiak B, Torp M, Skjerve E, Thrane U (2003) The prevalence and distribution of *Fusarium* species in Norwegian cereals: a survey. Acta Agric Scand B Soil Plant Sci 53:168–176

Kouri K, Lemmens M, Lemmens-Gruber R (2003) Beauvericin-induced channels in ventricular myocytes and liposomes. BBA-Biomembr 1609: 203–210

Kuiper-Goodman T, Scott PM, Watanabe H (1987) Risk assessment of the mycotoxin zearalenone. Regul Toxicol Pharmacol 7:253–306

Kumar S, Stack RW, Friesen TL, Faris JD (2007) Identification of a novel *Fusarium* head blight resistance quantitative trait locus on chromosome 7A in tetraploid wheat. Phytopathology 97:592–597

Kvas M, Marasas WFO, Wingfield BD, Wingfield MJ, Steenkamp ET (2009) Diversity and evolution of *Fusarium* species in the *Gibberella fujikuroi* complex. Fungal Divers 34:1–21

Langevin F, Eudes F, Comeau A (2004) Effect of trichothecenes produced by *Fusarium graminearum* during *Fusarium* head blight development in six cereal species. Eur J Plant Pathol 110:735–746

Lee T, Han YK, Kim KH, Yun SH, Lee YW (2002) Tri13 and Tri7 determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. Appl Environ Microbiol 68:2148–2154

Lee J, Chang IY, Kim H, Yun SH, Leslie JF, Lee YW (2009) Genetic diversity and fitness of *Fusarium graminearum* populations from rice in Korea. Appl Environ Microbiol 75:3289–3295

Lee J, Kim H, Jeon JJ, Kim HS, Zeller KA, Carter LL, Leslie JF, Lee YW (2012) Population structure and mycotoxin production of *Fusarium graminearum* from maize in Korea. Appl Environ Microbiol 78:2161–2167

Lemmens M, Haim K, Lew H, Ruckenbauer P (2004) The effect of nitrogen fertilization on *Fusarium* head blight development and deoxynivalenol contamination in wheat. J Phytopathol 152:1–8

Lemmens M, Scholz U, Berthiller F, Dall'Asta C, Koutnik A, Schuhmacher R, Adam G, Buerstmayr H, Mesterhazy A, Krska R, Ruckenbauer P (2005) The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus for *Fusarium* head blight resistance in wheat. Mol Plant Microbe Interact 18:1318–1324

Leslie JF, Bowden RL (2008) *Fusarium graminearum*: when species concepts collide. Cereal Res Commun 36:609–615

Leslie JF, Summerell BA (2006) The *Fusarium* laboratory manual. Blackwell Professional, Ames

Leslie JF, Anderson LL, Bowden RL, Lee YW (2007) Inter- and intra-specific genetic variation in *Fusarium*. Int J Food Microbiol 119:25–32

Levesque CA, Rahe JE (1992) Herbicide interactions with fungal root pathogens, with special reference to glyphosate. Annu Rev Phytopathol 30:579–602

Li HP, Zhang JB, Shi RP, Huang T, Fischer R, Liao YC (2008) Engineering *Fusarium* head blight resistance in wheat by expression of a fusion protein containing a *Fusarium*-specific antibody and an

antifungal peptide. Mol Plant Microbe Interact 21:1242–1248

Li YS, Wang ZH, Beier RC, Shen JZ, De Smet D, De Saeger S, Zhang SX (2011) T-2 Toxin, a trichothecene mycotoxin: review of toxicity, metabolism, and analytical methods. J Agric Food Chem 59:3441–3453

Lienemann K (2002) Auftreten von *Fusarium*-Arten an Winterweizen im Rheinland und Möglichkeiten der Befallskontrolle unter besonderer Berücksichtigung der Weizensorte. Dissertation, Institut für Pflanzenkrankheiten, Rheinische Friedrich-Wilhelms-Universität, Bonn

Liu X, Fu J, Yun Y, Yin Y, Ma Z (2011) A sterol C-14 reductase encoded by *FgERG24B* is responsible for the intrinsic resistance of *Fusarium graminearum* to amine fungicides. Microbiology 157: 1665–1675

Löffler M, Schön CC, Miedaner T (2009) Revealing the genetic architecture of FHB resistance in hexaploid wheat (*Triticum aestivum* L.) by QTL meta-analysis. Mol Breed 23:473–488

Logrieco A, Mule G, Moretti A, Bottalico A (2002) Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. Eur J Plant Pathol 108:597–609

Lori GA, Sisterna MN, Sarandon SJ, Rizzo I, Chidichimo H (2009) *Fusarium* head blight in wheat: impact of tillage and other agronomic practices under natural infection. Crop Prot 28:495–502

Lowe A, Harris S, Ashton P (2004) Ecological genetics: design, analysis, and application. Wiley-Blackwell, Hoboken

Lu QX, Szabo-Hever A, Bjornstad A, Lillemo M, Semagn K, Mesterhazy A, Ji F, Shi JR, Skinnes H (2011) Two major resistance quantitative trait loci are required to counteract the increased susceptibility to *Fusarium* head blight of the *Rht-D1b* dwarfing gene in wheat. Crop Sci 51: 2430–2438

Mackintosh CA, Lewis J, Radmer LE, Shin S, Heinen SJ, Smith LA, Wyckoff MN, Dill-Macky R, Evans CK, Kravchenko S, Baldridge GD, Zeyen RJ, Muehlbauer GJ (2007) Overexpression of defense response genes in transgenic wheat enhances resistance to *Fusarium* head blight. Plant Cell Rep 26:479–488

Magan N, Hope R, Colleate A, Baxter ES (2002) Relationship between growth and mycotoxin production by *Fusarium* species, biocides and environment. Eur J Plant Pathol 108:685–690

Maier FJ, Miedaner T, Hadeler B, Felk A, Salomon S, Lemmens M, Kassner H, Schäfer W (2006) Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (*Tri5*) gene in three field isolates of different chemotype and virulence. Mol Plant Pathol 7:449–461

Makandar R, Essig JS, Schapaugh MA, Trick HN, Shah J (2006) Genetically engineered resistance to *Fusarium* head blight in wheat by expression of *Arabidopsis NPR1*. Mol Plant Microbe Interact 19:123–129

Maldonado-Ramirez SL, Schmale DG, Shields EJ, Bergstrom GC (2005) The relative abundance of viable spores of *Gibberella zeae* in the planetary boundary layer suggests the role of long-distance transport in regional epidemics of *Fusarium* head blight. Agric Forest Meteorol 132:20–27

Mao SL, Wei YM, Cao W, Lan XJ, Yu M, Chen ZM, Chen GY, Zheng YL (2010) Confirmation of the relationship between plant height and *Fusarium* head blight resistance in wheat (*Triticum aestivum* L.) by QTL meta-analysis. Euphytica 174:343–356

Matarese F, Sarrocco S, Gruber S, Seidl-Seiboth V, Vannacci G (2012) Biocontrol of *Fusarium* head blight: interactions between *Trichoderma* and mycotoxigenic *Fusarium*. Microbiology 158:98–106

McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential, and durable resistance. Annu Rev Phytopathol 40:349–379

McMullen M, Jones R, Gallenberg D (1997) Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis 81:1340–1348

Mesterhazy A (1995) Types and components of resistance to *Fusarium* head blight of wheat. Plant Breed 114:377–386

Mesterhazy A (2002) Role of deoxynivalenol in aggressiveness of *Fusarium graminearum* and *F. culmorum* and in resistance to *Fusarium* head blight. Eur J Plant Pathol 108:675–684

Mesterhazy A (2003) Control of *Fusarium* head blight of wheat by fungicides. In: Leonard KJ, Bushnell WR (eds) *Fusarium* head blight of wheat and barley. APS Press, St. Paul, pp 363–380

Mesterhazy A, Bartok T, Mirocha CG, Komoroczy R (1999) Nature of wheat resistance to *Fusarium* head blight and the role of deoxynivalenol for breeding. Plant Breed 118:97–110

Miedaner T, Geiger HH (1996) Estimates of combining ability for resistance of winter rye to *Fusarium culmorum* head blight. Euphytica 89:339–344

Miedaner T, Korzun V (2012) Marker-assisted selection for disease resistance in wheat and barley breeding. Phytopathology 102:560–566

Miedaner T, Reinbrecht C (2001) Trichothecene content of rye and wheat genotypes inoculated with a deoxynivalenol- and a nivalenol-producing isolate of *Fusarium culmorum*. J Phytopathol 149: 245–251

Miedaner T, Schilling AG (1996) Genetic variation of aggressiveness in individual field populations of *Fusarium graminearum* and *Fusarium culmorum* tested on young plants of winter rye. Eur J Plant Pathol 102:823–830

Miedaner T, Voss HH (2008) Effect of dwarfing *Rht* genes on *Fusarium* head blight resistance in two sets of near-isogenic lines of wheat and check cultivars. Crop Sci 48:2115–2122

Miedaner T, Ludwig WF, Geiger HH (1995) Inheritance of foot rot resistance in winter rye. Crop Sci 35: 388–393

Miedaner T, Gang G, Reinbrecht C, Geiger HH (1997) Lack of association between *Fusarium* foot rot and head blight resistance in winter rye. Crop Sci 37: 327–331

Miedaner T, Reinbrecht C, Schilling AG (2000) Association among aggressiveness, fungal colonization, and mycotoxin production of 26 isolates of *Fusarium graminearum* in winter rye head blight. J Plant Dis Prot 107:124–134

Miedaner T, Schilling AG, Geiger HH (2001) Molecular genetic diversity and variation for aggressiveness in populations of *Fusarium graminearum* and *Fusarium culmorum* sampled from wheat fields in different countries. J Phytopathol 149:641–648

Miedaner T, Moldovan M, Ittu M (2003a) Comparison of spray and point inoculation to assess resistance to *Fusarium* head blight in a multienvironment wheat trial. Phytopathology 93:1068–1072

Miedaner T, Schneider B, Geiger HH (2003b) Deoxynivalenol (DON) content and *Fusarium* head blight resistance in segregating populations of winter rye and winter wheat. Crop Sci 43:519–526

Miedaner T, Schneider B, Oettler G (2006a) Means and variances for *Fusarium* head blight resistance of F-2-derived bulks from winter triticale and winter wheat crosses. Euphytica 152:405–411

Miedaner T, Wilde F, Steiner B, Buerstmayr H, Korzun V, Ebmeyer E (2006b) Stacking quantitative trait loci (QTL) for *Fusarium* head blight resistance from non-adapted sources in an European elite spring wheat background and assessing their effects on deoxynivalenol (DON) content and disease severity. Theor Appl Genet 112:562–569

Miedaner T, Cumagun CJR, Chakraborty S (2008) Population genetics of three important head blight pathogens *Fusarium graminearum F. pseudograminearum* and *F. culmorum*. J Phytopathol 156: 129–139

Miedaner T, Wilde F, Korzun V, Ebmeyer E, Schmolke M, Hartl L, Schön CC (2009) Marker selection for *Fusarium* head blight resistance based on quantitative trait loci (QTL) from two European sources compared to phenotypic selection in winter wheat. Euphytica 166:219–227

Miedaner T, Talas F, Longin F (2011) Sources of resistance to *Fusarium* head blight within Syrian durum wheat landraces. Plant Breed 130:398–400

Miller JD, Arnison PG (1986) Degradation of deoxynivalenol by suspension-cultures of the *Fusarium* head blight resistant wheat cultivar Frontana. Can J Plant Pathol 8:147–150

Miller JD, Greenhalgh R, Wang YZ, Lu M (1991) Trichothecene chemotypes of 3 *Fusarium* species. Mycologia 83:121–130

Mishra PK, Fox RTV, Culham A (2003) Inter-simple sequence repeat and aggressiveness analyses revealed high genetic diversity, recombination and long-range dispersal in *Fusarium culmorum*. Ann Appl Biol 143:291–301

Mishra PK, Tewari JP, Clear RM, Turkington TK (2004) Molecular genetic variation and geographical structuring in *Fusarium graminearum*. Ann Appl Biol 145:299–307

Mishra PK, Tewari JP, Clear RM, Turkington TK (2006) Genetic diversity and recombination within populations of *Fusarium pseudograminearum* from western Canada. Int Microbiol 9:65–68

Muthomi JW, Schütze A, Dehne HW, Mutitu EW, Oerke EC (2000) Characterization of *Fusarium culmorum* isolates by mycotoxin production and aggressiveness to winter wheat. J Plant Dis Prot 107:113–123

Naef A, Defago G (2006) Population structure of plant-pathogenic *Fusarium* species in overwintered stalk residues from Bt-transformed and non-transformed maize crops. Eur J Plant Pathol 116:129–143

Nakajima T, Yoshida M, Tomimura K (2008) Effect of lodging on the level of mycotoxins in wheat, barley, and rice infected with the *Fusarium graminearum* species complex. J Gen Plant Pathol 74:289–295

Neubert K, Mendgen K, Brinkmann H, Wirsel SGR (2006) Only a few fungal species dominate highly diverse mycofloras associated with the common reed. Appl Environ Microbiol 72:1118–1128

Nganje W, Johnson D (2003) Economic impact of *Fusarium* head blight in malting barley: blending margins and firm-level risk. Curr Agric Food Resour Issues 4:16–26

Nganje WE, Bangsund DA, Leistritz FL, Wilson WW, Tiapo NM (2002) Estimating the economic impact of a crop disease: the case of *Fusarium* head blight in U.S. wheat and barley. In: Canty SM, Lewis J, Siler L, Ward RW (eds) National *Fusarium* Head Blight Forum. U.S. Wheat & Barley Scab Initiative, Holiday Inn Cincinnati-Airport, Erlanger, pp 275–281

Nganje WE, Bangsund DA, Leistritz FL, Wilson WW, Tiapo NM (2004) Regional economic impacts of *Fusarium* head blight in wheat and barley. Rev Agric Econ 26:332–347

Nielsen LK, Jensen JD, Nielsen GC, Jensen JE, Spliid NH, Thomsen IK, Justesen AF, Collinge DB, Jorgensen LN (2011) *Fusarium* head blight of cereals in Denmark: species complex and related mycotoxins. Phytopathology 101:960–969

Nightingale MJ, Marchylo BA, Clear RM, Dexter JE, Preston KR (1999) *Fusarium* head blight: effect of

fungal proteases on wheat storage proteins. Cereal Chem 76:150–158

Nyvall RF, Percich JA, Mirocha CJ (1999) *Fusarium* head blight of cultivated and natural wild rice (*Zizania palustris*) in Minnesota caused by *Fusarium graminearum* and associated *Fusarium* spp. Plant Dis 83:159–164

O'Donnell K, Kistler HC, Tacke BK, Casper HH (2000) Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proc Natl Acad Sci USA 97:7905–7910

O'Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T (2004) Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet Biol 41:600–623

O'Donnell K, Ward TJ, Aberra D, Kistler HC, Aoki T, Orwig N, Kimura M, Bjornstad S, Klemsdal SS (2008) Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the *Fusarium graminearum* species complex from Ethiopia. Fungal Genet Biol 45:1514–1522

Obanor F, Erginbas-Orakci G, Tunali B, Nicol JM, Chakraborty S (2010) *Fusarium culmorum* is a single phylogenetic species based on multilocus sequence analysis. Fungal Biol 114:753–765

Obst A, Gleissenthall JL, Beck R (1997) On the etiology of *Fusarium* head blight of wheat in south Germany – preceding crops, weather conditions for inoculum production and head infection, proneness of the crop to infection and mycotoxin production. Cereal Res Commun 25:699–703

Oettler G, Heinrich N, Miedaner T (2004) Estimates of additive and dominance effects for *Fusarium* head blight resistance of winter tritical. Plant Breed 123:525–530

Okubara PA, Blechl AE, McCormick SP, Alexander NJ, Dill-Macky R, Hohn TM (2002) Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. Theor Appl Genet 106:74–83

Oliver RE, Cal X, Friesen TL, Halley S, Stack RW, Xu SS (2008) Evaluation of *Fusarium* head blight resistance in tetraploid wheat (*Triticum turgidum* L.). Crop Sci 48:213–222

Otto CD, Kianian SF, Elias EM, Stack RW, Joppa LR (2002) Genetic dissection of a major *Fusarium* head blight QTL in tetraploid wheat. Plant Mol Biol 48:625–632

Paillard S, Schnurbusch T, Tiwari R, Messmer M, Winzeler M, Keller B, Schachermayr G (2004) QTL analysis of resistance to *Fusarium* head blight in Swiss winter wheat (*Triticum aestivum* L.). Theor Appl Genet 109:323–332

Palazzini JM, Ramirez ML, Torres AM, Chulze SN (2007) Potential biocontrol agents for *Fusarium* head blight and deoxynivalenol production in wheat. Crop Prot 26:1702–1710

Palazzini JM, Ramirez ML, Alberione EJ, Torres AM, Chulze SN (2009) Osmotic stress adaptation, compatible solutes accumulation and biocontrol efficacy of two potential biocontrol agents on *Fusarium* head blight in wheat. Biol Control 51:370–376

Pandeya R, Graf R (2006) Advancement in FHB resistant winter wheat cultivar development using FRONTANA as the resistance donor parent. In: Ban T, Lewis JM, Phipps EE (eds) The global *Fusarium* initiative for international collaboration: a strategic planning workshop. CIMMYT, El Batan, Mexico, pp 77–81

Parry DW, Jenkinson P, Mcleod L (1995) *Fusarium* ear blight (scab) in small-grain cereals – a review. Plant Pathol 44:207–238

Paul PA, Lipps PE, Madden LV (2006) Meta-analysis of regression coefficients for the relationship between *Fusarium* head blight and deoxynivalenol content of wheat. Phytopathology 96:951–961

Paul PA, Lipps PE, Hershman DE, McMullen MP, Draper MA, Madden LV (2008) Efficacy of triazole-based fungicides for *Fusarium* head blight and deoxynivalenol control in wheat: a multivariate meta-analysis. Phytopathology 98:999–1011

Paul PA, McMullen MP, Hershman DE, Madden LV (2010) Meta-analysis of the effects of triazole-based fungicides on wheat yield and test weight as influenced by *Fusarium* head blight intensity. Phytopathology 100:160–171

Paulitz TC (1996) Diurnal release of ascospores by *Gibberella zeae* in inoculated wheat plots. Plant Dis 80:674–678

Pereyra SA, Dill-Macky R (2008) Colonization of the residues of diverse plant species by *Gibberella zeae* and their contribution to *Fusarium* head blight inoculum. Plant Dis 92:800–807

Pereyra SA, Dill-Macky R, Sims AL (2004) Survival and inoculum production of *Gibberella zeae* in wheat residue. Plant Dis 88:724–730

Pestka JJ, Smolinski AT (2005) Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 8:39–69

Pirgozliev SR, Edwards SG, Hare MC, Jenkinson P (2003) Strategies for the control of *Fusarium* head blight in cereals. Eur J Plant Pathol 109:731–742

Poppenberger B, Berthiller F, Lucyshyn D, Sieberer T, Schuhmacher R, Krska R, Kuchler K, Glossl J, Luschnig C, Adam G (2003) Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. J Biol Chem 278:47905–47914

Proctor RH, Hohn TM, McCormick SP (1995) Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Mol Plant Microbe Interact 8:593–601

Proctor RH, McCormick SP, Alexander NJ, Desjardins AE (2009) Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relo-

cation during evolution of the filamentous fungus *Fusarium*. Mol Microbiol 74:1128–1142

Puri KD, Zhong S (2010) The 3ADON population of *Fusarium graminearum* found in North Dakota is more aggressive and produces a higher level of DON than the prevalent 15ADON population in spring wheat. Phytopathology 100:1007–1014

Purss GS (1971) Pathogenic specialization in *Fusarium graminearum*. Aust J Agric Res 22:553–661

Qu B, Li HP, Zhang JB, Xu YB, Huang T, Wu AB, Zhao CS, Carter J, Nicholson P, Liao YC (2008) Geographic distribution and genetic diversity of *Fusarium graminearum* and *F. asiaticum* on wheat spikes throughout China. Plant Pathol 57:15–24

Ramirez ML, Chulze S, Magan N (2004) Impact of environmental factors and fungicides on growth and deoxinivalenol production by *Fusarium graminearum* isolates from Argentinian wheat. Crop Prot 23:117–125

Rohacik T, Hudec K (2005) Influence of agro-environmental factors on *Fusarium* infestation and population structure in wheat kernels. Ann Agric Environ Med 12:39–45

Rossi V, Languasco L, Pattori E, Giosue S (2002) Dynamics of airborne *Fusarium* macroconidia in wheat fields naturally affected by head blight. J Plant Pathol 84:53–64

Salameh A, Buerstmayr M, Steiner B, Neumayer A, Lemmens M, Buerstmayr H (2011) Effects of introgression of two QTL for *Fusarium* head blight resistance from Asian spring wheat by marker-assisted backcrossing into European winter wheat on *Fusarium* head blight resistance, yield and quality traits. Mol Breed 28:485–494

Sarver BA, Ward TJ, Gale LR, Broz K, Kistler CH, Aoki T, Nicholson P, Carter J, O'Donnell K (2011) Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. Fungal Genet Biol 48:1096–1107

Schaafsma AW (2002) Economic changes imposed by mycotoxins in food grains: case study of deoxynivalenol in winter wheat. In: DeVries JW, Trucksess MW, Jackson LS (eds) Mycotoxins and food safety. Kluwer Academic/Plenum Publishing, New York, pp 271–276

Schaafsma AW, Tamburic-Ilinic L, Miller JD, Hooker DC (2001) Agronomic considerations for reducing deoxynivalenol in wheat grain. Can J Plant Pathol 23:279–285

Scherm B, Orru M, Balmas V, Spanu F, Azara E, Delogu G, Hammond TM, Keller NP, Migheli Q (2011) Altered trichothecene biosynthesis in *TRI6*-silenced transformants of *Fusarium culmorum* influences the severity of crown and foot rot on durum wheat seedlings. Mol Plant Pathol 12:759–771

Schisler DA, Khan NI, Boehm MJ, Lipps PE, Slininger PJ, Zhang S (2006) Selection and evaluation of the potential of choline-metabolizing microbial strains to reduce *Fusarium* head blight. Biol Control 39:497–506

Schmale DG, Leslie JF, Zeller KA, Saleh AA, Shields EJ, Bergstrom GC (2006) Genetic structure of atmospheric populations of *Gibberella zeae*. Phytopathology 96:1021–1026

Schmale DG, Wood-Jones AK, Cowger C, Bergstrom GC, Arellano C (2011) Trichothecene genotypes of *Gibberella zeae* from winter wheat fields in the eastern USA. Plant Pathol 60:909–917

Schmolke M, Zimmermann G, Buerstmayr H, Schweizer G, Miedaner T, Korzun V, Ebmeyer E, Hartl L (2005) Molecular mapping of *Fusarium* head blight resistance in the winter wheat population Dream/Lynx. Theor Appl Genet 111:747–756

Schroeder HW, Christensen JJ (1963) Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. Phytopathology 53:831–838

Schweiger W, Boddu J, Shin S, Poppenberger B, Berthiller F, Lemmens M, Muehlbauer GJ, Adam G (2010) Validation of a candidate deoxynivalenol-inactivating UDP-glucosyltransferase from barley by heterologous expression in yeast. Mol Plant Microbe Interact 23:977–986

Scott JB, Chakraborty S (2006) Multilocus sequence analysis of *Fusarium pseudograminearum* reveals a single phylogenetic species. Mycol Res 110:1413–1425

Semagn K, Skinnes H, Bjornstad A, Maroy AG, Tarkegne Y (2007) Quantitative trait loci controlling *Fusarium* head blight resistance and low deoxynivalenol content in hexaploid wheat population from 'Arina' and NK93604. Crop Sci 47:294–303

Shaner G (2003) Epidemiology of *Fusarium* head blight of small grain cereals in North America. In: Leonard KJ, Bushnell WR (eds) *Fusarium* head blight of wheat and barley. APS Press, St. Paul, pp 84–119

Shin SY, Mackintosh CA, Lewis J, Heinen SJ, Radmer L, Dill-Macky R, Baldridge GD, Zeyen RJ, Muehlbauer GJ (2008) Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*. J Exp Bot 59:2371–2378

Shin S, Torres-Acosta JA, Heinen SJ, McCormick S, Lemmens M, Kovalsky-Paris MP, Berthiller F, Adam G, Muehlbauer GJ (2012) Transgenic *Arabidopsis thaliana* expressing a barley UDP glucosyltransferase exhibit resistance to the mycotoxin deoxynivalenol. J Exp Bot 63:4731–4740

Simpson DR, Weston GE, Turner JA, Jennings P, Nicholson P (2001) Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain. Eur J Plant Pathol 107:421–431

Snijders CHA (1990a) Genetic-variation for resistance to *Fusarium* head blight in bread wheat. Euphytica 50:171–179

Snijders CHA (1990b) The inheritance of resistance to head blight caused by *Fusarium culmorum* in winter wheat. Euphytica 50:11–18

Somers DJ, Thomas J, DePauw R, Fox S, Humphreys G, Fedak G (2005) Assembling complex genotypes to

resist *Fusarium* in wheat (*Triticum aestivum* L.). Theor Appl Genet 111:1623–1631

Somers DJ, Fedak G, Clarke J, Cao WG (2006) Mapping of FHB resistance QTLs in tetraploid wheat. Genome 49:1586–1593

Srinivasachary, Gosman N, Steed A, Simmonds J, Leverington-Waite M, Wang Y, Snape J, Nicholson P (2008) Susceptibility to *Fusarium* head blight is associated with the Rht-D1b semi-dwarfing allele in wheat. Theor Appl Genet 116:1145–1153

Stack RW (1989) A comparison of the inoculum potential of ascospores and conidia of *Gibberella zeae*. Can J Plant Pathol 11:137–142

Stack RW (2003) History of *Fusarium* head blight with emphasis on North America. In: Leonard KJ, Bushnell WR (eds) *Fusarium* head blight of wheat and barley. APS Press, St. Paul, pp 1–34

Starkey DE, Ward TJ, Aoki T, Gale LR, Kistler HC, Geiser DM, Suga H, Toth B, Varga J, O'Donnell K (2007) Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. Fungal Genet Biol 44:1191–1204

Stenglein SA (2009) *Fusarium poae*: a pathogen that needs more attention. J Plant Pathol 91:25–36

Stenglein SA, Rodriguero MS, Chandler E, Jennings P, Salerno GL, Nicholson P (2010) Phylogenetic relationships of *Fusarium poae* based on EF-1a and mtSSU sequences. Fungal Biol 114:96–106

Stepien L, Chelkowski J (2010) *Fusarium* head blight of wheat: pathogenic species and their mycotoxins. World Mycotoxin J 3:107–119

Suga H, Karugia GW, Ward T, Gale LR, Tomimura K, Nakajima T, Miyasaka A, Koizumi S, Kageyama K, Hyakumachi M (2008) Molecular characterization of the *Fusarium graminearum* species complex in Japan. Phytopathology 98:159–166

Sugita-Konishi Y, Kubosaki A, Takahashi M, Park BJ, Tanaka T, Takatori K, Hirose M, Shibutani M (2008) Nivalenol and the targeting of the female reproductive system as well as haematopoietic and immune systems in rats after 90-day exposure through the diet. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25:1118–1127

Summerell BA, Leslie JF, Liew ECY, Laurence MH, Bullock S, Petrovic T, Bentley AR, Howard CG, Peterson SA, Walsh JL, Burgess LW (2011) *Fusarium* species associated with plants in Australia. Fungal Divers 46:1–27

Sutton JC (1982) Epidemiology of wheat blight and maize ear rot caused by *Fusarium graminearum*. Can J Plant Pathol 4:195–209

Talas F, Longin F, Miedaner T (2011a) Sources of resistance to *Fusarium* head blight within Syrian durum wheat landraces. Plant Breed 130:398–400

Talas F, Parzies HK, Miedaner T (2011b) Diversity in genetic structure and chemotype composition of *Fusarium graminearum* sensu stricto populations causing wheat head blight in individual fields in Germany. Eur J Plant Pathol 131:39–48

Talas F, Kalih R, Miedaner T (2012a) Within-field variation of *Fusarium graminearum* isolates for aggressiveness and deoxynivalenol production in wheat head blight. Phytopathology 102:128–134

Talas F, Wuerschum T, Reif JC, Parzies HK, Miedaner T (2012b) Association of single nucleotide polymorphic sites in candidate genes with aggressiveness and deoxynivalenol production in *Fusarium graminearum* causing wheat head blight. BMC Genet 13:14

Tamburic-Ilincic L, Somers D, Fedak G, Schaafsma A (2009) Different quantitative trait loci for *Fusarium* resistance in wheat seedlings and adult stage in the Wuhan/Nyubai wheat population. Euphytica 165:453–458

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC (2000) Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21–32

Tonshin AA, Teplova VV, Andersson MA, Salkinoja-Salonen MS (2010) The *Fusarium* mycotoxins enniatins and beauvericin cause mitochondrial dysfunction by affecting the mitochondrial volume regulation, oxidative phosphorylation and ion homeostasis. Toxicology 276:49–57

Torp M, Nirenberg HI (2004) *Fusarium langsethiae sp. nov.* on cereals in Europe. Int J Food Microbiol 95:247–256

Toth B, Mesterhazy A, Nicholson P, Teren J, Varga J (2004) Mycotoxin production and molecular variability of European and American isolates of *Fusarium culmorum*. Eur J Plant Pathol 110:587–599

Toth B, Kaszonyi G, Bartok T, Varga J, Mesterhazy A (2008) Common resistance of wheat to members of the *Fusarium graminearum* species complex and *F. culmorum*. Plant Breed 127:1–8

Trail F (2009) For blighted waves of grain: *Fusarium graminearum* in the postgenomics era. Plant Physiol 149:103–110

Trail F, Common R (2000) Perithecial development by *Gibberella zeae*: a light microscopy study. Mycologia 92:130–138

Trail F, Xu HX, Loranger R, Gadoury D (2002) Physiological and environmental aspects of ascospore discharge in *Gibberella zeae* (anamorph *Fusarium graminearum*). Mycologia 94:181–189

Trail F, Gaffoor I, Vogel S (2005) Ejection mechanics and trajectory of the ascospores of *Gibberella zeae* (anamorph *Fuarium graminearum*). Fungal Genet Biol 42:528–533

Tschanz AT, Horst RK, Nelson PE (1976) Effect of environment on sexual reproduction of *Gibberella zeae*. Mycologia 68:327–340

Ueno Y (1985) The toxicology of mycotoxins. Crc Cr Rev Toxicol 14:99–132

Uhlig S, Jestoi M, Parikka P (2007) *Fusarium avenaceum* – the North European situation. Int J Food Microbiol 119:17–24

Van Eeuwijk EA, Mesterhazy A, Kling CI, Ruckenbauer P, Saur L, Buerstmayr H, Lemmens M, Keizer LCP, Maurin N, Snijders CHA (1995) Assessing nonspe-

cificity of resistance in wheat to head blight caused by inoculation with European strains of *Fusarium culmorum*, *F. graminearum* and *F. nivale* using a multiplicative model for interaction. Theor Appl Genet 90:221–228

Vanderplank JE (1984) Disease resistance in plants. Academic, London

Verstraete F (2008) European Union legislation on mycotoxins in food and feed. Overview of the decision-making process and recent and future developments. In: Leslie JF, Bandyopadhyay R, Visconti A (eds) Mycotoxins: detection methods, management, public health and agricultural trade. CABI, Wallingford, pp 77–99

von der Ohe C, Miedaner T (2011) Competitive aggressiveness in binary mixtures of *Fusarium graminearum* and *F. culmorum* isolates inoculated on spring wheat with highly effective resistance QTL. J Phytopathol 159:401–410

von der Ohe C, Ebmeyer E, Korzun V, Miedaner T (2010a) Agronomic and quality performance of winter wheat backcross populations carrying non-adapted *Fusarium* head blight resistance QTL. Crop Sci 50:2283–2290

von der Ohe C, Gauthier V, Tamburic-Ilincic L, Brule-Babel A, Fernando WGD, Clear R, Ward TJ, Miedaner T (2010b) A comparison of aggressiveness and deoxynivalenol production between Canadian *Fusarium graminearum* isolates with 3-acetyl and 15-acetyldeoxynivalenol chemotypes in field-grown spring wheat. Eur J Plant Pathol 127:407–417

Voss HH, Holzapfel J, Hartl L, Korzun V, Rabenstein F, Ebmeyer E, Coester H, Kempf H, Miedaner T (2008) Effect of the Rht-D1 dwarfing locus on *Fusarium* head blight rating in three segregating populations of winter wheat. Plant Breed 127: 333–339

Voss HH, Bowden RL, Leslie JF, Miedaner T (2010) Variation and transgression of aggressiveness among two *Gibberella zeae* crosses developed from highly aggressive parental isolates. Phytopathology 100:904–912

Waalwijk C, Kastelein P, de Vries I, Kerenyi Z, van der Lee T, Hesselink T, Kohl J, Kema G (2003) Major changes in *Fusarium* spp. in wheat in the Netherlands. Eur J Plant Pathol 109:743–754

Walker SL, Leath S, Hagler WM, Murphy JP (2001) Variation among isolates of *Fusarium graminearum* associated with *Fusarium* head blight in North Carolina. Plant Dis 85:404–410

Walter S, Nicholson P, Doohan FM (2010) Action and reaction of host and pathogen during *Fusarium* head blight disease. New Phytol 185:54–66

Wan YF, Yen C, Yang JL (1997) The diversity of head-scab resistance in Triticeae and their relation to ecological conditions. Euphytica 97:277–281

Wang YZ (1997) Epidemiology and management of wheat scab in China. In: Dubin HJ, Gilchrist L, Reeves J, McNab A (eds) *Fusarium* head scab: global status and future prospects. CIMMYT, El Batan, Mexico, pp 97–105

Wang JH, Wieser H, Pawelzik E, Weinert J, Keutgen AJ, Wolf GA (2005) Impact of the fungal protease produced by *Fusarium culmorum* on the protein quality and breadmaking properties of winter wheat. Eur Food Res Technol 220:552–559

Wang H, Hwang SF, Eudes F, Chang KF, Howard RJ, Turnbull GD (2006) Trichothecenes and aggressiveness of *Fusarium graminearum* causing seedling blight and root rot in cereals. Plant Pathol 55:224–230

Wanyoike MW, Walker F, Buchenauer H (2002) Relationship between virulence, fungal biomass and mycotoxin production by *Fusarium graminearum* in winter wheat head blight. J Plant Dis Prot 109:589–600

Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K (2002) Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proc Natl Acad Sci USA 99:9278–9283

Ward TJ, Clear RM, Rooney AP, O'Donnell K, Gaba D, Patrick S, Starkey DE, Gilbert J, Geiser DM, Nowicki TW (2008) An adaptive evolutionary shift in *Fusarium* head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. Fungal Genet Biol 45:473–484

Wilde F, Miedaner T (2006) Selection for *Fusarium* head blight resistance in early generations reduces the deoxynivalenol (DON) content in grain of winter and spring wheat. Plant Breed 125:96–98

Wilde F, Korzun V, Ebmeyer E, Geiger HH, Miedaner T (2007) Comparison of phenotypic and marker-based selection for *Fusarium* head blight resistance and DON content in spring wheat. Mol Breed 19:357–370

Willyerd KT, Li C, Madden LV, Bradley CA, Bergstrom GC, Sweets LE, McMullen M, Ransom JK, Grybauskas A, Osborne L, Wegulo SN, Hershman DE, Wise K, Bockus WW, Groth D, Dill-Macky R, Milus E, Esker PD, Waxman KD, Adee EA, Ebelhar SE, Young BG, Paul PA (2012) Efficacy and stability of integrating fungicide and cultivar resistance to manage *Fusarium* head blight and deoxynivalenol in wheat. Plant Dis 96:957–967

Windels CE (2000) Economic and social impacts of *Fusarium* head blight: changing farms and rural communities in the Northern Great Plains. Phytopathology 90:17–21

Xu XM (2003) Effects of environmental conditions on the development of *Fusarium* ear blight. Eur J Plant Pathol 109:683–689

Xu X, Nicholson P (2009) Community ecology of fungal pathogens causing wheat head blight. Annu Rev Phytopathol 47:83–103

Xu XM, Parry DW, Nicholson P, Thomsett MA, Simpson D, Edwards SG, Cooke BM, Doohan FM, Brennan JM, Moretti A, Tocco G, Mule G,

Hornok L, Giczey G, Tatnell J (2005) Predominance and association of pathogenic fungi causing *Fusarium* ear blight in wheat in four European countries. Eur J Plant Pathol 112:143–154

Xu XM, Monger W, Ritieni A, Nicholson P (2007) Effect of temperature and duration of wetness during initial infection periods on disease development, fungal biomass and mycotoxin concentrations on wheat inoculated with single, or combinations of, *Fusarium* species. Plant Pathol 56:943–956

Xue AG, Voldeng HD, Savard ME, Fedak G (2009) Biological management of *Fusarium* head blight and mycotoxin contamination in wheat. World Mycotoxin J 2:193–201

Yang ZP, Yang XY, Huang DC (1999) Comparison of evaluation methods for selection of resistance to *Fusarium* head blight in a recurrent selection programme in wheat (*Triticum aestivum* L.). Plant Breed 118:289–292

Yang L, van der Lee T, Yang X, Yu D, Waalwijk C (2008) *Fusarium* populations on Chinese barley show a dramatic gradient in mycotoxin profiles. Phytopathology 98:719–727

Yang F, Jensen JD, Spliid NH, Svensson B, Jacobsen S, Jorgensen LN, Jorgensen HJ, Collinge DB, Finnie C (2010) Investigation of the effect of nitrogen on severity of *Fusarium* head blight in barley. J Proteomics 73:743–752

Yang F, Svensson B, Finnie C (2011) Response of germinating barley seeds to *Fusarium graminearum*: the first molecular insight into *Fusarium* seedling blight. Plant Physiol Biochem 49:1362–1368

Yi C, Kaul HP, Kubler E, Schwadorf K, Aufhammer W (2001) Head blight (*Fusarium graminearum*) and deoxynivalenol concentration in winter wheat as affected by pre-crop, soil tillage and nitrogen fertilization. J Plant Dis Prot 108:217–230

Yin Y, Liu X, Li B, Ma Z (2009) Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. Phytopathology 99: 487–497

Yli-Mattila T (2010) Ecology and evolution of toxigenic *Fusarium* species in cereals in Northern Europe and Asia. J Plant Pathol 92:7–18

Yli-Mattila T, Gagkaeva T, Ward TJ, Aoki T, Kistler HC, O'Donnell K (2009) A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian Far East. Mycologia 101:841–852

Yoshida M, Nakajima T, Arai M, Suzuki F, Tomimura K (2008) Effect of the timing of fungicide application on *Fusarium* head blight and mycotoxin accumulation in closed-flowering barley. Plant Dis 92:1164–1170

Zeller KA, Bowden RL, Leslie JF (2003) Diversity of epidemic populations of *Gibberella zeae* from small quadrats in Kansas and North Dakota. Phytopathology 93:874–880

Zeller KA, Bowden RL, Leslie JF (2004) Population differentiation and recombination in wheat scab populations of *Gibberella zeae* from the United States. Mol Ecol 13:563–571

Zhang JB, Li HP, Dang FJ, Qu B, Xu YB, Zhao CS, Liao YC (2007) Determination of the trichothecene mycotoxin chemotypes and associated geographical distribution and phylogenetic species of the *Fusarium graminearum* clade from China. Mycol Res 111:967–975

Zhang H, Zhang Z, van der Lee T, Chen WQ, Xu J, Xu JS, Yang L, Yu D, Waalwijk C, Feng J (2010a) Population genetic analyses of *Fusarium asiaticum* populations from barley suggest a recent shift favoring 3ADON producers in Southern China. Phytopathology 100:328–336

Zhang Z, Zhang H, van der Lee T, Chen WQ, Arens P, Xu J, Xu JS, Yang LJ, Yu DZ, Waalwijk C, Feng JE (2010b) Geographic substructure of *Fusarium asiaticum* isolates collected from Barley in China. Eur J Plant Pathol 127:239–248

Zhang H, Van der Lee T, Waalwijk C, Chen W, Xu J, Zhang Y, Feng J (2012) Population analysis of the *Fusarium graminearum* species complex from wheat in China show a shift to more aggressive isolates. PLoS One 7:e31722

Zinedine A, Soriano JM, Molto JC, Manes J (2007) Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem Toxicol 45:1–18

9 Ecological and Economical Importance of Parasitic Zoosporic True Fungi

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CONTENTS

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I. Introduction

Many species of zoosporic true fungi have been observed and described in terrestrial, freshwater, and marine ecosystems (Sparrow 1960; Karling 1977; Powell 1993; Barr 2001). Most of these species are considered to be well-adapted to moist soil or freshwater habitats (Gleason et al. 2010a, b). The research on the ecology of these fungi prior to the early 1960s was thoroughly reviewed by Sparrow (1960, 1968). Yet during the next five decades, the ecology of these fungi has been poorly studied. The reasons for the lack of research are complex; however, they include difficulty in observing these microorganisms on their substrates with conventional microscopic procedures, the fact that few researchers are trained to recognize and do research with these microorganisms, and a perception that these microorganisms are relatively unimportant ecologically and economically. Even research on well-known plant and animal diseases caused by zoosporic fungal pathogens has historically been poorly funded.

Fortunately, these perceptions are slowly changing with the recent progress in research, especially with the discovery, using modern pyrosequencing approaches, of a wide variety of rumen chytrids (Neocallimastigales; Li et al. 1993) in the digestive systems of herbivorous vertebrates (Liggenstoffer et al. 2010), and

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chytridiomycosis in amphibians (Longcore et al. 1999; Fisher et al. 2009). Although many of the species listed by Sparrow (1960) have not been observed with the light microscope recently, DNA sequences putatively assigned to these groups of microorganisms are currently being documented widely in environmental surveys (Tuckwell et al. 2005; Stock et al. 2009; Edwards et al. 2008; Lara et al. 2010; Liggenstoffer et al. 2010; Jones et al. 2011a).

In this chapter, we review research on some of the zoosporic parasites of algae, animals, and higher plants, which are classified as true fungi and assigned to the Chytridiomycota *sensu lato*, and comprises all chitinous zoosporic fungi (Barr 2001; Voigt and Kirk 2011; Ebersberger et al. 2012). The taxonomy of zoosporic fungi was recently reviewed (Voigt 2012). Zoosporic fungal parasites are well-adapted to many types of aquatic habitats (Sparrow 1960; Powell 1993; Gleason et al. 2010a, 2011; Nascimento et al. 2011; Marano et al. 2011). They have propagules (zoospores) which are thought to sense environmental gradients because they swim toward their hosts (Gleason and Lilje 2009). They also have resistant structures which can survive environmental extremes (Gleason et al. 2010b). Some species are thought to be highly virulent and are considered to be emerging infectious diseases (Fisher et al. 2012), although data supporting this hypothesis are generally unavailable or incomplete.

Members in the Blastocladiomycota, Chytridiomycota, and *Olpidium* clade are included in this chapter. There are no known parasites in the Neocallimastigomycota and the Monoblepharomycota. We focus on zoosporic fungi in the Blastocladiomycota and Chytridiomycota which are parasites of primary producers (flowering plants and phytoplankton) in freshwater ecosystems, although some parasites of animal hosts are also included. Zoosporic fungal parasites in marine ecosystems and in invertebrate animals have recently been reviewed (Gleason et al. 2010a, 2011). Zoosporic parasites of freshwater phytoplankton mostly infect diatoms and green algae (Canter and Lund 1953; Canter 1969; Barr and Hickman 1967a, b) but have also been found parasitizing cyanobacteria (Canter 1972; Sen 1988a). In addition, theoretical concepts useful for understanding the general ecology and the potential ecological roles of these fungi are discussed. We believe that parasitism by zoosporic true fungi is significantly affected by environmental deterioration and climate change.

II. Phylogeny: Evolutionary Implications of Zoosporic Fungi and Their Phylogenetic Position Within the Fungal Kingdom

A. Phylogenetic Trees and Phylogenetic Novelties

Baldauf (2003) constructed a tree of life with branching supergroups based on nucleotide sequences of ribosomal genes to replace the five kingdoms. This concept has been extended by Adl et al. (2005) and James et al. (2006a) and others more recently to include more groups of fungi and protists (Steenkamp et al. 2005; Porter et al. 2008; Jones et al. 2011a, b; Capella-Gutiérrez et al. 2012). The tree that Baldauf originally constructed was designed to indicate pathways of evolution and relationships between phylogenetic groups, namely the sister group relationship between true fungi (Mycota) and multicellular animals (Metazoa), whose protistan origins were later phylogenetically investigated by Steenkamp et al. (2005). Even with data currently available from sequences of many genes in public data bases, there is still much to be learned about the evolutionary relationships. Traditionally, the zoosporic true fungi [Chytridiomycota as defined by Barr (2001)] are considered as the most basal fungal phylum of the kingdom of Fungi. Both molecular and morphological data (James et al. 2006b) have provided **evidence for divergence of the chytrids into five clades, four of which have been given the rank of phyla; these are: Blastocladiomycota** (James et al. 2006b)**, Chytridiomycota *s. str.*, and Neocallimastigomycota** (Hibbett et al. 2007)**, and the Monoblepharomycota** (Doweld 2001). The *Olpidium* group remains as clade at

present, though *Olpidium* was found to form a monophyletic group with taxa of those terrestrial fungi traditionally classified in the phylum Zygomycota (Sekimoto et al. 2011). The Neocallimastigomycota differ in their capability to tolerate oxygen and the ecological niches they inhabit. Whilst Blastocladiomycota and Chytridiomycota *s.str.* are aerobic microorganisms occuring mainly as saprobionts (saprotrophs) or parasites of plants, animals, protists, or algae inhabiting aquatic environments, the Neocallimastigomycota encompass a unique and phylogenetically coherent group of anaerobic microorganisms which inhabit the digestive systems of herbivorous mammals and reptiles. The phylogenetic relationships between these zoosporic phyla are not well-resolved when single or a few multiple genes are used for phylogenetic reconstruction (James et al. 2006a, b). However, recent phylogenomic studies based on more than 100 orthologous, protein-coding genes provided evidence for the **re-unification of Blastocladiomycota, Chytridiomycota *s.str.*, Neocallimastigomycota, and the Monoblepharomycota** into one phylogenetically coherent group named the Chytridiomycota *s.l.* for zoosporic true fungi as a whole. These fungi **share the production of zoospores with posteriorly directed whiplash flagella** and mitochondria, with flattened cristae as a common morphological feature with other opisthokonts (James et al. 2006b; Ebersberger et al. 2009a, 2012). The resolution of the deep fungal branches was solely made possible by **the reconstruction of a robust backbone of the fungal phylogenies using a multitude of genes, which were strongly selected for orthology applying novel bioinformatic tools** (Ebersberger et al. 2009b). That novel approach, HaMStR, combines a profile hidden Markov model search with subsequent BLAST search to extend the existing ortholog cluster with sequences from additional taxa, in order to mine EST and genomic data for the presence of orthologs to a curated set of genes (Ebersberger et al. 2009b). The phylogeny of zoosporic true fungi still requires resolution.

The development of a rigid chitin-rich cell wall and the osmotrophic uptake of nutrients during key phases of the life cycle are the primary defining features of all phyla accepted as true fungi (Voigt 2012). The zoosporic true fungi and all members and subgroups of its clades are osmotrophic in the major (vegetative) phases of their life cycle. Because of the potential of some species (though this is not experimentally proven yet) to become phagotrophic during the short zoosporic phase, the zoosporic true fungi can be considered as a transitional link between fungal (exclusively osmotrophic) and non-fungal (exclusively phagotrophic) **Opisthokonta.** Members of the non-fungal Opisthokonta (Fig. 9.1a) are organisms with opisthokont-flagellate stages in some phases of their life cycle; **nutrition uptake is phagotrophic, and chitin is non-structural.** Although chitin has been reported in dormant stages from a number of phylogenetically differing protists, e.g., diatoms, chrysophytes, ciliates, diplomonads and encysting *Entamoeba invadens* (Herth and Zugenmaier 1977; Herth et al. 1977; Arroyo-Begovich and Cárabez-Trejo 1982; Ward et al. 1985; Greco et al. 1990; Mulisch 1993), chitin is not the major structural component of the protistan cell wall. A rigid chitin-rich cell wall, which is present in key phases of the life cycle, is mandatory for the taxonomic placement of a given taxon in the kingdom Fungi.

In the past, the delimitation of the fungi was based on various aspects: classical approaches to identify fungi ranging from comparisons with the fossil record (Hawksworth et al. 1995), the use of growth physiological (Pitt 1979) and biochemical markers (Bridge 1985; Paterson and Bridge 1994), the composition of the cell wall (Bartnicki-Garcia 1970, 1987), and isoenzyme patterns (Maxson and Maxson 1990; Kohn 1992), the existence of pigments (Besl and Bresinsky 1997), and secondary metabolite profiles (Frisvad and Filtenborg 1990) to observations on the ultrastructure (Kimbrough 1994; James et al. 2006b). Genomic approaches utilize comparative genomics aimed at the elucidation of biosynthetic gene clusters and other gene syntenies known to be typical for fungi, e.g., chitin synthesis (James and Berbee 2011) or whole genome analyses resulting in the reconstruction of phylomes, which represent phylogenetic networks based on single trees (Capella-Gutiérrez et al. 2012). The true Fungi *sensu stricto* comprise a heterogenous, often

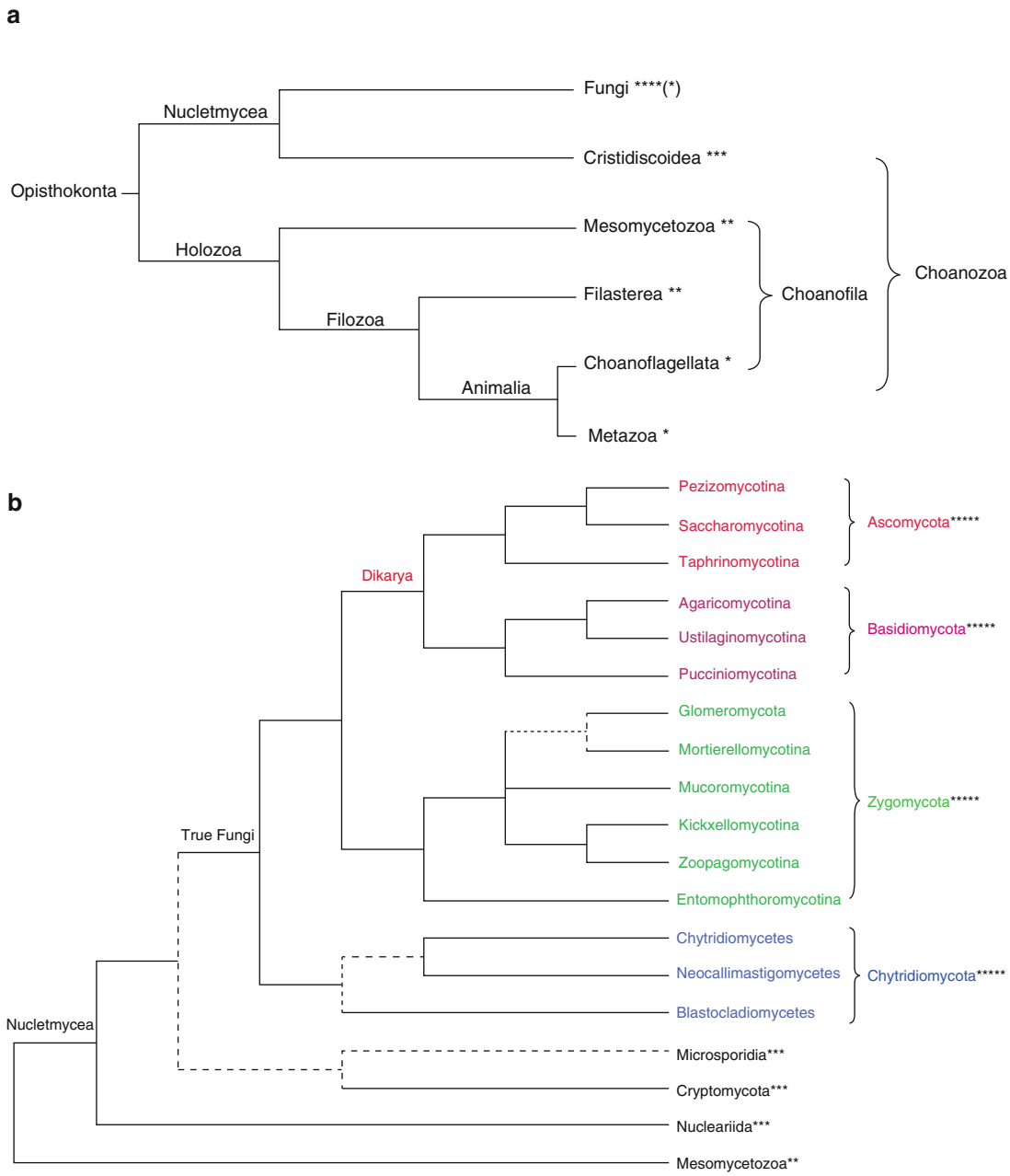

Fig. 9.1. Higher taxon-level phylogeny of the Opisthokonta (**a**) and the Fungi (**b**). (**a**) Cladogram shows the upper taxon-level systematics of the Opisthokonta, based mainly on Shalchian-Tabrizi (2008). The Nucletmycea consists of Fungi and Cristidiscoidia (Brown et al. 2009) and is equivalent to the Holomycota (Steenkamp et al. 2005). For lower taxon level phylogeny of the Fungi see (**b**). The Cristidiscoidia comprises only one class, which is the class Discicristoidea with two orders, Nucleariida and Fonticulida (Cavalier-Smith 2009). The class Filasterea possesses only one order, Ministeriida (Shalchian-Tabrizi et al. 2008). In the cladogram, the Mesomycetozoa comprises two classes: (1) the class Ichthyosporea with the orders Aphelidida, Dermocystida and Ichthyophonida, and (2) the class Corallochytrea with the order Corallochytrida. (**b**) Dendrogram of 17 fungal lineages showing consensus relationships. Phylogenetic relationships with high levels of uncertainty are indicated by *stippled lines*. The traditionally accepted node for delineating Fungi is marked, but there are current attempts to accept the term Fungi in a broader sense as indicated by 'F' (Lara et al. 2010;

inconspicuous group of microorganisms which (i) are primarily heterotrophic with an (ii) osmotrophic style of nutrition containing (iii) chitin and its derivatives in the cell wall. True fungi synthesize the amino acid lysine via α-aminoadipic acid instead of α, ε-diaminopimelic acid compared to plants, green algae, and oomycetes (Vogel 1961).

The zoosporic fungi represent the most basal, and thus most ancient group, of the fungal lineages producing chitin, as a structural component of the assimilative cell wall and converting the phagotrophic to an osmotrophic mode of nutrient uptake, throughout the main phases of their life cycle (Table 9.1). Whether or not zoospores are able to perform phagotrophic uptake of nutrients has not yet been clarified.

Therefore, the phylum referred to as "Chytridiomycota" *s.l.* as a coherent clade is employed to make clear that the term is being used in a colloquial and broader sense, for instance the inclusion of all basal lineages of aquatic chitinous fungi with the potential to form opisthokont flagellate zoospores or sharing any other of the plesiomorphic character of the re-unified clade, which may be designated to a phylum or even to a superphylum. Likewise, and in order not to contradict any other phylogenetic concepts, each of the four classes, **Blastocladiomycetes** (Doweld 2001)**, Chytridiomycetes** (Cavalier-Smith 1998)**, Neocallimastigomycetes** (Hibbett et al. 2007) **and Monoblepharidomycetes** (Schaffner 1909)**, appears to be monophyletic clades** (Fig. 9.1b). The systematics recently published by Voigt (2012) is applied, which implements the use of the term Chytridiomycota to summarize these four classes.

Of the five clades (Blastocladiomycetes, Chytridiomycetes, *Olpidium insertae sedis*, Neocallimastigomycetes, and Monoblepharidomycetes) which are described within the Opisthokonta superkingdom (Fig. 9.1a), parasites are only known in the Blastocladiomycetes, Chytridiomycetes, and *Olpidium* clade [Olpidiaceae (reviewed by Voigt 2012)]. The phylogenetic position of *Olpidium* is far from being entirely resolved. A concatenated multigene phylogeny based on maximum likelihood and Bayesian analyses of the elongation factor-2 (*Ef-2*), RNA polymerase subunits 1 and 2 (*RPB1*, *RPB2)* and actin (*act)* loci placed *Olpidium* within the terrestrial fungi, forming a monophyletic group with the taxa traditionally classified in the phylum Zygomycota (Sekimoto et al. 2011). Neither the monophyly of the *Olpidium* species and any other clades of zoosporic fungi nor the placement of *Olpidium* at the base of terrestrial fungi was supported by topology tests. Because of the lack of proper phylogenomic information, the chytridiomycetous ancestry of the *Olpidium* clade is retained, and thus *Olpidium* is classified with the Chytridiomycetes in this chapter. A completion of taxon sampling by the exploration of new zoosporic fungal taxa will largely increase the resolving power of phylogenetic analyses.

Perhaps ultimately some parasites will be discovered in the other two classes, the Monoblepharidomycetes and the Neocallimastigomycetes, which invariably encompass saprobic and symbiotic individuals respectively. The Cryptomycota (synonym: Rozellida) and the Mesomycetozoa (Ichthyosporea) are considered to be basal to the Fungi (Fig. 9.1a). The ecological roles of organisms in other supergroups (e.g., heterotrophic straminipiles and plasmodiophorids) are often similar, though entirely unrelated to the fungi and their opisthokont allies. Since many groups of phylogenetically unrelated organisms interact in the same environments, we must study organisms from a global perspective which includes morphological, phylogenetic, biogeographical, and ecological perspectives in order to fully understand function.

Fig. 9.1. (continued) Jones et al. 2011a, b; James and Berbee 2011; Capella-Gutiérrez et al. 2012; Schoch et al. 2012). References: * Group 1, ** Group 2, *** Group 3, **** Group 4, ***** Group 5 from Table 1. The term Chytridiomycota is used twice in the figure. There are two definitions: narrow definition (James et al. 2006a, b) and broad definiton (Barr 2001). Please refer to discussion in section II on the phylogeny and section II.A on phylogenetic trees and phylogenetic novelties. The term Rozellida has been superseded by Cryptomycota (Jones et al. 2011b). Both Mesomycetozoa and Ichthyosporea are in common usage

Table 9.1. Types of propagules in each of the genetic clades within the Opisthokonta supergroup based on Fig. 9.1a, b. See references in Fig. 9.1a, b

Groups	Type of propagules	Phylogenetic clades
Group 1 (*): Animals	Sometimes asexual reproduction can occur by fission. No unicellular propagules are produced	Choanoflagellata Metazoa
Group 2 (**): Animal-related organisms	Propagules are motile, posteriorly directed, uniflagellate zoospores, amoebae, or nonmotile walled endospores, phylogenetically basal to the Animalia. Chitin is found in some species of the Mesomycetozoa, but it is not a dominant part of the cell-wall structure	P. Mesomycetozoa (Cl. Dermatocystida—some some produce zoospores) and Ichthyophonida (some produce amoebae), Filasterea (O. Ministeriida)
Group 3 (***): Fungus-related organisms	Propagules are motile, posteriorly directed, uniflagellate zoospores, amoebae, or nonmotile walled endospores; static, chitinous hyphal stages do not occur or have not been observed during major stages of the life cycle. Chitin has been found in the resistant spores of Cryptomycota, phylogenetically basal to the true Fungi	Cristidiscoidea (Cl. Discicristoidea with O. Nucleariida and Fonticulida) (amoebae produce cysts) P. Microsporidia (produce walled spores) P. Cryptomycota (= Rozellida) (produce uniflagellate zoospores, chitinous resistant spores)
Group 4 (****): Zoosporic (planosporic) true Fungi	Propagules are motile, posteriorly directed, uniflagellate zoospores or nonmotile, static walled resistant sporangia (rarely amoebae or multiflagellate zoospores), chitinous hyphal stages do occur during major stages of the life cycle (rhizoids, rhizomycelium), lifestyle aquatic or aquatic habitat-dependent	P. Blastocladiomycota (Cl. Blastocladiomycetes) P. Chytridiomycota (Cl. Chytridiomycetes) P. Neocallimastigomycota (Cl. Neocallimastigomycetes) Monoblepharidomycetes Olpidium clade = Olpidiaceae *insertae sedis* (provisorily classified in the Chytridiomycetes)
Group 5 (*****): Higher (aplanosporic) true Fungi	Nonmotile walled spores (sporangiospores or conidia), chitinous hyphal stages do occur during major stages of the life cycle (rhizoids, rhizomycelium), life style terrestrial, in rare cases secondarily aquatic	P. Zygomycota P. Glomeromycota P. Ascomycota P. Basidiomycota

B. Number of Fungal Species

The number of fungal species described so far ranges between 72,000 and 120,000 (Hawksworth and Rossman 1997; Hawksworth 2001), which is less than 10 % of an estimated 1.5 million extant fungal species (Hawksworth 1991, 2001). Kirk et al. (2008) report 64,163, 31,515, 1,065, and just 724 from Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota respectively, which is a total of 97,957 fungal species. If environmental samples based on metagenomics data are taken into account, the estimated number of fungal species may be increased to 3.5 million (O'Brien et al. 2005). The progress in species recognition and description of the Ascomycota, Basidiomycota, and Glomeromycota was surveyed by Hibbett et al. (2009). The overall rate of new species description has been fairly low during the past 10 years, with an average of only 223 species per year, mostly Ascomycota. More than 90 % of the fungi remain unrecognized or unidentified, the majority of which may be found in undisturbed areas or in associations with plants, insects, and animals, or as lichen-forming fungi, particularly in the tropics (Hawksworth 2001). About 3,000 fungal species, including 576 species of chytrids, have been reported from aquatic habitats (Shearer et al. 2007).

The current number of obligate marine fungi or planktonic-derived fungi isolated from soils, sand, water or the deep sea (537 species) is a gross underestimate (Burgaud et al. 2009; Jones 2011). In accounting for these potential sources, the number of marine fungi might be in excess of 10,000 taxa (Jones 2011).

Another portion of undescribed fungi might be hidden species (cryptic species or taxa with similar morphology), which were either from environmental sources and deposited in public sequence databases without taxonomic delimitation to a species (Nagy et al. 2011), or previously considered as belonging to species already described (for an overview see Gherbawy and Voigt 2010). The discovery of zoosporic fungi or flagellate fungus-like organisms (such as the Cryptomycota) in natural habitats may easily double the size of the fungal kingdom and its allied groups (Jones et al. 2011a). Thus, the discovery of zoosporic fungi and their allies will become an increasing issue in mycology in the near future.

III. Pathosystems and Coevolution: Their Implications for Disease Development

The concept of **pathosystems** has been useful for the study of host–parasite interactions, especially in plant pathology, since the publication of *Plant Pathosystems*. Robinson (1976) defines **a plant pathosystem as a sub-division of the ecosystem which involves parasitism.** According to this definition, a pathosystem can include all hosts and all parasites in the ecosystem, but usually the pathosystem model has been applied to one population of parasites and one population of hosts. However, parasitic relationships at the ecosystem level are often more complex, i.e., one parasite may infect a range of hosts and one host may be infected by more than one species of parasite, as in the case of *Zygorhizidium planktonicum* infecting *Asterionella formosa*, and *Synedra acus,* and *A. formosa* infected by *Zygorhizidium planktonicum, Z. affluens*, and *Rhizophydium planktonicum* (Canter and Lund 1953; Canter 1969). Recently, de Souza et al. (unpublished data) modified **the concept of multiple pathosystems to include the entire range of parasite species that infect multiple species of hosts inhabiting similar ecological niches and which are interrelated through parasitism.**

Pathosystems involve populations, not individuals, and are dynamic systems (Robinson 1976). The word "system" refers to the part of the biosphere under study, and therefore depends on the scale of analysis. Conveniently the biosphere is often divided into "the system" and "the environment", which is outside the system. However, in the case of studying pathosystems at the ecosystem level, the environment should also be considered as part of the system.

As dynamic systems, pathosystems can remain stable only if they retain the balance or equilibrium which is achieved by systems controls (Robinson 1976). These controls involve interactions between the component parts of the pathosystem, which includes populations of hosts and parasites and the environment. Since pathosystems are open systems, any change in these components can result in a loss in equilibrium. In general, natural systems oscillate around a central point (steady or stationary state). Over time, the equilibrium point can shift if virulence of the pathogen, susceptibility of the host and/or environmental factors change (metastability condition).

The interaction between host and parasite populations often, but not always, results in clearly identifiable symptoms of disease. Robinson (1976) discussed the use of the disease triangle for analysis of the dynamics of pathosystems. Hosts, parasites, and the environment are placed at the corners of an equilateral triangle. The centre is labelled with **disease**. For pathosystems involving zoosporic parasites and phytoplankton hosts it is appropriate to label the centre with **prevalence** instead of disease, and also to include additional modifications to the disease triangle (Fig. 9.2). The **prevalence** of infection is defined as the proportion of live host cells infected by live parasites (Bruning et al. 1992).

Virulence can be defined as the ability of the parasite to decrease host fitness (de Bruin 2006). **Virulence factors** in parasites are thought to control the progression of a disease, while hosts are thought to have some form of

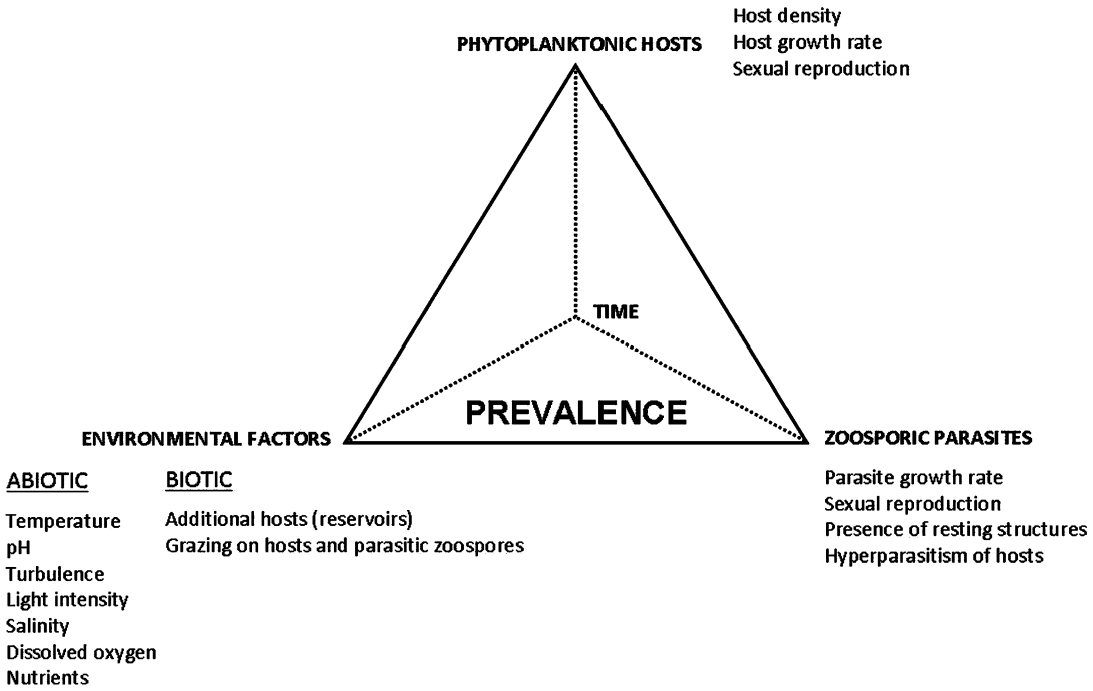

Fig. 9.2. Graphical representation of zoosporic parasite–phytoplanktonic host pathosystem based on the classical disease triangle

defense against parasitism. **Virulence and immunity are therefore opposing factors which determine the equilibrium in pathosystems at steady state.** Parasites develop mechanisms to maximize virulence, while hosts try to maximize defense. Virulence appears to evolve in response to the density of host. Short generation times and large populations generally operate to maximize virulence in parasites, while new defense mechanisms in the host might be acquired by recombination of genes in chromosomes during meiosis. This is known as the "sex against parasites" in the Red Queen hypothesis (de Bruin 2006). In the case of phytoplankton, resistence to infection, hypersensitivity responses, chemical defense, and maintenance of a high genetic diversity have been suggested as mechanisms of algal defense against parasites (Ibelings et al. 2004; Kagami et al. 2007a). Hypersensitivity response in algae operates through the rapid death of the host following the contact of parasitic zoospores with the host cell. In this way, further development of fungal zoosporangia is prevented, and therefore infection is halted. This type of response has been observed in *Asterionella formosa* parasitized by *Rhizophydium planktonicum* (Canter and Jaworski 1979). Sexual reproduction has never been observed in *A. formosa*. Therefore, sexual recombination as a mechanism of maintaining high genetic diversity and obtaining resistance against parasites does not appear to operate in *A. formosa* populations. Other mechanisms not related to sexuality are apparently involved in the great genetic variability observed in *A. formosa*. However, these mechanisms have not yet been elucidated (de Bruin 2006). Immune systems in flowering plant hosts have recently been reviewed by Spooel and Dong (2012).

Zoosporic parasites exert strong selective pressure on hosts and hosts do so on zoosporic parasites that might lead to potential **coevolution** (Sønstebø and Rohrlack 2011), in which constant changes and reciprocal adaptations are essential for coexistence of host and parasites. This process requires long-term coexistence of host and parasite species (Ibelings et al. 2003), and particularly involves evolution of host resistence and parasite infectivity (de

Bruin 2006). The main conditions for coevolution of host–parasite pathosystems are that the parasite must inflict damage to the host and depend on the host to survive and reproduce. In addition, genetic variation in parasite infectivity and host resistance must be present (de Bruin 2006). Coevolution might therefore result in changes in gene frequencies over time, and affect the genetic structure of populations through differential success of infected vs uninfected host genotypes. Therefore, the impact of frequency-dependent selection on genetic polymorphism in host and parasite populations may maintain genetic variation (the Red Queen Hypothesis; van Valen 1973). Algal host and zoosporic parasites relationships tend to be species-specific. The density of resistant hosts might increase when parasites with a narrow host range infect the dominant susceptible hosts. In some cases, host diversity might also increase because parasites force competing hosts to coexist. In other cases, the specialization of parasites might be reduced with the adoption of a generalist strategy of resources exploitation.

Sønstebø and Rohrlack (2011) observed that infection of *Planktothrix* hosts by fungal parasites was chemotype-dependent. The existence of different *Planktothrix* chemotypes and of zoosporic parasites with chemotype preferences (narrow host range) is compatible with general ecological concepts such as the 'Kill-the-winner' concept and Red Queen Hypothesis (Sønstebø and Rohrlack 2011). High specificity of zoospores for a particular host occurs during encystment of the zoospores (Holfeld 2000a), and appears to be mediated by glycoproteins at the zoospore surface (Powell 1994). *Planktothrix* species are known to release protease inhibitors, which possibly protect them from the serine proteases produced by the rhizoids of fungal parasites (Sønstebø and Rohrlack 2011).

Barr and Hickman (1967a) studied the parasitism of isolates of *Spirogyra* by *Rhizophydium sphaerocarpum* with both field- and laboratory-based research. *Rhizophydium sphaerocarpum* is a monocentric fungus with an epibiotic sessile sporangium and endobiotic rhizoids. Both the parasite and the hosts could be grown easily in pure culture. Although ecotypes of *R. sphaerocarpum* have been reported to infect a wide range of species in the genera *Spirogyra*, *Oedogonium*, and *Mougeotia*, the isolate that the authors chose infected only a very restricted range of hosts. Sixteen isolates of *Spirogyra* were tested: three were very susceptible, four were moderately susceptible, and nine were immune to infection. Parasitism by zoosporic fungi on a wide variety of planktonic hosts were extensively studied by Sen (1987a, b, c; 1988a, b).

IV. Abiotic Factors

A. Physical and Chemical Factors Affecting the Dynamics of Host–Parasite Relationships

In natural ecosystems, the environment in which parasites and hosts live is never completely homogeneous; rather, it consists of a complex set of gradients in time (temporal scales) and in space (spacial scales) that need to be defined at each scale of analysis. The ranges within which each physical factor permits growth and survival of parasites and hosts and their interactions can be different. For facultative parasites, these ranges can be measured separately for the parasite, the host, and the interaction in the laboratory. For biotrophic parasites, the parasite can only be grown with the host, so that the ranges for the parasite and the interactions cannot be determined separately.

Studies on the effect of physical factors on a few of the host–parasite relationships have been conducted with monocentric parasites and unicellular hosts. Techniques for measuring population parameters in zoosporic true fungi have recently been reviewed by Marano et al. (2012). One of the best measurements of the degree of infection is *prevalence*.

Important physical and chemical factors influencing the prevalence of infection include temperature, pH, turbulence, salinity, dissolved oxygen, and nutrient concentration (Fig. 9.2). If changes in the range of these and other factors present in an ecosystem occur with environmental deterioration and climate change, we would expect the equilibrium between parasite and host populations in the pathosystem to

shift. If growth of the parasite is favoured, we would expect the population sizes of the host to decrease. With extreme changes in the range of physical and chemical factors, is there a theoretical threshold (minimum host population size) for survival, or do both the host and the parasite become extinct?

Fisher et al. (2012) proposed a mathematical model for interspecies dynamics involving fungal parasites. These dynamics might lead to a significant loss of biodiversity in some situations, and even extinction in extreme cases. Several special features of parasites contribute to increase the emergence of infectious disease. (1) Parasites with high reproductive potentials and high virulence (prevalence of infection) can quickly infect and kill all individuals in a large host population. These parasites use an **r survival strategy**. (2) A theoretical threshold (minimum) host population size necessary for survival does not always prevent extinction. (3) Parasites with long-lived infection stages have an increased potential to cause loss in diversity and sometimes extinction. Some of these parasites have resting stages which are tolerant of extreme environmental conditions, they can live as saprotrophs on detritus or during growth they can tolerate a wide range of environmental conditions (**s survival strategy**). (4) The host range is broad, and includes a range of both susceptible and tolerant species in the host population or they can parasitize alternative host species. In the study of epidemiology, it is important to define host specificity including primary and alternative hosts and reservoirs. We have added one more characteristic to this model. (5) The infectious stages of parasites have mechanisms for rapid and efficient dispersal, such as by chemotactic zoospores. Some zoosporic true fungi discussed in the present review have characteristics which fit this model. Some of these characteristics have been discussed further by Gleason et al. (2011) and Gleason and Marano (2011).

Many factors that govern the blooming of the algal host are also involved in the germination of resting spores of their zoosporic parasites. Periods of increased lake turbulence appear to be related to fungal epidemics (Doggett and Porter 1996). Epidemics are more likely to occur if conditions are particularly unfavourable for the host (Kagami and Urabe 2002). However, the effects of turbulence can be twofold. On the one hand, it can resuspend fungal resting structures from the sediments which might germinate if conditions are appropriate. On the other, turbulence might diminish the amount of incident light in the euphotic zone, which is unfavourable for growth of both parasite and host (de Bruin 2006). In addition, it might interfere in the gradients of extracellular substances released by the algae during photosynthesis which act as attractants in chemotaxis. Therefore, it can be more difficult for zoospores to detect the algal host (Kühn and Hofmann 1999), and epidemics fail to develop.

Barr and Hickman (1967b) selected three isolates of *Spyrogyra* for testing the effects of temperature, pH, and light intensity on parasitism by *Rhizophydium sphaerocarpum*. The optimum temperature for growth of the parasite was 30 °C. One of the hosts could not grow at this temperature. Some of the most resistant host species could grow at higher temperatures than the parasite. Some of the hosts grew over a wider pH than the parasite. Using growth rates and infection rates as dependent variables and the three physical factors as independent variables, the authors documented differences in responses of the parasite, the hosts, and the interaction (parasitism) to these physical factors. This is an excellent model for the study of parasitism, but the results were preliminary and much more research is needed.

Bruning (1991a, b, c, d) and Bruning et al. (1992) developed a mathematical model for studying the *Rhizophydium planktonicum–Asterionella formosa* pathosystem during two epidemics in Lake Maarsseveen. The parasite, *R. planktonicum*, is a monocentric, epibiotic, and biotrophic zoosporic fungus. Infection always results in the death of the host cell. The host, *A. formosa*, is a colonial, pennate diatom which is commonly found in many freshwater ecosystems. The rhizoids of the parasite penetrate the host cell through the girdle lamellae between the epitheca and the hypotheca which compose the silica skeleton (Beakes et al. 1993).

Many important parameters including prevalence, which was outlined previously in this chapter, were carefully characterized by Bruning et al. (1992). The **development time of the parasite** is defined as the elapsed time between the beginning of infection by a zoospore and the release of zoospores from the sporangia of the parasite (sporulation). Some

Table 9.2. Some of the growth parameters in the Bruning et al. (1992) model

Parameter	Impact on host/parasite	Effect of environmental factors:		
		Phosphate limitation	Temperature	Light
Specific growth rate of uninfected host	Host	+	+	+
Specific growth rate of parasite	Both	+	+	+
Rate of zoospore production	Parasite	+	+	+
Infectivity constant	Host	n.d.	n.d.	n.d.
Development time of sporangium	Parasite	Slight	+	−
Number of zoospores/sporangium	Parasite	+	+	+
Search time for zoospores	Parasite	+	n.d.	n.d.
Infective lifetime of zoospores	Parasite	n.d.	+	−
Specific rate of loss due to infection	Host	n.d.	n.d.	n.d.
Prevalence of infection	Both	+	n.d.	n.d.
Critical prevalence	Both	n.d.	n.d.	n.d.

n.d. not determined

physiological activities of the host cell, such as photosynthesis, continue for some time after the moment of infection, and are necessary for zoospore production. The **critical prevalence value** is defined as the maximum prevalence of infection tolerated by host populations just prior to the point when the loss rates due to infection and other factors exceed the growth rates of uninfected cells, and the host population begin to decline.

In Bruning's model, the dependent variable was prevalence (Bruning 1991a, b, c, d). The first independent variable was time of incubation. Bruning generated a series of growth curves from his data. The second independent variable was the presence or absence of parasites. He measured the rate of growth of host cells without infection and the rate of loss of uninfected cells with infection. All other variables were held constant, at a temperature of 6 °C and in a 15.9 h light/dark cycle with constant light intensity during the light phase and without turbulence.

Later, other independent environmental factors were added to the model: temperature, light intensity, and limitation of phosphate. Five temperatures (2 °C, 6 °C, 11 °C, 16 °C and 21 °C) and six light intensities were tested. The specific growth rate of the host is affected by all three variables, and potentially by other variables such as silicon limitation, pH, salinity, oxygen tension, etc. which are not included in Bruning's model. Low light intensity is known to depress infectivity of zoospores of many parasitic species. Silicon is required for the synthesis of the silica skeleton of diatoms.

The number of uninfected host cells in the infected population increases only by the reproduction of uninfected cells and decreases due to the infection process and to factors other than parasitism (e.g., grazing, sedimentation). The rate of loss of uninfected cells is dependent on parameters which affect the growth rate of the parasite. The specific growth rate of *R. planktonicum* is a function of: (1) host density (specific growth rate of the host), (2) the number of zoospores per mature sporangium, (3) the development time of the sporangia, (4) the infective lifetime of the zoospores, and (5) the infectivity constant *i* (efficiency mechanism that enables zoospores to find and infect host cells).

For an epidemic to develop the specific growth rate of the parasite must exceed the specific growth rate of the host above the threshold value for epidemic development. Once the model was developed, Bruning et al. (1992) tested the effects of physical factors on the interactions between the parasite and the host (Table 9.2).

Ibelings et al. (2011) examined the dynamics of host–parasite interactions between *Asterionella formosa* (the host) and two parasites, *Rhizophydium planktonicum* and *Zygorhizidium planktonicum*, in Lake Maarsseveen over a 30-year period. During cold winters with temperatures frequently below 3 °C, these parasites do not infect the host, but during warm winters with temperatures mostly above 3 °C, the parasites reduce the size of the host population prior to the spring bloom.

Fernández et al. (2012) studied the *Rhizophydium couchii-Closterium aciculare* pathosystem in Paso de las Piedras Reservoir in Argentina. In the research design, population density of the hosts and prevalence of infection were the dependent variables. Time of year was the independent variable. Water temperature, solar radiation, phosphate concentration, nitrogen concentration, and P/N ratio were measured as a function of time of year, and then correlated with population density and prevalence. The authors documented major

changes in water temperature and solar radiation during the year. There was a significant positive correlation between the density of *C. aciculare* and water temperature and solar radiation, as well as a highly significant negative correlation between the prevalence of infection and water temperature and solar radiation. The infection correlated with low light intensity and temperature, conditions that also affected negatively the growth of *C. aciculare*, but to a lesser extent. The growth of the algal host population was not limited by either nitrogen or phosphate in this ecosystem.

B. Environmental Gradients

Zoosporic true fungi are normally characterized as freshwater, estuarine, or marine. Yet there is no clear boundary between these arbitrary categories. Microbial species live within a range of salinities along environmental gradients. Most species of zoosporic true fungi are found exclusively in soil or freshwater ecosystems (Sparrow 1960; Powell 1993; Marano et al. 2011). Very few species of zoosporic true fungi are estuarine or marine (Gleason et al. 2011). Logares et al. (2009) and Heger et al. (2010) have recently proposed that transitions of eukaryotic microorganisms between marine and freshwater ecosystems are rare on an evolutionary scale. Sharp differentiation into freshwater and marine forms is one of the most distinctive features of biodiversity in most groups of organisms (Logares et al. 2009; Heger et al. 2010).

Gradients in osmotic pressure and ion concentrations are among the most important barriers in preventing migration between freshwater and marine ecosystems. Many zoosporic true fungi have specialized mechanisms which allow them to adapt to gradual changes in salinity, but these may not be sufficient for large, sudden, or permanent osmotic changes. Therefore, well-defined changes in the composition of communities along a salinity gradient would be expected (Logares et al. 2009).

Temperature, light and oxygen gradients also determine the location (range) of populations within the ecosystem in three-dimensional space. The composition of communities will change over time, and must be monitored at both micro and macro scales. Large bodies of water such as freshwater lakes are known to have steep gradients. For example, temperature, light, and oxygen gradients extend vertically from the top surface to the bottom. The producers must remain near the surface in order to use high-intensity light for photosynthesis. Most producers have adaptations to bring them to the surface. Both heat and oxygen diffuse downward slowly in lakes when there is no mixing. Oxygen is removed by community respiration.

Canter et al. (1992) discussed an interesting adaptation to gradients in Lake Windermere (English Lake District). Cyanobacteria have gas vacuoles which bring their cells to the surface. Canter (1972) described a variety of common zoosporic true fungal parasites of cyanobacteria, for example species of *Chytridium, Rhizidium, Rhizophydium*, and *Rhizosiphon*. These are attached to the host cells. In addition, large numbers of various species of Vorticellids can be attached to the surface of the host cells. Vorticellids do not feed on large cyanobacteria, but can feed on the small bacteria and zoospores in the water near the cyanobacterial colonies. Vorticellids are motile, and move the host colonies, such as *Anabaena*, around with their cilia, making them appear to swim. The gas vacuoles therefore are capable of bringing the host cells along with the attached microorganisms to the surface. Most fungi and ciliates are aerobic, and grow faster at the surface and at higher temperatures. Larger ciliates and other protozoa and metazoa graze on the floating communities. **A complex food web** results. If the host cyanobacteria were allowed to sink into deep water with reduced dissolved oxygen concentrations, the communities would not grow well.

V. Biotic Factors

A. Trophic Modes and Symbiotic Relationships

The primary mode of nutrition is one determining factor for the ecological placement of microorganisms according to function. Most heterotrophic microorganisms are currently assigned to the arbitrary categories according to their primary modes of nutrition, and many

species have an intimate and often interdependent relationship (symbiosis) with other species because of nutrition.

However recent research on the interactions between organisms has revealed that **symbiotic relationships are frequently not exclusively saprotrophic, mutualistic, or parasitic, but are frequently a mixture of trophic modes depending on environmental factors** (McCreadie et al. 2011). The nature and magnitude of interspecific interactions may vary through time and space, because this relationship is fluid in nature. The degree of parasitism is determined by the virulence of the parasite and the resistance of the host, and by environmental factors. Some virulent parasites control the population sizes of their host. The precise nature of these interactions and hence the ecological functions of the species involved, can only be understood with intensive metagenomic (e.g., transcriptomic profiles, monitoring gene expression levels), physiological (e.g., monitoring of nutrient uptake, metabolic products), and morphological investigations.

Parasites can roughly be divided into three groups: biotrophic, necrotrophic, and facultative parasites (Gleason et al. 2010a).

Biotrophic parasites depend on a living host for their growth and propagation. Obligate (biotrophic) parasites are never capable of growth without a host. These organisms are therefore host-dependent, where infectivity is limited to a narrow host range. They are also host-specific.

Necrotrophic parasites first kill living host cells by releasing toxins which stimulate apoptosis of the host cells. Digestive enzymes are then excreted by the parasite to consume the host tissue. They can grow well either as saprotrophs or parasites, and these organisms often have a wide host range.

Facultative parasites are usually non-pathogenic in the natural environment and survive with the saprotrophic mode of nutrition, but can become pathogenic when unfavourable conditions occur for the host (stressful environment) or when the hosts become immunocompromised.

B. Ecological Roles of Zoosporic Parasites

1. Maintenance of Genetic Polymorphism and Increase in Biodiversity of Hosts

Parasitism is one of the most significant agents of natural selection. There is selection pressure on hosts for resistance to parasites and equally on parasites to overcome host defenses. This might alter host gene frequencies and reciprocally determine the fitness of genotypes of the parasite. As a consequence of selective parasitism, the infected host populations and the parasites might become genetically heterogeneous, and potentially result in the acquisition of new defence mechanisms by the host against fast-evolving parasites and parasite weaponry (coevolution). In this sense, exposure to narrow host-range parasites might ultimately select for an increase in biodiversity (Sønstebø and Rohrlack 2011).

2. Control of Sizes of Host Populations

Fungal parasitism could be a particularly important factor in population dynamics of phytoplankton, especially with species which are resistant to grazing such as large diatoms and inedible cyanobacteria (Sommer 1987; Sen 1987b, c; 1988a). The development of an epidemic depends on the relationship between growth rates of host and parasite populations (Kagami et al. 2007a). Therefore, an epidemic will occur only if the parasite grows faster than the host (van Donk and Bruning 1992). Factors that limit algal growth (unfavourable conditions of light and temperature among others) or favour fungal growth (high host density) will contribute to this phenomenon (Gsell et al. 2012).

The conditions for survival of the parasite and development of an epidemic were studied by Bruning et al. (1992). There are two threshold (minimum) values for host density. The first is the **threshold value for survival of the parasite**. This is defined as the host density below which the parasite is unable to maintain its population size. The second is the **threshold value for epidemic development**. This is defined as the host density below which an epidemic cannot develop. These values provide criteria for survival or epidemic development. There are **three phases of development of the parasite along the host density gradient: extinction, persistence, and epidemic.**

During epidemics, host populations are often highly reduced. The appearance of algal blooms can be delayed, or the dimension of blooms can be minimized by mortality of hosts via epidemic parasitism. Infection by zoosporic fungi reduces host growth and reproduction and in most cases, kills the host cell (Canter and Lund 1951). However, part of the host population is able to escape from zoosporic epidemics in refuges (e.g., lake sediments). Turbulence might resuspend algal resting stages (as in the case of fungal resting spores), which in turn can restock populations when conditions are appropiate. This behaviour has been observed for *A. formosa* and its parasite *Z. planktonicum* (de Bruin 2006).

Rhizophydium couchii is a common parasite of the freshwater desmid *Closterium aciculare* (Fernández et al. 2012). The population densities of the parasite and the host changed with the season. Prevalence was determined by measuring the presence of encysted zoospores or sporangia on the surface of the host cells. The highest abundance of *C. aciculare* occurred in summer, and it remained high until the beginning of winter. The prevalence of infection was very low until April, then increased from fall to winter, at which time there was a sharp decline in the density of *C. aciculare*. There was a highly significant negative correlation between the density of *C. aciculare* and the prevalence of infection during this decline. In conclusion, the parasitism by *R. couchii* played an important role in the dynamics of the host population.

3. Regulation of Phytoplankton Succession

Most studies on phytoplankton have only considered the relationship of environmental parameters and herbivory to changes in phytoplankton succession. Parasitism has been traditionally neglected as a regulation factor of phytoplankton dynamics. Even so, parasitism by zoosporic fungi can be an important factor controlling seasonal succession of phytoplankton. Zoosporic fungi appear to more commonly infect algae that are resistant to grazing by zooplankton (Sommer 1987). Infection of one algal species by zoosporic parasites (selective parasitism) may favour the development of other algal species influencing seasonal succession. The consequences of this can operate directly (species eliminated by host-specific parasites are replaced by ecologically similar species according to species inoculum availability and growth rate) or indirectly as a result of **interspecific competition.**

Canter and Lund (1951) observed that infected populations of *Asterionella formosa* were replaced by *Fragilaria crotonensis* and *Tabellaria fenestrata*. Parasitism is particularly relevant in the case of algae that do not dominate the phytoplankton, such as desmids whose abundance is significantly reduced as a result of fungal infection. For example, parasitism of *A. formosa* by *Zygorhizidium planktonicum* favors the increase in the number of cells of *Stephanodiscus atraea*, a typically subdominant species in the phytoplankton assemblage (Reynolds 1973; Youngman et al. 1976). Van Donk and Ringelberg (1983) also observed that infection of *A. formosa* resulted in an increase in the dominance of *Fragilaria crotonensis*, *S. hantzschii*, and *S. astraea*.

4. Control of Parasite Populations Through Hyperparasitism

The zoosporic parasite itself can be infected by other species of zoosporic fungi, as in the case of *Rozella parva* which has been frequently observed infecting the algal parasite *Zygorhizidium affluens* (Canter 1969). As previously shown, parasites play major roles in regulating the sizes and compositions of phytoplankton assemblages, and if hyperparasites regulate the sizes of parasite populations, then interaction between parasites and hyperparasites might add even more complexity to food webs. **Hyperparasitism can therefore control the population size of algal parasites and indirectly stimulate growth of algal blooms.** The magnitude of the impact of hyperparasitism in the dynamics of food webs is yet unknown.

5. Increase in Susceptibility of Infected Algae to Grazing

Parasitism by zoosporic true fungi is not restricted to weakened or moribund algae, but impacts healthy, actively dividing algal cells (de Bruin 2006). Holfeld (2000b) reported that the quality of the food available for zooplankters decreased during an epidemic. Potentially, the susceptibility of edible and infected phytoplanktonic algae to grazing might also be altered. However, there is no evidence supporting this hypothesis.

6. Trophic Upgrading

Free-living zoospores of parasites provide high quality food resources for zooplanktonic grazers, especially when inedible diatoms and cyanobacteria are the dominant species in the phytoplankton during spring or summer blooms. These species are either protected by silica cell walls or are too large to be eaten by grazers. Because free-living zoospores contain large reserves of lipids which are essential nutrients such as cholesterol and polyunsaturated fatty acids (PUFAs), they represent one of the most important food resources for grazers such as *Daphnia* (Kagami et al. 2004, 2007b, 2012). The sources of zoospores might be either saprotrophic or parasitic chytrids. **When predators eat zoospores of parasites, the severity of infection of the hosts might be reduced.** This has been documented for *Batrachochytrium dendrobatidis,* a serious pathogen of amphibia (Buck et al. 2011). It has also been well-documented that the species of *Daphnia* can reduce the rate of infection of phytoplankton, and prevent the transmission of chytridiomycosis by grazing zoospores (Kagami et al. 2004; Buck et al. 2011). In general, the growth of grazers is facilitated by the consumption of zoospores because of their high nutritional quality (Kagami et al. 2012). Since fungal zoospores can be abundant in water, they can represent an important flow of carbon from inedible phytoplankton to zooplankters. Recently, Grami et al. (2011) showed that the inclusion of zoosporic true fungi in food webs greatly impacted the channeling of carbon from primary producers to grazers via "mycoloop". In general, phytoplankton sinks to the bed of a body of water without being grazed by zooplankton. Phytoplankton infected by parasitic zoosporic fungi, however, is consumed by the chytrids before sinking so that the chytrids are partially eaten by zooplankton and enter the food web. Thus, the phytoplankton is indirectly consumed by zooplankton via chytrid zoospores. This pathway is named 'mycoloop' (Kagami 2012). In addition, loss of carbon because of sedimentation of large inedible diatoms is substantially reduced when zoosporic parasites are included in food webs. The importance of carbon fluxes mediated by zoosporic parasites, particularly in pelagic food webs of lentic habitats, should therefore not be neglected.

7. Addition of Trophic Levels, Branching and Length of Food Chains

In general, it has been postulated that parasites increase species richness, trophic levels, connectance, and the length of food webs. This topic has been reviewed in detail by Gleason et al. (2008, 2011, 2012a), Sime-Ngando et al. (2011) and Lafferty et al. (2008).

VI. Economical Relevance of Parasitism by Zoosporic Fungi

Many genera of zoosporic true fungi are known as parasites of algae, plants, animals, and other fungi (Table 9.3). However, a few genera, which are discussed below, have recognized economical relevance.

1. *Synchytrium*

Several species of *Synchytrium* are known to be significant parasites of flowering plants. For example, *S. endobioticum* is the causative agent of wart disease of potato (Hampson et al. 1997). This holocarpic, endobiotic pathogen produces galls on stem tissues of *Solanum tuberosum*. Since this fungus is a biotroph, it must be grown in dual culture with potato plants. Cycloheximide and benomyl inhibit the release of zoospores, and these fungicides have been tested for chemical control of wart disease (Gleason and Marano 2011). However, resting

Table 9.3. Parasitic genera of the zoosporic fungi and their principal hosts, using the classification system in current usage based on Sparrow (1960) and Karling (1977) supplemented by the genera listed in section IV

Parasite genus	Taxonomic affiliation [Phylum–Order–Family]	Example species	Host phylum or subphylum	Reference
1. Parasites of algae, straminopiles and plants				
Achlyogeton	Chytridiomycetes–Chytridiales–Sirolpidiaceae	*A. entophytum*	Chlorophyta (*Cladophora*)	Karling (1977)
Blyttiomyces	Chytridiomycetes–Chytridiales–Chytridiaceae	*B. spinosus* *B. vaucheriae*	Chlorophyta (*Spirogyra*) Xanthophyta (*Vaucheria*)	Blackwell et al. (2011)
Canteria	Chytridiomycetes–Chytridiales–Endochytriaceae	*C. apophysata*	Chlorophyta (*Mougeotia*)	Karling (1977)
Coralliochytrium	Chytridiomycetes–Chytridiales–*Insertae sedis*	*C. scherffellii*	Chlorophyta (*Zygnema)*	Karling (1977)
Chytridium	Chytridiomycetes–Chytridiales–Chytridiaceae	*C. polysiphoniae*	Phaeophyta, Rhodophyta	Müller et al. (1999) Gleason et al. (2011)
Diplochytridium	Chytridiomycetes–Chytridiales–Chytridiaceae	*D. aggregatum, D. gibbosum*	Chlorophyta (*Oedogonium, Cladophora*)	Karling (1977)
Endocoenobium	Chytridiomycetes–Chytridiales–Chytridiaceae	*E. eudorinae*	Chlorophyta (*Eudorina)*	Karling (1977)
Endodesmidium	Chytridiomycetes–Chytridiales–Synchytriaceae	*E. formosum*	Chlorophyta	Karling (1977)
Entophlyctis	Chytridiomycetes–Chytridiales–Endochytriaceae	*E. bulbigera* *E. apiculata*	Chlorophyta (*Spirogyra*) *Chlamydomonas*	Karling (1977) Shin et al. (2001)
Loborhiza	Chytridiomycetes–Chytridiales–Chytridiaceae	*L. metzneri*	Chlorophyta (*Volvox*)	Karling (1977)
Micromycopsis	Chytridiomycetes–Chytridiales–Synchytriaceae	*M. intermedia*	Chlorophyta (e.g. *Zygnema, Netrium)*	Karling (1977)
Olpidium	Chytridiomycetes–*Incertae sedis*–Olpidiaceae	*O. brassicae* *O. utriculiforme*	Brassicaceae (e.g. lettuce) Chlorophyta (*Cosmarium*)	Karling (1977)
Paraphysoderma	Blastocladiomycetes–Blastocladiales–Physodermataceae	*P. sedebokerensis*	Chlorophyta (*Haematococcus pluvialis*)	Gutman et al. (2009)
Plasmophagus	Chytridiomycetes–Chytridiales–*Insertae sedis*	*P. oedogoniorum*, *P. coleochaetes*	Chlorophyta (*Tribonema*, *Oedogonium, Coleochaete*)	Karling (1977)
Phlyctochytrium	Chytridiomycetes–Chytridiales–Chytridiaceae	*P. hydrodictyi, P. bullatum, P. dentiferum, P. planicorne*	Chlorophyta (*Hydrodiction, Oedogonium, Cladophora*)	Karling (1977)
Podochytrium	Chytridiomycetes–Chytridiales–Chytridiaceae	*P. lanceolatum*	Chrysophyta (*Melosira*)	Karling (1977)
Physoderma	Blastocladiomycetes–Blastocladiales–Physodermataceae	*P. maydis, P. leproides, P. alfalfae*	Corn, Beet, Lucerne and clover	Sparrow 1960 Lange and Olson (1980)

Polyphagus	Chytridiomycetes–Chytridiales–Chytridiaceae	*P. asymmetricus, P. starrii*	Xanthophyta (*Botrydiopsis*), Chlorophyta (Volvocales)	Karling (1977)
Rhizidium	Chytridiomycetes–Chytridiales–Chytridiaceae	*R. braunii, R. variabile*	Chrysophyta (*Pinnularia*), Chlorophyta (*Spirogyra*)	Karling (1977)
Rhizophydium	Chytridiomycetes–Rhizophydiales–Rhizophydiaceae	*R. graminis*	Roots of Poales, Zea mays, Solanaceae and other vegetable crops	Ledingham (1936), McFarlane (1970), Barr (1973)
Septolpidium	Chytridiomycetes–hytridiales–*Insertae sedis*	*S. lineare*	Chrysophyta (*Synedra*)	Karling (1977)
Solutoparies	Chytridiomycetes–Chytridiales–Chytridiaceae	*S. pythii*	Oophyta–'Oomycota' (*Pythium*)	Karling (1977)
Sparrowia	Chytridiomycetes–Chytridiales–Chytridiaceae	*S. parasitica*	Oophyta–'Oomycota' (*Pythium*)	Karling (1977)
Synchytrium (syn. *Chrysophlyctis*, *Pycnochytrium*, *Woroninella*)	Chytridiomycetes–Chytridiales–Synchytriaceae	*S. desmodii* *S. endobioticum* *S. solstitiale*	Legumes (*Desmodium ovaliforlium*) Solanaceae (*Solanum tuberosum*) *Centaurea solstitialis*	Lenné (1985) Hampson et al. (1997) Bruckart et al. (2011)
Zygorhizidium	Chytridiomycetes–Chytridiales–Chytridiaceae	*Z. affluens*	Bacillariophyta (*Asterionella*)	Sen (1987a), Kagami et al. (2007b)
2. Parasites of animals				
Batrachochytrium	Chytridiomycetes–Rhizophydiales–*Insertae sedis*	*B. dendrobatidis*	Amphibia	Longcore et al. (1999), Fisher et al. (2009)
Catenaria	Blastocladiomycetes–Blastocladiales–Catenariaceae	*C. uncinata*	Nematodes and midges	Singh et al. (2007), Martin (1981)
Coelomomyces	Blastocladiomycetes–Blastocladiales–Coelomycetaceae		Diptera Crustacea (planktonic)	Federici and Lucarotti (1986) Whisler et al. (2009)
Olpidium	Chytridiomycetes–*Incertae sedis*–Olpidiaceae	*O. vermicola* *O. rotiferum*	Nematodes and their eggs Rotifers	Karling (1977)
Polycaryum	Chytridiomycetes–*Incertae sedis*	*P. leave*	*Daphnia* and other cladocerans	Johnson et al. (2006, 2009)
Sorochytrium	Blastocladiomycetes–Blastocladiales–Sorochytriaceae	*S. milnesiophthora*	Tardigrades (e.g., *Milnesium*)	Joines (1984)
3. Parasites of fungi				
Blyttiomyces	Chytridiomycetes–Chytridiales–Chytridiaceae	*B. rhizophyctidis*	Chytridiomycota (*Rhizophlyctis*)	Karling (1977)
Caulochytrium	Chytridiomycetes–Spizellomycetales–Caulochytriaceae	*C. gloeosporii* *C. protostelioides*	Ascomycota (*Gloeosporium)* Ascomycota (*Cladosporium)*	Karling (1977) Olive (1980)
Mastigochytrium	Chytridiomycetes–Chytridiales–Chytridiaceae	*M. saccardiae*	Ascomycota (*Saccardia*)	Karling (1977)

continued

Table 9.3. (continued)

Parasite genus	Taxonomic affiliation [Phylum–Order–Family]	Example species	Host phylum or subphylum	Reference
Olpidium	Chytridiomycetes–*Incertae sedis*–Olpidiaceae	*O. uredinis* *O. synchytrii*	Pucciniomycotina Chytridiomycota (*Synchytrium*)	Karling (1977)
Septosperma	Chytridiomycetes–Chytridiales–Chytridiaceae	*S. rhizophydii, S. anomala*	Chytridiomycota	Karling (1977)
Spizellomyces	Chytridiomycetes–Spizellomycetales–Spizellomycetaceae	*S. punctatus*	Glomeromycota	Paulitz and Menge (1984)
4. Parasites on protists				
Olpidium	Chytridiomycetes–*Incertae sedis*–Olpidiaceae	*O. vampyrellae*	Amoebozoa (e.g., filose cercozoid amoebae)	Karling (1977)
5. Parasites on bacteria				
Rhizosiphon	Chytridiomycetes–Chytridiales–*Insertae sedis*	*R. anabaenae*	Cyanobacteria (*Anabaena*)	Karling (1977)
Rhizidium	Chytridiomycetes–Chytridiales–Chytridiaceae	*R. microcystidis*	Cyanobacteria (*Microcystis aeruginosa*)	Sen (1988a)

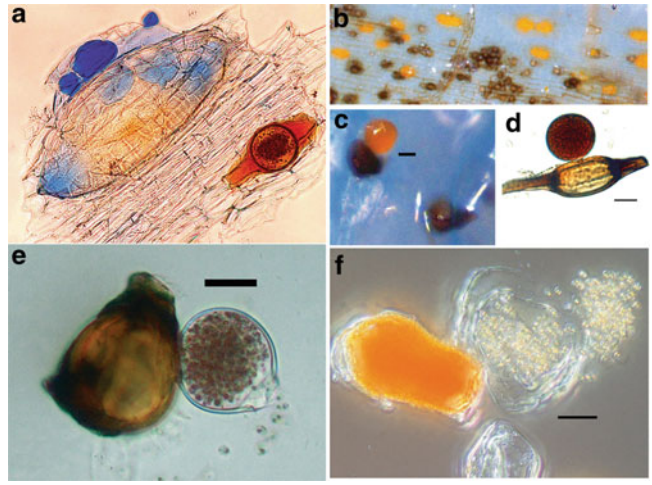

Fig. 9.3. Lightmicroscopical images from *Synchytrium solstitiale*. (**a**) A single sporangial gall (*upper left*) with sori (stained with *aniline blue*) and a single resting spore gall (rs, *lower right*). (**b**) *Orange* sporangial galls and *brown* resting spore galls in an epidermal strip of *yellow* starthistle leaf tissue. (**c**) A resting spore with an *orange* sorus (*arrow*). Bar = 20 μm. (**d**) Resting spore with a sorus that has transformed into a single sporangium (*arrow*). Bar = 20 μm. (**e**) Resting spore with sporangium and zoospore (z) release via pore. Bar = 20 μm. (**f**) Two sporangia from a sporangial gall, one *orange* and immature (*left*), and the other hyaline and ruptured, with released zoospores (z). Bar = 20 μm (Reprint with permission from: Mycologia, 103(4), 2011, pp. 775–778. DOI: 10.3852/10-286 2011 by The Mycological Society of America, Lawrence, KS 66044–8897)

spores are known to remain viable in the soil for many years (Hampson 1980; Laidlaw 1985). Another species, *Synchytrium desmodii*, causes wart disease in the tropical pasteur legume *Desmodium ovaliforlium* in Colombia (Lenné 1985).

S. solstitiale is the causative agent of false rust of yellow star thistle. The fungus is endobiotic and holocarpic, and forms sporangial and resting spore galls with sori-releasing zoospores (Fig. 9.3). This pathogen is also a biotroph (Bruckart et al. 2011). The host, *Centaurea solstitialis*, is an important weed pest in western USA, and the parasite, *S. solstitiale*, has been considered for use in biological control of this weed. The resting spores are known to survive for more than two years on dried leaves. In France, *Centaurea solstialis* has also been observed to be the host for *S. solstitiale* (Widmer and Guermanche 2006). Two kinds of galls are produced by this fungus on star thistle, sporangial galls and resting spore galls. The orange sporangial galls are multicellular and contain multiple sori. Each of the sori produce multiple sporangia (5–25). Zoospores are released when the sporangia split. The brown resting spore galls contain a single sorus with a single sporangium. The zoospores are released through a pore. The morphology of the sporangia is an important character in classification of species of *Synchytrium*.

2. *Rhizophydium graminis*

Rhizophydium graminis was originally described as a parasite of wheat roots (Ledingham 1936). It has also been observed growing on roots of barley, several other species of grasses, corn, tomatoes, and other vegetable crops (McFarlane 1970; Barr 1973). No obvious damage to infected root systems has been evident. Resistant structures of this fungus survived for nine years in air-dried soil stored in a greenhouse. Although possibly ubiquitous on roots of flowering plants, this fungus has rarely been studied.

3. *Chytridium polysiphoniae*

Chytridium polysiphoniae is a common biotroph of many species of brown and red

algae in marine coastal ecosystems (Müller et al. 1999; Gleason et al. 2011). This parasite causes epidemics, especially in populations of filamentous brown algae along the coasts of Northern Europe. Nothing is known about the genetic diversity within this species. This parasite can substantially reduce productivity of some populations of brown algae. Although this parasite can also infect red algae, the disease symptoms have not been studied. But it is possible that this parasite may become a problem for the mariculture industry.

4. *Batrachochytrium dendrobatidis*

The only documented case of a zoosporic fungus parasitizing vertebrates is *Batrachochytrium dendrobatidis*, one of the most prominent species of the Rhizophydiales (Chytridiomycetes). This species is a highly destructive parasite causing chytridiomycosis in many species of amphibians (frogs and salamanders) (Longcore et al. 1999; Fisher et al. 2009). The pathogen infects over 350 species of amphibians, and is found on all continents except Antarctica, and therefore is thought to have caused considerable decline in some populations of amphibians worldwide (Fisher et al. 2009). Goka et al. (2009) found considerable genetic diversity among the isolates of this fungus. The pathogen load (the number of zoospores present) is positively correlated with the incidence of the disease (Voyles et al. 2012). No resistant thick-walled sporangia have ever been observed in any of the isolates studied, but the populations of parasites can be maintained in long-lived tadpoles, which are infected but which show no symptoms of disease (Briggs et al. 2010). The epidermis of healthy amphibians is known to regulate osmotic balance. Both laboratory and field studies indicate that during infection, electrolyte balance across the epidermis in adults is inhibited, and plasma electrolyte concentrations (particularly potassium, sodium, and choride) are reduced (Voyles et al. 2009, 2012). Asystolic cardiac arrest follows, and results in the death of post-metamorphic frogs.

5. Parasites of Phytoplankton

Many phytoplankton species are susceptible to infection by zoosporic parasites. Holfeld (2000a) and Kagami et al. (2007a) provide a long list of hosts and their parasites. These relationships tend to be species-specific. *Rhizidium microcystidis* is a highly specific parasite of the nuisance planktonic cyanobacterium *Microcystis aeruginosa* (Sen 1988a), and *Entophlyctis apiculata* appears to be restricted to the genus *Chlamydomonas* (Shin et al. 2001). Many other examples of these parasites have been discussed previously in this review or are listed in Table 9.3.

6. *Physoderma* and *Paraphysoderma*

Several species of *Physoderma* are common pathogens which cause diseases in important species of flowering plants used in agriculture, such as brown spot of corn and beet tumor caused by *Physoderma maydis* and *P. leproides* respectively (Sparrow 1960; Lange and Olson 1980). Another species, *P. alfalfa*, causes the crownwart disease in lucerne and clover. Although these pathogens have often been listed recently in agricultural publications, there has been very little research on the diseases which they cause. Another species is parasitic on the aquatic sedge *Dulichium* (Johns 1966). However, a closely related obligate parasite of *Haematococcus pluvialis* (Chlorophyta), *Paraphysoderma sedebokerensis,* has been studied in the laboratory (Gutman et al. 2009). The motile propagule of this parasite is an amoeba without flagella. This parasite significantly reduces population densities of its host in cultures in the laboratory and in commercial facilities for production of *H. pluvialis*.

7. *Coelomomyces*

The ecology of *Coelomomyces* has been reviewed by Whisler (1985) and more recently by Gleason et al. (2011). The species in this genus are all obligate parasites (biotrophs) of many species of mosquitos, other dipterans, and planktonic crustaceans (Federici and

Lucarotti 1986; Whisler et al. 2009). The life cycles of some species of *Coelomomyces* involve a full alternation of haploid and diploid generations and an obligate alternation between two hosts, one of which is a mosquito and the other is either a copepod or an ostracod. Other species of *Coelomomyces* can also infect other dipteran species such as midges, but the complete details of the life cycles are not known. Several species of *Coelomomyces* have been considered for use in biological control of mosquito larvae. The effects of physical factors on the interactions between the parasites and hosts in these groups are not understood, but some preliminary data have been provided by Apperson et al. (1992). The incidence of this disease is seasonal, and light and temperature affect the growth of both the parasites and their hosts, but further experiments are necessary to provide more detailed information. Research on the host–parasite interactions with species of *Coelomomyces* has been difficult because these parasites cannot be grown in pure culture without the host.

8. *Catenaria*

Some species of *Catenaria* are common facultative parasites of nematodes and midges (Martin 1981; Singh et al. 2007). Several *Catenaria* species have been considered for use in the biological control of species of nematodes, which are parasites of agriculturally important plants. Martin (1981) provided evidence that *Catenaria uncinata* plays a role in natural control of population sizes of midge larvae in freshwater ecosystems. Since *Catenaria* can easily be grown in culture, the host–parasite interactions can be studied in the laboratory (Martin 1981; Deacon and Saxena 1997).

9. *Polycaryum*

Johnson et al. (2006, 2009) studied the natural regulation of host population dynamics of *Daphnia pulicaria* in freshwater lake ecosystems. Infection by *Polycaryum laeve* was highly specific to *D. pulicaria*, but could occasionally be observed in other cladocerans also *P. laeve* caused declines of up to 99 % in host population densities during epizootics. Infection prevalence was seasonal, highest during late winter and early spring, and lowest in late summer. Infected individuals produced no eggs, molted less frequently, and died sooner than uninfected individuals. Because of their altered appearance, infected *Daphnia* were also more susceptible to visual predators such as fish (Johnson et al. 2006).

10. *Olpidium*

Species of *Olpidium* are ubiquitous intercellular parasites of the roots of flowering plants. The infection occurs mostly symptomless, without any damage to serve the parasite's benefit, but coinfection often occurs with several important soil-borne plant viruses. These fungi are known to vector a wide range of plant viruses, such as big-vein virus in lettuce and necrosis virus in melons, from at least four virus families (Campbell 1996; Rochon et al. 2001). ITS sequence data from infected brassicas, melons, carrots, cucumbers, and lettuce plants originating from four continents revealed genetic differences between *Olpidium* parasites (Hartwright et al. 2010; Herrera-Vásquez et al. 2010a, b). Other species of *Olpidium* infect several species of phytoplankters (Sparrow 1960; Gleason et al. 2011), rotifers (Glockling 1998), and nematode eggs (Barron and Szijarto 1986), but little is known about the symptoms of the disease in these pathosystems. In addition, parasitic species of fungi (plant pathogenic rust fungi) and protists are known (Table 9.3). Therefore, the members of the genus *Olpidium* (provisionally classified into the family Olpidiaceae) account for one zoosporic fungal genus expressing the largest and widest host spectra spanning plants, animals, and fungi, and thus contribute significantly to natural biocontrol systems. For an overview of parasitic genera, see Table 9.3.

VII. Conclusions

Out of a total of about 100,000 fungal species described so far, approximately 700 (0.7 %) are zoosporic true fungi. Estimates forecast a dramatic increase of up to 1.5–3.5 million fungal species, of which zoosporic fungi or flagellate fungus-like organisms may comprise the

majority. Therefore, zoosporic fungi represent a highly underestimated group of fungi, which have not been greatly studied in the past but deserve higher attention in all respects. They significantly contribute to the control of food webs, disease cycles, and management of ecological balances by, for example, reduction of certain harmful populations due to parasitism in order to favour the more beneficial populations. Because of their ubiquitous appearance and wide variety of life styles (generalist and specialist), zoosporic fungi enter the food web at any stage right from the bottom to the top. Zoosporic true fungi as a whole are an ecologically as well as a phylogenetically coherent group, and have successfully adapted primarily to conditions in soil and other freshwater habitats. Most species are described as saprotrophs, while only a few species are described as facultative or obligate parasites (biotrophs). The largest number of species are parasites of phytoplanktonic diatoms, green algae, and cyanobacteria. The parasitism of phytoplankton by zoosporic fungi is particularly relevant, not only because it can control the blooming of nuisance algae, but also because zoospores provide a considerable amount of the biomass to higher trophic levels and enhance the transfer efficiency in food webs. This chapter: (i) draws attention to an increased discovery of novel zoosporic fungal clades and to the necessity to resolve phylogenetic inconsistencies caused by incomplete taxon sampling arising by obligate parasitic and thus non-culturable taxa, (ii) reviews the mode and mechanisms of their action and participation in the control of ecosystem processes, and (iii) highlights economic implications. The distinction between economic and ecological importance of parasitism by zoosporic true fungi and the degree of importance is very difficult to define because of a lack of quantitative data. The examples of parasitic zoosporic fungi we have included in this chapter were chosen because studies with these parasitic fungi have been published, and the authors considered their research to be particularly important. These fungi, which were chosen as examples, are causative agents of important emerging infectious diseases (EID). An EID can be defined as an infectious disease that has recently appeared in a population, or that has been known for some time but is rapidly increasing in incidence or geographic range (Fisher et al. 2012). Two of the examples presented in this chapter, the chytridiomycosis caused by *B. dendrobatidis* and the potato wart disease by *S. endobioticum*, are already considered EIDs, and are listed as quarantine diseases world-wide (Daszak et al. 1999; Johnson and Speare 2003; Niepold and Stachewicz 2004). In our opinion, there are many other economically and ecologically important pathosystems awaiting future research. The examples we included are useful for understanding basic concepts in ecology and refer to the pathosystem concept. The pathosystem concept provides a framework for understanding the complexity of all host–parasite interactions on a large scale of analysis. This concept has been discussed in the context of zoosporic true fungal parasites here. The model for emerging infectious diseases is also applicable to ecological studies with some species of zoosporic true fungi, such as *Batrachochytrium dendrobatidis* (Fisher et al. 2012). Although the ecological roles of *Batrachochytrium* and other zoosporic fungal parasites considered in this chapter have long been accepted by ecologists, we have highlighted the significance of these roles in research with zoosporic true fungal parasites. Historically, the economic roles of fungi have been emphasized, while the ecological roles have been unappreciated. In general, zoosporic true fungi have been traditionally poorly sampled and described. We expect that there are many more species of parasites which will be discovered in the future. With the increasing importance of research into global climate change, the ecological roles of all groups of fungi need re-evaluation.

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References

Adl SM, Simpson AGB, Farmer MA, Andersen RA, Anderson OR, Barta JA, Bowser SS, Bragerolle G, Fensome RA, Fredericq S, James TY, Karpov S, Kugrens P, Krug J, Lane CE, Lewis LA, Lodge J, Lynn DH, Mann DG, McCourt RM, Mendoza L, Moestrup Ø, Mozley-Standridge SE, Nerad TA, Shearer CA, Smirnov AV, Spiegel FW, Taylor MFJR (2005) The new higher level classification of Eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol 52:399–451

Apperson CS, Federici BA, Tarver FR, Stewart W (1992) Biotic and abiotic parameters associated with an epizootic of *Coelomomyces punctatus* during in a larval population of the mosquito *Anopheles quadrimaculatus*. J Invertebr Pathol 60:219–228

Arroyo-Begovich A, Cárabez-Trejo A (1982) Location of chitin in the cyst wall of *Entamoeba invadens* with colloidal gold racers. J Parasitol 68:253–258

Baldauf SL (2003) The deep roots of Eukaryotes. Science 300:1703–1706

Barr DJS (1973) *Rhizophydium graminis* (Chytridiales): morphology, host range, and temperature effect. Can Plant Dis Survey 53:191–193

Barr DJS (2001) Chytridiomycota. In: McLaughlin DL, McLaughlin EG, Lemke PA (eds) The mycota, vol VII, Part A. Springer, New York, pp 93–112

Barr DJS, Hickman CJ (1967a) Chytrids and algae I. Host-substrate range, and morphological variation of species of *Rhizophydium*. Can J Bot 45:423–430

Barr DJS, Hickman CJ (1967b) Chytrids and algae II. Factors influencing parasitism of *Rhizophydium sphaerocarpum* on *Spirogyra*. Can J Bot 45:431–440

Barron GL, Szijarto E (1986) A new species of *Olpidium* parasitc in nematode eggs. Mycologia 78:972–975

Bartnicki-Garcia S (1970) Cell wall composition and other biochemical markers in fungal phylogeny. In: Harbone JB (ed) Phytochemical phylogeny. Academic, London, pp 81–103

Bartnicki-Garcia S (1987) The cell wall in fungal evolution. In: Rayner ADM, Brasier CM, Moore D (eds) Evolutionary biology of the fungi. Cambridge University Press, New York, pp 389–403

Beakes GW, Canter HM, Jaworski GHM (1993) Sporangium differentiation and zoospore fine-structure of the chytrid *Rhizophydium planktonicum*, a fungal parasite of *Asterionella formosa*. Mycol Res 97:1059–1074

Besl H, Bresinsky A (1997) Chemosystematics of *Suillaceae* and *Gomphidiaceae* (suborder *Suillineae*). Plant Syst Evol 206:223–242

Blackwell WH, Letcher PM, Powell MJ (2011) The occurrence of *Blyttiomyces spinosus* in Alabama and Argentina, and comments on the genus *Blyttiomyces* (Chytridiomycota). Phytologia 93:304–315

Bridge PD (1985) An evaluation of some physiological and biochemical methods as an aid to the characterization of species of *Penicillium* subsection Fasciculata. J Gen Microbiol 131:1887–1895

Briggs CJ, Knapp R, Vrendenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proc Natl Acad Sci USA 107:9695–9700

Brown MW, Spiegel FW, Silberman JD (2009) Phylogeny of the "forgotten" cellular slime mold, *Fonticula alba*, reveals a key evolutionary branch within Opisthokonta. Mol Biol Evol 26:2699–2709

Bruckart WL, Eskandari FM, Widmer TL (2011) *Synchytrium solstitiale*: reclassification based on the function and role of resting spores. Mycologia 103:775–778

Bruning K (1991a) Effects of phosphorus limitation on the epidemiology of a chytrid phytoplankton parasite. Freshwater Biol 25:409–417

Bruning K (1991b) Effects of temperature and light on the population-dynamics of the *Asterionella-Rhizophydium* association. J Plankton Res 13:707–719

Bruning K (1991c) Infection of the diatom *Asterionella* by a chytrid. 1. Effects of light on reproduction and infectivity of the parasite. J Plankton Res 13:103–117

Bruning K (1991d) Infection of a diatom *Asterionella* by a chytrid. II. Effects of light on survival and epidemic development of the parasite. J Plankton Res 13:119–129

Bruning K, Lingeman R, Ringelberg J (1992) Estimating the impact of fungal parasites on phytoplankton populations. Limnol Oceanogr 37:252–260

Buck J, Truong L, Blaustein A (2011) Predation by zooplankton on *Batrachochytrium dendrobatidis*: biological control of the deadly amphibian chytrid fungus? Biodivers Conserv 20:3549–3553

Burgaud G, Le Calvez T, Arzur D, Vandenkoornhuyse P, Barbier G (2009) Diversity of culturable marine filamentous fungi from deep-sea hydrothermal vents. Appl Environ Microbiol 75:6415–6421

Campbell RN (1996) Fungal transmission of plant viruses. Annu Rev Phytopathol 34:87–108

Canter HM (1969) Studies on British chytrids. XXIX. A taxonomic revision of certain fungi found on the diatom *Asterionella*. Bot J Linn Soc 62:267–278

Canter HM (1972) A guide to the fungi occurring on planktonic blue-green algae. In: Desikachary TV (ed) Taxonomy and biology of blue-green algae. University of Madras, Madras, pp 145–158

Canter HM, Jaworski GHM (1979) The occurrence of a hypersensitive reaction in the planktonic diatom *Asterionella formosa* Hassall parasitized by the chytrid *Rhizophydium planktonicum* Canter emend. in culture. New Phytol 82:187–206

Canter HM, Lund JWG (1951) Studies on plankton parasites. III. Examples of the interaction between parasitism and other factors determining the growth of diatoms. Ann Bot 15:359–372

Canter HM, Lund JWG (1953) Studies on plankton parasites. II. The parasitism of diatoms with

special reference to lakes in the English Lake District. Trans Br Mycol Soc 36:13–37
Canter HM, Walsby AE, Kinsman R, Ibelings BW (1992) The effect of attached Vorticellids on the buoyancy of the colonial cyanobacterium *Anabaena lemmermannii*. Brit Phycol J 27:65–74
Capella-Gutiérrez S, Marcet-Houben M, Gabaldón T (2012) Phylogenomics supports microsporidia as the earliest diverging clade of sequenced fungi. BMC Biol 10:47. doi:10.1186/1741-7007-10-47
Cavalier-Smith T (1998) A revised six-kingdom of life. Biol Rev 73:203–266
Cavalier-Smith T (2009) Megaphylogeny, cell body plans, adaptive zones: causes and timing of Eukaryote basal radiations. J Eukaryot Microbiol 56:26–33
Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R (1999) Emerging infectious diseases and amphibian population declines. Emerg Infect Dis 5:735–748
De Bruin A (2006) The potential for coevolution in a host-parasite system. Dissertation, Radboud University Nijmegen, Netherlands Institute of Ecology of the Royal Academy of Arts and Sciences, Amsterdam, The Netherlands
Deacon JW, Saxena G (1997) Oriented zoospore attachment and cyst germination in *Catenaria anguillulae*, a facultative endoparasite of nematodes. Mycol Res 101:513–522
Doggett MS, Porter D (1996) Sexual reproduction in the fungal parasite, *Zygorhizidium planktonicum*. Mycologia 88:720–732
Doweld A (2001) Prosyllabus tracheophytorum – tentamen systematis plantarum vascularium (Tracheophyta). Goes MMI, Moscow, pp 1–111
Ebersberger I, Gube M, Strauss S, Kupczok A, Eckart M, Voigt K, Kothe E, von Haeseler A (2009a) A stable backbone for the fungi. Nature precedings: hdl:10101/npre.2009.2901.1. Accessed 19 Sept 2012
Ebersberger I, Strauss S, von Haeseler A (2009b) HaMStR: profile hidden Markov model based. BMC Evol Biol. doi:10.1186/1471-2148-9-157
Ebersberger I, de Matos Simoes R, Kupczok A, Gube M, Kothe E, Voigt K, von Haeseler A (2012) A consistent phylogenetic backbone for the fungi. Mol Biol Evol 29:1319–1334
Edwards JE, Huws SA, Kim EJ, Kingston-Smith AH, Jimenes HR, Skot KP, Griffith GW, McEwan NR, Theodorou MK (2008) Dynamics of initial colonozation of nonconserved perennial ryegrass by anaerobic fungi in the bovine rumen. FEMS Microbiol Ecol 66:537–545
Federici BA, Lucarotti CJ (1986) Structure and behavior of the meiospore of *Coelomomyces dodgei* during encystment on the copepod host *Acanthocyclops vernalis*. J Invert Pathol 48:259–268
Fernández C, Parodi ER, Cáceres EJ (2012) Impact of the fungal parasite *Rhizophydium couchii* (Chytridiomycota) on the population dynamics of the freshwater alga *Closterium aciculare* (Chlorophyta). Nova Hedwigia 93:97–106
Fisher MC, Garner TWJ, Walker SF (2009) Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annu Rev Microbiol 63:291–310
Fisher MC, Henk DA, Briggs CJ, Brownsteiin JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186–194
Frisvad JC, Filtenborg O (1990) Secondary metabolites as consistent criteria in *Penicillium* taxonomy and a synoptic key to *Penicillium* subgenus *Penicillium*. In: Samson RA, Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus* classification. Plenum, New York, pp 373–384
Gherbawy Y, Voigt K (2010) Molecular identification of fungi. Springer, Berlin/Heidelberg/New York
Gleason FH, Lilje O (2009) Structure and function of fungal zoospores: ecological implications. Fungal Ecol 2:53–59
Gleason FH, Marano AV (2011) The effects of antifungal substances on zoosporic fungi. Hydrobiologia 659:81–92
Gleason FH, Kagami M, Lefèvre E, Sime-Ngando T (2008) The ecology of chytrids in aquatic ecosystems: roles in food web dynamics. Fungal Biol Rev 2:17–25
Gleason FH, Marano AV, Johnson P, Martin WW (2010a) Blastocladian parasites of invertebrates. Fungal Biol Rev 24:56–67
Gleason FH, Schmidt SK, Marano AV (2010b) Can zoosporic true fungi grow or survive in extreme or stressful environments? Extremophiles 14:417–425
Gleason FH, Amon JP, Küpper FC, Gachon CMM, Marano AV, Sime-Ngando T, Lilje O (2011) Zoosporic true fungi in marine ecosystems. Mar Freshwater Res 62:383–393
Gleason FH, Crawford JW, Neuhauser S, Henderson L, Lilje O (2012a) Resource seeking strategies of zoosporic true fungi in soils. Soil Biol Biochem 45:79–88
Gleason FH, Carney LT, Lilje O, Glockling SL (2012b) Ecological potentials of species of *Rozella* (Cryptomycota). Fungal Ecol 5:651–656
Glockling SL (1998) Isolation of a new species of rotifer-attacking *Olpidium*. Mycol Res 102:206–208
Goka K, Yokoyama J, Une Y, Kuroki T, Suzuki K, Nakahara M, Kobayashi A, Inaba S, Mizutani T, Hyatt AD (2009) Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. Mol Ecol 18:4757–4774
Grami B, Rasconi S, Niquil N, Jobard M, Saint-Béat B (2011) Functional effects of parasites on food web properties during the spring diatom bloom in Lake Pavin: a linear inverse modeling analysis. PLoS One 6:e23273. doi:10.1371/journal.pone.0023273
Greco N, Bussers JC, Van Daele Y, Goffinet G (1990) Ultrastructural localization of chitin in the cystic wall of *Euplotes muscicola* Kahl (Ciliata, Hypotrichia). Europ J Protistol 26:75–80
Gsell AS, De Senerpont Domis LN, Naus-Wiezer SMH, Helmsing NR, van Donk E, Ibelings BW (2012)

Spatiotemporal variation in the distribution of chytrid parasites in diatom host populations. Freshw Biol. doi:10.1111/j.1365-2427.2012.02786.x

Gutman J, Zarka A, Boussiba S (2009) The host-range of *Paraphysoderma sedebokerensis*, a chytrid that infects *Haematococcus fluvialis*. European J Phycol 44:509–514

Hampson MC (1980) Pathogenesis of *Synchytrium endobioticum*: 2. Effect of soil amendments and fertilization. Can J Plant Pathol 2:148–151

Hampson MC, Coombes JW, Debnath SC (1997) Dual culture of *Solanum tuberosum* and *Synchytrium endobioticum* (Pathotype 2). Mycologia 89:772–776

Hartwright LM, Hunter PJ, Walsh JA (2010) A comparison of *Olpidium* isolates from a range of host plants using internal transcribed spacer sequence analysis and host range studies. Fungal Biol 114:26–33

Hawksworth DL (1991) The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol Res 95:641–655

Hawksworth DL (2001) The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol Res 105:1422–1432

Hawksworth DL, Rossman AY (1997) Where are all the undescribed fungi? Phytopathol 87:888–891

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995) Ainsworth & Bisby's dictionary of the fungi, 8th edn. International Mycological Institute, Kew/ Surrey

Heger TJ, Mitchell EAD, Todorov M, Golemansky V, Lara E, Leander BS, Pawlowski J (2010) Molecular phylogeny of euglyphid testate amoebae (Cercozoa: Euglyphida) suggests transitions between marine supralittoral and freshwater/terrestrial environments are infrequent. Mol Phyl Evol 55:113–122

Herrera-Vásquez JA, Cebrián MdC, Alfaro-Fernández A, Córdoba-Sellés MC, Jordá C (2010a) Multiplex PCR assay for simultaneous detection and differentiation of *Olpidium bornovanus, O. brassicae* and *O. virulentus*. Mycol Res 113:602–610

Herrera-Vásquez JA, Córdoba-Sellés MC, Cebrián MdC, Rosselló JA, Alfaro-Fernández A, Jordá C (2010b) Genetic diversity of melon necrotic spot virus and *Olpidium* isolates from different origins. Plant Pathol 59:240–251

Herth W, Zugenmaier P (1977) Ultrastructure of the chitin fibrils of the centric diatom *Cyclotella cryptica*. J Ultrastructure Res 61:230–239

Herth W, Kuppel A, Schnepf E (1977) Chitinous fibrils in the lorica of the flagellate chrysophyte *Poteriochromonas stipitata* (syn. *Ochromonas malhamensis*). J Cell Biol 73:311–321

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson O, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch T, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde K, Köljalb U, Kurtzman CP, Larsson K-H, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto R, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schuessler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang A, Weir A, Weiss M, White M, Winka K, Yao Y-J, Zhang N (2007) A higher-level phylogenetic classification of the fungi. Mycol Res 111:509–547

Hibbett DS, Ohman A, Kirk PM (2009) Fungal ecology catches fire. New Phytol 184:279–282

Holfeld H (2000a) Relative abundance, rate of increase, and fungal infections of freshwater phytoplankton. J Plankton Res 22:987–995

Holfeld H (2000b) Infection of the single-celled diatom *Stephanodiscus alpinus* by the chytrid *Zygorhizidium*: parasite distribution within host population, changes in host cell size, and host-parasite size relationship. Limnol Oceang 45:1440–1444

Ibelings BW, de Bruin A, van Donk E (2003) Parasitic fungi of freshwater phytoplankton. In: Tsui CKM, Hyde KD (eds) Freshwater mycology, vol 10, Fungal diversity research series. Fungal Diversity Press, Hong Kong, pp 11–50

Ibelings BW, de Bruin A, Kagami M, Rijkeboer M, Brehm M, van Donk E (2004) Host parasite interactions between freshwater phytoplankton and chytrid fungi (Chytridiomycota). J Phycol 40:437–453

Ibelings BW, Gsell AS, Mooij WM, van Donk E, van Wyngaert S, de Senerpont Domis LN (2011) Chytrid infections and diatom spring blooms: paradoxical effects of climate warming on fungal epidemic in Lakes. Freshwater Biol 56:754–766

James TY, Berbee ML (2011) No jacket required – new fungal lineage defies dress code: recently described zoosporic fungi lack a cell wall during trophic phase. Bioessays 34:94–102

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J, Lumbsch HT, Rauhut A, Reeb V, Arnold AE, Amtoft A, Stajich JE, Hosaka K, Sung GH, Johnson D, O'Rourke B, Crockett M, Binder M, Curtis JM, Slot JC, Wang Z, Wilson AW, Schüler A, Longcore JE, O'Donnell K, Mozley-Standridge S, Porter D, Letcher PM, Powell MJ, Taylor JW, White MM, Griffith GW, Davies DR, Humber RA, Morton JB, Sugiyama J, Rossman AY, Rogers JD, Pfister DH, Hewitt D, Hansen K, Hambleton S, Shoemaker RA, Kohlmeyer J, Volkmann-Kohlmeyer B, Spotts RA, Serdani M, Crous PW, Hughes KW, Matsuura K, Langer E, Langer G, Untereiner WA, Lücking R, Büdel B, Geiser DM, Aptroot A, Diederich P, Schmitt I, Schultz M, Yahr R, Hibbett DS, Lutzoni F, McLaughlin DJ, Spatafora JW, Vilgalys R (2006a) Reconstructing the

early evolution of the fungi using a six-gene phylogeny. Nature 443:818–822
James TY, Letcher PM, Longcore JE, Mozley-Strandridge SE, Porter D, Powell MJ, Griffith GW, Vilgalys R (2006b) A molecular phylogeny of the flagellated fungi (*Chytridiomycota*) and description of a new phylum (*Blastocladiomycota*). Mycologia 98:860–871
Johns RM (1966) Morphological and ecological study of *Physoderma dulichii*. Amer J Bot 53:34–45
Johnson ML, Speare R (2003) Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. Emerg Infect Dis 9:922–925
Johnson PTJ, Longcore JE, Stanton DE, Carnegie RB, Shields JD, Preu ER (2006) Chytrid infections of *Daphnia pulicaria*: development, ecology, pathology and phylogeny of *Polycaryum laeve*. Freshwater Biol 51:634–648
Johnson PTJ, Ives AR, Lathrop RC, Carpenter SR (2009) Longterm disease dynamics in lakes: causes and consequences of chytrid infections in *Daphnia* populations. Ecology 90:132–144
Joines JD (1984) Observations on the fungus *Sorochytrium milnesiophthora* sp. nov., an endoparasite of the tardigrade *Milnesium tardigradum* Doyere: M.Sc. thesis, Appalachian State University
Jones EBG (2011) Are there more marine fungi to be described? Botanica Marina 54:343–354
Jones MDM, Forn I, Gadelha C, Egan MJ, Bass D, Massana R, Richards TA (2011a) Discovery of novel intermediate forms redefines the fungal tree of life. Nature 474:200–203
Jones MDM, Richards TA, Hawksworth DL, Bass D (2011b) Validation and justification of the phylum name *Cryptomycota* phyl. nov. IMA Fungus 2:173–175
Kagami M (2012) Mycoloop. http://www.lab.toho-u.ac.jp/sci/env/kagami/english/research/index.html. Accessed 19 Sept 2012
Kagami M, Urabe J (2002) Mortality of the planktonic desmid, *Staurastrum dorsidentiferum*, due to interplay of fungal parasitism and low light conditions. Verh Int Verein Limnol 28:1001–1005
Kagami M, van Donk E, de Bruin A, Rijkeboer M, Ibelings BW (2004) *Daphnia* can protect diatoms from fungal parasitism. Limnol Oceanogr 49:680–5685
Kagami M, de Bruin A, Ibelings BW, van Donk E (2007a) Parasitic chytrids: their effects on phytoplankton communities and food-web dynamics. Hydrobiologia 578:113–129
Kagami M, von Elert E, Ibelings BW, de Bruin A, van Donk E (2007b) The parasitic chytrid, *Zygorhizidium*, facilitates the growth of the cladoceran zooplankter, *Daphnia*, in cultures of the inedible alga, *Asterionella*. Proc Royal Soc B 274:1561–1566
Kagami M, Amano Y, Ishii N (2012) Community structure of planktonic fungi and the Impact of parasitic chytrids on phytoplankton in Lake Inba, Japan. Microb Ecol 63:358–368
Karling JS (1977) Chytridiomycetarum Iconographia. J Cramer, Vaduz
Kimbrough JW (1994) Septal ultrastructure and ascomycete systematics. In: Hawksworth DL (ed) Ascomycete systematics: problems and perspectives in the nineties. Plenum, New York, pp 127–141
Kirk PM, Cannon PF, David DW, Stalpers JA (2008) Dictionary of the fungi, 10th edn. CSIRO, Wallingford
Kohn LM (1992) Developing new characters for fungal systematics: an experimental approach for determining the rank of resolution. Mycologia 84:139–153
Kühn SF, Hofmann M (1999) Infection of *Coscinodiscus granii* by the parasitoid nanoflagellate *Pirsonia diadema*: III. Effects of turbulence on the incidence of infection. J Plankton Res 21:2323–2340
Lafferty KD, Allesina S, Arim M, Briggs CJ, De Leo G, Dobson AP, Dunne JA, Johnson PTJ, Kuris AM, Marcogliese DJ, Martinez ND, Memmott J, Marquet PA, McLaughlin JP, Mordecai EA, Pascual M, Poulin R, Thieltges DW (2008) Parasites in food webs: the ultimate missing links. Ecol Let 11:533–546
Laidlaw WMR (1985) A method for detection of the resting sporangia of potato wart disease (*Synchytrium endobioticum*) in the soil of old outbreak sites. Potato Res 28:223–232
Lange L, Olson LW (1980) Germination of the resting sporangia of *Physoderma maydis*, the causal agent of *Physoderma* disease of maize. Protoplasma 102:323–342
Lara E, Moreira D, López-García P (2010) The environmental clade LKM11 and *Rozella* form the deepest branching clade of fungi. Protist 161:116–121
Ledingham GA (1936) *Rhizophydium graminis* n. sp., a parasite of wheat roots. Can J Res 14:117–121
Lenné JM (1985) *Synchytrium desmodii*, cause of wart disease of the tropical legume *Desmodium ovalifolium* in Colombia. Plant Dis 69:806–808
Li J, Heath IB, Packer L (1993) The phylogenetic relationships of the anaerobic chytridiomycetous gut fungi (Neocallimasticaceae) and the Chytridiomycota. II. Cladistic analysis of structural data and description of Neocallimasticales ord. nov. Can J Bot 71:393–407
Liggenstoffer AS, Youssef NH, Couger MB, Elshahed MS (2010) Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME J 4:1225–1235
Logares R, Brate J, Bertilsson S, Clasen JL, Shalchian-Tabrizi K, Rengefors K (2009) Infrequent marine-freshwater transitions in the microbial world. Trends Microbiol 17:414–422
Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219–227
Marano AV, Pires-Zottarelli CLA, Gleason FH, Neuhauser S, Steciow MM (2011) Chapter 1: Assemblages of zoosporic true fungi, heterotrophic stramenipiles and plasmodiophorids in freshwater ecosys-

tems. In: Browne SA (ed) Aquatic ecosystems. Nova, New York, pp 1–56
Marano AV, Gleason FH, Bärlocher F, Pires-Zottarelli CLA, Schmidt SK, Rasconi S, Lilje O, Kagami M, Telesphore S-N, Barrera MD, Boussiba S, de Souza JI, Edwards JE (2012) Quantitative methods for the analysis of zoosporic fungi. J Microbiol Meth 89:22–33
Martin WW (1981) The natural regulation of midge populations by aquatic fungi in Virginia. J Elisha Mitchell Sci Soc 97:162–170
Maxson LR, Maxson RD (1990) Proteins II. Immunological techniques. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer Associates, Sunderland, pp 127–155
McCreadie JW, Adler PH, Beard CE (2011) Ecology of symbiotes of larval black flies (Diptera: Simuliidae): distribution, diversity and scale. Environ Entomol 40:289–302
McFarlane I (1970) *Lagena radicicola* and *Rhizophydium graminis*, two common and neglected fungi. Trans Br Mycol Soc 55:113–116
Mulisch M (1993) Chitin in protistan organisms: distribution, synthesis and deposition. Eur J Protistol 29:1–18
Müller DG, Küpper FC, Küpper H (1999) Infection experiments reveal broad host ranges of *Eurychasma dicksonii* (Oomycota) and *Chytridium polysiphoniae* (Chytridiomycota), two eukaryotic parasites in marine brown algae (Phaeophyceae). Phycol Res 47:217–223
Nagy LG, Petkovits T, Kovács GM, Voigt K, Vágvölgyi C, Papp T (2011) Where is the unseen fungal diversity hidden? A study of *Mortierella* reveals a large contribution of reference collections to the identification of fungal environmental sequences. New Phytol 19:789–794
Nascimento de Almeida C, Gomes EP, Pires-Zottarelli CL (2011) Occurrence and distribution of zoosporic organisms in water bodies from Brazilian Cerrado. Mycologia 103:261–272
Niepold F, Stachewicz H (2004) PCR-detection of *Synchytrium endobioticum* (Schilb.) Perc. J Plant Dis Protect 111:313–321
O'Brien HE, Parrent JL, Jackson JA, Moncalvo JM, Vilgalys R (2005) Fungal community analysis by large-scale sequencing of environmental samples. Appl Environ Microbiol 71:5544–5550
Olive LS (1980) *Caulochytrium protostelioides* sp. nov., a new chytrid with aerial sporangia. Amer J Bot 67:568–574
Paterson RRM, Bridge PD (1994) Biochemical techniques for filamentous fungi. CAB International, Wallingford
Paulitz TC, Menge JA (1984) Is *Spizellomyces punctatum* a parasite or saprophyte of vesicular-arbuscular mycorrhizal fungi? Mycologia 76:99–107
Pitt JI (1979) The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic, London
Porter TM, Schadt CW, Rizvi L, Martin AP, Schmidt SK, Scott-Denton L, Vilgalys R, Moncalvo JM (2008) Widespread occurrence and phylogenetic placement of a soil clone group adds a prominent new branch to the fungal tree of life. Mol Phyl Evol 46:635–644
Powell MJ (1993) Looking at mycology with a Janus face: a glimpse at chytridiomycetes active in the environment. Mycologia 85:1–20
Powell MJ (1994) Production and modifications of extra cellular structures during development of Chytridiomycetes. Protoplasma 181:123–141
Reynolds CS (1973) The seasonal periodicity of planktonic diatoms in a shallow eutrophic lake. Freshwater Biol 3:89–110
Robinson RA (1976) Plant pathosystems. Springer, Berlin
Rochon D, Kakani K, Robbins M, Reade R (2001) Molecular aspects of plant virus transmission by *Olpidium* and plasmodiophorid viruses. Annu Rev Phytopathol 42:211–241
Schaffner JH (1909) The classification of plants, IV. Ohio Naturalist 9:446–455
Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA 109:6241–6246
Sekimoto S, Rochon D, Long JE, Dee JM, Berbee ML (2011) A multigene phylogeny of *Olpidium* and its implications for early fungal evolution. BMC Evol Biol 11:331. doi:10.1186/1471-2148-11-331
Sen B (1987a) Fungal parasitism of planktonic algae in Shearwater. I. Occurrence of *Zygorhizidium affluens* Canter on *Asterionella formosa* Hass in relation to the seasonal periodicity of the alga. Arch Hydrobiol 76:101–127
Sen B (1987b) Fungal parasitism of planktonic algae in Shearwater. II. A study of the chytrid parasites of the diatom *Fragilaria crotonensis* Kitton. Arch Hydrobiol 76:129–144
Sen B (1987c) Fungal parasitism of planktonic algae in Shearwater. III. Fungal parasites of centric diatoms. Arch Hydrobiol 79:167–175
Sen B (1988a) Fungal parasitism of planktonic algae in Shearwater. IV. Parasitic occurrence of a new chytrid species on the bluegreen alga *Microcystis aeruginosa* Kuetz emend. Elenkin. Archiv Hydrobiol 79:177–184
Sen B (1988b) Fungal parasitism of planktonic algae in Shearwater. V. Fungal parasites of the green algae. Arch Hydrobiol 79:185–205
Shalchian-Tabrizi K, Minge MA, Espelund M, Orr R, Ruden T, Jakobsen KS, Cavalier-Smith T (2008) Multigene phylogeny of Choanozoa and the origin of animals. PLoS One 3:e2098. doi:10.1371/journal.pone.0002098
Shearer CA, Descals E, Kohlmeyer B, Kohlmeyer J, Marvanova L, Padgett D, Porter D, Raja HA, Schmit JP, Thorton HA, Voglymayr H (2007) Fungal biodiversity in aquatic habitats. Biodivers Conserv 16:49–67

Shin W, Boo SM, Longcore J (2001) *Entophlyctis apiculata*, a chytrid parasite of *Chlamydomonas* sp. (Chlorophyceae). Can J Bot 79:1083–1089

Sime-Ngando T, Lefévre E, Gleason FH (2011) Hidden diversity among aquatic heterotrophic flagellates: ecological potentials of zoosporic fungi. Hydrobiologia 659:5–22

Singh KP, Jaiswal RK, Kumar N (2007) *Catenaria anguillulae* Sorokin: a natural biocontrol agent of *Meloidogyne graminicola* causing root knot disease of rice (*Oryza sativa* L.). World J Microb Biot 23:291–294

Sommer U (1987) Factors controlling the seasonal variation in phytoplankton species composition – a case study for a deep, nutrient rich lake. Prog Phycol Res 5:124–178

Sønstebø JH, Rohrlack T (2011) Possible implications of chytrid parasitism for population subdivision in freshwater cyanobacteria of the genus *Planktothrix*. Appl Environ Microbiol 77:1344–1351

Sparrow FK Jr (1960) Aquatic phycomycetes, 2nd edn. University of Michigan Press, Ann Arbor

Sparrow FK Jr (1968) Ecology of freshwater fungi. In: Ainsworth GC, Sussman AS (eds) The fungi: an advanced treatise. Academic, New York, pp 41–93

Spoel SH, Dong X (2012) How do plants achieve immunity? Defence without specialized immune cells. Nat Rev Immunol 12:89–100

Steenkamp ET, Wright J, Baldauf SL (2005) The protistan origins of animals and fungi. Mol Biol Evol 23:93–106

Stock A, Jürgens K, Bunge J, Stoeck T (2009) Protistan diversity in suboxic and anoxic waters of the Gotland Deep (Baltic Sea) as revealed by 18S rRNA clone libraries. Aquat Microb Ecol 55:267–284

Tuckwell DS, Nicholson MJ, McSweeney CS, Theodorou MK, Brookman JL (2005) The rapid assignement of ruminal fungi to presumptive genera using ITS1 and ITS2 RNA secondary structures to produce group-specific fingerprints. Microbiology 151:1557–1567

van Donk E, Bruning K (1992) Ecology of aquatic fungi in and on algae. In: Reisser W (ed) Algae and symbioses. Biopress, Bristol, pp 567–592

van Donk E, Ringelberg J (1983) The effect of fungal parasitism on the discussion of diatoms in Lake Maarsseveen I (The Netherlands). Freshwater Biol 13:241–251

van Valen L (1973) A new evolutionary law. Evol Theor 1:1–30

Vogel HJ (1961) Lysine synthesis and phylogeny of lower fungi: some chytrids versus *Hyphochytrium*. Nature 189:1026–1027

Voigt K (2012) Chytridiomycota. In: Engler A, Frey W (eds) Syllabus of plant families, Vol 1/1: blue-green algae, myxomycetes and myxomycete-like organisms, phytoparqasitic protists, heterotrophic heterokontobionta and fungi. Borntraeger, Stuttgart, pp 106–129

Voigt K, Kirk PM (2011) Recent developments in the taxonomic affiliation and phylogenetic positioning of fungi: impact in applied microbiology and environmental biotechnology. Appl Microbiol Biotechnol 90:41–57

Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, Cook D, Webb R, Alford RA, Skerratt LF, Speare R (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326:582–585

Voyles J, Vredenburg VT, Tunstall TS, Parker JM, Briggs CJ, Rosenblum EB (2012) Pathophysiology in mountain yellow-legged frogs (*Rana muscosa*) during a chytridiomycosis outbreak. PLoS One 7: e35374. doi:10.1371/journal.pone.0035374

Ward HHD, Alroy J, Lev BI, Keusch GT, Pereira MEA (1985) Identification of chitin as a structural component of *Giardia* cysts. Infect Immun 49:629–634

Whisler HC (1985) Life history of species of *coelomomyces*. In: Couch JN, Bland CE (eds) The genus *Coelomomyces*. Academic, New York, pp 9–22

Whisler HC, Sabwa Karanja DM, Shemanchuk JA, Zebold SL, Romney SV, Nielsen LT (2009) The life history and in vivo culture of *Coelomomyces utahensis* (Blastocladiomycetes). J Invert Pathol 100:40–43

Widmer TL, Guermanche F (2006) Factors affecting infection of yellow starthistle (*Centaurea solstitialis*) by *Synchytrium solstitale*, causal agent of false rust disease. Plant Dis 90:425–428

Youngman RE, Johnson D, Farley MR (1976) Factors influencing phytoplankton growth and succession in Farmoor Reservoir. Freshwater Biol 6:253–263

Symbiontic Fungi and Mycorrhiza

10 New Insights into Ectomycorrhizal Symbiosis Evolution and Function

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CONTENTS

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I. Introduction

There is increasing interest world-wide in tree plantations, because of their use in the economy (wood, paper, resin), ecology (carbon sequestration), and bioenergy (source of heat and conversion of cellulose in biofuel) (Biswas et al. 2011; Harfouche et al. 2011; Oliver et al. 2009; Seguin 2011). The wood industry is interested in highly productive trees, as the growth time before harvesting trees is long, often as much as 50–60 years (even longer for oaks). In this context, every component impacting growth rate, i.e., tree productivity, is studied. In nature, roots of trees are associated with both endomycorrhizal and ectomycorrhizal soil-borne fungi (Smith and Read 2008). This symbiotic association is crucial for forest ecosystems, and is an adaptation to low fertility of forest soils in terms of their physio-chemical properties and, in particular, the low bioavailability of nutrients for plants (Tibbett 2000). In addition, forest soils are inhabited by a vast complexity of microorganisms and fauna, and the root system of an adult tree is associated with a cortège of ECM fungal species (Newton and Haigh 1998). **Development and implementation of environmentally friendly tree plantations (i.e., with limited or even no use of nitrogen fertilizers) has to be mastered by optimizing the ecosystem services provided by ECM fungi.**

Despite their ecological importance, symbiosis between eukaryotic organisms, such as that occurring between plant cells and ECM fungi, is far less understood than bacterial symbiosis, partly because of the complexity of eukaryotic cells and their multicellularity. The mutualistic

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interaction is driven by a fair-trade exchange of nutrients (Martin and Nehls 2009). The hyphal networks assimilate organic nitrogen and phosphorous trapped in organic polymers from the soils, and supply the plant root cells with their nitrogen and phosphorous needs (Chalot and Plassard 2011). In return, the plant cells provide carbon derived from their photosynthesis activity, up to 20 % of photosynthesis products (Leake et al. 2004; Nehls et al. 2010). Building a functional ectomycorrhizal organ depends on physical and cellular changes on both sides. Hyphal cells are active in division, aggregate to form the mantle, and penetrate within the apoplast of the root cells to develop the Hartig net. The root system in contact with hyphae displays an increased number of lateral roots, and mantle-embedded root tips arrest their growth. At the same time, plant cells in direct contact with the fungus loosen their connection to one another (for a review see Martin et al. 2007). Proper development of the ECM organ depends on hormones produced by the fungi or the plant such as auxins and ethylene (Felten et al. 2009; Jambois et al. 2005; Splivallo et al. 2009).

Molecular mechanisms driving ECM development and functioning are receiving renewed attention because of the enormous effort in fungal genome sequencing (Martin et al. 2011). As of now, analysis of the genomes of two non-phylogenetically related ECM-fungi, *L. accaria bicolor* (Basidiomycotina, Agaricomycotina, Agaricales, Hydnangiaceae) and *Tuber melanosporum* (Ascomycotina, Pezizomycotina, Pezizales, Tuberaceae), have been published (Martin et al. 2008, 2010). There is a growing number of saprotrophic and ECM fungal genomes becoming publicly available (Grigoriev et al. 2011; Martin et al. 2011). The present paper aims to highlight **how comparative genomics using saprotrophic, pathogenic, and mutualistic fungi as well as functional genomics have either strengthened or changed our understanding of the molecular mechanisms driving ECM development and functioning.** In this chapter, we summarize answers to several key questions: (i) how did mycorrhizal symbiosis evolve within the fungal kingdom? (ii) do symbiotic fungal genomes share hallmarks or what are the trade-offs? (iii) how are symbiotic fungi recognized as favorable intruders? and (iv) how can hyphae proliferate in host roots without eliciting plant defenses and do they control nutrient fluxes?

II. Evolution of the ECM Symbiosis Lifestyle Within the Fungal Kingdom

A. When Did the ECM Lifestyle Arise During Evolution?

Roots from the most ecologically and/or economically important tree species from Pinaceae, Fagaceae, to Fabaceae located in boreal, temperate, Mediterranean, and tropical forests interact with ECM fungi, often with several ECM species within the same root system. This type of symbiosis is more recent than the endomycorrhizal association involving Glomeromycota (arbuscular mycorrhizal fungi AMF). Symbiotic glomales within the fossil plant genus Rhynia have been recorded in the Early Devonian period (around 400 million years ago) (Remy et al. 1994) (Fig. 10.1). Mosses (the largest living group of bryophytes) displayed endophytic glomalean fungi within their thallus, as no real roots are formed in these plants (Rabatin 1980; Read et al. 2000). In addition, genes required for the formation of arbuscular mycorrhiza have been identified in all studied embryophyte lineages (Wang et al. 2010: 514–525). These data suggest that endomycorrhizal symbiosis played a crucial role in plant adaptation to land (Simon et al. 1993). It is therefore likely that the first terrestrial fungal organisms colonized land before plants did.

ECM fungi include at least 6,000 species, primarily belonging to the Dikarya clade (Basidiomycetes and Ascomycetes). True ECM fossils

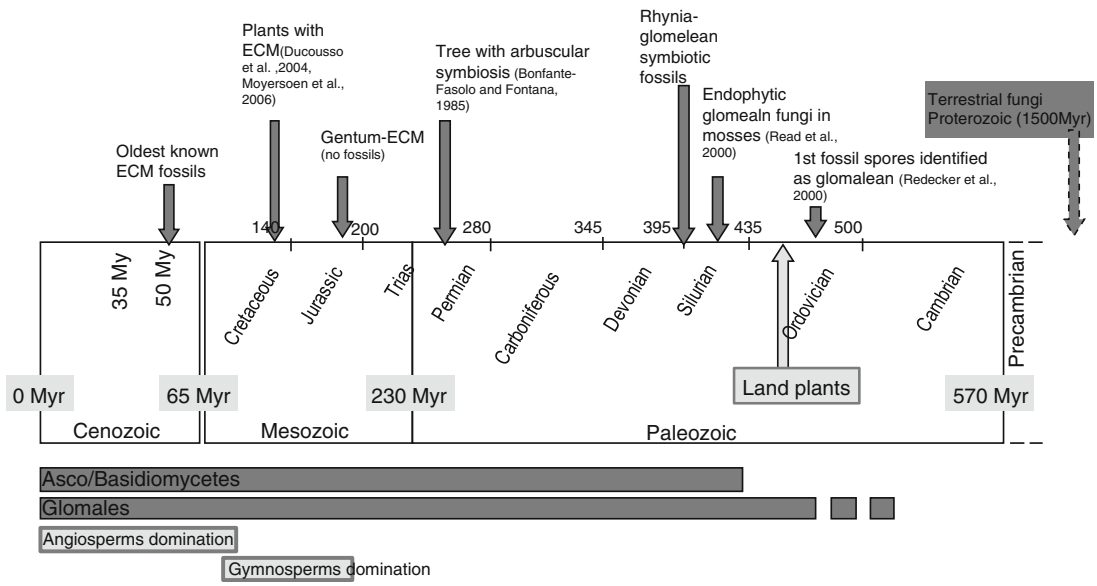

Fig. 10.1. Evolution of plant–fungi symbiosis. Approximate ages of plant and fungal lineages from fossil evidence and molecular phylogenies are indicated (Adapted from Brundrett (2002))

date from the Middle and Lower Eocene periods (lasting from 56 to 34 million years ago) (LePage et al. 1997; Beimforde et al. 2011) (Fig. 10.1). However, several studies suggest that ECM association may be older (around 150 million years ago). First, ECM fungi have been described within the Gnetales plant lineage (Brundrett 2002). Secondly, Hibbett and Matheny, using Bayesian relaxed molecular clock analyses, proposed that the first ECM interactions appeared between the Pinaceae gymnosperms and Boletales and/or Russulales fungi (Hibbett and Matheny 2009). Gnetales and Pinacae plant lineages existed in the early Jurassic (190 Myr) and early Cretaceous (120 Myr) eras respectively. In addition, one study suggests that the common ancestor of Dipterocarpoideae and Sarcolaenaceae was probably an ECM plant, and could be dated to before the separation of Madagascar from the India–Seychelles block, i.e., around 88 Myr (Ducousso et al. 2004). Another study presents evidence of an ECM status of a neotropical genus of Dipterocarpaceae, indicating that ECM fungi possibly evolved before the separation of South America and Africa that occurred 135 million years ago (Moyersoen 2006). It is highly probable that a wide diversification of ECM fungi appeared during the period of rapid angiosperm radiation in the Cretaceous (Brundrett 2002) (Fig. 10.1).

B. How Has ECM Symbiosis Evolved Within the Rhizospheric Network?

The ECM fungi are polyphyletic and interspersed with their saprotrophic or pathogenic relatives. In forest ecosystems, all of these fungi play integrated roles. Saprotrophic fungi are essential for degradation of cellulosic and lignin-rich material, and are composed of wood-decay fungi (white- and brown-rot fungi) and humicolous fungi (leaf litter decomposers). ECM fungi are essential for the nitrogen cycle (Smith and Read 2008) and improve plant nutrition, which impacts tree productivity, whereas pathogenic fungi could have a deleterious impact on forest health. The existence of a **continuum** between these three **fungal ways of life** was proposed nearly a decade ago (Jones and Smith 2004), and the enormous effort made in fungal genome sequencing confirms this trend (Veneault-Fourrey and Martin 2011; Plett and Martin 2011). As a consequence, distinct evolutionary scenarios exist to explain the origin of symbiosis across the fungal kingdom.

Research has been more thorough within the Agaricomycetes, as they contain the major nutritional modes (white and brown rot as well as ECM fungi). To be brief, several phylogenetic analyses conclude that the Agaricomycete is ancestrally saprotrophic, and multiple gains and losses of the ECM lifestyle occurred (Hibbett et al. 2000; James et al. 2006). A second scenario also infers that the Agaricomycete is ancestrally saprotrophic, but that only gains of the ECM lifestyle occurred (Tedersoo et al. 2010). A recent multi-gene phylogeny performed on the *Amanita* genus confirms this second scenario of stability of the ECM lifestyle. Indeed, in this study the authors showed that ECM *Amanita* species never reverted towards saprotrophy (Wolfe et al. 2012). The third scenario suggests that the ECM lifestyle could have been the ancestral condition of the Agaricomycetes and that many fungi reversed towards saprotrophy (Weiss et al. 2004). The authors investigated in detail the phylogeny of the Sebacinales, as this clade is the most ancient containing mycorrhizal fungi, and almost all representatives are ECM fungi (Weiss et al. 2004). Since their study, the genome of the Sebacinale mutualistic fungi *Piriformospora indica* has been released (Zuccaro et al. 2011).

Interestingly, the genome of *P. indica* presents similarities with several biotrophic fungal genomes (e.g., a reduction of secondary metabolism, presence of small secreted proteins during interaction, absence of nitrate uptake) and other characteristics common to saprotrophic fungi (e.g., expansion of plant-cell-wall-degrading enzymes, metalloproteases and the presence of carbohydrate-binding domain containing proteins) (Zuccaro et al. 2011). Therefore, *P. indica* might be an ancestral/endophytic fungus leading to saprotrophic or mutualistic ones. (Veneault-Fourrey and Martin 2011; Jones and Smith 2004)

Recently, comparative genomic analyses have been performed using the genomes of white rot, brown rot and ECM fungi (Eastwood et al. 2011; Martin et al. 2008, 2010; Martinez et al. 2009; Tang et al. 2012), and we are now able to have a clearer view of how ECM fungi have evolved. Brown-rot and ECM fungal genomes display parallel contractions of genes encoding enzymes acting on plant cell wall polysaccharides (Carbohydrate Active Enzymes: CAZymes), in particular enzymes able to degrade cellulose and xylans as well as class II peroxidases (lignin peroxidase, manganese peroxidase, versatile peroxidase, and "basal" low redox potential peroxidase), which are able to depolymerize lignin. A recent comparative genomic analysis specifies the reconstructed ancestor of the Agaricomycete as a white-rot fungus containing lignin-degrading peroxidases (Floudas et al. 2012). Peroxidase gene families have then been independently contracted or even extinguished within the brown-rot fungi clade and the ECM clade (Floudas et al. 2012). To conclude, **ECM fungi experienced extensive gene loss of lignocellulose decay machinery through the losses of extracellular enzymes required for efficient saprotrophic ability such as CAZymes and lignin-degrading enzymes**. Consequently, these fungi are probably dependent on plant associations for their carbon needs, and reversals from an ECM way of life towards a saprotrophic one, as suggested in the past, are thus unlikely to occur.

III. Evolution Towards Symbiosis Through Transposon Proliferation and Gene Duplication?

Both genomes of the ECM fungi *L. bicolor* and *T. melanosporum*, with 60 and 125 MB respectively, are amongst the largest fungal genomes, and display an increased size when compared to saprotrophic fungal genomes (Raffaele and Kamoun 2012). **This increased size of genome is due to transposon proliferation**, making up 25 % of the *L. bicolor* genome and 58 % of the *T. melanosporum* genome. Interestingly, transposon proliferation seems to be a common trend in the genomes of plant associated biotrophic fungi (Baxter et al. 2010; Duplessis et al. 2011; Spanu et al. 2010; Spanu 2012). It has been hypothesized that expansion of transposable elements (TEs) may be a way of accelerating evolution by allowing gene expansion (Spanu et al. 2010), diversification of genes involved in the interaction (Rouxel et al. 2011), and gene deletion, and/or even in some cases it may be at the origin of horizontal gene transfer (Keeling

and Palmer 2008). With regard to symbiotic fungal genomes, transcriptomic analyses has shown that some TEs are expressed and thus still active in both symbiotic tissues and mature fruiting bodies. The question of the exact role of transposable elements in the evolution and regulation of "symbiotic genes" remains to be addressed. Population genomic approaches leading to an estimate of the intraspecific diversity are required, and should help to assess this proposed genetic plasticity (Hollister et al. 2011).

In addition to a large number of transposons, the size of the *L. bicolor* genome, containing around 23,000 protein-encoding genes, is due to the **expansion of lineage specific multigene families** (Martin et al. 2008). Involvement of transposable elements in gene duplication has been suggested for the *L. bicolor* hydrophobin-encoding genes (Plett et al. 2012). Strikingly, gene family expansions have been found within genes involved either in protein–protein interactions (e.g., ankyrin repeats) or in signaling (e.g., Tyrosine-like kinases, RAS small GTPase) (Martin and Tunlid 2009). In addition, expression of some of these lineage multi-gene families is induced in symbiotic tissues (Martin et al. 2008), making them good candidates to be "symbiotic genes". These symbiosis-related genes may have appeared after neo-functionalization or sub-functionalization of the duplicated genes. However, while the *L. bicolor* genome has evolved towards lineage-specific multi-gene families (probably containing SR genes), the *T. melanosporum* genome contains a limited set of genes with 7,496 protein-encoding genes, and only very few genes that arise from duplication (Martin et al. 2010). Interestingly, genomes of ascomycete biotrophic fungi with a restricted number of plant hosts also display a very limited gene repertoire (Spanu et al. 2010; Spanu 2012). This restricted number of genes may thus be a hallmark of restricted host specificity within Ascomycetes. It has been suggested that fungal species lacking gene duplication may be more constrained in adapting to changing environments (Cuomo et al. 2007). However, in yeast cells, mutations in the number of tandem repeats found within promoters of genes may affect their gene expression, leading to a rapid response to variable environments (Vinces et al. 2009). Along with this study, Murat et al., showed that in the *T. melanosporum* genome, the vast majority of SSRs are found within non-coding regions (Murat et al. 2010). It would be of interest to test the same hypothesis as in yeast in the regulation of *T. melanosporum* gene expression.

At this point, it is tempting to speculate that **genome evolution towards symbiosis is different between Ascomycete and Basidiomycete fungi, the former displaying an extensive number of transposons and the latter favoring gene duplications.** In addition, the degree of gene duplication within genomes may explain the spectra of plant hosts with limited gene duplication correlating with narrow plant host spectra (e.g., T. *melanosporum*, host-specific symbiont) and vice-versa (e.g., *L. bicolor* as a generalist symbiont). The recent release of several ECM Agaricales genomes (*Hebeloma cylindrosporum*, *Paxillus involutus*, *Laccaria amethystina*, *Piloderma croceum*, etc.: http://mycor.nancy.inra.fr/genomeResources.php) reveals that the vast majority of them encode a high number of genes with multigene families (Francis Martin, personal communication).

Large-scale fungal genome sequencing projects will soon provide novel insights into symbiosis evolution and traits associated with the lifestyle of ECM fungi, but there is still limited information available on host responses to mycorrhizal fungi in infection.

IV. How Are Symbiotic Fungi Recognized as Favorable Intruders?

Establishment of ectomycorrhiza requires a switch from saprotrophic free-living mycelium in soil to hyphae in intimate contact with the apoplast of root cells–two contrasted ways of life. First, fungal hyphae from the soil grow towards host root cells and encompass short lateral roots to form the mantle. Mycelia then

colonize the apoplastic space forming the Hartig net, the symbiotic interface where efficient nutrient exchanges as well as molecular dialogue take place (Peterson and Massicotte 2004).

Plant cell walls consists of a complex network of polysaccharides, composed mainly of cellulose, hemicellulose (xyloglucan, galactomannan, arabinoxylan), and pectins (homogalacturonan, rhamnogalacturonan). On the other hand, fungal cell walls are composed of chitin, beta-1,6-glycans, and mannoproteins.

Some of these compounds, as well as their hydrolysis products, are able to elicit plant defense responses (Hahn 1996). For example, chitin and its hydrolysis products are considered as microbial associated molecular patterns (MAPMs) that induce plant defenses via chitin-receptor-like kinases (Monaghan and Zipfel 2012; Schwessinger and Ronald 2012). During fungal growth, ECM fungi continuously produce chitin-related elicitors as demonstrated using the ECM fungus *Hebeloma crustuliniforme* in contact with spruce cells (Salzer et al. 1996; Sirrenberg et al. 1995). The plant defense responses are similar to those produced during attack by a pathogenic microbe, but the intensity of the responses are weaker. In addition, several transcriptomic analyses during ectomycorrhiza formation have revealed a transient and low-level induction of plant defense responses (Duplessis et al. 2005; Larsen et al. 2011; Le Quéré et al. 2005; Morel et al. 2005; Voiblet et al. 2001). Consequently, **ECM fungi have developed strategies to both avoid as much as possible the trigger of plant defense responses and to suppress or evade those induced host responses.** Here, we will review evidence we obtained to explain the methods ECM fungi use to successfully silence their entry into roots, and how they probably control the plant immune system and nutrient transport (Fig. 10.2).

A. Cell Wall Integrity: "Sentinels" for Symbiosis Development andFunctioning?

Mycelia colonize the apoplastic space to form the Hartig net, the interface where efficient nutrient exchanges as well as molecular dialogue take place. Microscopic analysis of the symbiotic interface has shown that fungal and plant cell walls are totally integrated and display alteration within their composition (Balestrini et al. 1996; Bonfante et al. 1998; Dexheimer and Pargney 1991; Tagu and Martin 1996). Thus, **both fungal and plant cell walls are probably remodeled to form the symbiotic interface.** Among the enzymes required for fungal and plant cell wall remodeling, carbohydrate-active enzymes (CAZymes) are the most important, as they are responsible for the biosynthesis, degradation, and modification of oligo- and polysaccharides as well as glycoconjugates. Recent genomic analyses have revealed that **ECM fungi have a reduced number of plant-cell-wall-degrading enzymes** (Martin et al. 2008, 2011). In particular, enzymes able to degrade cellulose and xylans (GH6, GH7) are absent from the *T. melanosporum* and *L. bicolor* genomes, suggesting that ECM fungi have poor or no saprotrophic growth on complex cellulosic materials. Interestingly, a decreased number of cellulose-degrading enzymes has been observed in the ECM fungus *Amanita bisporigera* (Nagendran et al. 2009) and in obligate plant-pathogens (Baxter et al. 2010; Duplessis et al. 2011; Spanu et al. 2010). The evolution of obligate ECM species in the *Amanita* genus correlates with the losses of genes encoding endoglucanases and cellobiohydrolases, and the subsequent inability to grow on such compounds (Wolfe et al. 2012). However, the *L. bicolor* genome contains only one carbohydrate- binding domain, CBM1 fused to a putative glucanase GH5, and its expression is induced during symbiosis (Martin et al. 2008, Veneault-Fourrey, unpublished). The *T. melanosporum* genome contains two CBM1 domains, with one fused to a candidate beta-glycosidase related to endoglucanase GH61. Both GH5 and GH61 might display weak cellulolytic activity. Cell wall integrity sensing is one mechanism by which plants may detect pathogen attack (as discussed above). In addition, *Arabidopsis thaliana* with a mutation to the cellulose synthase gene CeSA3 displayed a defect in cellulose content that led to constitutive plant defense responses, in particular,

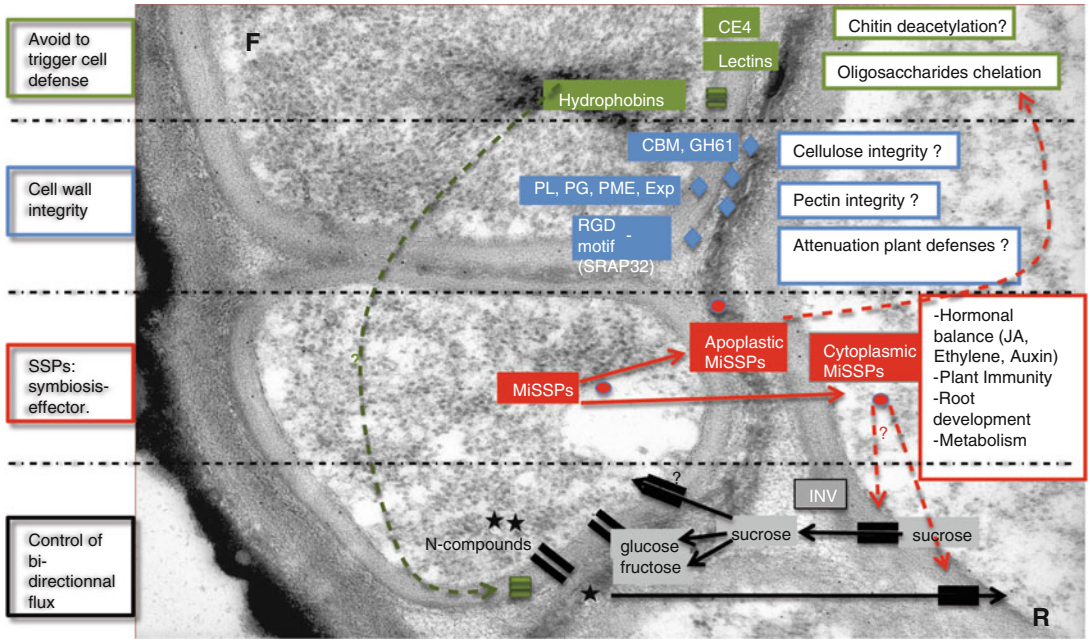

Fig. 10.2. Development and functioning of ECM: outline of how fungal molecules and/or proteins control the plant immune system and nutrient transport. (**a**) Different fungal proteins may be useful in order to avoid the trigger of plant defense responses: the enzyme chitin deacetylase changing fungal cell wall composition and lectins and hydrophobins, supposed to chelate oligosaccharides that may elicit plant defense responses. (**b**) Fungal comparative genomics highlight the few number of CAZymes acting on plant cell wall in ECM fungi. However, a few probably acting on middle lamella are present (*PL*: pectin and pectate lyase; *PG*: polygalacturonase; *PME*: Pectin methylesterase; *Exp*: expansin), and may be required to help fungal colonization within the apoplast. In addition, several enzymes acting on cellulose are still present, and led to the question of the role of cellulose integrity within mutualistic symbiosis. (**c**) Symbiotic fungi use effectors called MiSSPs that can be either apoplastic or cytoplasmic. It still remains to be proved which plant cellular processes are targeted, and how these MiSSPs control the development and functioning of ECM. Recent data in *L. bicolor* suggest a control of hormonal balance and root development. (**d**) ECM symbiosis is characterized by a reciprocal nutrient exchange. Nitrogen-compounds are transferred from the fungal cells in a form assimilable by the plant cells. Sucrose derived from plant photosynthesis activity is clived by invertase (*INV*) in the symbiotic interface, and fungal hexoses uptake the released glucose. Putative crosstalk between these four mechanisms are indicated by *arrows in dotted-line*. *F*: fungal cells. *R*: Root cells

jasmonic acid and ethylene signaling (Ellis et al. 2002). This constitutes the first study linking hormonal balance and plant cell wall integrity. A similar role has been demonstrated in rice (Vega-Sanchez et al. 2012).

Cell wall integrity is also maintained through connections that hold the plasma membrane and plant cell wall together. Addition of Arg-Gly-Asp (RGD) peptides disrupts the adhesion between the plasma membrane and plant cell wall, leading to a decrease in plant defense responses (Canut et al. 1998; Gao et al. 2007; Schindler et al. 1989). In RGD-treated cowpea plants, a decrease in plant defense responses is correlated to better fungal colonization efficiency (Mellersh and Heath 2001). Interestingly, a symbiosis-regulated acidic polypeptide (SRAP32) containing the RGD motif from *Pisolithus tinctorius* is highly expressed 3 days post-contact between fungal hyphae and *Eucalyptus globus* roots (Laurent et al. 1999). At that time, the authors proposed that SRAP32 might be involved in the hyphae adhesion and aggregation. However, the corresponding proteins also localized in fungal cell walls of the Hartig-net-forming hyphae, suggesting a possible role in diminishing plant defense responses. The RGD motif is found in extracellular proteins such as the mammalian

glycoproteins fibronectin and vitronectin that reside in the extracellular matrix. Such extracellular proteins bind to transmembrane receptors such as integrins, relaying information from extracellular areas into intracellular signals. SRAPs might then have a dual role, adhesion of fungal hyphae to the host and communicator-relay to attenuate plant immunity, but no experimental evidence exists to give clues to their precise role. SRAPs are thus probably the first symbiotic effectors (discussed below) discovered.

Both the *T. melanosporum* and *L. bicolor* genomes are lacking in pectin and pectate lyase genes (PL1, PL3, PL4, PL9 and PL11 families), but still contain candidate genes for polygalacturonase (GH28) and pectine methylesterase (CE8) (Martin et al. 2008, 2010), suggesting a **possible progression of ECM fungi within pectin-rich middle lamella**. In addition, the *T. melanosporum* genome contains hemicellulases from the GH10 and GH43 families that are absent in *L. bicolor*. Instead, *L. bicolor* contains 12 expansin-encoding genes, hypothesized to loosen the non-covalent links between hemicellulose strands on cellulose microfibrils. Eight expansin-encoding genes of the 12 are regulated throughout ectomycorrhiza formation (Veneault-Fourrey et al., unpublished data). This trend is confirmed in other publicly available genomes of ECM fungi (Francis Martin, personal communication, http://mycor.nancy.inra.fr/genomeResources.php).

All together, the current view is that **biotrophic interaction with plant cells leads to convergent losses of CAZymes acting on plant cell walls (mainly cellulose or hemi-cellulose), and leaves a minimal enzymatic arsenal for intercellular colonization.** We hypothesized that this may be in order to **maintain plant cell wall integrity as much as possible, to avoid trigger of plant defense responses either by the release of elicitors or by unbalancing cellulose content.** However, a continuum in gene number exists among ECM taxa, with some having a few members of lignocellulose decay enzyme machinery, and others experiencing a complete loss. It is not yet determined whether the maintained genes (which are expressed in ECM tissues or free-living mycelium in the case of *L. bicolor*) confer some capacity to attack lignocellulose or other not yet discovered functions. **Complete loss of the plant cell wall decay machinery would lead to a high dependence of the fungal symbiont on plant cells in order to retrieve carbon, and thus might lead to obligate ECM fungal species.**

B. Avoiding the Triggering of Plant Defense Responses

To avoid the triggering of plant defense responses, ECM fungi may first convert surface-exposed chitin into chitosan, as chitosan is less sensitive to plant chitinase action. Such a conversion may be mediated by fungal chitin deacetylase (CE4), the expression of which is induced in *L. bicolor* and *T. melanosporum* during ECM formation (Martin et al. 2008, 2010). In addition to this possible conversion, it has been demonstrated that plant chitinases secreted into the apoplastic space may inactivate chitin elicitors produced by ECM fungi by cleaving them into monomeric N-acetyl glucosamine units (Salzer et al. 1996, 1997a, b; for a review see Salzer et al. 2001). This suggests a possible feed-back loop on the part of the plant, to reduce its own defense response when in contact with beneficial organisms. More recently, De Jonge et al. showed that Ecp6 produced by the plant pathogenic fungus *Cladosporium fulvum* is able to bind oligomeric chitin fragments, leading to ligand competition between Ecp6 and plant receptors, and consequently preventing host immune activation (de Jonge et al. 2010). Fixation of Ecp6 to chitin fragments occurs through its LysM domains. Ecp6 homologues also bind chitin and block chitin-inducible plant defenses (Marshall et al. 2011; Mentlak et al. 2012), suggesting conserved mechanisms amongst plant pathogenic fungi. A LysM protein from *T. melanosporum* is up-regulated during ECM formation, and might play a similar role. LysM proteins found in both microbes and plants are known to mediate binding to GlcNAc-containing glycans (Buist et al. 2008; Gust et al. 2012), and may act as a ligand or may promote modification of GlcNAc-containing substrates when this domain is linked to secreted enzyme. In the same line, lectins and lectin-like proteins can perturb

host immunity by chelating oligosaccharides released from the cell walls of both partners, and in this way may promote fungal progression within plant tissues. For example, the lectin produced by the ECM fungus *Lactarius deterrimus* has identified receptor sites in spruce root cells, in particular root hairs and the tips of lateral roots (Giollant et al. 1993). Transcriptomic analysis in ECM tissues revealed an over-expression of C-lectins in both *L. bicolor* and *T. melanosporum* (Martin et al. 2008, 2010).

Other molecules such as hydrophobins are localized on the outer surface of fungal cell walls as a result of their biochemical properties. Hydrophobins confer water-repellent properties on fungal structures as their hydrophobic side is exposed while their hydrophilic surface is bound to the cell wall. Indeed, hydrophobins are known to bind to fungal beta-1,3-D-glucans and chitins (Sharp et al. 1984a, b, Roby et al. 1987; Templeton et al. 1994), and thus may act as 'stealth' factors, protecting the invading fungal hyphae from active plant defenses (Aimanianda et al. 2009; Spanu 1997). Hydrophobins are ubiquitous proteins, as all fungi possess them in differing quantities.

"Interestingly, two hydrophobins from the ECM fungus *Pisolithus microcarpus* (formerly *P. tinctorius*), *hydPt-1* and *hydPt-2*, are up-regulated during the early stages of plant colonization (Duplessis et al. 2001, 2005; Tagu and Martin 1996; Voiblet et al. 2001). Similarly, increased accumulation of hydrophobin transcripts was observed in *Paxillus involutus/Betula pendula* ectomycorrhiza (Le Quéré et al. 2005). In addition, involvement of hydrophobins in plant pathogenicity has been demonstrated (Kershaw and Talbot 1998)." The genome of *L. bicolor* S238N-H82 encodes 12 hydrophobin genes with characteristics of class I hydrophobin genes and no chimeric class I/class II hydrophobins (Plett et al. 2012). Some of these genes are up-regulated during ECM formation, but a different set is expressed depending on the nature of the plant host (high versus low mycorrhiza ability), suggesting an adaptation of the hydrophobin repertoire to the plant hosts.

The exact roles of hydrophobins in symbiosis remain to be elucidated, and other additional hypotheses could be proposed. For example, hydrophobins may also be required for hyphae adhesion to the host surface as well as for hyphae aggregation (Keshaw et al. 1998; Whiteford and Spanu 2002; Whiteford et al. 2004; Wosten 2001), and thus they may be important to maintain the cohesion of mantle-forming mycelium. In the context of lichen symbiosis, it has been proposed that the fungal partner secretes the hydrophobin monomers, which may diffuse into the apoplast and thus, according to their hydrophobic properties, surround both partners in the symbiosis. The authors hypothesized that this hydrophobic layer may allow for efficient apoplastic transport of water and solutes between the two partners of the symbiosis, as well as permit optimal gas exchanges (Wessels 2000). Exploring such a role for fungal hydrophobins in ECM formation would be of interest in order to assess what makes an ECM functional, i.e., an organ leading to fair-trade exchanges.

We cannot as yet conclude which **strategies ECM fungi use to avoid triggering of plant defense responses**, but can only speculate on different hypotheses: **deacetylation of chitin, or use of lectins or small-secreted proteins able to bind oligosaccharides. Extensive functional analysis is required to address the exact role of hydrophobins: whether they are involved in fungal cell attachment, or in the development of a functional symbiotic interface.**

C. How Do Symbiotic Fungi Control Plant Immunity?

Even though ECM fungi display several mechanisms to avoid recognition by the plant cell and release of elicitors as described above, a transient expression of plant defense responses occurs during the first steps of ectomycorrhiza formation. This suggests that an additional level of control has been developed to control plant immunity. It is now well-known that plant pathogenic microbes use a wide range of small secreted proteins called effectors, either acting in the apoplast or entering plant cells to alter metabolism and immunity in order to enhance microbe growth and survival within plant tissues (Rafiqi et al. 2012; Zamioudis and Pieterse 2012).

Recent studies have demonstrated that similar effectors exist also in mutualistic fungi such as the ectosymbiont *L. bicolor* and the endosymbiont *Glomus intraradices* (*Rhizophagus irregulare)* (Kloppholz et al. 2011; Plett et al. 2011).

When the genome of *L. bicolor* was deciphered, a dozen small secreted proteins upregulated expression during symbiosis were discovered, and were called MiSSPs for 'mycorrhiza-induced small secreted proteins' (Martin et al. 2008). Interestingly, the arsenal of LbMiSSPs is different depending on the associated plant host, suggesting that *L. bicolor* is able to receive signals from the host, and adapts its arsenal to initiate molecular dialogue and symbiosis establishment. The first functional characterization was carried out on MiSSP7, the most highly regulated MiSSP in *L. bicolor,* during establishment and maintenance of mycorrhizal root tips. MiSSP7 is a 7 kDa protein bearing no homology to any characterized protein. MiSSP7 expression is triggered by root exudates (Plett and Martin 2011). MiSSP7 RNA-silencing strains of *L. bicolor* are impaired in fungal colonization and ECM formation on both poplar and Douglas pine. Upon secretion of MiSSP7, it enters the plant cell via PI-3-P mediated endocytosis and localizes in nuclei (Plett et al. 2011). Poplar genes encoding for proteins involved in cell-wall modifications and oxidative stress are regulated by the MiSSP7 localized in nuclei (Plett et al. 2011). Recent findings indicate that MiSSP7 interacts with the jasmonic acid signaling pathway through its direct action on the jasmonate receptor protein JAZ (jasmonate zim domain) (Plett et al., unpublished).

Interaction of a symbiotic effector with hormonal balance was also found for the more ancient AM fungi *G. intraradices* and its protein SP7 (Kloppholz et al. 2011). SP7 enters the plant cell and localizes to the plant nucleus, where it interacts with ethylene response factor19 (ERF19) to repress plant defense signaling (Kloppholz et al. 2011). When SP7 is expressed in the hemibiotrophic fungus *Magnaporthe oryzae*, the length of the biotrophic phase is prolonged, and hypersensitive response is suppressed. Interestingly, recent studies highlight the conserved role of plant hormones, in particular ethylene, in mutualistic interactions. First, Camehl and co-workers showed that ethylene signaling components are required for symbiosis establishment between *Arabidopsis thaliana* and *P. indica* (Camehl et al. 2009). At the same time, the ethylene-signaling network is required for auxin to help in fungal colonization of lateral roots (Splivallo et al. 2010). Thus, the role of ethylene in mutualistic interactions seems to be complex, and the cross-talk between plant hormones when roots are challenged with mutualistic infection still needs to be addressed.

These studies first reveal that **ECM** and AM **fungi use small secreted proteins in order to control plant immunity, as plant pathogenic microbes do**. Secondly, **mutualistic effectors are likely to control plant immunity by governing hormonal balance.** In addition to effectors being able to enter the plant cell, other MiSSPs have been characterized in *L. bicolor* with one, MiSSP8, being apoplastic and also required for symbiosis development (Veneault-Fourrey, unpublished). Because of their localization, these apoplastic effectors probably constitute the first line of effectors, and should receive renewed attention in the next few years. All of these analyses reveal that **both mutualistic and pathogenic fungi use the same ways of communication through SSPs with plants**, even if the outcome of the interaction is different. It remains then a challenge to discover key molecular players leading to fair-trade nutrient exchanges, as exemplified in symbiosis versus unidirectional nutrient fluxes in pathogenic interactions.

D. How to Control Efficient and Bi-directional Nutrient Exchanges

Nutrient shuffling between symbiotic partners has been extensively studied in the past years (for a more recent review see, Chalot and Plassard 2011; Doidy et al. 2012; Nehls et al. 2010); however, the form(s) of nitrogen compounds and carbohydrates used for nutrient transfer is/are still under debate (Chalot et al. 2006; Doidy

et al. 2012), and identification of the key fungal and plant transporters is still lacking. In this section, we will summarize the data and hypotheses about how the fair-trade exchange is maintained between ECM fungi and their host plants.

Identification in *Medigaco truncatula* of a phosphate transport protein MtPT4 indispensable for acquisition of inorganic orthophosphate (Pi) by the plant cell, but also for arbuscule maintenance within root cells, suggests that Pi delivery by the fungal endosymbiont is a signal to establish and maintain arbuscular symbiosis (Javot et al. 2007). Interestingly, in *mtpt4* mutants, arbuscules degenerate prematurely, even if the fungus still has access to carbon. However, if the *mtpt4* mutants encounter nitrogen limitation, arbuscule cell death is suppressed (Javot et al. 2011), suggesting a regulation of the function of endomycorrhiza by nitrogen and Pi availability. It is thus likely that when the plant experiences low Pi and N availability, the N delivered by the fungal endosymbiont is sufficient to trigger the responses leading to functional endomycorrhiza. The regulation by both partners of the mutualistic interaction has been recently highlighted by several studies. Indeed, it has been shown that plants can recognize arbuscular mycorrhizal fungi that transfer more phosphate, and reward them by giving them more carbon while, reciprocally, fungi can give more phosphate to plants that transfer more carbon (Kiers et al. 2011). In accordance with this, the authors showed that N uptake and transport are enhanced when C, in the form of sucrose, is delivered from the plant through the symbiotic interface (Fellbaum et al. 2012). Examining what regulates such a cooperative exchange in ECM symbiosis will greatly advance our understanding of ECM functioning. Up to now, we only have evidence that ectomycorrhiza are not established with silenced nitrate–reductase *L. bicolor* strains (Kemppainen et al. 2009), suggesting that symbiosis establishment requires efficient N-supply to the plant.

The symbiotic organ is viewed as a sink by the plant, as it uses up to 20 % of the photosynthate products. It is still unclear whether the sucrose can be taken up directly by the ECM fungi, or if hydrolysis is required (for a review, see Doidy et al. 2012). However, fructose and glucose (hydrolysis products of sucrose) can be accessed at lower concentrations than the sucrose itself. Most of the ECM taxa contain invertase encoding-genes, but they contain genes with homology to fungal sucrose-transporters (Parrent et al. 2009; Veneault-Fourrey and Martin 2011). A large percentage of epiphytic bacteria activate plant cell wall invertases through the production of 3-indole acetic acid (auxin) (Lindow and Brandl 2003). It is well-known that ECM fungi also produce auxin or analogues, and we can speculate that one role of this fungal auxin may be to control cell-wall invertases in order to get access to hexose sugars. A recent study shows that effectors produced and secreted through the type-II secretion system by the pathogenic bacteria *Pseudomonas syringae* pv. tomato strain DC3000 are able to regulate expression of SWEET genes, identified as glucose importers or exporters (Chen et al. 2010). The authors suggest that these effectors may control glucose efflux towards plant pathogenic bacteria (Chen et al. 2010). Interestingly, poplar SWEET orthologous genes are induced during ECM development (Brun et al., unpublished results).

Tight and reciprocal controls of nutrient exchanges through the regulation of key transporters or the production of molecules or proteins (effectors) by both partners is likely to occur to maintain long-term association between ECM fungi and root tips. The next research area will be to identify key molecular players in order to decipher the cellular response from both sides.

V. How Useful Are ECM Fungal Inoculums in Eco-biotechnology Applications?

A. Use in Forestry to Help Establishment and Growth of Tree Plantation and as Biofertilizers

There is renewed attention to tree planting and reforestation programs due to international policies of reducing our oil needs and replacing it with biomass resources such as wood. In addition,

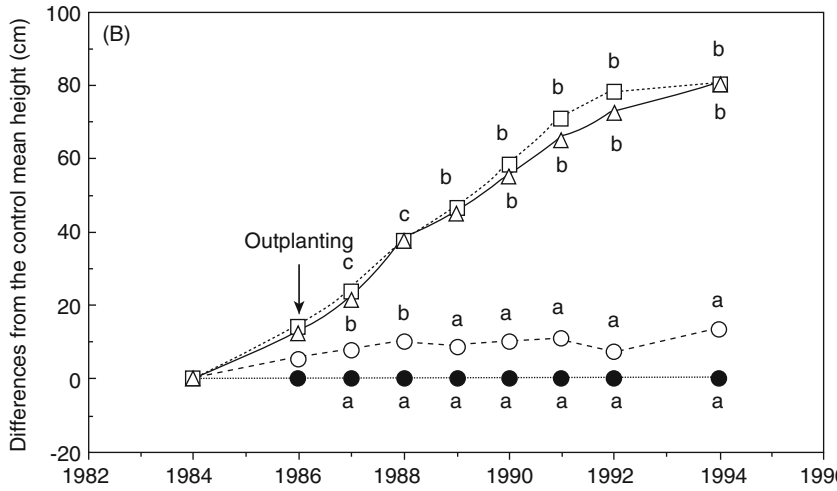

Fig. 10.3. Effect of ECM inoculum on tree growth. Growth of Douglas fir in nursery and 8 years after outplanting in different inoculation conditions: (○) native ECM fungi without soil fumigation; (•) native ECM fungi after soil fumigation; (Δ) seedlings inoculated with *L. bicolor* 81206 (French strain); (□) seedlings inoculated with S238N (American strain) (From Selosse et al. (2000), "© Canadian Science Publishing or its licensors"

wood constitutes an enormous sink for carbon sequestration. In nature, ECM fungi naturally interact with tree roots, improving both their mineral nutrition and resistance to pests, and leading to an enhanced tree growth rate (Smith and Read 2008). As a consequence, **ECM fungi have been used in nurseries in order to provide ectomycorrhizal seedlings with improved survival and growth rates in the field.**

Mycorrhization can be an advantage in **overcoming transplanting stress**, because of the protection of a seedling's roots from desiccation and the ability to explore a greater volume of soil for water and nutrient acquisition (Garbaye 2000; Smith and Read 2008). In addition, ectomycorrhizal seedlings grow better than non-inoculated ones, leading to a reduced time in producing seedlings suitable for outplanting. The first experiments showing the positive impact of ECM fungi in survival and plant growth (Fig. 10.3) were obtained with pines, as they are a broadly distributed and economically important forest species. Eight years after transplanting, Douglas fir (*Pseudotsuga menziesii)* seedlings inoculated with the ECM fungus *Laccaria bicolor* (Maire) P.D. Orton S238N (American strain) and *L. bicolor* 81306 (French strain) produced more wood (increase by 60 % of the total volume) than the control mycorrhized with natural inoculant (Selosse et al. 1998). However, an optimization is required between substrates and each tested ECM fungal species with *Quercus ilex*, in order to obtain a real impact on plant height and phosphorous content in leaves (Oliveira et al. 2012b). Studies on *Pinus pinea* and *Pinus pinaster* using either *Laccaria laccata*, *Pisolithus tinctorius*, or *Melanogaster ambiguus* showed that obtaining ECM seedlings requires an optimization for each tree–fungal species association, and that growth effects were dependent on both fertilization method and fungal species inoculated (Rincon et al. 2005, 2007). For example, inoculation with the ECM *Laccaria proxima* and *Thelephora terrestris* resulted in growth depressions of Sitka spruce 6 years after out-planting into natural soils with low additions of phosphate (Le Tacon et al. 1992), as the fungal strains may not have been well-adapted to the transplantation soil. The use of a mixture of ECM fungal species as inoculum on *Pinus pinaster* (maritime pine) increased the biomass of the plants (Oliveira et al. 2012a; Sousa et al. 2012a), suggesting that such a mix may be an alternative to evade the required optimization mentioned above. **The best field-planting results have been obtained on adverse sites** such as coal spoils and poor reforestation

sites, or in semi-arid regions (Mediterranean climate) or burned soils (Castellano 1994; Sousa et al. 2011).

ECM fungi are also known to improve nitrogen nutrition of their host plants, and are able to use organic sources of nitrogen not available to plants. Consequently, **ECM fungi may be used in order to decrease fertilization inputs and should be included in the management of nursery practices** (Sousa et al. 2012a; Oliveira et al. 2012a, b). In addition, production of selected tree genotypes requires, in some cases, efficient vegetative propagation. Several studies have shown the potential of using ECM fungi in conifer vegetative propagation (for a review, see Niemi et al. 2004), as ECM fungi facilitate root formation and subsequent root branching, as well as increasing resistance to water deficiency encountered during ex-vitro transfer.

As a conclusion, ECM fungi may improve tree performance following planting for reforestation. However, success depends on the ecological context and requires optimization between tree and fungal genotypes as well as substrates.

B. Production of Edible Fruiting-Bodies

Most of the ECM fungi display a sexual reproduction phase, leading to the production of fruiting bodies. Some fruiting bodies may be used as food products (Boletus, Truffles, Lactarius, Cantharellus, etc.), whereas others are toxic for animals and humans. The most famous application of controlled mycorrhization involves the "truffières", fields planted with oaks and/or hazelnuts mycorrhized with Tuber species (e.g., *T. melanosporum* for the delicate Périgord truffles). In contrast to the highly demanding "Périgord Truffle" (*T. melanosporum)*, the Burgundy truffle (*T. uncinatum*) can be cultivated in cold climates and soils with high C/N ratio, and a pH between 7.0 and 8.0 or higher. In addition, *T. uncinatum* is less host-specialized, as it can be associated with roots of *Quercus petraea*, *Q. pubescens*, *Carpinus betulus*, *Ostrya carpinifolia* (in Italy), *Corylus avellana*, *C. colurna*, *Tilia* spp., or *Pinus nigra*. In addition, experiments in the field reveal that it is of interest to mix trees that rapidly produce truffles, such as hazels, with trees in which mycorrhizas will produce fruit-bodies for longer, such as oaks (Chevalier et al. 2002; Chevalier 2010). **The authors suggest that it is possible to reconcile reforestation programs with the cultivation of truffles, unless the soils do not contain a high level of concurrent ECM fungal propagules that might compete with the truffle mycelium** (Chevalier et al. 2002). Studies over 10 years of a Tuber plantation in Northern France reveal that free-living mycelium of the introduced ECM fungus is still growing within the soil. While the entire root system displays ectomycorrhiza, only 2 % are from Tuber mycelium. However, this is sufficient to still produce the fruiting bodies (truffles) (La Tacon, personal communication).

Trials have been conducted for chanterelle mushrooms (Danell 2002, 2005) and the authors have been able to obtain fruitbodies in the green-house. However, no commercial applications are available (Pilz et al. 2003). It seems that the main problem consists of the persistence of the fungal inoculum within the soils as a result of harsh competition either by other native ECM fungi or other microbes.

C. ECM Fungi as Helpers for Bioremediation

As ECM fungi are native soil-borne fungi, they may be confronted by soils contaminated either by metals or organic chemicals. The potential role of ectomycorrhizal associations for bioremediation has been discussed over the years and the conclusions are still conflicting.

ECM fungi isolated from unpolluted soils are able to degrade various POPs such as PCBs, polyaromatic hydrocarbons, and TNT (Meharg and Cairney 2000) during axenic growth, but only a small number of ECM species have been tested (Meharg and Cairney 2000). Growth under symbiotic conditions may lead to enhanced degradation of these pollutants as shown for the degradation of 2,4-dichlorophenol by *Pinus sylvestris* root tips colonized by *Paxillus involutus* or *Suillus variegatus* (Meharg et al. 1997). However, this is not the general case (Genney et al. 2004; Finlay 2008), and degradation of such

compounds may be due to degrader bacteria, as ECM mycelium may be a good matrix to support biofilms of such bacteria (Sarand et al. 1998).

It has been demonstrated that ECM fungi may grow as free-living mycelium on media containing heavy metals. The metal-tolerant fungi are able to solubilize metal better that the non-tolerant ones (Fomina et al. 2005). In addition to a higher capacity of solubilization, other extracellular and cellular mechanisms promoting metal tolerance in fungi have been proposed (Bellion et al. 2006; Blaudez et al 2000). Studies conducted on the influence of ECM fungi in metal uptake and translocation to plants revealed their ability to influence the process (Gadd 2007; Harms et al. 2011; Sousa et al. 2012b). However, it is not clear which mechanisms are employed during symbiosis, and how they can help the host plant to overcome metal stress. It has been suggested that ECM fungi may decrease the plant's uptake of toxic compounds and thus may serve as a biological filter/barrier, or alternatively they may increase the plant's metal uptake thus diminishing pollutant concentration in the soil (Leyval et al. 1997). **Improving reforestation programs aiming to use trees for phytoremediation needs to be based on the use of specific indigenous trees and associated ECM fungi**.

D. Selection of ECM Strains and Ecological Concerns

Whatever the application, selection of well-adapted fungal strains native to the soil condition (pH, C/N ratio, physico-chemical properties, and concentration of organic pollutants or heavy metals etc.) is required (Teste et al. 2004). **Selection of ectomycorrhizal strains for application in forestry is mostly based on the evaluation of symbiotic performance in small-scale experiments.** However, these strains are not necessarily well-adapted to the properties of the transplanted soils. For example, a high rate of mortality in ECM-inoculated seedlings of spruce may be observed after transplantation. In order to diminish this rate of mortality, Pennanen and co-workers tested ECM fungi isolated from 2- to 8-year-old healthy, well-growing spruce seedlings for their capacity to promote seedling growth in the early years of planting, and identified five well-adapted isolates (Pennanen et al. 2010 IMC9, Edinburgh, 2010). The same strategy is required in order to select the best-adapted ECM fungi in polluted areas. Indeed, distinct tolerance and accumulation of cadmium has been demonstrated for ECM fungi such as *Scleroderma citrinum (Sc)* isolated from polluted sites and two strains of *Pisolithus tinctorius (Pt*) commonly used in remediation but isolated from unpolluted sites. At high cadmium concentration, *Sc* displayed a higher biomass than the two strains of *Pt*. Whereas the tolerance index is higher for *Sc* than *Pt*, a higher accumulation of cadmium within *Pt* hyphae has been observed (Carrillo-González and González-Chavez 2012). In addition, mycorrhiza ability is plant-genotype-dependent, as demonstrated by quantitative trait loci associated with mycorrhization ability of poplar with *L. bicolor* performed in a F1 pedigree of a *Populus deltoides* (low mycorrhization ability) × P*opulus trichocarpa* (high mycorrhization ability) (Labbé et al. 2011). In the F1 population, a whole range exists with regard to mycorrhization ability. This indicates that genetic programs may improve finding of the best plant–fungal associations.

After selection of favorable fungi (for example, the ones that improve tree productivity), monitoring of ECM fungal persistence, competition, and succession dynamics between selected ECM fungi and the native fungal community is required. Attempts to introduce micro-organisms with biocontrol or bioremediation properties often fail because the inoculum encounters harsh competition within the ecosystem. Thus, controlled mycorrhization with the aim of improving tree growth needs to be limited to soils without a high level of natural inoculum, at least during the first years after transplanting. Indeed, the root systems of older trees display a cortège of different ECM fungal species, with a high level of functional diversity (Courty et al. 2010; Rineau and Courty 2010), and tree growth requires such a functional complex. Interestingly, 10 years after transplantation of Douglas fir inoculated with

L. bicolor S238N or 81306, the root system displayed very diverse ECM associations. The primary inoculum is still present and dominant, but it did not prevent association with other native ECM fungi.

Introduction of a fungal inoculum or plant in non-native areas may impact the ecosystem, as introduced species may become invasive (Jairus et al. 2011), or may be fertile with indigenous microbes impacting the genetic diversity. Several scientists in a joint publication have proposed that fungal isolates should be chosen from among the native ones or, if that is not possible, that ECM isolates should be selected for different traits such as a high ability to increase host-plant vigor, a high-level of host specificity, and a low ability for saprotrophic growth, in order to build a sustainable ecosystem (Schwartz et al. 2006).

VI. Conclusion and Future Research

As a result of large-scale genomic programs on symbiotic, pathogenic, and saprotrophic fungi, our understanding of ECM symbiosis evolution as well as ECM fungal biology have been improved. Comparative genomics have led to the conclusion that ECM and brown-rot fungi display convergent evolution towards a loss of lignocellulolytic decay machinery from a white-rot ancestral fungus. These losses in ECM fungi are unlikely to be reversible, and consequently reversion from symbiosis towards saprotrophy is unlikely to occur, in contrast with what was previously believed. We now think that ECM fungi derived from saprotrophic relatives. In particular, it was predicted that the reconstructed ancestor of the Agarycomycete would have been a white-rot fungus. ECM genomes also display a high number of transposable elements, as shown in the genomes of plant-pathogenic fungi. The exact role within evolution of these TEs is a difficult question to address. It is likely that genome resequencing on different strains under different selection pressure of the same fungal species may help to answer this question.

In addition to the fungal genome features, we are now in the middle of accumulating data on either the transcriptomic, metabolomic, proteomic, or nutrient fluxes covering all fungal life stages from the free-living mycelium to the mature ECM root tips, as well as the mantle and the Hartig net mycelium. We now need to interpret this massive amount of data and link them together using systems biology tools, to obtain models of functioning fungi and predict their evolution (for example, in a context of global changes). However, groups of candidate genes, molecules probably involved in symbiosis development and functioning, have been identified from these data, and need further investigation. In particular, lineage-specific mycorrhizal effector proteins have been discovered, and might be key molecules controlling either plant immunity, plant hormonal balance, remodeling of cell walls and/or nutrient fluxes. Research developed within the next decade will aim to decipher how symbiotic effectors take control of their hosts, and how the plant cells could react to avoid fungal over-colonization of the roots. In addition, the arsenal of MiSSPs expressed can differ from one host plant to another (e.g., different MiSSPs are expressed between *P. trichocarpa/L.bicolor* and *Douglas/L. bicolor* ECM root tips). Such molecules are thus good candidates to address the larger question of what the molecular basis of host specificity is small-secreted proteins are found within the genomes of saprotrophic and ECM fungi, indicating that these SSPs are probably required for the establishment of an interaction with the plant cells. This leads to the question of the ancestral role(s) and function(s) of these proteins in fungi.

Future research will thus bridge “omics” to ecology and system functioning to improve the efficiency of ECM mutualistic interactions, in order to maintain the sustainable development of forest ecosystems.

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References

Aimanianda V, Bayry J, Bozza S, Kniemeyer O, Perruccio K et al (2009) Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature 460:1117–1121

Balestrini R, Hahn MG, Bonfante P (1996) Location of cell-wall components in ectomycorrhizae of Corylus avellana and Tuber magnatum. Protoplasma 191:55–69

Baxter L, Tripathy S, Ishaque N, Boot N, Cabral A, Kemen E, Thines M, Ah-Fong A, Anderson R, Badejoko W et al (2010) Signatures of adaptation to obligate biotrophy in the Hyaloperonospora arabidopsidis genome. Science 330:1549–1551

Beimforde C, Schaefer N, Doerfelt H, Nascimbene PC, Singh H, Heinrichs J, Reitner J, Rana RS, Schmidt AR (2011) Ectomycorrhizas from a Lower Eocene angiosperm forest. New Phytol 192:988–996

Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol Lett 254:173–181

Biswas B, Scott PT, Gresshoff PM (2011) Tree legumes as feedstock for sustainable biofuel production: opportunities and challenges. J Plant Physiol 168:1877–1884

Blaudez D, Jacob C, Turnau K, Colpaert JV, Ahonen-Jonnarth U, Finlay R, Botton B, Chalot M (2000) Differential responses of ectomycorrhizal fungi to heavy metals in vitro. Mycol Res 104:1366–1371

Bonfante P, Balestrini R, Martino E, Perotto S, Plassard C, Mousain D (1998) Morphological analysis of early contacts between pine roots and two ectomycorrhizal Suillus strains. Mycorrhiza 8:1–10

Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. New Phytol 2002(154):275–304

Buist G, Steen A, Kok J, Kuipers OP (2008) LysM, a widely distributed protein motif for binding to (peptido)glycans. Mol Microbiol 68:838–847

Camehl I, Sherameti I, Venus Y, Bethke G, Varma A, Lee J, Oelmuller R (2009) Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. New Phytol 185:1062–1073

Canut H, Carrasco A, Galaud JP, Cassan C, Bouyssou H, Vita N, Ferrara P, Pont-Lezica R (1998) High affinity RGD-binding sites at the plasma membrane of Arabidopsis thaliana links the cell wall. Plant J 16:63–71

Carrillo-González R, González-Chavez MCA (2012) Tolerance to and accumulation of cadmium by the mycelium of the fungi *Scleroderma citrinum* and *Pisolithus tinctorius*. Biol Trace Elem Res 146:388–395

Castellano MA (1994) Current status of outplanting studies using ectomycorrhiza-inoculated forest trees. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. APS Press, St. Paul

Chalot M, Plassard C (2011) Ectomycorrhiza and nitrogen provision to the host tree. In: Polacco JC, Todd CD (eds) Ecological aspects of nitrogen metabolism in plants, Wiley, Hoboken. doi:10.1002/9780470959404.ch4

Chalot M, Blaudez D, Brun A (2006) Ammonia: a candidate for nitrogen transfer at the mycorrhizal interface. Trends Plant Sci 11:263–266

Chen LQ, Hou BH, Lalonde S, Takanaga H, Hartung ML, Qu XQ, Guo WJ, Kim JG, Underwood W, Chaudhuri B et al (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. Nature 468:527–532

Chevalier G (2010) The truffle of Europe (Tuber aestivum): geographic limits, ecology and possibility of cultivation. Osterreichische Zeitschrift fur Pilzkunde 19:249–259

Chevalier G, Gregori G, Frochot H, Zambonelli A (2002) The cultivation of the Burgundy truffle. In: Proceedings of the second international conference on edible mycorrhizal fungi, Christchurch, pp 0–12

Courty P-E, Franc A, Garbaye J (2010) Temporal and functional pattern of secreted enzyme activities in an ectomycorrhizal community. Soil Biol Biochem 42:2022–2025

Cuomo CA, Guldener U, Xu JR, Trail F, Turgeon BG, Di Pietro A, Walton JD, Ma LJ, Baker SE, Rep M et al (2007) The Fusarium graminearum genome reveals a link between localized polymorphism and pathogen specialization. Science 317:1400–1402

Danell E (2002) Current research on chanterelle cultivation in Sweden. In: Proceedings of the second international conference on edible mycorrhizal fungi, Christchurch, pp 0–4

Danell E (2005) The chanterelle: an edible ectomycorrhizal mushroom. In: Deshmukh SK, Rai MK (eds) Biodiversity of fungi: their role in human life. Science, Enfield, pp 329–339

de Jonge R, van Esse HP, Kombrink A, Shinya T, Desaki Y, Bours R, van der Krol S, Shibuya N, Joosten MH, Thomma BP (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science 329:953–955

Dexheimer J, Pargney JC (1991) Comparative anatomy of the host-fungus interface in mycorrhizas. Experientia 47:312–321

Doidy J, Grace E, Kuhn C, Simon-Plas F, Casieri L, Wipf D (2012) Sugar transporters in plants and in their interactions with fungi. Trends Plant Sci 17:413–422

Ducousso M, Bena G, Bourgeois C, Buyck B, Eyssartier G, Vincelette M, Rabevohitra R, Randrihasipara L, Dreyfus B, Prin Y (2004) The last common ancestor of Sarcolaenaceae and Asian dipterocarp trees was ectomycorrhizal before the India-Madagascar separation, about 88 million years ago. Mol Ecol 13:231–236

Duplessis S, Sorin C, Voiblet C, Palin B, Martin F, Tagu D (2001) Cloning and expression analysis of a new hydrophobin cDNA from the ectomycorrhizal basidiomycete Pisolithus. Curr Genet 39:335–339

Duplessis S, Courty PE, Tagu D, Martin F (2005) Transcript patterns associated with ectomycorrhiza development in Eucalyptus globulus and Pisolithus microcarpus. New Phytol 165:599–611

Duplessis S, Cuomo CA, Lin YC, Aerts A, Tisserant E, Veneault-Fourrey C, Joly DL, Hacquard S, Amselem J, Cantarel BL et al (2011) Obligate biotrophy features unraveled by the genomic analysis of rust fungi. Proc Natl Acad Sci USA 108:9166–9171

Eastwood DC, Floudas D, Binder M, Majcherczyk A, Schneider P, Aerts A, Asiegbu FO, Baker SE, Barry K, Bendiksby M et al (2011) The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. Science 333:762–765

Ellis C, Karafyllidis I, Wasternack C, Turner JG (2002) The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. Plant Cell 14:1557–1566

Fellbaum CR, Gachomo EW, Beesetty Y, Choudhari S, Strahan GD, Pfeffer PE, Kiers ET, Bucking H (2012) Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 109:2666–2671

Felten J, Kohler A, Morin E, Bhalerao RP, Palme K, Martin F, Ditengou FA, Legue V (2009) The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and *Arabidopsis* through auxin transport and signaling. Plant Physiol 151:1991–2005

Finlay RD (2008) Ecological aspects of mycorrhizal symbiosis: with special emphasis on the functional diversity of interactions involving the extraradical mycelium. J Exp Bot 59:1115–1126

Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B, Martinez AT, Otillar R, Spatafora JW, Yadav JS et al (2012) The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336:1715–1719

Fomina MA, Alexander IJ, Colpaert JV, Gadd GM (2005) Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi. Soil Biol Biochem 37:851–866

Gadd GM (2007) Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol Res 111:3–49

Gao H, Gong Y-W, Yuan Y-J (2007) RGD-dependent mechanotransduction of suspension cultured Taxus cell in response to shear stress. Biotechnol Prog 23:673–679

Garbaye J (2000) The role of ectomycorrhizal symbiosis in the resistance of forest to water stress. Outlook Agric 29:63–69

Genney DR, Alexander IJ, Killham K, Meharg AA (2004) Degradation of the polycyclic aromatic hydrocarbon (PAH) fluorene is retarded in a Scots pine ectomycorrhizosphere. New Phytol 163:641–649

Giollant M, Guillot J, Damez M, Dusser M, Didier P, Didier E (1993) characterization of a lectin from *Lactarius-deterrimus* – research on the possible involvement of the fungal lectin in recognition between mushroom and spruce during the early stages of mycorrhizae formation. Plant Physiol 101:513–522

Grigoriev IV, Cullen D, Hibbett D, Goodwin SB, Jeffries TW, Kubiek CP, Kuske C, Magnuson J, Martin F, Spatafora JW, Tsang A, Baker S (2011) Fueling the future with fungal genomics. Mycology 2 (3):192–209

Gust AA, Willmann R, Desaki Y, Grabherr HM, Nurnberger T (2012) Plant LysM proteins: modules mediating symbiosis and immunity. Trends Plant Sci. doi:10.1016/j.tplants.2012.04.003

Hahn MG (1996) Microbial elicitors and their receptors in plants. Annu Rev Phytopathol 34:387–412

Harfouche A, Meilan R, Altman A (2011) Tree genetic engineering and applications to sustainable forestry and biomass production. Trends Biotechnol 29:9–17

Harms H, Schlosser D, Wick LY (2011) Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Microbiol 9:177–192

Hibbett DS, Matheny PB (2009) The relative ages of ectomycorrhizal mushrooms and their plant hosts estimated using Bayesian relaxed molecular clock analyses. BMC Biol 7:13

Hibbett DS, Gilbert LB, Donoghue MJ (2000) Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. Nature 407:506–508

Hollister JD, Smith LM, Guo YL, Ott F, Weigel D, Gaut BS (2011) Transposable elements and small RNAs contribute to gene expression divergence between Arabidopsis thaliana and Arabidopsis lyrata. Proc Natl Acad Sci USA 108:2322–2327

Jairus T, Mpumba R, Chinoya S, Tedersoo L (2011) Invasion potential and host shifts of Australian and African ectomycorrhizal fungi in mixed eucalypt plantations. New Phytol 192:179–187

Jambois A, Dauphin A, Kawano T, Ditengou F, Bouteau F, Legué et F, Lapeyrie F (2005) Competitive antagonism between IAA and indole alkaloid hypaphorine must contribute to regulate ontogenesis. Physiol Plant 123:120–129

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J et al (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature 443:818–822

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ (2007) A Medicago truncatula phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 104:1720–1725

Javot H, Penmetsa RV, Breuillin F, Bhattarai KK, Noar RD, Gomez SK, Zhang Q, Cook DR, Harrison MJ (2011) Medicago truncatula *mtpt4* mutants reveal a role for nitrogen in the regulation of arbuscule degeneration in arbuscular mycorrhizal symbiosis. Plant J 68:954–965

Jones MD, Smith SE (2004) Exploring functional definitions of mycorrhizas: are mycorrhizas always mutualisms? Can J Bot 82:1089–1109

Keeling PJ, Palmer JD (2008) Horizontal gene transfer in eukaryotic evolution. Nat Rev Genet 9:605–618

Kemppainen M, Duplessis S, Martin F, Pardo AG (2009) RNA silencing in the model mycorrhizal fungus *Laccaria bicolor*: gene knock-down of nitrate reductase results in inhibition of symbiosis with *Populus*. Environ Microbiol 11:1878–1896

Kershaw MJ, Talbot NJ (1998) Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis. Fungal Genet Biol 23:18–33

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A et al (2011) Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. Science 333:880–882

Kloppholz S, Kuhn H, Requena N (2011) A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. Curr Biol 21:1204–1209

Labbé J, Jorge V, Kohler A, Vion P, Marcais B, Bastien C et al (2011) Identification of quantitative trait loci affecting ectomycorrhizal symbiosis in an interspecific F1 poplar cross and differential expression of genes in ectomycorrhizas of the two parents: *Populus deltoides* and *Populus trichocarpa*. Tree Genet Genomes 7:617–627

Larsen PE, Sreedasyam A, Trivedi G, Podila GK, Cseke LJ, Collart FR (2011) Using next generation transcriptome sequencing to predict an ectomycorrhizal metabolome. BMC Syst Biol 5:70

Laurent P, Voiblet C, Tagu D, de Carvalho D, Nehls U, De Bellis R, Balestrini R, Bauw G, Bonfante P, Martin F (1999) A novel class of ectomycorrhiza-regulated cell wall polypeptides in Pisolithus tinctorius. Mol Plant Microbe Interact 12:862–871

Le Quéré A, Wright DP, Söderström B, Tunlid A, Johansson T (2005) Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (Betula pendula Roth.) and Paxillus involutus (Batsch) Fr. Mol Plant Microbe Interact 18:659–673

Le Tacon F, Alvarez IF, Bouchard D, Henrion B, Jackson RM, Luff S, Parladé JI, Pera J, Stenström E, Villeneuve N, Walker C (1992) Variations in field response of forest trees to nursery ectomycorrhizal inoculation in Europe. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CAB International, Oxford, pp 119–134

Leake JR, Johnson D, Donnelly DP, Muckle G, Boddy L, Read D (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Can J Bot 82:1016–1045

LePage BA, Currah RS, Stockey RA, Rothwell GW (1997) Fossil ectomycorrhizae from the middle Eocene. Am J Bot 84:410–412

Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 7:139–153

Lindow SE, Brandl MA (2003) Microbiology of the phyllosphere. Appl Environ Microbiol 69:1875–1883

Marshall R, Kombrink A, Motteram J, Loza-Reyes E, Lucas J, Hammond-Kosack KE, Thomma BP, Rudd JJ (2011) Analysis of two in planta expressed LysM effector homologs from the fungus Mycosphaerella graminicola reveals novel functional properties and varying contributions to virulence on wheat. Plant Physiol 156:756–769

Martin F, Nehls U (2009) Harnessing ectomycorrhizal genomics for ecological insights. Curr Opin Plant Biol 12:508–515

Martin F, Tunlid A (2009) The ectomycorrhizal symbiosis: a marriage of convenience. In: Deising HB (ed) The Mycota, vol 5. Springer, Berlin, pp 237–257

Martin F, Kohler A, Duplessis S (2007) Living in harmony in the wood underground: ectomycorrhizal genomics. Curr Opin Plant Biol 10:204–210

Martin F, Aerts A, Ahrn D, Brun A, Danchin EGJ et al (2008) The genome sequence of the basidiomycete fungus *Laccaria bicolor* provides insights into the mycorrhizal symbiosis. Nature 452:88–92

Martin F, Kohler A, Murat C, Balestrini R, Coutinho PM, Jaillon O, Montanini B, Morin E, Noel B, Percudani R et al (2010) Perigord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature 464:1033–1038

Martin F, Cullen D, Hibbett D, Pisabarro AG, Spatafora JW, Baker SE, Grigoriev I (2011) Sequencing the fungal tree of life. New Phytol 190(4):818–821

Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Duenas FJ, Martinez AT, Kersten P et al (2009) Genome, transcriptome, and secretome analysis of wood decay fungus Postia placenta supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci USA 106:1954–1959

Meharg AA, Cairney JWG (2000) Ectomycorrhizas – extending the capabilities of rhizosphere remediation? Soil Biol Biochem 32:1475–1484

Meharg AA, Dennis GR, Cairney JWG (1997) Biotransformation of 2,4,6-trinitrotoluene (TNT) by ectomycorrhizal basidiomycetes. Chemosphere 35:513–521

Mellersh DG, Heath MC (2001) Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell 13:413–424

Mentlak TA, Kombrink A, Shinya T, Ryder LS, Otomo I, Saitoh H, Terauchi R, Nishizawa Y, Shibuya N, Thomma BP et al (2012) Effector-mediated suppression of chitin-triggered immunity by magnaporthe oryzae is necessary for rice blast disease. Plant Cell 24:322–335

Monaghan J, Zipfel C (2012) Plant pattern recognition receptor complexes at the plasma membrane. Curr Opin Plant Biol. doi:dx.doi.org/10.1016/j.pbi.2012.05.006

Morel M, Jacob C, Kohler A, Johansson T, Martin F, Chalot M, Brun A (2005) Identification of genes differentially expressed in extraradical mycelium and ectomycorrhizal roots during Paxillus

involutus-Betula pendula ectomycorrhizal symbiosis. Appl Environ Microbiol 71:382–391

Moyersoen B (2006) Pakaraimaea dipterocarpacea is ectomycorrhizal, indicating an ancient Gondwanaland origin for the ectomycorrhizal habit in Dipterocarpaceae. New Phytol 172:753–762

Murat C, Riccioni C, Belfiori B, Cichocki N, Labbe J, Morin E, Tisserant E, Paolocci F, Rubini A, Martin F (2010) Distribution and localization of microsatellites in the Perigord black truffle genome and identification of new molecular markers. Fungal Genet Biol 48(6):592–601

Nagendran S, Hallen-Adams HE, Paper JM, Aslam N, Walton JD (2009) Reduced genomic potential for secreted plant cell-wall-degrading enzymes in the ectomycorrhizal fungus Amanita bisporigera, based on the secretome of Trichoderma reesei. Fungal Genet Biol 46(5):427–435

Nehls U, Gohringer F, Wittulsky S, Dietz S (2010) Fungal carbohydrate support in the ectomycorrhizal symbiosis: a review. Plant Biol 12:292–301

Newton AC, Haigh JM (1998) Diversity of ectomycorrhizal fungi in Britain: a test of the species-area relationship, and the role of host specificity. New Phytol 138:619–627

Niemi K, Scagel C, Haggman H (2004) Application of ectomycorrhizal fungi in vegetative propagation of conifers. Plant Cell Tissue Organ Cult 78:83–91

Oliveira RS, Franco AR, Castro PML (2012a) Combined use of *Pinus pinaster* plus and inoculation with selected ectomycorrhizal fungi as an ecotechnology to improve plant performance. Ecol Eng 43:95–103

Oliveira RS, Franco AR, Vosatka M, Castro PML (2012b) Management of nuresery pratices for efficient ectomycorrhizal fungi application in the production of *Quercus ilex*. Symbiosis 52:125–131

Oliver RJ, Finch JW, Taylor G (2009) Second generation bioenergy crops and climate change: a review of the effects of elevated atmospheric CO_2 and drought on water use and the implications for yield. Glob Change Biol Bioenergy 1:97–114

Parrent JL, James TY, Vasaitis R, Taylor AF (2009) Friend or foe? Evolutionary history of glycoside hydrolase family 32 genes encoding for sucrolytic activity in fungi and its implications for plant-fungal symbioses. BMC Evol Biol 9:148

Pennanen T, Garbaye J, Piri T, Timonen S, Rajala T, Müller M et al (2010) Ectomycorrhizal fungi on Norway spruce- a seedling growth rate perspective. In: 9th international mycological congress, Edinburgh

Peterson RL, Massicotte HB (2004) Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. Can J Bot (Revue Canadienne De Botanique) 82:1074–1088

Pilz D, Norvell L, Danell E, Molina R (2003) Ecology and management of commercially harvested chanterelle mushrooms. General Technical Report – Pacific Northwest Research Station, USDA Forest Service

Plett JM, Martin F (2011) Blurred boundaries: lifestyle lessons from ectomycorrhizal fungal genomes. Trends Genet 27:14–22

Plett JM, Kemppainen M, Kale SD, Kohler A, Legue V, Brun A, Tyler BM, Pardo AG, Martin F (2011) A secreted effector protein of Laccaria bicolor is required for symbiosis development. Curr Biol 21:1197–1203

Plett JM, Gibon J, Kohler A, Duffy K, Hoegger PJ, Velagapudi R, Han J, Kuees U, Grigoriev IV, Martin F (2012) Phylogenetic, genomic organization and expression analysis of hydrophobin genes in the ectomycorrhizal basidiomycete Laccaria bicolor. Fungal Genet Biol 49:199–209

Rabatin S (1980) The occurence of the vesicular-arbuscular mycorrhizal fungus Glomus tenuis with moss. Mycologia 72:191–195

Raffaele S, Kamoun S (2012) Genome evolution in filamentous plant pathogens: why bigger can be better. NatRev Microbiol 10:417–430

Rafiqi M, Ellis JG, Ludowici VA, Hardham AR, Dodds PN (2012) Challenges and progress towards understanding the role of effectors in plant-fungal interactions. Curr Opin Plant Biol 15(4):477–482

Read DJ, Duckett JG, Francis R, Ligrone R, Russell A (2000) Symbiotic fungal associations in 'lower' land plants. Philos Trans R Soc Lond B Biol Sci 355:815–830

Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843

Rincon A, Parlade J, Pera J (2005) Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerised *Pinus pinea* L. seedlings produced in a commercial nursery. Ann Forest Sci 62:817–822

Rincon A, Parlade J, Pera J (2007) Influence of the fertilisation method in controlled ectomycorrhizal inoculation of two Mediterranean pines. Ann Forest Sci 64:577–583

Rineau F, Courty P-E (2010) Secreted enzymatic activities of ectomycorrhizal fungi as a case study of functional diversity and functional redundancy. Ann Forest Sci 68:69–80

Roby D, Gadelle A, Toppan A (1987) Chitin oligosaccharides as elicitors of chitinase activity in melon plants. Biochem Biophys Res Comm 143:885–892

Rouxel T, Grandaubert J, Hane JK, Hoede C, van de Wouw AP, Couloux A, Dominguez V, Anthouard V, Bally P, Bourras S et al (2011) Effector diversification within compartments of the Leptosphaeria maculans genome affected by Repeat-Induced Point mutations. Nat Commun 2:202

Salzer P, Hebe G, Reith A, ZitterellHaid B, Stransky H, Gaschler K, Hager A (1996) Rapid reactions of spruce cells to elicitors released from the ectomycorrhizal fungus Hebeloma crustuliniforme, and inactivation of these elicitors by extracellular spruce cell enzymes. Planta 198:118–126

Salzer P, Hebe G, Hager A (1997a) Cleavage of chitinous elicitors from the ectomycorrhizal fungus Hebeloma crustuliniforme by host chitinases prevents induction of K+and Cl− release, extracellular alkalinization and H_2O_2 synthesis of *Picea abies* cells. Planta 203:470–479

Salzer P, Hubner B, Sirrenberg A, Hager A (1997b) Differential effect of purified spruce chitinases and beta-1,3-glucanases on the activity of elicitors from ectomycorrhizal fungi. Plant Physiol 114:957–968

Salzer P, Wiemken A, Boller T (2001) Different roles of plant chitinases in mycorrhizae and pathogenic interactions. In: Muzzarelli RAA (ed) Chitin enzymology 2001. Atec Edizioni, Grottammare, pp 31–40

Sarand I, Timonen S, Nurmiaho-Lassila EL, Koivula T, Haahtela K, Romantschuk M, Sen R (1998) Microbial biofilms and catabolic plasmid harbouring degradative fluorescent pseudomonads in Scots pine mycorrhizospheres developed on petroleum contaminated soil. FEMS Microbiol Ecol 27:115–126

Schindler M, Meiners S, Cheresh DA (1989) rgd-dependent linkage between plant-cell wall and plasma-membrane – consequences for growth. J Cell Biol 108:1955–1965

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515

Schwessinger B, Ronald PC (2012) Plant innate immunity: perception of conserved microbial signatures. Ann Rev Plant Biol 63:451–482. %U http://www.annualreviews.org/doi/abs/410.1146/annurev-arplant-042811-105518

Seguin A (2011) How could forest trees play an important role as feedstock for bioenergy production? Curr Opin Environ Sustain 3:90–94

Selosse MA, Bouchard D, Martin F, Le Tacon F (2000) Effect of *Laccaria bicolor* strains inoculated on Douglas-fir (Pseudotsuga menziesii) several years after nursery inoculation. Can J Forest Res (Revue Canadienne De Recherche Forestiere) 30:360–371

Sharp JA, McNeil M, Albersheim P (1984a) The primary structure of one elicitor active and seven elicitor-inactive hexa [ß-D-glucopyranolsyl]-D-glucitols isolated from the mycelia walls of *Phytophthora megasperma* f.sp. *glycinea*. J Biol Chem 25:11321–11326

Sharp JA, Valent B, Albersheim P (1984b) Purification and partial characterization of a ß-glucan fragment that elicits phytoalexin accumulation in soybean. J Biol Chem 25:11312–11320

Simon L, Bousquet J, Levesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69

Sirrenberg A, Salzer P, Hager A (1995) Induction of mycorrhiza-like structures and defense reactions in dual cultures of spruce callus and ectomycorrhizal fungi. New Phytol 130:149–156

Smith SE, Read JR (2008) Mycorrhizal symbiosis. Academic, San Diego

Sousa NR, Franco AR, Ramos MA, Oliveira RS, Castro PML (2011) Reforestation of burned stands: the effect of ectomycorrhizal fungi on Pinus pinaster establishment. Soil Biol Biochem 43:2115–2120

Sousa NR, Franco AR, Oliveira RS, Castro PML (2012a) Ectomycorrhizal fungi as an alternative to the use of chemical fertilisers in nursery production of *Pinus pinaster*. J Environ Manage 95:S269–S274

Sousa NR, Ramos MA, Marques APGC, Castro PML (2012b) The effect of ectomycorrhizal fungi forming symbiosis with Pinus pinaster seedlings exposed to cadmium. Sci Total Environ 414:63–67

Spanu P (1997) HCF-1, a hydrophobin from the tomato pathogen *Cladosporium fulvum*. Gene 193:89–96

Spanu P (2012) The genomics of Obligate (and nonobligate) biotrophs. Annu Rev Phytopathol 50:5.1–5.19

Spanu PD, Abbott JC, Amselem J, Burgis TA, Soanes DM, Stuber K, Loren V, van Themaat E, Brown JK, Butcher SA, Gurr SJ et al (2010) Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. Science 330:1543–1546

Splivallo R, Fischer U, Gobel C, Feussner I, Karlovsky P (2009) Truffles regulate plant root morphogenesis via the production of auxin and ethylene. Plant Physiol 150:2018–2029

Tagu D, Martin F (1996) Molecular analysis of cell wall proteins expressed during the early steps of ectomycorrhiza development. New Phytol 133:73–85

Tang JD, Perkins AD, Sonstegard TS, Schroeder SG, Burgess SC, Diehl SV (2012) Short-read sequencing for genomic analysis of the brown rot fungus Fibroporia radiculosa. Appl Environ Microbiol 78:2272–2281

Tedersoo L, May TW, Smith ME (2010) Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20:217–263

Templeton MD, Rikkerink EHA, Beever RE (1994) Small, cysteine-rich proteins and recognition in fungal-plant interactions. Mol Plant-Microbe Inter 7:320–325

Teste FP, Schmidt MG, Berch SM, Bulmer C, Egger KN (2004) Effects of ectomycorrhizal inoculants on survival and growth of interior Douglas-fir seedlings on reforestation sites and partially rehabilitated landings. Can J Forest Res (Revue Canadienne De Recherche Forestiere) 34:2074–2088

Tibbett M (2000) Roots, foraging and the exploitation of soil nutrient patches: the role of mycorrhizal symbiosis. Funct Ecol 14:397–399

Vega-Sanchez ME, Verhertbruggen Y, Christensen U, Chen X, Sharma V, Varanasi P, Jobling SA, Talbot M, White RG, Joo M et al (2012) Loss of Cellulose Synthase-Like F6 function affects mixed-linkage glucan deposition, cell wall mechanical properties, and defense responses in vegetative tissues of rice. Plant Physiol 159:56–69

Veneault-Fourrey C, Martin F (2011) Mutualistic interactions on a knife-edge between saprotro-

phy and pathogenesis. Curr Opin Plant Biol 14:444–450

Vinces MD, Legendre M, Caldara M, Hagihara M, Verstrepen KJ (2009) Unstable tandem repeats in promoters confer transcriptional evolvability. Science 324:1213–1216

Voiblet C, Duplessis S, Encelot N, Martin F (2001) Identification of symbiosis-regulated genes in *Eucalyptus globulus-Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. Plant J 25:181–191

Wang B, Yeun LH, Xue J-Y, Liu Y, Ane J-M, Qiu Y-L (2010) Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. New Phytol 186:514–525

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Wessels JGH (2000) Hydrophobins, unique fungal proteins. Mycologist 14:153–159

Whiteford JR, Spanu PD (2002) Hydrophobins and the interactions between fungi and plants. Mol Plant Pathol 3:391–400

Whiteford JR, Lacroix H, Talbot NJ, Spanu PD (2004) Stage-specific cellular localisation of two hydrophobins during plant infection by the pathogenic fungus *Cladosporium fulvum*. Fungal Genet Biol 41:624–634

Wolfe BE, Tulloss RE, Pringle A (2012) The irreversible loss of a decomposition pathway marks the single origin of an ectomycorrhizal symbiosis. PLoS One 7(7):e39597

Wosten HAB (2001) Hydrophobins: multipurpose proteins. Annu Rev Microbiol 55:625–646

Zamioudis C, Pieterse CM (2012) Modulation of host immunity by beneficial microbes. Mol Plant Microbe Interact 25:139–150

Zuccaro A, Lahrmann U, Gueldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M et al (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont Piriformospora indica. PLoS Pathog 7(10):e1002290

11 Ectomycorrhiza-Specific Gene Expression

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CONTENTS

I. Introduction

The **mutualistic symbioses** of mycorrhiza (Frank 1885) between plant roots and fungi provide a dominant ecosystem function. Although known already for a long time, the molecular understanding that will make it possible to assess these functions is still rare, specifically for the ectomycorrhizal (ECM) associations. This is partially due to the slow development of tools for basidiomycetes, the main group of ECM fungi, and partly associated with the slow growth of these fungi in cultivation systems, providing in vitro access to axenic cultures of ECM (Fig. 11.1). The growth pattern may be linked to strategies in environmental spreading, with faster growing ***early-stage*** ECM fungi, such as **r-strategists**, expected to show a broader host range and to occur at disturbed sites in an early succession, and ***late-stage*** fungi showing slow growth and high host specificity typical for **K-strategy** organisms. Thus, it should be carefully assessed whether there are differences between the two types in specific gene expression in ECM.

In addition, functional characterization of genes identified seems important to explain differences in ECM formation, transfer of nutrients and water, exchange of signaling molecules, or the protective role of ECM fungi to link them up with the wealth of available environmental data. These include the stages of development in ECM formation, the diversity of ECM fungi in forest soils generated by **morphotyping** of ECM root tips (Agerer 1987/2002), characterization of extraradical hyphae or rhizomorphs in the soil, or sequencing approaches mostly based on the internal transcribed spacer (ITS) region of fungal rDNA (see UNITE database at http://unite.ut.ee) to characterize diversity in pristine or environmentally disturbed forest ecosystems. A better understanding will allow **application of ECM in environmental clean-up strategies, renewable energy production, and forestry.**

The ECM root–fungus interface is characterized by the exchange of molecules between the two partners, with carbohydrates and vitamins delivered by the plant, which receives soil minerals, water, nitrogen, and phosphorus in return. Furthermore, **the plant profits by increased drought and heavy metal tolerance, and resistance against phytopathogens** (Martin and Nehls 2009). To form the interface, fungal hyphae enter the root and grow within the apoplast, where they form the intraradical **Hartig' net**, while a **hyphal mantle** formed at the root's surface offers protection (see Fig. 11.1). Most ECM root-tips have a short lifetime, and are replaced every year. The hyphae expanding into the surrounding soil functionally replace the root hairs, and may provide long distance transport of water and

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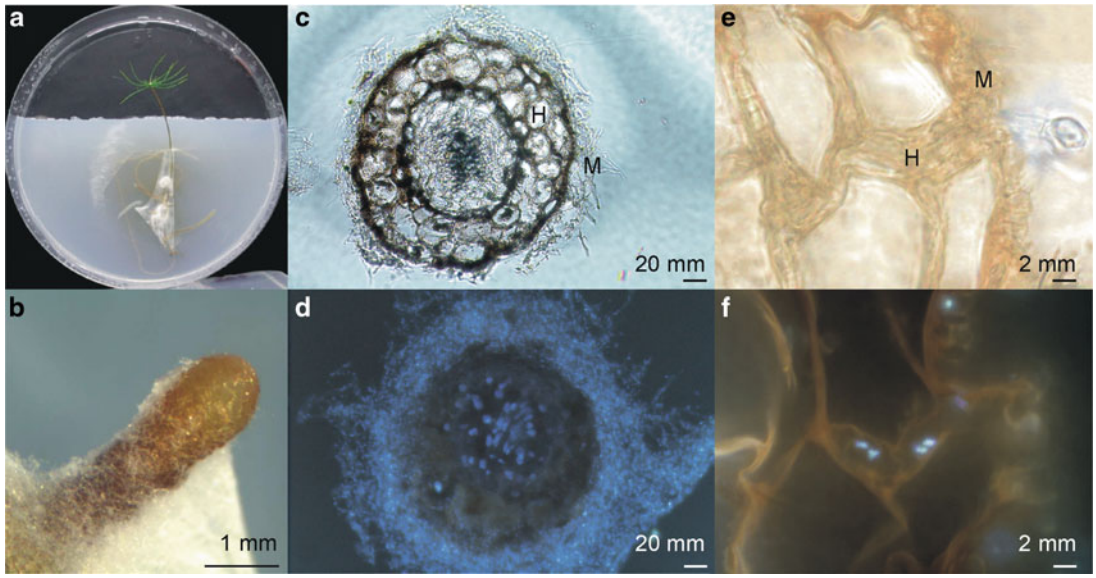

Fig. 11.1. Axenic cultivation of ECM. (**a**) Petri dish culture of *Tricholoma vaccinum*-spruce interaction after 3 months of co-culture. (**b**) ECM short root cross-section with hyphal mantle (*M*) and Hartig' net (*H*). (**c**, **d**) Bright field and dark field image with DAPI-stained nuclei showing dikaryotic hyphae penetrating between cortical plant cells respectively. (**e**, **f**) Bright field and dark field image with DAPI-stained nuclei of Hartig' net

nutrients via morphologically distinct hyphal bundles, the **rhizomorphs**. From the amount and length of hyphae spreading into the surrounding soil, a **differentiation ranging from contact types of ECM to long-distance exploration types** has been proposed, which may explain differences in ecosystem function (Agerer 2001). Extraradical hyphae connect the **mushroom sporocarps** with different host trees, forming 'fungal networks' which can stabilize the entire forest ecosystem against, for example, drought stress (Simard et al. 1997; Smith and Read 2008).

ECM is formed by 5,000–6,000 fungal species (Molina et al. 1992) of **basidiomycetes**, but also by some **ascomycetes** and few **zygomycetes**, meaning that ECM symbionts have evolved repeatedly and convergently from saprotrophic ancestors (Hibbett et al. 2000). The host plants of ECM fungi are mainly woody perennials, around 3 % of phanerogams (Meyer 1973). Plants show a low level of host specificity. While **mycotrophic plants** such as pine and beech are associated with tens to hundreds of symbiotic fungi, low mycotrophy ones such as maple are rarely colonized (Trappe 1962). ECM is, nevertheless, an important form of interaction between plant and fungi because of its wide distribution, especially in boreal forests, large biomass with ECM mycelia constituting one third of total soil microbial biomass, and for its economic importance in mushroom production (Smith and Read 2008). The development of sporocarps is observed only in symbiotic interaction with compatible partners, making axenic cultivation inaccessible for mushroom growers (Trappe 1962). In axenic co-cultures, sporocarp formation was shown only in a limited number of cases pointing, again, to the need for a molecular understanding of host specificity of the mycobiont partners observed in many ECM fungi in varying degrees, ranging from fruiting bodies being produced only with one host tree species to an array of trees including both deciduous and coniferous trees as hosts for one fungus (Krause 2005; Ohta 1998; Sanmee et al. 2010).

The interaction is initiated when the saprotrophic fungus is attracted towards a host tree by **root exudates** or **tree volatiles**, which induce directional growth and reinforced branching of hyphae (Fries et al. 1987; Horan and Chilvers 1990; Weiss et al. 1997). The branched hyphae

Table 11.1 Summarized view of gene groups involved in ECM development and functioning

Extraradical hyphae		Plant–fungus interface	
Soil interface	Rhizomorphs	Mantle	Hartig' net
Soil specificity for phosphate uptake Presymbiotic signaling Cytoskeleton for branching Metallothioneins in metal-rich soils	Cell wall for hyphal aggregates Cytoskeleton for transport along hyphae	Tissue development with signaling Cell wall and cytoskeleton	Signaling to host Host specificity Response to plant stressors Tissue development with cell wall and cytoskeleton remodeling Membrane interface and transport to/from host Carbohydrate and nitrogen metabolism

form aggregates at the surface of **short roots** developing into the **pseudoparenchymatic tissue** of the hyphal mantle (Kottke and Oberwinkler 1986). Since variation is observed for different partners, both in the arrangement of hyphae or hyphal layers and color, **recognition between the two partners** must be involved in this step. By changes in the fungal and plant cell wall composition and structure, hyphae penetrate into the intercellular spaces of the root and form the Hartig' net (Barker et al. 1998; Martin et al. 1999). The interaction of plant and mycobiont can be resolved by investigating signals, regulation processes, or the **communication between mycobiont and host** (Table 11.1).

The following sections will summarize transcription analyses of ECM fungi to identify recurring classes of genes regulated in ECM, and then investigate examples for such genes or gene groups. A separate section will be devoted to the currently available genetic modifications for functional analysis of genes identified. Potentially, proteome analyses should be interesting as well. However, owing to the extremely small amounts of biomass available from axenic ECM in vitro cultivation, such attempts are still lacking.

II. Genome-Wide Expression Profiling

Recently, three ECM fungal **genome sequences** have been published: the basidiomycetes *Laccaria bicolor* and *Paxillus involutus,* as well as the ascomycete truffle *Tuber melanosporum* (Le Quere et al. 2002; Martin et al. 2008, 2010), with genome sizes of 65 Mb with ca. 19,000 protein-encoding genes, 23 Mb with 7,700 genes and 125 Mb with only 7,500 protein-coding genes, respectively. Using different tissues or developmental stages, the annotations enable **transcriptome** analyses to identify sets of genes involved in symbiosis. In addition, differential display, EST (**expressed sequence tags**)-sequencing and **(micro)array analyses** have been performed to identify genes up-regulated during mycorrhizal interaction (Krause and Kothe 2006; Küster et al. 2007; Peter et al. 2003; Tagu and Martin 1995). Different model systems have been used which necessitate a thorough comparison of their biology when identifying, for example, plant response. A faster-growing fungus such as *Pisolithus* will most likely exert a stronger **plant defense response**, and thus has to cope with specifically strong stressors, while the slow-growing *Tricholoma* may, after the initial phase, see less attack. Nevertheless, these approaches are providing a basis for detecting gene families with distinct roles in the fungal mycelium.

In presymbiotic interaction of *Hydnangium* with eucalypt trees lacking direct contact, carbohydrate and amino acid metabolism, energy metabolism, expression machinery signal transduction, and communication, as well as stress response, are regulated (da Silva Coelho et al. 2010). Another ECM of eucalypt, ***Pisolithus*** ECM, has been used as a model for some time now (Duplessis et al. 2005; Tagu and Martin 1995; Tagu et al. 1993). Here, cell-wall proteins have been identified to be one major group of regulated genes (Martin et al. 1999). This is also the case for *Pinus sylvestris–**Laccaria bicolor*** ECM, where specifically general defense and cell-wall synthesis in the plant were down-regulated

(Heller et al. 2008). The hydrophobins associated with cell-wall properties and cross-connection of hyphae in the pseudoparenchymatic tissues of ECM have also been identified as strongly regulated genes, not only in the fast-growing ECM fungi discussed so far, but also with the slow-growing ECM fungi (Tagu et al. 1996). In a differential display of highly host-specific ***Tricholoma vaccinum***–spruce ECM, up-regulation of genes with a function in stress response, signal transduction, nutrient exchange, and growth of the fungus in the plant were observed. Here, it was possible to show in situ a host-specific induction of expression of a hydrophobin within the Hartig' net (Krause and Kothe 2006; Mankel et al. 2002).

In fully developed ECM, **carbohydrate metabolism** is expected to play an important role. Hence, genes induced in *Laccaria* ECM, including glycolysis and trehalose/mannitol metabolism related genes, have been studied in more detail (Deveau et al. 2008). This group of genes was also up-regulated in ***Paxillus involutus***–birch ECM, where additionally stress response and signal transduction were identified as being up-regulated, while lipid metabolism and secondary metabolites were down-regulated (Johansson et al. 2004, see also Le Quere et al. 2005). Indeed, sugar transport and metabolism can be identified as a major functions regulated differentially within the Hartig' net, as the supply of carbohydrates from the plant is a major part of physiological interactions with mycorrhiza.

In rhizomorphs, specifically nitrogen metabolism and cytoskeleton were found to be regulated (Wright et al. 2005) which is in accordance with the transport function of these structures. Since these **extraradical structures** connect different trees in the forest ecosystem, the transfer of nutrients in the ecosystem has received some attention, and genes involved in partitioning have been identified (Wiemken and Boller 2002). In extraradical hyphae, the ion uptake for nutrients is the dominant function.

This already shows that different tissues in ECM may show variations on the theme of gene expression. The analysis of gene expression in the interaction of *Betula pendula* with *Paxillus involutus* and *Eucalyptus globulus* with *Pisolithus microcarpus* yielded an **increased expression of cell-wall synthesis genes, defense, and stress in both partners**, which, however, was higher on the fungal side during host colonization. **During mantle formation, genes of carbohydrate and hormone metabolism showed higher expression**, while the amounts of these transcripts were reduced **during Hartig' net formation. In this tissue, the expression of genes of amino acid metabolism and protein folding was increased** (Martin et al. 2007).

In summary, groups of genes with general relevance in ECM can be identified, and a preliminary picture which allows to define genes of interest with respect to different fungal compartments in ECM will emerge (Table 11.1). Additional data are needed to complete this picture.

III. ECM Carbon Metabolism

The **main benefit of the mycobiont in ECM is the carbohydrate supply from the plant**. This certainly is at a cost to the plant, which is delivering up to 30 % of its net fixed carbon via glucose and fructose (Pfeffer et al. 2001; Söderström and Read 1987). This is balanced by an increase of up to 29 % in photosynthesis rates (Rousseau and Reid 1991; Wright et al. 2000). Under field conditions, around 15 % of assimilated C is transferred to the fungi, indicating that mycorrhized plants nevertheless profit from the symbiosis (Finlay and Söderström 1992). Smith and Read (2008) suggested that the maximum C allocation to below-ground systems occurs at the end of the growing season, at the time of bud set, and during fungal fruiting body production. However, ECM fungi are not obligate biotrophs. Rather, they live **saprotrophically** and are involved in **plant litter mineralization** and nutrient cycling in forest ecosystems.

ECM fungi generally show only limited ligninolytic and cellolytic abilities as compared to wood-decomposing fungi or ericoid mycorrhizal fungi (Bending and Read 1997; Haselwandter et al. 1990; Perotto et al. 1995; Varma and Bonfante 1994). **Pectinolytic activity, starch- and disaccharide-degrading ability, and utilization of glucose, mannose, and fructose are well-developed** (Smith and Read 2008). Thus, during mycorrhization the fungi

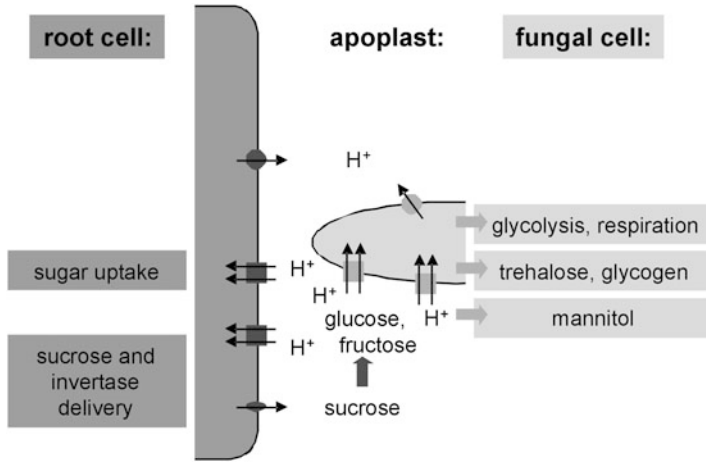

Fig. 11.2 Sugar partitioning and metabolism in the Hartig' net. The root cell (*dark grey*) as well as the fungal cell (*light grey*) produce ATPase to provide a proton gradient leading to an apoplast pH of 4.5. Transporters for both fructose and glucose compete for monosaccharides released from plant sucrose by plant invertase (*dark grey arrow*). Upon uptake, sugars are catabolized and transported, and storage compounds trehalose and glycogen are produced from glucose, while fructose in converted to mannitol (*light grey arrows*)

are able to penetrate the root and to use cellobiose as carbon source from the converted root cell walls, while not producing too many elicitors to provoke a strong plant-defense reaction. The enzymes merely allow the fungus, and hence indirectly the host plant, to utilize the humus-bound plant material in case of non-symbiotic growth (Burke and Cairney 1997). Even during symbiosis, ECM biomass may contain 2 % C originating from litter degradation (Treseder et al. 2006). A partitioning of carbohydrates can be seen. Excised ECM from *Fagus* showed characteristic carbohydrates from plant and fungus: plant-localized sucrose and starch, fungus-localized trehalose, mannitol, and glycogen, and common to both, glucose and fructose (Smith and Read 2008).

During symbiosis, **sucrose from the apoplast is the main fungal C source.** However, in contrast to phytopatogens and ECM ascomycetes such as *Tuber* spp., investigated basidiomycete ECM fungi, e.g., *Amanita muscaria* and *Hebeloma crustiliniforme*, are dependent on plant invertase for disaccharide hydrolysis (Nehls et al. 2010; Salzer and Hager 1991). Nehls et al. (1998) identified the first two **monosaccharide transporter genes** in *Amanita muscaria, mst1* and *mst2*. While Mst1 preferentially imports glucose, Mst2 has a higher capacity for fructose transport. A proton-dependent transport is supported by ATPase activity in the membranes of both partners in the Hartig' net (Lei and Dexheimer 1988). Both plant and mycobiont thus compete for the monosaccharides, which might explain the sustained mutual symbiosis. Monosaccharide concentrations of more than 2 mM led to 4-fold up-regulation of both fungal transporter genes. A tissue-specific expression of *mst1* was seen in poplar–*Amanita* ECM, with low expression in hyphae of the fungal mantle and 6-fold induction in the Hartig' net (Nehls et al. 2001a). Since, a glucose gradient is likely, fructose is supposed to be an extra C source (Nehls et al. 2010).

The importance of **hexose transport** is underlined by the fact that six of the 15 annotated monosaccharide transporters in the *Laccaria bicolor* genome showed strong up-regulation in ECM (Fajardo Lopez et al. 2008). In *Populus tremula* ECM, the plant sugar transporter gene *mst3.1* was up-regulated, suggesting the ability of the host to restrict sugar supply to the fungus in adverse environmental conditions, avoiding fungal parasitsm (Grunze et al. 2004). In Fig. 11.2, a hypothetical scheme of the sugar transfer at the plant fungus interface, followed by the conversion of glucose to glycogen and trehalose and of fructose into mannitol as fungal storage compounds, is given (Smith and Read 2008). The fungal long-term storage compound **glycogen** is stored in large, non-mobile granules in cytoplasm, whereas the mobile, short-term storage compound **trehalose** is well-suited

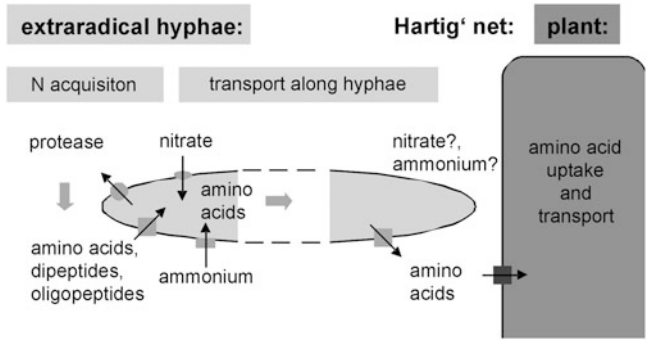

Fig. 11.3 Partitioning of N uptake and assimilation. Extraradical hyphae (*light grey*, at *left*) gain access to inorganic N or via excretion of proteases (*light grey arrow at far left*) to organic N, which is taken up by specific systems, transported through the mycelium and delivered to the Hartig' net, most likely by excretion of amino acids from the hyphae (*light grey*, *middle part*). These can be taken up into the plant cells (*dark grey*) and distributed throughout the plant via phloem transport

for long-distance transport into the external mycelium (Nehls et al. 2010). In addition, transcriptome studies have shown **increased catabolism as well as gluconeogenesis** (see above).

IV. ECM Nitrogen and Amino Acid Metabolism

Nitrate, ammonium, amino acids, dipeptides, or oligopeptides serve as N sources, taken up by specific transporters into fungal cells regardless of whether saprotrophic growth or ECM is considered, with a preferred use of ammonium, the main N source in forest soils (Marschner and Dell 1994). Genes for **ammonium transporters**, *amt1*, **nitrite reductase**, *nir1*, **nitrate transporter**, *nrt2* and **nitrate reductase**, *nar1*, have been analyzed from *Hebeloma spp.*, and an ammonium transporter was identified in *Amanita muscaria* (Jargeat et al. 2000, 2003; Plassard et al. 1991, 1994; Willmann et al. 2007). Ammonium is incorporated into glutamine and glutamate by glutamine (**GS-GOGAT pathway**, *gs* genes) and glutamate dehydrogenases (*gdh* genes). High ammonium concentrations repress *amt1, amt2*, and *gdhA*, while low levels of ammonium induce transcription of *amt1, amt2, amt3, glnA*, and *gdhA* in *H. cylindrosporium*, resulting in increased uptake and metabolism of ammonium (Javelle et al. 2003a, b, 2004).

Proteins can be used as N source, distinguishing the '**protein fungi**' *A. muscaria*, *Cenococcum geophilum*, *Paxillus involutus*, *Rhizopogon roseolus*, *Suillus bovines*, and *Hebeloma crustiliniforme* from the '**non-protein fungi**' *Laccaria laccata* and *Lactarius rufus* (Abuzinadah and Read 1986a, b). Nehls et al. (2001b) identified two **aspartic proteases** of *A. muscaria*, Prot1 and Prot2, released in a pH-dependent manner from the hyphae. A regulation of *prot1* was exerted by both, N starvation (threefold to fourfold) and in absence of C (12-fold), which led to the expectation of a **cross-pathway control** of endogenous C availability on proteolytic activity. As a result of proteolytic activity, amino acids are available for uptake. In *A. muscaria,* the **amino acid transporter** gene *aap1* shows low expression in the presence of amino acids and up-regulation during N starvation (Nehls et al. 1999). This is true even in the presence of nitrate or phenylalanine, which are non-N substrates for *A. muscaria*. Wipf et al. (2002) isolated the **amino acid permease** *gapI* of *H. cylindrosporum* with a broad substrate spectrum for all 20 proteinogenic amino acids. This transporter shows no expression in mycorrhiza, suggesting a function in uptake of amino acids from soil. In addition, two **di- and tripeptide transporters**, *ptr2A* and *ptr2B*, of *H. cylindrosporum* have been identified. While *ptr2A* was up-regulated during N deficiency or in the presence of a second N source, *ptr2B* was constitutively expressed (Benjdia et al. 2006) which led the authors to suggest a regulation of peptide uptake by mechanisms sensing extracellular and intracellular N sources. Müller et al. (2007) proposed a function in peptide uptake from soil for *ptr2A*

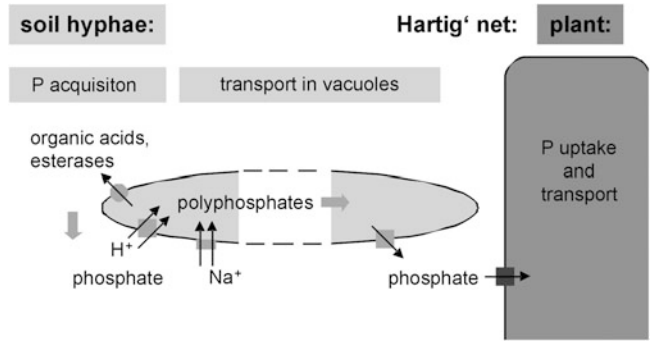

Fig. 11.4 Partitioning of P uptake and assimilation. The release of organic acids and esterases mobilizes phosphate, which is taken up by specific fungal transporters and allows an improved supply of phosphate of the fungus (*light grey*). P is transported through the hyphae into the root, where it may be delivered to the plant partner (*dark grey*) in ECM

under stress conditions such as nitrogen starvation and for *ptr2B* in the constitutive uptake.

The supply of N to the host tree is still poorly understood (Chalot et al. 2006; Müller et al. 2007, Fig. 11.3), with the recent identification of ESTs corresponding to a poplar amino acid transporter, *aap12,* in ECM roots indicating **exchange via amino acid excretion from the fungus and uptake by the plant** (Couturier et al. 2010).

V. ECM Phosphorous and Water Supply

Most woody habitats, especially highly weathered, acidic or calcareous soils, are characterized by limited **bioavailability of P** (see for review: Plassard and Dell 2010). ECM enables plants to accumulate P at higher rates than without this symbiosis (Fig. 11.4). P is mostly present in **organic phosphomonoesters**, e.g., inositol hexaphosphate, or **phosphodiesters**, such as nucleic acids and phospholipids. Increased **phosphomonoesterase** activity has been reported for pure cultures of ECM fungi, ECM roots, and rhizomorphs (Dinkelaker and Marschner 1992; Louche et al. 2010). Studies dealing with 'ectomycorrhizal weathering' show that some ECM fungi (including species of the genera *Cortinarius*, *Lactarius*, *Paxillus*, *Piloderma*, *Pisolithus* and *Suillus*) are able to **solubilize P from minerals, mainly from apatite**, by acidification and production of low-molecular-weight organic acids such as oxalic acid (Courty et al. 2010; Cromack et al. 1979; Hagerberg et al. 2003; Rosling et al. 2004). After dissolution and mobilization of P by excreting organic acids, active, **high affinity uptake and storage of polyphosphate in vacuoles** is a prerequisite for P transfer into the plant root. For *Pisolithus tinctorius,* polyphosphate could be identified in vacuoles, and co-occurrence with K^+ and Mg^{2+} was hypothesized (Ashford et al. 1994; Orlovich and Ashford 1993). Five genes possibly encoding the necessary **phosphate transporter** were identified in the genome of *Laccaria bicolor* (Martin et al. 2008), and Tatry et al. (2009) characterized two transporters of *Hebeloma cylindrosporum: pt1* with high expression in phosphate starvation and *pt2* with up-regulation in mycorrhized roots of soils with high phosphate availability as compared to pure cultures of the fungus. Additionally, *pt9* and *pt12,* two poplar high-affinity P_i transporter genes of the Pht1 family show preferential expression in ECM root tips (Loth-Pereda et al. 2011).

The fungal high-affinity phosphate transporters have been investigated in the yeast *Saccharomyces cerevisiae* and the filamentous ascomycete *Neurospora crassa* (Bun-Ya et al. 1991; Mann et al. 1989; Martinez and Persson 1998; Versaw 1995). From the gene families identified in both systems, a **proton symporter** (similar to *PHO84* in

S. cerevisiae and *pho-5* in *N. crassa*) and a **sodium symporter subfamily** member (similar to *PHO89* in *S. cerevisiae* and *pho-4* in *N. crassa*) each have been investigated in *Tricholoma vaccinum* and *T. terreum*. These transporters have been implied in soil preference, mainly because proton symport with phosphate is easy at neutral to alkaline environmental pH, the environment preferred by *Tricholoma terreum*, while a sodium symport shows better function at low pH, which is the environmental condition in which *T. vaccinum* is found (Kothe et al. 2002). In comparison to the *N. crassa* genes, these could be re-annotated by re-evaluating intron positions, and the structures of both proteins were predicted (Fig. 11.5, Terpitz and Kothe, unpublished). Since phosphate acquisition in extraradical hyphae has been linked to ambient soil pH, it may also be considered part of the **fungal soil preference** (see below).

In ECM plants, an improved supply with water was discussed divergently (see review Lehto and Zwiazek 2011). However, plant aquaporins increased water transport capacity by 57 % in roots of ECM plants (Marjanović et al. 2005a, b), and in poplar–*Amanita muscaria* ECM, three of seven **aquaporin** genes showed an increased expression. Two of the genes were highly expressed during drought stress.

VI. Host Specificity

The formation of the symbiosis between fungus and plant is a complex process depending on extensive **signal exchange**. The chronological sequence for the formation of ECM differs depending on the growth strategy of the fungus. In axenic cultures, faster-growing r-strategists like *Pisolithus tinctorius* show fully developed ECM structures after 7 days, while slow-growing K-strategists like *Tricholoma terreum* and *T. vaccinum* need 1.5–5 months for the formation of ECM (Asiimwe et al. 2012; Malajczuk et al. 1990; Mankel et al. 2002). Specific **root exudates** may be involved prior to colonization, exhibiting parallels to flavonoid signaling in plant interactions of *Agrobacterium* or *Rhizobium* (Hirsch and Kapulnik 1998). In addition, the presence of '**mycorrhiza helper bacteria**' stimulates ECM formation (Bonfante and Anca 2009; Garbaye 1994).

The phytohormones **auxin** (indole-3-acetic acid, IAA; Gay and Debaud 1987) and **cytokinin** (like zeatin; Lagrange et al. 2001) are reported to influence **hyphal branching and growth**. The **synthesis and release of IAA by fungi** shows a synthetic pathway different from plant synthesis, and starts from tryptophan, which may be delivered to the fungi since it is a typical root exudate. In the pathway, the **aldehyde dehydrogenase** gene *ald1* shows up-regulation in the presence of the IAA precursor indole-3-acetaldehyde in pure cultures of *Tricholoma vaccinum*, as well as in ECM (Asiimwe et al. 2012; Krause and Kothe 2006). Gea et al. (1994) observed a hypertrophic Hartig' net in the ECM association between an IAA-overproducing transformant of *Hebeloma cylindrosporum* and *Pinus pinaster* seedlings, and suggested that fungal IAA affects the physiology of the host for the development of the Hartig' net. However, no correlation between the IAA-synthesizing ability of the IAA-overproducing *H. cylindrosporum* and their mycorrhizal activity could be observed (Gay et al. 1994). Charvet-Candela et al. (2002) hypothesized that IAA acts as a signal that induces differential expression of genes involved in mycorrhiza differentiation, and Felten et al. (2009) reported on the stimulation of lateral root formation by *Laccaria bicolor* through auxin transport and signaling.

Zeatin exposure is followed by the accumulation of metabolites such as the indole alkaloid **hypaphorine** in *Pisolithus microcarpus* hyphae, which is found during ECM development (Beguiristain and Lapeyrie 1997) and is regarded as an IAA antagonist, involved in fine-tuning plant development (Jambois et al. 2005).

In further ECM development, the contact between root and fungus involves fungal **hydrophobins**. These small proteins have a broad range of functions, such as protection of aerial hyphae and conidiospores, are involved in the formation of pseudoparenchyma, and promote the contact of hyphae with plant surfaces. Tagu et al. (1996, 2001, 2002) and Plett et al. (2012) showed the up-regulation of *hydPt-2* and *hydPt-3* in *Eucalyptus–Pisolithus* ECM and of *LbH5* in *Laccaria* ECM and in fruiting bodies. Furthermore, in *Tricholoma terreum* ECM, **host-specific accumulation of hydrophobin in the Hartig' net** was observed (Mankel et al. 2000, 2002). The ECM fungi *Hebeloma crustiliniforme* and *Pisolithus tinctorius* show a broad host range, while

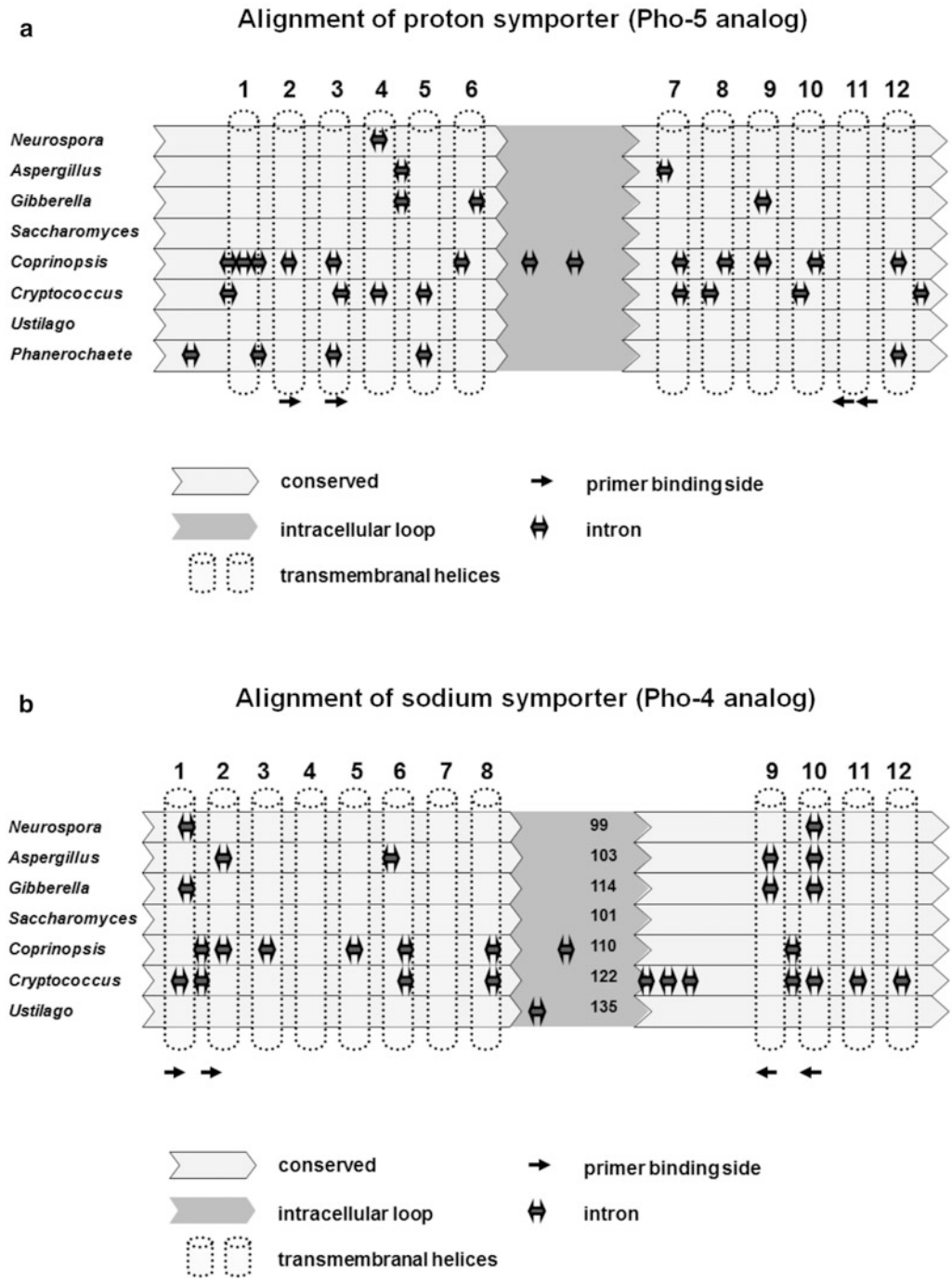

Fig. 11.5 Schematic presentation of the predicted structure of high-affinity phosphate transporter subfamilies. (**a**) Proton symporters (*Neurospora crassa* Pho-5/XM_957445, *Aspergillus fumigatus* XP_746548, *Gibberella zeae* PH-1/XM_388070, *Saccharomyces cerevisiae* PHO84/NC_001145, *Coprinopsis cinereus* XP_001840941, *Cryptococcus neoformans* XP_569629, *Ustilago maydis* XP_762637, *Phanerochaete chrysosporium* scaffold_63: 94009–95937). (**b**) Sodium symporters (*Neurospora crassa* Pho-4/AAA33607, *Aspergillus fumigatus* XP_748875, *Gibberella zeae* PH-1/XP_382602, *Saccharomyces cerevisiae* PHO89/CBK39373, *Coprinopsis cinereus* XP_001830199, *Cryptococcus neoformans* XP_568082, *Ustilago maydis* XP_759622)

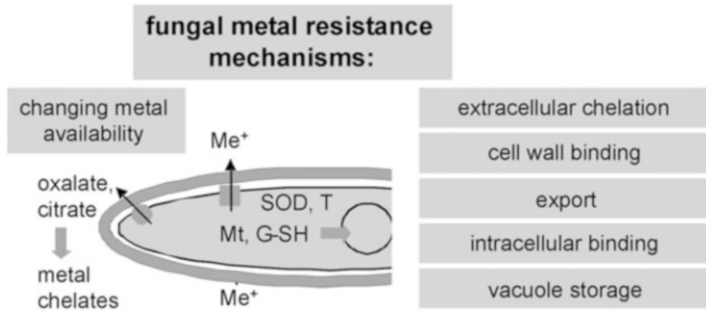

Fig. 11.6 Mechanisms of metal stress response in ECM fungi. The excretion of chelating acids alters environmental metal availability, while cell-wall sequestration reduces potential uptake. Exporters for heavy metal (Me^+) extrusion and intracellular metallothionein (*Mt*) or glutathione (*G-SH*) binding and transport into subcellular compartments such as the vacuole, as well as superoxide dismutase (*SOD*) and thioredoxin (*T*), lead to a filter function of the mycelia in ECM

species of genus *Tricholoma* are host specific, with fruiting bodies of *Tricholoma terreum* preferentially being found with pine. Only in the Hartig' net of this host-specific interaction was hydrophobin detectable, while expression was missing in the interaction with spruce, which is not the native host.

Cytoskeleton alteration in ECM was investigated using immunolabeling techniques and expression analyses. A different organization of **cortical microtubules** in comparison to control plants could be shown (Kuga-Uetake et al. 2004), and specifically **thin microtubular tracks were visible in the inner mantle and a reticulate structure in the Hartig' net** (Timonen and Peterson 2002). Small **cytoplasmic actin patches** were visible in the fungal external mycelium, while in Hartig' net tissue, actin could not be visualized at septa, in tips, or with patches. However, actin gene expression was not differentially regulated during mycorrhization (Schrey et al. 2007; Tarkka et al. 2000; Timonen and Peterson 2002). Gorfer et al. (2001) characterized two *Suillus bovinus* **small GTPases of the Rho subfamily** known to be involved in actin cytoskeleton regulation (Rac1 and Cdc42). Cdc42 was localized at the membrane of Hartig' net hyphae and at the tips and forming septa of external hyphae. Thus, Timonen and Peterson (2002) argued that actin is involved in the polarization of growth, and hence contributes to the differentiation of mycorrhizal tissue.

VII. Soil Specificity

Different processes such as use of fossil fuel, mining, or acid rain result in the accumulation of heavy metals in high concentrations, or in changed bioavailability of metal ions in ECM habitats. Although some heavy metal ions are essential in low concentrations, higher concentrations cause toxic symptoms by inactivating enzymes and proteins via reaction with metal-sensitive SH- and histidyl-groups, via binding to DNA or inducing stress by the formation of free radicals or reactive oxygen species (Brunner 2001; Hall 2001). **ECM fungi can reduce toxic effects on their host plant**, which has been reported for Al, Cd, Cu, Pb, Ni, and Zn (Smith and Read 2008). Fungi interact with metals and minerals, altering their physical and chemical state depending on fungal species and strain, the type of metal, metal concentration, and pH in rhizosphere (Cairney 1999; Jentschke and Godbold 2000; Leyval et al. 1997). This, in turn, affects growth, activity, and survival of both partners (Gadd 2010). Adaptation to higher metal concentrations can be seen with different mechanisms involved in **metal tolerance** (Fig. 11.6, Bellion et al. 2006; Meharg 2003). **Extracellular redox reactions** by excreted enzymes, **chelation by excreted ligands** (e.g., low molecular weight organic acids such as citrate and oxalate), **cell-wall binding** to carboxyl, hydroxyl, amine, phosphate, and sulfhydryl

groups of chitin and pigments, and **enhanced efflux rates** can reduce the amount of heavy metals in the cell, while **intracellular binding** to metallothioneins/phytochelatins or glutathione, as well as **sequestration in the vacuole**, are mechanisms leading to higher metal tolerance of the fungus (for review: Bellion et al. 2006; Colpaert 2008). In ECM fungi, extracellular binding is supposed to exert a filter function both at the mantle surface and within the Hartig' net by catching toxic ions before they reach the plant (Jentschke and Godbold 2000; Leyval et al. 1997).

After entering hyphal cells, metal ions might be sequestered and not transported onwards to the root cells. Cd phytochelatins have been detected in *Boletus edulis* after Cd exposition (Collin-Hansen et al. 2007), and Cu and Cd application resulted in the **up-regulation of metallothionein** in *P. involutus*. Additionally, Cd stress led to an increased content of **glutatione and γ-glutamine cysteine** (Bellion et al. 2007; Courbot et al. 2004). Post-translational as well as transcriptional up-regulation of **superoxide dismutase** (SOD), as well as up-regulation of other EST tags has been observed (Jacob et al. 2001, 2004). In *Paxillus involutus*, thioredoxins (small, heat-stable oxidoreductases), SODs, and strongly induced glutathione synthesis were observed upon Cd treatment (Bellion et al. 2006; Schützendübel and Polle 2002). Microarrays of *Cadophora finlandica* (Gorfer et al. 2009) revealed expression changes for a broad range of genes involved in transport, nutrition, signaling, and stress response. The mechanisms discussed in different reviews are depicted in Fig. 11.6 (see also: Bellion et al. 2006; Gherghel and Krause 2012).

In addition, soil specificity may be reflected in preferred expression of phosphate uptake systems (see above). A link to metal resistance may be assumed, since metal deposition in polyphosphates within the fungal vacuoles has been shown by differential staining methods (e.g., see Terpitz and Kothe 2006).

VIII. Transformation of Ectomycorrhizal Fungi

Molecular genetic manipulation is an important tool to unravel the biological functions of genes identified in genome-wide expression profiling during ECM establishment and maintenance. Over-expression, promoter studies, or localization of (fluorescently labeled, GFP) tagged gene products in situ, as well as inhibition of expression would be desirable for model ECM fungi, all depending on a stable transformation system. Gene deletion does not seem a valid approach so far, since usually **dikaryons** are found in ECM, and hence a knock-out would be needed in both nuclei before functional analysis is possible. However, **knock-down or RNA** interference assays seem possible ways out of this dilemma.

PEG-mediated **protoplast fusion**, first established for the filamentous ascomycete *Neurospora crassa* (Mishra and Tatum 1973), has been adapted to *Hebeloma cylindrosporum* using the glycerinaldehyde-3-phosphatdehydrogenase (*gpd*) promotor and tryptophan biosynthesis (*trpC)* terminator, both of the ascomycete *Aspergillus nidulans* (Marmeisse et al. 1992; Hebraud and Fevre 1988). The same construct was used to transform protoplasts of *Laccaria laccata* (Barrett et al. 1990) after stable protoplasts had been generated for ECM fungi (Kropp and Fortin 1986; Barrett et al. 1989). However, the method proved tedious and did not yield satisfying results for a number of fungi. In 1995, ***Agrobacterium tumefaciens* mediated transformation** (ATMT) was used to transform *Saccharomyces cerevisiae* (Bundock et al. 1995). An adaptation of this method led to *Suillus bovinus, H. cylindrosporum*, and *Paxillus involutus* transformation systems (Hanif et al. 2002; Pardo et al. 2002; Michielse et al. 2005). The transformation of *S. bovinus* was performed using a phleomycin resistance cassette containg the *gpd* promoter of *Schizophyllum commune,* and the hygromycin B resistance gene (*hph*) using the *gpd* promotor of *Agaricus bisporus*. In contrast to *S. commune* requiring intron sequences in the transformed genes for high expression, ECM fungi such as *Tuber borchii, Pisolithus tinctorius*, or *H. cylindrosporum* show intron-independent expression (Grimaldi et al. 2005; Rodríguez-Tovar et al. 2005; Müller et al. 2006; Rekangalt et al. 2007).

Transformation efficiency using ATMT varies for different fungi. *Hebeloma cylindrosporium, Tuber borchii*, and *Tricholoma vaccinum* showed only 6–18 %, 20 % and 30 % transformation efficiency, while *S. bovinus* and *P. involutus* achieved 67 % and up to 80 %, respectively (Hanif

et al. 2002; Pardo et al. 2002; Grimaldi et al. 2005; Müller et al. 2006; Asiimwe et al. 2012). In addition to the initial transformation efficiency, stability of transformants is essential if planning a long-term mycorrhization experiment, which might well need 3–6 months of co-cultivation before a fully developed Hartig' net is obtained. Since multicopy integration is often observed, a trade-off between higher stability and impairment of mycorrhization capacity might be expected, as a result of mutations at multiple integration loci. Hence, both traits should be carefully checked before performing *in planta* assays. Transformed hyphae of *Pisolithus tinctorius* showed 90 % **mitotic stability** after up to 10 months cultivation without the antibiotic hygomycin B (Rodríguez-Tovar et al. 2005). All transformants of *S. bovinus* were stable for 2 months (Hanif et al. 2002), and *T. vaccinum* transformants showed 100 % mitotic stability for *hph* and 70 % for *egfp* after 5 years of cultivation, with 20–50 % of multicopy integrations in independent transformation assays (Asiimwe et al. 2012). For *S. bovinus*, *T. vaccinum*, and *P. tinctorius,* transformants were able to form mycorrhiza (Pardo et al. 2002; Hanif et al. 2002; Rodríguez-Tovar et al. 2005; Asiimwe et al. 2012).

Recently, investigation of gene function using ATMT in ECM fungi has been reported for *Hebeloma cylindrosporum* overexpressing a heterologous, *Paxillus involutus* metallothionein (Bellion et al. 2007). Also, overexpression of an aldehyde dehydrogenase in *T. vaccinum* (Asiimwe et al. 2012) and a gene silencing approach in *Laccaria laccata* (Kemppainen and Pardo 2010) have been described.

IX. Conclusions

Recent work has extended the knowledge on ECM with regard to plant nutrition, and it has been possible to clearly establish the advantage of this symbiosis on a molecular level. The ECM fungal species differ in their abilities to metabolize specific substrates, which is reflected in different associations. **Pioneers of disturbed soil,** such as *Hebeloma* ssp., are able to use the N source nitrate besides ammonium, and nitrate transporter, nitrate reductase, and nitrite reductase genes have been analyzed. In contrast, the slow-growing *Amanita muscaria* did not reveal orthologs of these genes. This example may serve to strengthen the point that fungi of different habitats should be investigated in more detail to reveal specific features. Only if a detailed understanding is reached can specific traits be used in, for example, re-forestation or remediation programs. Another important point for future development in mycorrhizal research is to **define the release, absorption, and transfer of macronutrients with regard to different ecotypes of ECM.** Both soil specificity and host specificity depend on different abiotic and biotic factors, including succession, and co-culture experiments in incubator chambers may serve as first models to approach complex, natural conditions. The inclusion of further partners such as 'mycorrhiza helper bacteria', additional fungi, or the influence of insects are research topics that might be tackled after the understanding of the binary symbiosis is soundly based on molecular process descriptions.

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References

Abuzinadah RA, Read DJ (1986a) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. Utilization of peptides and proteins by ectomycorrhizal fungi. New Phytol 103:481–493

Abuzinadah RA, Read DJ (1986b) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. III. Protein utilization by *Betula*, *Picea* and *Pinus* in mycorrhizal association with *Hebeloma crustiliniforme*. New Phytol 103:507–514

Agerer R (1987/2002) Colour atlas of ectomycorrhizae. Einhorn, Schwäbisch Gmünd

Agerer R (2001) Exploration types of ectomycorrhizae – a proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11:107–114

Ashford AE, Ryde S, Barrow KD (1994) Demonstration of a short chain polyphosphate in *Pisolithus tinctorius* and the implicationsfor phosphorus transport. New Phytol 126:239–247

Asiimwe T, Krause K, Schlunk I, Kothe E (2012) Modulation of ethanol stress tolerance by aldehyde dehydrogenase in the mycorrhizal fungus *Tricholoma vaccinum*. Mycorrhiza 22:471–484

Barker SJ, Tagu D, Delp G (1998) Regulation of root and fungal morphogenesis in mycorrhizal symbioses. Plant Physiol 116:1201–1207

Barrett V, Lemke PA, Dixon RK (1989) Protoplast formation from selected species of ectomycorrhizal fungi. Appl Microbiol Biotechnol 30:381–387

Barrett V, Dixon RK, Lemke PA (1990) Genetic transformation of a mycorrhizal fungus. Appl Microbiol Biotechnol 33:313–316

Beguiristain T, Lapeyrie F (1997) Host plant stimulates hypaphorine accumulation in *Pisolithus tinctorius* hyphae during ectomycorrhizal infection while excreted fungal hypaphorine controls root hair development. New Phytol 136(3):525–532

Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol Lett 254(2):173–181

Bellion M, Courbot M, Jacob C, Guinet F, Blaudez D, Chalot M (2007) Metal induction of a *Paxillus involutus* metallothionein and its heterologous expression in *Hebeloma cylindrosporum*. New Phytol 174(1):151–158

Bending GD, Read DJ (1997) Lignin and soluble phenolic degradation by ectomycorrhizal and ericoid mycorrhizal fungi. Mycol Res 101(11):1348–1354

Benjdia M, Rikirsch E, Müller T, Morel M, Corratge C, Zimmermann S, Chalot M, Frommer WB, Wipf D (2006) Peptide uptake in the ectomycorrhizal fungus *Hebeloma cylindrosporum*: characterization of two di- and tri-peptide transporters (HcPTR2A and B). New Phytol 170:401–410

Bonfante P, Anca I-A (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annu Rev Microbiol 63:363–383

Brunner I (2001) Ectomycorrhizas: their rhole in forest ecosystems under the impact of acidifying pollutants. Perspect Plant Ecol 4(1):13–27

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J 14(13):3206–3214

Bun-Ya M, Nishimura M, Harashima S, Oshima Y (1991) The *PHO84* Gene of *Saccharomyces cerevisiae* encodes an inorganic phosphate transporter. Mol Cell Biol 11:3229–3238

Burke RM, Cairney JWG (1997) Carbohydrolase production by the ericoid mycorrhizal fungus *Hymenoscyphus ericae* under solid-state fermentation conditions. Mycol Res 101(9):1135–1139

Cairney JWG (1999) Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza 9: 125–135

Chalot M, Blaudez D, Brun A (2006) Ammonia: a candidate for nitrogen transfer at the mycorrhizal interface. Trends Plant Sci 11:263–266

Charvet-Candela V, Hitchin S, Ernst D, Sandermann H, Marmeisse R, Gay G (2002) Characterization of an Aux/IAA cDNA upregulated in *Pinus pinaster* roots in response to colonization by the ectomycorrhizal fungus *Hebeloma cylindrosporum*. New Phytol 154(3):769–777

Collin-Hansen C, Pedersen SA, Andersen RA, Steinnes E (2007) First report of phytochelatins in a mushroom: induction of phytochelatins by metal exposure in *Boletus edulis*. Mycologia 99(2):161–174

Colpaert JV (2008) Heavy metal pollution and genetic adaptations in ectomycorrhizal fungi. In: Avery S, Stratford M, van West P (eds) Stress in yeasts and filamentous fungi. Elsevier, Amsterdam, pp 157–173

Courbot M, Diez L, Ruotolo R, Chalot M, Leroy P (2004) Cadmium-responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*. Appl Environ Microbiol 70(12):7413–7417

Courty PE, Buée M, Diedhou AG, Frey-Klett P, Le Tacon F, Rineau F, Turpault M-P, Uroz S, Garbaye J (2010) The role of ectomycorrhizal communities in forest ecosystem processes: new perspectives and emerging concepts. Soil Biol Biochem. doi:10.1016/j.soilbio.2009.12.006

Couturier J, Fay E, Fitz M, Wipf D, Blaudez D, Chalot M (2010) PtAAP11, a high affinity amino acid transporter specifically expressed in differentiating xylem cells of poplar. J Exp Bot 61(6):1671–1682

Cromack K, Sollins P, Granstein WC (1979) Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus *Hysterangium crassum*. Soil Biol Biochem 11:463–468

da Silva Coelho I, de Queiroz MV, Costa MD, Kasuya MCM, de Araújo EF (2010) Identification of differentially expressed genes of the fungus *Hydnangium* sp. during the pre-symbiotic phase of the ectomycorrhizal association with *Eucalyptus grandis*. Mycorrhiza 20:531–540

Deveau A, Kohler A, Frey-Klett P, Martin F (2008) The major pathways of carbohydrate metabolism in the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N. New Phytol 180(2):379–390

Dinkelaker B, Marschner H (1992) In vivo demonstration of acid phosphatase activity in the rhizosphere of soil-grown plants. Plant Soil 144:199–205

Duplessis S, Courty PE, Tagu D, Martin F (2005) Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globus* and *Pisolithus microcarpus*. New Phytol 165:599–611

Fajardo Lopez M, Dietz S, Grunze N, Bloschies J, Weiss M, Nehls U (2008) The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil growing hyphae. New Phytol 180:365–378

Felten J, Kohler A, Morin E, Bhalerao RP, Palme K, Martin F, Ditengou FA, Legue V (2009) The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and *Arabidopsis* through auxin transport and signaling. Plant Physiol 151(4): 1991–2005

Finlay RD, Söderström B (1992) Mycorrhiza and carbon flow to the soil. In: Allen M (ed) Mycorrhiza functioning. Chapman and Hall, London, pp 134–160

Frank AB (1885) Über die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. Ber Dtsch Bot Ges 3:128–145

Fries N, Serck-Hanssen K, Häll Dimberg L, Theander O (1987) Abietic acid an activator of basidiospore germination in ectomycorrhizal species of the genus *Suillus* (Boletaceae). Exp Mycol 11:360–363

Gadd GM (2010) Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology 156:609–643

Garbaye J (1994) Helper bacteria – a new dimension to the mycorrhizal symbiosis. New Phytol 128(2): 197–210

Gay G, Debaud JC (1987) Genetic study on indole-3-acetic acid production by ectomycorrhizal *Hebeloma* species: inter- and intraspecific variability in the homo- and dikaryotic mycelia. Appl Microbiol Biotechnol 26:141–146

Gay G, Normand L, Marmeisse R, Sotta B, Debaud JC (1994) Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnési have increased mycorrhiza activity. New Phytol 128:645–657

Gea L, Normand L, Vian B, Gay G (1994) Structural aspects of ectomycorrhiza of *Pinus pinaster* (Ait.) Sol. formed by an IAA-overproducer mutant of *Hebeloma cylindrosporum* Romagnési. New Phytol 128:659–670

Gherghel F, Krause K (2012) Role of mycorrhiza in reforestation at heavy metal-contaminated sites. In: Kothe E, Varma A (eds) Bio-geo interactions in metal-contaminated soils, vol 31, Soil biology. Springer, Berlin, pp 183–199

Gorfer M, Tarkka MT, Hanif M, Pardo AG, Laitiainen E, Raudaskoski M (2001) Characterization of small GTPases Cdc42 and Rac and the relationship between Cdc42 and actin cytoskeleton in vegetative and ectomycorrhizal hyphae of *Suillus bovines*. Mol Plant Microbe Interact 14(2):135–144

Gorfer M, Persak H, Berger H, Brynda S, Bandian D, Strauss J (2009) Identification of heavy metal regulated genes from the root associated ascomycete *Cadophora finlandica* using a genomic microarray. Mycol Res 113:1377–1388

Grimaldi B, de Raaf MA, Filetici P, Ottonello S, Ballario P (2005) *Agrobacterium*-mediated gene transfer and enhanced green fluorescent protein visualization in the mycorrhizal ascomycete *Tuber borchii*: a first step towards truffle genetics. Curr Genet 48(1): 69–74

Grunze N, Willmann M, Nehls U (2004) The impact of ectomycorrhiza formation on monosaccaride transporter gene expression on poplar roots. New Phytol 164:147–155

Hagerberg D, Thelin G, Wallander H (2003) The production of ectomycorrhizal mycelium in forests: relation between forest nutrient status and local mineral sources. Plant Soil 252:279–290

Hall JL (2001) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53(366): 1–11

Hanif M, Pardo AG, Gorfer M, Raudaskoski M (2002) T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker. Curr Genet 41:183–188

Haselwandter K, Bobleter O, Read DJ (1990) Degradation of C-14-labeled lignin and dehydropolymer of coniferyl alcohol by ericoid and ectomycorrhizal fungi. Arch Microbiol 153(4):352–354

Hebraud M, Fevre M (1988) Protoplast production and regeneration from mycorrhizal fungi and their use for isolation of mutants. Can J Microbiol 34:157–161

Heller G, Adomas A, Li GS, Osborne J, van Zyl L, Sederoff R, Finlay RD, Stenlid J, Asiegbu FO (2008) Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus Laccaria bicolor. BMC Plant Biol 8(19). doi:10.1186/1471-2229-8-19

Hibbett DS, Gilbert LB, Donoghue MJ (2000) Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. Nature 407(6803):506–508

Hirsch AM, Kapulnik Y (1998) Signal transduction pathways in mycorrhizal associations: comparisons with the *Rhizobium*-legume symbiosis. Fungal Genet Biol 23:205–212

Horan DP, Chilvers GA (1990) Chemotropism; the key to ectomycorrhizal formation? New Phytol 116: 297–301

Jacob C, Courbot M, Brun A (2001) Molecular cloning, characterization and regulation by cadmium of a superoxide dismutase from the ectomycorrhizal fungus *Paxillus involutus*. Eur J Biochem 268: 3223–3232

Jacob C, Courbot ML, Martin F, Brun A, Chalot M (2004) Transcriptomic responses to cadmium in the ectomycorrhizal fungus *Paxillus involutus*. FEBS Lett 576(3):423–427

Jambois A, Dauphin A, Kawano T, Ditengou FA, Bouteau F, Legue V, Lapeyrie F (2005) Competitive antagonism between IAA and indole alkaloid hypaphorine must contribute to regulate ontogenesis. Physiol Plant 123(2):120–129

Jargeat P, Gay G, Debaud JC, Marmeisse R (2000) Transcription of a nitrate reductase gene isolated from the symbiotic basidiomycete fungus *Hebeloma cylindrosporum* does not require induction by nitrate. Mol Gen Genet 263:948–956

Jargeat P, Rekangalt D, Verner MC, Gay G, Debaud JC, Marmeisse R, Fraissinet-Tachet L (2003) Characterisation and expression analysis of a nitrate transporter and nitrite reductase genes, two members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma cylindrosporum*. Curr Genet 43:199–205

Javelle A, Andre B, Marini AM, Chalot M (2003a) High-affinity ammonium transporters and nitrogen sensing in mycorrhizas. Trends Microbiol 11:53–55

Javelle A, Morel M, Rodriguez-Pastrana BR, Botton B, Andre B, Marini AM, Brun A, Chalot M (2003b)

Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP–GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mol Microbiol 47:411–430

Javelle A, Chalot M, Brun A, Botton B (2004) Nitrogen transport and metabolism in mycorrhizal fungi and mycorrhizas. In: Varma A, Abbot L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, pp 393–429

Jentschke G, Godbold DL (2000) Metal toxicity and ectomycorrhizas. Physiol Plant 109:107–116

Johansson T, Le Quere A, Ahren D, Soderstrom B, Erlandsson R, Lundeberg J, Uhlen M, Tunlid A (2004) Transcriptional responses of *Paxillus involutus* and *Betula pendula* during formation of ectomycorrhizal root tissue. Mol Plant Microbe Interact 17(2): 202–215

Kemppainen MJ, Pardo AG (2010) pHg/pSILBA gamma vector system for efficient gene silencing in homobasidiomycetes: optimization of ihpRNA-triggering in the mycorrhizal fungus *Laccaria bicolor*. Microb Biotechnol 3:178–200

Kothe E, Muller D, Krause K (2002) Different high affinity phosphate uptake systems of ectomycorrhizal *Tricholoma* species in relation to substrate specificity. J Appl Bot 76:127–132

Kottke I, Oberwinkler F (1986) Mycorrhiza of forest trees – structure and function. Trees 1:1–24

Krause K (2005) Wirtsspezifität und spezifische Genexpression in Mykorrhiza-Pilzen der Gattung *Tricholoma*. Dissertation, Jena

Krause K, Kothe E (2006) Use of RNA fingerprinting to identify fungal genes specifically expressed during ectomycorrhizal interaction. J Basic Microbiol 46(5): 387–399

Kropp BR, Fortin JA (1986) Formation and regeneration of protoplasts from the ectomycorrhizal basidiomycete *Laccaria bicolor*. Can J Bot 64:1224–1226

Kuga-Uetake Y, Purich M, Massicotte HB, Peterson RL (2004) Host microtubules in the Hartig net region of ectomycorrhizas, ectendomycorrhizas, and monotropoid mycorrhizas. Can J Bot 82(7):938–946

Küster H, Becker A, Firnhaber C, Hohnjec N, Manthey K, Perlick AM, Bekel T, Dondrup M, Henckel K, Goesmann A, Meyer F, Wipf D, Requena N, Hildebrandt U, Hampp R, Nehls U, Krajinski F, Franken P, Pühler A (2007) Development of bioinformatic tools to support EST-sequencing, in silico- and microarray-based transcriptome profiling in mycorrhizal symbioses. Phytochemistry 68:19–32

Lagrange H, Jay-Allgmand C, Lapeyrie F (2001) Rutin, the phenolglycoside from eucalyptus root exudates, stimulates *Pisolithus* hyphal growth at picomolar concentration. New Phytol 149(2):349–355

Le Quere A, Johansson T, Tunlid A (2002) Size and complexity of the nuclear genome of the ectomycorrhizal fungus *Paxillus involutus*. Fungal Genet Biol 36:234–241

Le Quere A, Wright DP, Soderstrom B, Tunlid A, Johansson T (2005) Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) fr. Mol Plant Microbe Interact 18(7):659–673

Lehto T, Zwiazek JJ (2011) Ectomycorrhizas and water relations of trees: a review. Mycorrhiza 21 (2):71–90

Lei J, Dexheimer J (1988) Ultrastructural localisation of ATPase activity in *Pinus sylvestris*/*Laccaria laccata* mycorrhizal association. New Phytol 108:329–334

Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 7(3):139–153

Loth-Pereda V, Orsini E, Courty P-E, Lota F, Kohler A, Diss L, Blaudez D, Chalot M, Nehls U, Bucher M, Martin F (2011) Structure and expression profile of the phosphate Pht1 transporter gene family in mycorrhizal *Populus trichocarpa*. Plant Physiol 156:2141–2154

Louche J, Ali MA, Cloutier-Hurteau B, Sauvage F-X, Quiquampoix H, Plassard C (2010) Efficiency of acid phosphatases secreted from the ectomycorrhizal fungus *Hebeloma cylindrosporum* to hydrolyse organic phosphorus in podzols. FEMS Microbiol Ecol 73(2):323–335

Malajczuk N, Lapeyrie F, Garbaye J (1990) Infectivity of pine and eucalypt isolates of *Pisolithus tinctorius* on roots of *Eucalyptus urophylla invitro*. New Phytol 4:627–631

Mankel A, Krause K, Genenger M, Kost G, Kothe E (2000) A hydrophobin accumulated in the Hartig' net of ectomycorrhiza formed between *Tricholoma terreum* and its compatible host tree is missing in an incompatible association. J Appl Bot 74:95–99

Mankel A, Krause K, Kothe E (2002) Identification of a hydrophobin gene that is developmentally regulated in the ectomycorrhizal fungus *Tricholoma terreum*. Appl Environ Microbiol 68(3):1408–1413

Mann BJ, Bowman BJ, Grotelueschen J, Metzenberg RL (1989) Nucleotide sequence of pho-4+, encoding phosphate-repressible phosphate permease of *Neurospora crassa*. Gene 83:281–289

Marjanović Z, Uehlein N, Kaldenhoff R, Zwiazek JJ, Weiss M, Hampp R, Nehls U (2005a) Aquaporins in poplar: what a difference a symbiont makes! Planta 222(2):258–268

Marjanović Z, Nehls U, Hampp R et al (2005b) Mycorrhiza formation enhances adaptive response of hybrid poplar to drought. In: Biophysics from molecules to brain: in memory of Radoslav K.

Andjus, vol 1048. New York Academy of Sciences, New York, pp 496–499
Marmeisse R, Gay G, Debaud JC, Casselton LA (1992) Genetic transformation of the symbiontic basidiomycete fungus *Hebeloma cylindrosporum*. Curr Genet 22(1):41–45
Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant Soil 159:89–102
Martin F, Nehls U (2009) Harnessing ectomycorrhizal genomics for ecological insights. Curr Opin Plant Biol 12(4):508–515
Martin F, Laurent P, de Carvalho D, Voiblet C, Balestrini R, Bonfante P, Tagu D (1999) Cell wall proteins of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*: Identification, function, and expression in symbiosis. Fungal Genet Biol 27:161–174
Martin F, Kohler A, Duplessis S (2007) Living in harmony in the wood underground: ectomycorrhizal genomics. Curr Opin Plant Biol 10(2):204–210
Martin F, Aerts A, Ahren D, Brun A, Danchin EGJ, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, Salamov A, Shapiro HJ, Wuyts J, Blaudez D, Buee M, Brokstein P, Canback B, Cohen D, Courty PE, Coutinho PM, Delaruelle C, Detter JC, Deveau A, DiFazio S, Duplessis S, Fraissinet-Tachet L, Lucic E, Frey-Klett P, Fourrey C, Feussner I, Gay G, Grimwood J, Hoegger PJ, Jain P, Kilaru S, Labbe J, Lin YC, Legue V, Le Tacon F, Marmeisse R, Melayah D, Montanini B, Muratet M, Nehls U, Niculita-Hirzel H, Oudot-Le Secq MP, Peter M, Quesneville H, Rajashekar B, Reich M, Rouhier N, Schmutz J, Yin T, Chalot M, Henrissat B, Kues U, Lucas S, Van de Peer Y, Podila GK, Polle A, Pukkila PJ, Richardson PM, Rouze P, Sanders IR, Stajich JE, Tunlid A, Tuskan G, Grigoriev IV (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature 452(7183):88–92
Martin F, Kohler A, Murat C, Balestrini R, Coutinho PM, Jaillon O, Montanini B, Morin E, Noel B, Percudani R, Porcel B, Rubini A, Amicucci A, Amselem J, Anthouard V, Arcioni S, Artiguenave F, Aury J-M, Ballario P, Bolchi A, Brenna A, Brun A, Buée M, Cantarel B, Chevalier G, Couloux A, Da Silva C, Denoeud F, Duplessis S, Ghignone S, Hilselberger B, Iotti M, Marçais B, Mello A, Miranda M, Pacioni G, Quesneville H, Riccioni C, Ruotolo R, Splivallo R, Stocchi V, Tisserant E, Viscomi AR, Zambonelli A, Zampieri E, Henrissat B, Lebrun M-H, Paolocci F, Bonfante P, Ottonello S, Wincker P (2010) Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature 464: 1033–1038
Martinez P, Persson BL (1998) Identification, cloning and characterization of a derepressible Na+−coupled phosphate transporter in *Saccharomyces cerevisiae*. Mol Gen Genet 258:628–638
Meharg AA (2003) The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. Mycol Res 107:1253–1265
Meyer FH (1973) Distribution of ectomycorrhizae in native and man-made forests. In: Marks GC, Kozlowski TT (eds) Ectomycorrhizae. Academic, New York, pp 79–105
Michielse CB, Hooykaas PJ, van den Hondel CA, Ram AF (2005) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr Genet 48(1):1–17
Mishra NC, Tatum EL (1973) Non-mendelian inheritance of DNA-induced inositol independence in *Neurospora*. Proc Natl Acad Sci USA 70:3875–3879
Molina R, Massicotte H, Trappe JM (1992) Specifity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: Allen MF (ed) Mycorrhizal functioning. Chapman and Hall, New York, pp 357–423
Müller T, Benjdia M, Avolio M, Voigt B, Menzel D, Pardo A, Frommer WB, Wipf D (2006) Functional expression of the green fluorescent protein in the ectomycorrhizal model fungus *Hebeloma cylindrosporum*. Mycorrhiza 16(6):437–442
Müller T, Avolio M, Olivi M, Benjdia M, Rikirsch E, Kasaras A, Fitz M, Chalot M, Wipf D (2007) Nitrogen transport in the ectomycorrhiza association: the *Hebeloma cylindrosporum-Pinus pinaster* model. Phytochemistry 68(1):41–51
Nehls U, Wiese A, Guttenberger M, Hampp R (1998) Carbon allocation in etomycorrhizas: identification and expression analysis of an *Amanita muscaria* monosaccharide transporter. Mol Plant Microbe Interact 11:167–176
Nehls U, Kleber R, Wiese J, Hampp R (1999) Isolation and characterization of a general amino acid permease from the ectomycorrhizal fungus *Amanita muscaria*. New Phytol 144:343–349
Nehls U, Bock A, Ecke M, Hampp R (2001a) Differential expression of the hexose-regulated fungal genes *amPAL* and *amMst1* within *Amanita*/*Populus* ectomycorrhizas. New Phytol 150:583–589
Nehls U, Bock A, Einig W, Hampp R (2001b) Excretion of two proteases by the ectomycorrhizal fungus *Amanita muscaria*. Plant Cell Environ 24:741–747
Nehls U, Göhringer F, Wittulsky S, Dietz S (2010) Fungal carbohydrate support in the ectomycorrhizal symbiosis: a review. Plant Biol 12(2):292–301
Ohta A (1998) Fruit-body production of two ectomycorrhizal fungi in the genus *Hebeloma* in pure culture. Mycoscience 39:15–19
Orlovich DA, Ashford AE (1993) Polyphosphate granules are an artifact of specimen preparation in the ectomycorrhizal fungus *Pisolithus tinctorius*. Protoplasma 173:91–102
Pardo AG, Hanif M, Raudaskoski M, Gorfer M (2002) Genetic transformation of ectomycorrhizal fungi mediated by *Agrobacterium tumefaciens*. Mycol Res 106:132–137
Perotto S, Peretto R, Faccio A, Schubert A, Varma A, Bonfante P (1995) Ericoid mycorrhizal fungi: cellu-

lar and molecular bases of their interactions with the host plant. Can J Bot 73:557–568
Peter M, Courty PE, Kohler A, Delaruelle C, Martin D, Tagu D, Frey-Klett P, Duplessis S, Chalot M, Podila G, Martin F (2003) Analysis of expressed sequence tags from the ectomycorrhizal basidiomycetes *Laccaria bicolor* and *Pisolithus microcarpus*. New Phytol 159(1):117–129
Pfeffer PE, Bago B, Sachar-Hill Y (2001) Exploring mycorrhizal function with NMR spectroscopy. New Phytol 150:543–553
Plassard C, Dell B (2010) Phosphorus nutrition of mycorrhizal trees. Tree Physiol 30:1129–1139
Plassard C, Scheromm P, Mousain D, Salsac L (1991) Assimilation of mineral nitrogen and ion balance in the two partners of ectomycorrhizal symbiosis: data and hypothesis. Experientia 47:340–349
Plassard C, Barry D, Eltrop L, Mousain D (1994) Nitrate uptake in maritime pine (*Pinus pinaster*) and the ectomycorrhizal fungus *Hebeloma cylindrosporum* – effect of ectomycorrhizal symbiosis. Can J Bot 72:189–197
Plett JM, Gibon J, Kohler A, Duffy K, Hoegger PJ, Velagapudi R, Han J, Kües U, Grigoriev IV, Martin F (2012) Phylogenetic, genomic organization and expression analysis of hydrophobin genes in the ectomycorrhizal basidiomycete *Laccaria bicolor*. Fungal Genet Biol 49(3):199–209
Rekangalt D, Verner MC, Kües U, Walser PJ, Marmeisse R, Debaud JC, Fraissinet-Tachet L (2007) Green fluorescent protein expression in the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. FEMS Microbiol Lett 268(1):67–72
Rodríguez-Tovar AV, Ruiz-Medrano R, Herrera-Martínez A, Barrera-Figueroa BE, Hidalgo-Lara ME, Reyes-Mrquez BE, Cabrera-Ponce JL, Valdés M, Xoconostle-Czares B (2005) Stable genetic transformation of the ectomycorrhizal fungus *Pisolithus tinctorius*. J Microbiol Methods 63(1):45–54
Rosling A, Lindahl BD, Taylor AFS, Finlay RD (2004) Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. FEMS Microbiol Ecol 47:31–37
Rousseau JVD, Reid CPP (1991) Effects of phosphorus and ectomycorrhizas on the carbon balance of loblolly pine seedlings. Forest Sci 36:101–112
Salzer P, Hager A (1991) Sucrose utilzation of the ectomycorrhizal fungi *Amanita muscaria* and *Hebeloma crustiliniforme* depends on the cell wall-bound invertase activity of their host *Picea abies*. Bot Acta 104:439–445
Sanmee R, Lumyong P, Dell B, Lumyong S (2010) In vitro cultivation and fruit body formation of the black bolete, *Phlebopus portentosus*, a popular edible ectomycorrhizal fungus in Thailand. Mycoscience 51:15–22
Schrey SD, Salo V, Raudaskoski M, Hampp R, Nehls U, Tarkka MT (2007) Interaction with mycorrhiza helper bacterium *Streptomyces* sp. AcH 505 modifies organisation of actin cytoskeleton in the ectomycorrhizal fungus *Amanita muscaria* (fly agaric). Curr Genet 52(2):77–85
Schützendübel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351–1365
Simard SW, Perry DA, Jones MD, Myrold DD, Durall DM, Molina R (1997) Net transfer of carbon between ectomycorrhizal tree species in the field. Nature 388:579–582
Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Academic, London
Söderström B, Read DJ (1987) Respiratory activity of intact and excised ectomycorrhizal mycelial systems growing in unsterilised soil. Soil Biol Biochem 19:231–236
Tagu D, Martin F (1995) Expressed sequence tags of randomly selected cDNA clones from *Eucalyptus globus–Pisolithus tinctorius* ectomycorrhizae. Mol Plant Microbe Interact 8:781–783
Tagu D, Python M, Crétin C, Martin F (1993) Cloning symbiosis related cDNAs from eucalypt ectomycorrhizas by PCR-assisted differential screening. New Phytol 125:339–343
Tagu D, Nasse B, Martin F (1996) Cloning and characterization of hydrophobins-encoding cDNAs from the ectomycorrhizal Basidiomycete *Pisolithus tinctorius*. Gene 168(1):93–97
Tagu D, De Bellis R, Balestrini R, De Vries OMH, Piccoli G, Stocchi V, Bonfante P, Martin F (2001) Immunolocalization of hydrophobin HYDPt-1 from the ectomycorrhizal basidiomycete *Pisolithus tinctorius* during colonization of *Eucalyptus globulus* roots. New Phytol 149(1):127–135
Tagu D, Lapeyrie F, Martin F (2002) The ectomycorrhizal symbiosis: genetics and development. Plant Soil 244(1–2):97–105
Tarkka MT, Vasara R, Gorfer M, Raudaskoski M (2000) Molecular characterization of actin genes from homobasidiomycetes: two different actin genes from *Schizophyllum commune* and *Suillus bovinus*. Gene 251(1):27–35
Tatry M-V, El Kassis E, Lambilliotte R, Corratgé C, Van Aarle I, Amenc LK, Alary R, Zimmermann S, Sentenac H, Plassard C (2009) Two differentially regulated phosphate transporters from the symbiotic fungus *Hebeloma cylindrosporum* and phosphorus acquisition by ectomycorrhizal *Pinus pinaster*. Plant J 57:1092–1102
Terpitz U, Kothe E (2006) Diversity and adaptation of soil fungi in an ecosystem with contamination originating form a phosphate fertilizer plant. J Appl Bot Food Qual 80:187–193
Timonen S, Peterson L (2002) Cytoskeleton in mycorrhizal symbiosis. Plant Soil 244:199–210
Trappe JM (1962) Fungus associates of ectotrophic mycorrhizae. Bot Rev 28(4):538–606
Treseder KK, Torn MS, Masiello CA (2006) An ecosystem-scale radiocarbon tracer to test use of

litter carbon by ectomycorrhizal fungi. Soil Biol Biochem 38:1077–1082

Varma A, Bonfante P (1994) Utilization of cell-wall related carbohydrates by ericoid mycorrhizal endophytes. Symbiosis 16(3):301–313

Versaw WK (1995) A phosphate-repressible, high-affinity phosphate permease is encoded by the pho-5+ gene of *Neurospora crassa*. Gene 153: 135–139

Weiss M, Mikolajewski S, Peipp H, Schmitt U, Schmidt J, Wray V, Strack D (1997) Tissue-specific and development-dependent accumulation of phenylpropanoids in Larch mycorrhizas. Plant Physiol 114:15–27

Wiemken V, Boller T (2002) Ectomycorrhiza: gene expression, metabolism and the wood-wide web. Curr Opin Plant Biol 5(4):355–361

Willmann A, Weiss M, Nehls U (2007) Ectomycorrhiza-mediated repression of the high-affinity ammonium importer gene *AmAMT2* in *Amanita muscaria*. Curr Genet 51(2):71–78

Wipf D, Benjdia M, Tegeder M, Frommer WB (2002) Characterization of a general amino acid permease from *Hebeloma cylindrosporum*. FEBS Lett 528: 119–124

Wright DP, Scholes JD, Read DJ, Rolfe SA (2000) Changes in carbon allocation and expression of carbon transporter genes in *Betula pendula* (Batsch) Fr. Plant Cell Environ 23:39–49

Wright DP, Johansson T, Le Quere A, Soderstrom B, Tunlid A (2005) Spatial patterns of gene expression in the extramatrical mycelium and mycorrhizal root tips formed by the ectomycorrhizal fungus *Paxillus involutus* in association with birch (*Betula pendula*) seedlings in soil microcosms. New Phytol 167(2): 579–596

Phytopathogenicity

12 Rust Fungi: Achievements and Future Challenges on Genomics and Host–Parasite Interactions

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I. Introduction

Rusts are plant diseases caused by **pathogenic fungi of the Pucciniales order (Basidiomycota)**. The term 'rust' refers to the yellow- or rust-colored spores, which are produced on infected plant organs. **Rust fungi are destructive parasites of cultivated plants from almost all families, encompassing more than 7,000 host species** (Kolmer et al. 2009). They are responsible for important yield losses in a variety of annual crops such as cereals (wheat, barley), flax, and soybean, but they also attack perennial plants such as pine, poplar, and coffee as well as ornamentals plants (rose, etc.). Interestingly, the model plants *Arabidopsis thaliana* and rice (*Oryza sativa*) are immune to rust fungi, which offers opportunities for studying the physiological and genetical mechanisms underlying non-host resistance in plant–rust fungi interactions (Mellersh and Heath 2003; Shafiei et al. 2007; Loehrer et al. 2008; Azinheira et al. 2010; Ayliffe et al. 2011).

Rust diseases have been known for centuries, and records are available for wheat stem rust since antiquity (Voegele et al. 2009). We need to look back just 100 years to the devastating **coffee orange rust** outbreak in Sri Lanka that led to a switch from coffee culture to tea to see how rust diseases may dramatically change the course of history (Waller et al. 2007). Presently, the emergence of new races of the **stem rust *Puccinia graminis* f. sp. *tritici*** (races of the Ug99 lineage) **is threatening wheat production in the world** (Singh et al. 2011), and the **Asian soybean rust (*Phakopsora pachyrhizi*) is an emerging disease of soybean (*Glycine max*)**

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(Goellner et al. 2010). Based on scientific and economic criteria, rust diseases of wheat, flax, and soybean are considered among the most important plant-pathogenic fungi (Dean et al. 2012). The **infective unit of rust fungi is the spore**, which adheres to the host surface, germinates and penetrates the plant directly through the epidermis or through natural openings (stomata). After successful colonization of plant tissues, millions of spores may be produced on an infected host and then wind-dispersed for long distances. On a small scale, spores may be spread through rain splashes from leaf to leaf or stem to stem. Rust fungi display complex life cycles, with several spore types that may be sometimes produced on different host species (see section III). **The most important diseases are caused by dikaryotic urediniospores** (Agrios 2005).

Rusts are obligate biotrophic fungi which depend on living host tissues for their growth and reproduction. A common feature is the formation of a **specialized structure called the haustorium** [from Latin: *haurire* (*haurio, hausi, haustum*), to drink, to draw; de Bari (1863)], which allows an intimate contact with the living plant cell to obtain nutrients from host tissues (O'Connell and Panstruga 2006; Voegele and Mendgen 2011). Major insights have emerged from studies on rust fungi for describing plant–parasite relationships. First, rust fungi are amenable to genetic crosses of selected isolates, and the genetic model of host–parasite relationships established from the observation of ***Melampsora lini*** **interactions with flax (*Linum usitatissimum*)** in the middle of the last century was a keystone in plant pathology (Flor 1956; see section IV). More recently, molecular studies on ***Uromyces fabae*** have indicated that the haustorium coordinates the uptake of host nutrients, and also signals between the host cell and the parasite through the invaginated plant cell plasma membrane (reviewed in Voegele and Mendgen 2011).

Pioneer molecular studies on purified haustoria of *M. lini* showed that **a number of small proteins are secreted by this structure, including avirulence proteins that trigger host resistance responses** (Dodds et al. 2004; Catanzariti et al. 2006). Plants employ sophisticated mechanisms to perceive and appropriately defend against pathogens. The **plant immune system** is able to detect foreign molecules called 'pathogen-associated molecular patterns' (PAMPs) to **activate PAMP-triggered immunity** (PTI) (Zipfel and Robatzek 2010). **Pathogens interfere with this response by secreting effector proteins to suppress defense responses** and enable successful colonization (for review, see O'Connell and Panstruga 2006; Stergiopoulos and De Wit 2009). In turn, plants can perceive such effectors through additional **receptors**—typically nucleotide-binding leucine-rich repeat (NB-LRR) proteins—to mount **a second layer of defense called effector-triggered immunity** (ETI) (Jones and Dangl 2006). For rust pathogens, subversion of host cellular organization and functions is thought to occur through the secretion of effector molecules from the haustoria, but also from spores and infection hyphae all along the infection cycle (reviewed in Duplessis et al. 2012). Until recently, identification of genes from obligate biotrophs was a major limitation to study rust fungi. Fundamental knowledge of the fungal genomes should significantly advance our understanding of the plant–rust fungi interaction. Next generation sequencing technologies now help in gaining new insights into the genome and the transcriptome of rust parasites expressed within their hosts.

II. Rust Genomes

Recent reports of plant pathogen genome sequences, including fungi and oomycetes with biotrophic, hemibiotrophic, or necrotrophic lifestyles, have opened new perspectives in the understanding of host–parasite relationships through **comparative genomics** (for recent reviews, see Raffaele and Kamoun 2012; Spanu 2012). **Rust genome sequencing projects** were launched in 2006 by the Broad Institute of MIT and Harvard, USA for the wheat stem rust fungus *P. graminis* f. sp. *tritici* (Fungal Genome

Initiative, public release 2007) and by the DOE Joint Genome Institute, USA for the poplar leaf rust fungus ***Melampsora larici-populina*** (Community Sequencing Program, public release 2008). However **the size and the complex composition of rust fungi genomes—particularly richness in repetitive and transposable elements (TE)—have hampered their analysis.** Manual annotation of gene families performed by the poplar rust and the wheat rust communities has unraveled specific **expansions and contractions of several biological functions** (Duplessis et al. 2011a). In addition, whole-genome oligoarray-based transcriptomics particularly helped in identifying **key genes expressed in planta** when the fungi are invading their respective hosts, which may be important in interactions with their hosts such as those encoding effectors (Duplessis et al. 2011a).

The draft genome (Illumina sequencing only) of the wheat leaf rust *Puccinia striiformis* f. sp. *tritici* has been released by the University of California (Cantu et al. 2011). Apart from these three rust genomes, other rust sequencing projects are ongoing in sequencing centers, such as the JGI (*Cronartium quercuum f. sp. fusiforme*) and the Broad Institute (*P. striiformis* f. sp. *tritici*, *Puccinia triticina*, *Puccinia* spp. strains resequencing), as well as in different research groups (coffee rust *Hemileia vastatrix*, Cenicafe, Columbia; flax rust *M. lini*, CSIRO, Australia; poplar rusts *Melampsora* spp. NRC, Canada; poplar rust *M. larici-populina* strains genome resequencing, INRA Nancy, France). In this section, we focus on complete genomes already published for rust fungi. Other rust genomic ressources are available in databases for a larger number of species, such as Expressed Sequence Tags (ESTs), protein or nucleotide sequences, including 167,170 ESTs (as of April 2012; four species cumulating >150,000 ESTs: *M. larici-populina*, 54,445 ESTs; *Phakopsora pachyrhizi*, 34,394 ESTs; *Puccinia triticina*, 44,407 ESTs and *Uromyces appendiculatus*, 19,480 ESTs) which provides valuable informations in terms of comparative genomics of rust fungi (see section III.C).

A. Description

1. Sequenced Rust Genomes

a) General Features of the Rust Genomes

The genomes of the polar rust *M. larici-poulina* and the wheat stem rust *P. graminis* f. sp. *tritici* were sequenced by using a whole genome shotgun (WGS) strategy with Sanger sequencing. Genomic libraries of DNA fragments of various sizes were used to help the genome assembly process. As obligate biotrophic pathogens, the genetic material was recovered from spores collected on the host; in the present case, dikaryotic ($2 \times n$) urediniospores, i.e., rust genome sequences are chimeras of the two haplotypes contained in collected urediniospores. The WGS strategy led to a rather good quality of genome assemblies with a few hundred scaffolds for both rust fungi, half of the genomes being contained in no more than 30 scaffolds (Table 12.1; N50 statistics), and gaps representing less than 10 % of the sequences. (Duplessis et al. 2011a)

The size of the rust genomes is quite large compared with other basidiomycetes, with 101 and 89 megabases (Mb) for *M. larici-populina* and *P. graminis* f. sp. *tritici* respectively. The large size of these genomes is mostly explained by TE invasion, as in each genome TE account for nearly half of the sequence (Table 12.1), and no evidence of genome-scale duplication could be detected. Interestingly, both the poplar rust and the wheat rust genomes contain similar **high number of predicted genes** (>16,000 genes; Table 12.1) compared to other basidiomycete pathogens (Duplessis et al. 2011a; Raffaele and Kamoun 2012). **These large numbers of genes are partly explained by gene family expansions** with the presence of numerous multigene families, particularly some containing more than 50 members (19 and 14 families in *M. larici-populina* and *P. graminis* f. sp. *tritici*, respectively, versus 13 families in the tree symbiotic fungus *Laccaria bicolor*; Duplessis et al. 2011a).

One of the most striking features of the rust fungi coding space is that less than 50 % of the predicted proteins showed significant homology to non-redundant protein database at GenBank (Table 12.1). However, homology searches between the two rust genomes showed significant matches for 57 % of the proteins, which indicates that the two rust species probably share genes specific to the Pucciniales group.

Table 12.1. Genome sequence statistics of Pucciniales

	Melampsora larici-populina genome (Duplessis et al. 2011a)	*Puccinia graminis* f. sp. *tritici* genome (Duplessis et al. 2011a)	*Puccinia striiformis* f. sp. *tritici* draft genome (Cantu et al. 2011)
Number of contigs/scaffolds	3,264/462	4,557/392	29,178 (contigs only)
Total assembly size of contigs/ scaffolds (% gaps)	97.7/101.1 Mb (3.4 %)	81.5/88.6 Mb (8 %)	64.8 Mb (estimated total genome size between 68.2 and 78.8 Mb)
Contigs N50/L50 (Kb)[a]	27/1.1 Mb	30/0.97 Mb	n.a./5.1 Kb
Scaffolds N50/L50 (Kb)[a]	265/112.3 Kb	546/39.5 Kb	n.a.
Coverage (x-fold)	6.9	12	>50
Number of predicted genes	16,399	17,773	20,423
Transposable elements (TE) coverage (% genome)	45[b]	43.7[b]	17.8[b]
Class I retrotransposons	11.6	13.4	8.2
LTR retrotransposons	8.12	12.4	7.2
Class II DNA transposons	15.2	11.7	8.2
Terminal inverted repeat (TIR)	13.8	11.7	
Not categorized	18.2	18.6	1.3
Predicted proteins showing similarity to documented proteins (BLASTP E-value ≤ 10–5)	41 %	35 %	n.a. (69 % showing similarity with *P. graminis* f.sp. *tritici* proteins)
Predicted secretome	1,848	1,522	1,088
Mitochondrion genome	79.5 kb (four scaffolds)	79.2 kb (six scaffolds)	84 kb (22 contigs)

n.a. not available

[a]N50 corresponds to the N largest contigs/scaffolds that capture half of the total sequence and L50 is the length of the smallest contig/scaffold in the N50 set

[b]TE analysis was performed using a strictly identical approach in *M. larici-populina* and *P. graminis* f. sp. *tritici*, whereas a distinct approach was considered for *P. striiformis* f. sp. *tritici* and most likely explains the differences observed

The draft genome of the rust fungus *P. striiformis* f. sp. *tritici* has an estimated size of 78.8 Mb in the range of the *P. graminis* f. sp. *tritici* genome, and about ~20,000 predicted genes (Cantu et al. 2011; the authors provide this number with caution, since only a single gene prediction method was used). Quite interestingly, 69 % of the *P. striiformis* f. sp. *tritici* predicted genes show significant homology to *P. graminis* f. sp. *tritici* genes, which strongly supports the existence of conserved genes at the level of the Pucciniaceae family within the Pucciniales.

Ongoing sequencing projects conducted for other rust species in diverse families will help to identify the precise extent of gene specificity in rust fungi. The **large number of genes of unknown function and without homology in international databases is a singularity of rust genomes** that is striking compared to other biotrophic fungal pathogens, like the ascomycete *Blumeria graminis* (barley powdery mildew), with a ~120 Mb genome and only 5,854 genes (Spanu et al. 2010) and the maize smut basidiomycetes *Ustilago maydis* and *Sporisorium reilianum*, with 18.7 and 20.5 Mb genomes respectively, and only 6,902 and 6,648 gene, respectively (Kämper et al. 2006; Schirawski et al. 2010).

b) Genome Organization

Singular genome organizations have been reported for several pathogenic fungi or oomycete infecting plants (Raffaele and Kamoun 2012). For instance, several AT-rich isochores regions have been detected in the genome of the oilseed rape pathogen *Leptosphaeria maculans*, in homogenic GC-content genomic regions (Rouxel et al. 2011). Interestingly, avirulence genes and candidate virulence effector genes were identified in these regions, suggesting that these isochores might be a niche for effector genes. In the case of plant pathogenic

oomycetes *Phytophthora* spp., very large gene-sparse regions were observed in the genome sequences, and interestingly, these regions contain avirulence genes and genes encoding putative effectors (Haas et al. 2009; Raffaele et al. 2010). In the case of the poplar and the wheat stem rusts, their genomes do not show any particular organization, and genes and TE are fairly evenly distributed along genomic scaffolds (Duplessis et al. 2011a). No significant association between TE and genes sharing the same *g*ene *o*ntologies (GO) could be detected in the rust genomes. Although a slightly higher number of flanking intergenic regions could be detected for a few genes encoding candidate effectors compared with all predicted genes in the genome of *M. larici-populina* (Saunders et al. 2012), no significant association with TE was found (Duplessis et al. 2011a). Nevertheless, careful examination of **large gene families encoding small secreted proteins** showed the presence of a few TE types close to some members of gene families, thus indicating that some duplication events in these gene families might be related to past TE activity (Duplessis et al. 2011a).

Comparison of synonymous substitution rates for paralogous and orthologous gene pairs in the two rust genomes suggests that duplications between the two genomes were older than within each rust genome. Only 39 synteny blocks could be found between the two genomes, with a maximum of six orthologous gene pairs on ~280 Kb genomic sequence, indicating a lack of synteny between the two rust fungi genomes (Duplessis et al. 2011a). Mirroring the few gene homologs found between the two gene complements of the poplar rust and the wheat rust fungi, these results suggest an old divergence between the Melampsoraceae and the Pucciniaceae lineages within the Pucciniales. Interestingly, the comparative analysis of the *P. graminis* f. sp. *tritici* genome sequence and the draft sequence of *P. striiformis* f. sp. *tritici* identified microsynteny between the two rust genomes (Cantu et al. 2011). An improved assembly of the latter genome and comparison with *P. triticina* would greatly help in determining the exact level of synteny within the Pucciniaceae. In addition, the forthcoming analyses of several *Melampsora* spp. genome sequences will greatly help to describe genome evolution in Melampsoraceae, and will provide the basis for extensive comparison of genome evolution in several families of rust fungi.

2. Data from Other Species

The size of other rust genomes in ongoing genome projects are in the same range as, or even larger than those reported for the poplar and the wheat stem rust fungi, whereas genomes of several species in the Pucciniomycotina out of the Pucciniales, are so far smaller (JGI Mycocosm website; http://www.jgi.doe.gov/fungi). In the Pucciniacceae family, the size of the *P. graminis* f. sp. *tritici* and the *P. striiformis* f. sp. *tritici* genomes are rather similar, whereas the genome of the wheat leaf rust *P. triticina* is estimated to be larger (>120 Mb; Puccinia Group Database at the Broad Institute). The size of the genome of the flax rust *M. lini* is estimated around 200 Mb, which is about twice as large as the size of the genome of *M. larici-populina*, a relatively close species in the Melamposoraceae family (P.N. Dodds, personal communication). In the Phakopsoraceae family, the size of the genome of the soybean rust fungus *P. pachyrhizi* was estimated to be 500–800 Mb after initial sequencing by the JGI, and genome complexity hindered quality assembly (I. Grigoriev, personal communication). Altogether, these published and preliminary informations indicate that the larger genome size observed in the Pucciniales lineage compared with most basidiomycete pathogens is a common trend.

Repetitive elements and TE seem to significantly account for the greater size of rust fungi genomes, similar to several reports concerning other fungal biotrophs (Spanu 2012; Raffaele and Kamoun 2012).

Comparative analysis of complete LTR TE in the genomes of *M. larici-populina* and *P. graminis* f. sp. *tritici* showed that insertion age of most *Gypsy*- and *Copia*-like elements was recent in both genomes (<1 Mya) (Duplessis et al. 2011a). Completion of more rust genome projects will help to determine more precisely by which mechanisms genome expansion occurred in this taxonomical group of fungi, and whether such bursts of TE activity are responsible for the larger genome size of rust fungi. Also, the report of TE invasion and genome size expansion in diverse obligate biotrophic pathogens suggest that these trends might be related to this particular lifestyle, i.e., by allowing the diversification of crucial genes for biotrophy. It would be interesting to determine whether TE activity, along with diversification of virulence-related genes

(i.e., effectors) differ in rust fungi that require a single or two different hosts to complete their biological cycles.

Apart from the important TE proportion, rust fungal genomes sequenced so far contain a large number of genes compared to other basidiomycetes, and in *M. larici-populina* and *P. graminis* f. sp. *tritici* the expansion of lineage-specific multigene families seems to also account for the greater size of these genomes. With the availability of more rust genomes, it will be possible to determine at which taxonomical level such gene family expansions occurred, and how they might have shaped the evolution of rust fungi.

B. Specific Features of the Rust Genomes

As already stated above, **repetitive elements and TE account for nearly half of poplar and wheat rust fungi genomes** (Table 12.1). Interestingly, examination of TE classes composition showed differences between the two genomes. Indeed, class II DNA transposons are more represented in the *M. larici-populina* genome, particularly the TIR type, whereas class I retrotransposons are more important in the *P. graminis* f. sp. *tritici* genome, particularly the LTR retrotransposon type (Table 12.1). In the two genomes, uncategorized TE types are also found in similar proportions (Duplessis et al. 2011a). Reports from the *P. striiformis* f. sp. *tritici* genome differ (Table 12.1), with similar proportions for classes I and II transposons; however, the methods used for TE annotation were not similar, and this most probably explains the reported differences (Cantu et al. 2011). Another striking feature of rust fungi genomes is the large number of genes with no known homology in databases and no functional annotation (Duplessis et al. 2011a). Gene annotation of the draft genome of *P. striiformis* f. sp. *tritici* confirms this trend, and the comparison of *M. larici-populina* and *P. graminis* f. sp. *tritici* gene complements with ESTs available for *Phakopsora* spp., *Uromyces* spp., and *Puccinia* spp. confirms the presence of distinct sets of lineage-specific genes in rust fungi (Puthoff et al. 2008; Posada-Buitrago and Frederick 2005; Cantu et al. 2011; Duplessis et al. 2011a; Xu et al. 2011; Fernandez et al. 2012). **Analysis of GO terms associated with expanded gene families** showed that similar GO were significantly over-represented in both genomes, namely **zinc ion binding and nucleic acid binding activities**. Functional annotations of the largest expanded families in each rust genome also indicated common expansions of **zinc-finger proteins encoding genes** (Duplessis et al. 2011a). **Oligopeptide and amino acid transporters** were also among expanded gene families in both rust genomes, suggesting they might have an important role to play in rust fungi biology. This observation confirms the importance of amino acid transporters for rust fungi infection, as previously reported (Struck et al. 2002, 2004; Voegele et al. 2009; see section III.C). Furthermore, the strong expression of several oligopeptide transporter genes during the colonization of host tissues indicates that they probably have an important role during biotrophic growth (Duplessis et al. 2011b). Oligopeptides generated by fungal peptidase secreted in the host apoplast or in the extrahaustorial matrix could be a major source of nutrients that will require further functional investigations. **Glycosyl hydrolases (carbohydrate active enzymes, CAZymes), lipases and peptidases encoding genes were also among expanded gene families in the two rust genomes** (Duplessis et al. 2011a). **Chitin deacetylases** are among expanded CAZyme genes, and they seem important to achieve host infection, particularly at early stages (see section III.C). Although some CAZyme categories are expanded in rust fungi genomes, the total number of CAZymes is lower than in saprotrophic or hemibiotrophic fungi (Duplessis et al. 2011a), but larger than in other biotrophic pathogens that have undergone massive loss of CAZymes (Spanu et al. 2010; Spanu 2012). **Most of the large multigene families have no known functions, and among them, several encode lineage-specific small secreted proteins** (Duplessis et al. 2011a). Many virulence effectors as well as avirulence factors of plant pathogens correspond to small secreted proteins of unknown function (Stergiopoulos and de wit 2009), which explains the particular attention given to this fraction of the secretome in pathogen genome reports (Martin and Kamoun 2012).

In each rust genome, **over a thousand small secreted protein encoding genes were identified** (Table 12.1; Duplessis et al. 2011a; Cantu et al. 2011). **Among these proteins, many possess typical features of pathogenic effectors, and homologs of flax rust avirulence proteins and haustorially expressed secreted proteins were found** (Duplessis et al. 2011a; Hacquard et al. 2012; Saunders et al. 2012; see Duplessis et al. 2012 for a review on rust fungi effectors). In *M. larici-populina*, clusters of paralogous genes encoding such small secreted proteins that are expressed during infection of poplar leaves showed robust evidence of **diversifying selection**, suggesting that they have evolved under the pressure of the interaction with the host plant (Hacquard et al. 2012). The fact that distinct groups of lineage-specific candidate effector genes are found in *M. larici-populina* and in *P. graminis* f. sp. *tritici* strengthens the difference between the two rust fungi, and could also illustrate the specialization of each fungus on its specific hosts. Apart from these gene expansions, several conserved fungal genes were not found in rust fungi, which might be related to their obligate biotrophic status. **In particular, some genes crucial for nitrate and sulphate assimilation are missing, as well as genes involved in secondary metabolites and toxins synthesis usually present in most hemibiotrophic and necrotrophic fungal pathogens** (Lebrun 2012). Interestingly, some of the genes missing in rust fungi are also absent from the genomes of several other obligate biotrophic pathogens such as the ascomycete *B. graminis*, or the oomycetes *Hyaloperonospora arabidopsidis* and *Albugo labachii* (Spanu et al. 2010; Baxter et al. 2010; Kemen et al. 2011), suggesting convergent loss of functions in distinct eukaryotic pathogens towards adaptation to obligate biotrophic lifestyle.

III. Fungal Biology and Plant Infection Process

A. Overview of Rust Fungi Life Cycles

Although all rust fungi follow an obligate biotrophic lifestyle in nature, **the level of complexity of the life cycle varies considerably from one species to another**, or even according to particular ecological conditions. At its most complex form, **rust fungi can produce five types of spores: basidiospores, pycniospores, aeciospores, urediniospores (or uredospores) and teliospores (or teleutospores).** Those that can go through the five spore stages are termed **macrocyclic rusts**, while those lacking some of these stages are **microcyclic rusts**. In parallel, rusts are termed **heteroecious** when two distinct host species (often taxonomically unrelated) are required for completion of the life cycle, and **autoecious** when the life cycle can be completed in a single host species. Examples of macrocyclic heteroecious rusts are several *Puccinia* spp. and *Melampsora* spp., while the faba bean rust pathogen (***U. fabae***) represents a typical example of macrocyclic autoecious rust fungus, and the coffee leaf rust fungus (***Hemileia vastatrix***) is an example of microcyclic rust.

The life cycle of rust fungi is also characterized by diverse nuclear and chromosomal organizations. On a typical macrocyclic lifestyle, haploid dikaryotic teliospores ($n+n$), overwintering (or oversummering) in telia (Fig. 12.1a) usually on dead host tissue, undergo karyogamy (nuclear fusion; 2n), producing a basidium (2n), from which in turn originate single nuclei haploid basidiospores (n) (Fig. 12.1b). Considering heteroecious rusts, basidiospores cannot infect the telial host, and therefore, must travel to the aecial host (or 'alternate host', although in plant pathology this term is frequently attributed to the host for which symptoms cause less important economic damage). Basidiospores infect host tissues (Fig. 12.1c) to produce structures of two (or more) mating types termed pycnia (Fig. 12.1d). Insects or moisture on host surface cross-transport pycniospores enabling fertilization, where two cells merge to form a single cell with two genetically distinct, haploid nuclei (a process also termed plasmogamy; n+n) (Fig. 12.1e). These dikaryotic cells differentiate aecia (Fig. 12.1f), where aeciospores are produced and dispersed, often at long distances, to invade the telial host (heteroecious species). Typically, aeciospores do so through the stomatal opening (Fig. 12.1g), producing another fruiting body termed uredinium (Fig. 12.1h). Urediniospores (n+n) are the only spore type capable of successive infection that will iteratively produce urediniospores (Fig. 12.1i), potentially generating exponential multiplications of inoculum, leading to severe epidemics. Under host senescence conditions, uredinia convert to telia and produce thick-walled teliospores, capable of surviving on host dead tissues. (Agrios 2005; Kolmer et al. 2009; Voegele et al. 2009)

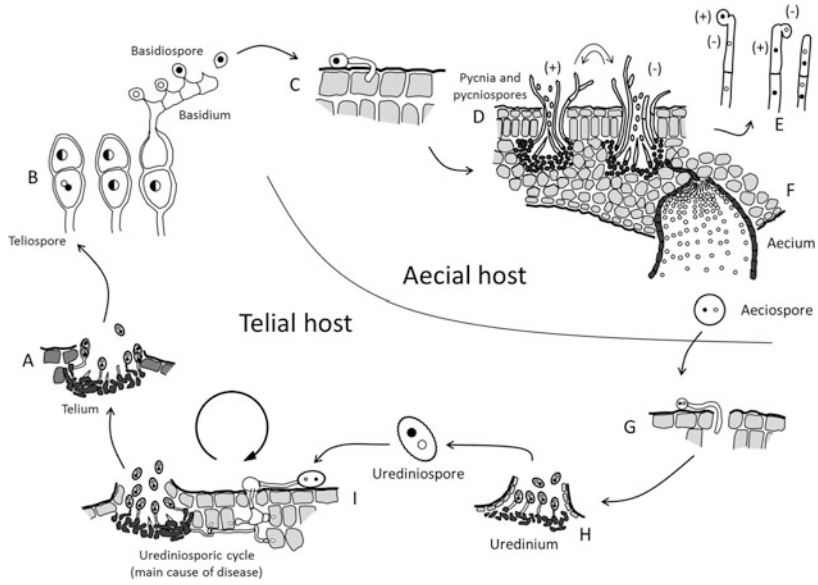

Fig. 12.1. A typical macrocyclic heteroecious rust lifestyle. (**a**) Telium on dead host tissue with haploid dikaryotic teliospores ($n+n$ chromosomes; haploid nuclei depicted by *black* and *white circles*). (**b**) Teliospores undergoing karyogamy (nuclear fusion; 2n), producing a basidium (2n), which in turn originates single nuclei haploid basidiospores (n). (**c**) Basidiospore infecting alternate (aecial) host by direct penetration. (**d**) Mycelia derived from basidiospore infection differentiate pycnia of different mating types. (**e**) Pycniospores (n) merge with hyphae of the alternate mating type (plasmogamy) to form a single cell with two genetically distinct haploid nuclei (n+n). (**f**) Haploid dikaryotic hyphae differentiate aecia (typically on the lower surface of the leaf), which produce aeciospores (n+n) that can be dispersed at long distances. (**g**) Aeciospores infect the telial host through the stomata. (**h**) Hyphae differentiate uredinia, where urediniospores (n+n) are released. (**i**) Urediniospores germinate on the host surface through a germ tube, which differentiates an appressorium over the stoma, and undergoes producing a penetration hypha into the substomatal chamber, a vesicle, haustoria mother cells close to host cells, which differentiate haustoria into the host cells, while the mesophyll is invaded by intercellular hyphae differentiating haustoria mother cells and haustoria, culminating with the production of uredinia and the repetition of the urediniospore infection cycle; on senescent host tissues, uredinia convert in telia where teliospores are formed, closing the macrocycle (Adapted from Alexopoulos et al. 1996; Agrios 2005; Kolmer et al. 2009; and Voegele et al. 2009)

B. Plant Infection Stages

1. Pre-penetration Stages

While basidiospores, urediniospores, and aeciospores are capable of infecting host plants, and teliospores and pycniospores are not, the infection arising from urediniospores has been studied in deeper detail, as this stage is important in terms of multiplication and dissemination in rust life cycles. Aeciospores are considered to behave like urediniospores in their the response to host surface topography, whereas basidiospores (which are thin-walled structures that rapidly lose viability through desiccation) infect host tissues by producing less differentiated structures compared with urediniospores (Voegele et al. 2009).

The following sections mostly focus on **host infection by urediniospores**. Given appropriate physical conditions, spores germinate through a germ pore to produce a **germ tube** [the number and position of germ pores are important morphological features for identification of rust species; e.g., Savile (1984)]. Specific **topographic signals** are then required so that the germ tube can differentiate appressorial infection structure. **On the host surface, appressoria are differentiated over stomata.** Topographic stimuli to produce appressoria in vitro were correlated to the height of the **stomata lips** for

several rust fungi in their respective host plants (Allen et al. 1991; Kemen et al. 2005a). In fact, some *Hordeum chilense* accessions are resistant to *Puccinia hordei* because **wax layers** over stomatal guard cells prevent topographic recognition, an early resistance mechanism termed *avoidance* (Vaz Patto and Niks 2001). The stimuli (topographic or other) required for appressoria differentiation vary from one rust fungus to another, and appressoria can differentiate in vitro on polyethylene membranes or on oil-collodion membranes (Staples et al. 1983; Azinheira et al. 2001; Kemen et al. 2005a; Vieira et al. 2012). However, urediniospores from some *Phakopsora* spp. can produce infection structures capable of direct leaf surface penetration (Allen et al. 1991). Upon appressorium formation, the cytoplasmic content is transferred from the germ tube into the appressorium, and a **septum** is formed between the germ tube and the appressorium. The appressorium differentiates a **penetration hypha into the stoma**, a process that involves the exertion of some **mechanical pressure**, e.g., 0.35 MPa for *U. appendiculatus*, which is considerably less than the pressure exerted by fungi that penetrate directly through the cuticle, such as *Magnaporthe grisea* or *Colletotrichum* spp. (Howard et al. 1991; Chen et al. 2004). Subsequently, the penetration hypha differentiates a septum-delimited **vesicle** in the substomatal chamber. Upon contact with a mesophyll cell, a septum-delimited **haustorial mother cell** is differentiated, into which most of the cytoplasmic content moves, with the previously differentiated structures remaining mostly vacuolated. Haustorial mother cells share some functional and structural similarities with the appressoria, as both engage on a combination of pressure exerted and lytic enzymes secreted to invade the host (for detailed review, see Voegele et al. 2009).

2. Post-penetration Stages

After penetration into host plant tissue, rust pathogens spread by differentiating **infection hyphae** that form haustorium mother cells involved in plant cell wall penetration and **haustorium** production (Fig. 12.2). **The haustorium is the hallmark of biotrophy, considered as a feeding structure, but also playing a role in the suppression of the host defense response and reprogramming of the host metabolism** (for a recent review see Voegele and Mendgen 2011).

> The haustorium is formed by breaching the plant cell wall and by invaginating the plant plasmalemma membrane without passing beyond the membrane. The composition of the host membrane surrounding the haustorium is modified, apparently lacking intramembranous particles and ATPase activity and exhibiting a dramatic reduction of sterols (Harder and Mendgen 1982; Harder and Chong 1991; Baka et al. 1995), and an extra-haustorial matrix, resembling an amorphous mixture mainly composed of carbohydrates and proteins, mostly of plant origin, is formed around the haustorium (Harder and Chong 1991). This matrix prompts an intimate contact between host and parasite, of key relevance for the biotrophic interaction, enabling the exchange of nutrients and information between the host cell and the haustorium. (Heath et al. 1997)

In fact, in hemibiotrophic fungi the initial biotrophic phase is also characterized by the presence of a contact zone between the fungus and the plant, which is lost after the switch to necrotrophy (Perfect and Green 2001; Mendgen and Hahn 2002). The **neck** at the base of the haustorium joins the plant and fungal plasma membranes, sealing the **extra-haustorial matrix** against the bulk apoplast. In compatible interactions, the fungus densely colonizes the host tissue by producing **inter-cellular infection hyphae**, haustoria mother cells, and haustoria in the mesophyll cells. **Uredinia** are then differentiated underneath the host cuticle, and urediniospores are released after the rupture of the cuticle or, in some cases, through the stomatal opening in **bouquet-shaped structures** (Agrios 2005; Kolmer et al. 2009; Voegele et al. 2009).

C. Fungal Gene Expression Throughout Rust Life Cycle

During the past decade, large-scale EST projects have generated a total of 167,170 Pucciniales ESTs (as of April 2012) available in

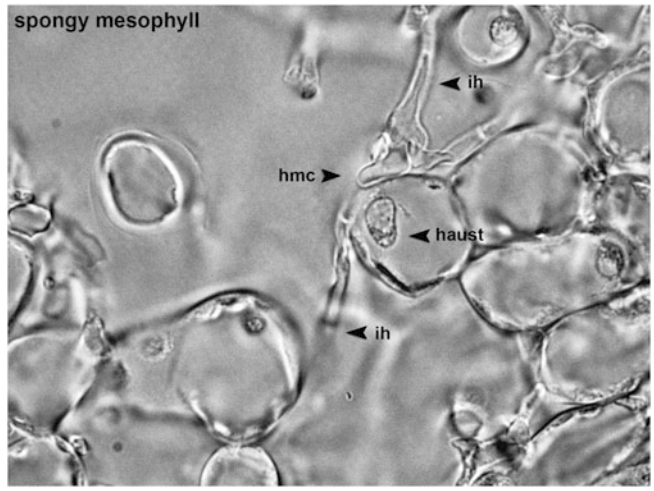

Fig. 12.2. Light transmission microscopy observation of *Melampsora larici-populina* invading hyphae (*ih*) between host cells; and haustorial mother-cell (*hmc*) and haustorium (*haust*) formed in contact of a host cell in the spongy mesophyll of a poplar leaf (BX41 Olympus microscope; picture by Sébastien Duplessis)

Table 12.2. Number of EST sequences for the Pucciniales currently published and/or available in public databases

Organism	Nr ESTs	References
Cronartium quercuum f. sp. *fusiforme*	1,236	Warren and Covert 2004; Baker et al. 2006
Hemileia vastatrix	6,763	Fernandez et al. 2012
Melampsora larici-populina	54,445	Joly et al. 2010; Duplessis et al. 2011a
Melampsora medusae f. sp. *deltoidis*	4,453	Joly et al. 2010
Melampsora medusae f. sp. *tremuloidis*	2,456	Joly et al. 2010
Melampsora occidentalis	2,567	Joly et al. 2010
Phakopsora pachyrhizi	34,394	Posada-Buitrago and Frederick 2005; Posada-Buitrago et al., unpublished (retrieved from NCBI database)
Puccinia coronata var. *lolii*	6	Dracatos et al. 2006
Puccinia graminis f. sp. *tritici*	261	Broeker et al. 2006; Fehser et al. 2010
Puccinia striiformis	2,864	Ling et al. 2007; Zhang et al. 2008; Yin et al. 2009; Cheng et al., unpublished (retrieved from NCBI database)
Puccinia triticina	44407	Hu et al. 2007; Xu et al. 2011
Uromyces appendiculatus	19,480	Cooper et al. 2007; Puthoff et al. 2008
Uromyces viciae-fabae	601	Jakupovic et al. 2006; Link and Voegele 2008

public databases (Table 12.2). Rust fungi life cycle stages represented comprise resting or germinating spores, appressoria, epiphytic material, infected leaf tissue containing haustoria and infection hyphae, and purified haustoria (Table 12.3). These data are crucial molecular investigations toward the understanding of the physiological processes involved in fungal development during host infection.

Over the years, many attempts to induce differentiation stages of rust fungi in the absence of the host have been conducted, particularly with the purpose of investigating the genetic programs controlling rust differentiation. **Urediniospore germination can occur**

Table 12.3. Number of EST sequences mentioned in Table 12.2 organized by sample type

Sample	Nr ESTs
Resting spores	21,052
Urediniospores	11,118
Pycniospores	4,869
Teliospores	3,703
Aeciospores	1,292
Germinating spores	94,501
Germinating urediniospores	93,278
Germinating basidiospores	1,223
Appressoria	5,404
Epiphytic material	11,876
Infected leaf tissue containing haustoria	26,586
Isolated haustoria	14,499

outside of the host on various types of substrate, even on water, **and appressorium formation can be induced on artificial membranes** (Hoch et al. 1987). A few rust fungi can eventually be cultivated axenically on complex rich culture media (e.g., Williams 1984; Fasters et al. 1993; Kaitera and Nuorteva 2010). Nevertheless, true functional haustoria as well as parasitic and sporulation structures can only be formed in planta, which is thought to be due to the lack of appropriate signals from the host plant in axenic culture (Heath 1990). With regard to these limitations, the method developed by Hahn and Mendgen (1992) for the isolation of haustoria from rust-infected leaves was a major breakthrough, enabling molecular and biochemical studies of biotrophy in different rust fungi (Hahn and Mendgen 1997; Jakupovic et al. 2006; Link and Voegele 2008; Yin et al. 2009; Joly et al. 2010; Puthoff et al. 2008; Xu et al. 2011). The use of laser capture microdissection to isolate in-planta rust structures such as uredinia (Tremblay et al. 2008; Hacquard et al. 2010) is another major advance to study molecular processes related to the biotrophic lifestyle of rust pathogens.

While resting urediniospores are metabolically largely inactive, many studies have reported the presence of a large number of transcripts, indicating the presence of a pool of mRNA available for critical functions upon germination (Hu et al. 2007; Ling et al. 2007). For instance, more than half of 13,093 *M. larici-populina* transcripts are detected at high expression levels in resting urediniospores with oligoarrays, a figure that rises only slightly (+137 transcripts) during germination (Duplessis et al. 2011b). In addition, the number of transcripts shared by resting and germinating urediniospores is larger than those shared by any other two differentiation stages (Hu et al. 2007; Duplessis et al. 2011b). On the other hand, genes expressed by distinct spore types seem to be drastically different. A study comparing *P. triticina* transcripts in urediniospores, teliospores, aeciospores, and pycniospores showed that 77–92 % of genes are unique to each spore type, and only one gene was common to all spore types (Xu et al. 2011). In total, 13,328 *P. triticina* genes were surveyed in this study, and some spore stages were represented by only over a thousand ESTs. With 17,773 and 22,815 genes in the closely-related species *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* respectively, more core genes expressed all along the life style might still be uncovered in future studies. For most species, it remains to be determined whether transcripts detected in resting urediniospores are also expressed at other stages of their life cycles. This will be a major future challenge to determine the complete developmental programs in these fungi, knowing that some stages are very hard to sample.

Studies in *P. pachyrhizi*, *M. larici-populina* and different *Puccinia* spp. have focused on ESTs **during urediniospores germination** (Liu et al. 1993; Posada-Buitrago and Frederick 2005; Hu et al. 2007; Zhang et al. 2008; Duplessis et al. 2011a, b), and revealed **a high proportion of genes involved in metabolism, gene/protein expression, cell division, cell signalling/communication, and cell structure and growth.** However, in these cases the most abundant transcript categories corresponded to unknown function, highlighting the preponderance of new genes in rust fungi compared with other fungi, as indicated in previous sections. A higher proportion of genes of unknown functions was reported in germinating urediniospores and appressoria compared to other differentiation stages of *P. triticina* (Hu et al. 2007). **Still, very little information is available for ESTs specifically expressed in appressoria.** Preliminary information suggests an increase in the number of genes involved in metabolism, translational activity, and production of new structures in *H. vastatrix* appressoria as compared to germinating urediniospores (Talhinhas et al., unpublished data).

Most EST sequencing studies in rust fungi have focused on biotrophic growth, either through isolation of haustoria from infected host tissues or directly from infected host tissues. The seminal study by Hahn and Mendgen (1997) enabled the identification of 31 in-planta induced genes (termed PIGs) in *U. fabae* purified haustoria, including thiamin biosynthesis genes (Thi1p and Thi2p), involved in carbon metabolism (Sohn et al. 2000), and transporters.

These include AAT1p, a broad-specificity amino acid secondary transporter with affinity for L-histidine and L-lysine (Struck et al. 2002), and AAT3p, an aminoacid transporter with preferences for L-leucine, L-methionine, and L-cysteine (Struck et al. 2004). The study by Voegele et al. (2001) on the *U. fabae* hexose transporter HXT1p provided the first conclusive evidence that rust haustoria are indeed nutrient uptake structures. Recently, different expression studies of HXT1p orthologues showed that these carbohydrate transporters were also detected in germinating urediniospores and appressoria (Duplessis et al. 2011a, b; Vieira et al. 2012) and in pycniospores (Xu et al. 2011). Substrate translocation is driven by secondary active transport systems, enabling a direct link to the proton gradient established by a plasma membrane H^+-ATPase, for which transcripts were induced in haustoria (Struck et al. 1996, 1998). Several enzymes involved in carbohydrate metabolism were subsequently identified in *U. fabae*, such as β-glucosidase (Haerter and Voegele 2004), invertase and NADP-dependent mannitol dehydrogenase (Voegele and Mendgen 2011), and NADP-dependent D-arabitol dehydrogenase (Link et al. 2005), and their expression in planta was confirmed by microarrays (Jakupovic et al. 2006). Since then, orthologues have been identified in other rust fungi, such as *P. triticina* (Thara et al. 2003), *P. graminis* f. sp. *tritici* (Broeker et al. 2006), *P. striiformis* f. sp. *tritici* (Ma et al. 2009; Yin et al. 2009), *Cronartium quercuum* f. sp. *fusiforme* (Warren and Covert 2004), *H. vastatrix* (Vieira et al. 2012), *M. lini* (Catanzariti et al. 2006), and other *Melampsora* spp. (Hacquard et al. 2010; Joly et al. 2010; Duplessis et al. 2011a)

Taken together, expression analyses of genes involved in **carbohydrate metabolism** enable the identification of efficient pathways for mobilization, uptake, conversion, storage, and use of energy sources (Voegele and Mendgen 2011). Besides being of utmost importance for nutrition, some of these enzymes might also be involved in the suppression of host defenses (Haerter and Voegele 2004; Voegele and Mendgen 2011). Another strategy of biotrophic fungi to **escape host defenses** is in the ability to modify the fungal cell wall composition during infection, e.g., conversion of chitin to chitosan by **chitin deacetylases**. In addition to the expansion of genes encoding such functions in the genomes of rust fungi (Duplessis et al. 2011a), expression of chitin deacetylases genes has been detected at pre- and early penetration stages in *M. larici-populina* and *H. vastatrix*, and orthologues have also been found in *P. pachyrhizi* and *P. striiformis* f. sp. *tritici* pre-penetration stage ESTs (Posada-Buitrago and Frederick 2005; Zhang et al. 2008; Duplessis et al. 2011b; Vieira et al. 2012). This suggests possible stealthing of the fungus at early infection stages. In addition to this, late expression of these genes has also been reported in *H. vastatrix*, *M. larici-populina*, *U. fabae*, and *P. striiformis* f. sp. *tritici* (Jakupovic et al. 2006; Zhang et al. 2008; Duplessis et al. 2011b), which might be related to the production of sporogenous hyphae and new urediniospores in the plant tissue. For instance, a *M. larici-populina* chitin deacetylase gene was reported among the most highly induced genes in microdissected uredinia compared to biotrophic structures in the palisade mesophyll (Hacquard et al. 2010). Transcriptome profiling of *M. larici-populina* genes showed distinct profiles for at least two chitin deacetylase genes (Duplessis et al. 2011b), suggesting a variety of expression profiles among this large multigene family.

In the near future, the new deep-sequencing approaches and genome-scale DNA chips will totally modify our understanding of rust-fungi biology. Particularly promising are the techniques for non-model species, such as *H. vastatrix*, for which a lot of new biological data have been gained (Fernandez et al. 2012).

IV. Host - Parasite Interactions

A. Plant Rust Resistance Genes and Flor's Gene-for-Gene Model

Rust fungi are good examples of plant and pathogen co-evolution, and the first genetic

statements of specific plant–pathogen relationships come from the genetic dissection of the interaction between the rust fungus *M. lini* with flax (*L. usitatissimum*) in the 1940s by Harold H. Flor. By crossing different flax rust races as well as available flax lines and scoring the reaction of the plant to the pathogen (immune, resistance, semi-resistant, susceptible), Flor found that both resistance in flax as well as avirulence (i.e., lack of virulence) in the rust pathogen were inherited, and that the specific interaction was controlled by pairs of dominant matching genes. **Flor then formulated what is now called the gene-for-gene hypothesis; that for every gene in the plant that confers resistance (*R* gene), there is a corresponding gene in the pathogen that confers avirulence (*Avr* gene)** (Flor 1956). The outcome of the infection (resistance or susceptibility) depends on the presence of the corresponding *R* gene in the plant and *Avr* gene in the pathogen.

Fifty years after this pioneering work, some *M. lini Avr* genes and flax *R* genes have been isolated (Anderson et al. 1997; Barrett et al. 2009; Catanzariti et al. 2006; Dodds et al. 2004, 2006; Ellis et al. 1999; Lawrence et al. 1995, 2010; see Ravensdale et al. 2011 for review). Induction of defense responses [cell death related to the **hypersensitive response (HR)**] when pairs of proteins were co-expressed in planta was consistent with the gene-for-gene basis of recognition (Dodds et al. 2004). In addition, the high level of amino acid polymorphism found between *M. lini* Avr proteins is consistent with a co-evolutionary scheme involving **diversifying selection** to escape from recognition with the R proteins (Dodds et al. 2004). In other plant–rust interactions studied so far, the gene-for-gene hypothesis applies. In wheat, where more than 50 leaf rust resistance (*Lr*) genes have been identified; most *Lr* genes confer race-specific resistance in a gene-for-gene manner (Kolmer 1996). In coffee, more than nine leaf rust resistance factors have been identified (Kushalappa and Eskes 1989), and in poplar eight qualitative resistances to *M. larici-populina* have been determined for cultivars used in plantations (Frey and Pinon 2004). All these *R* genes condition incompatible interactions with corresponding pathogens, characterized by a HR (Heath 1999) and the induction of defense-related genes that impair the pathogen growth (Fofana et al. 2007; Rinaldi et al. 2007; Ramiro et al. 2009). To date, some barley and wheat *R* genes have been cloned (Feuillet et al. 2003; Huang et al. 2003) and no rust *Avr* gene from other species than *M. lini* has yet been isolated. **The discovery that some flax R proteins and their cognate rust effectors interact directly** (Dodds et al. 2006) **is an important confirmation of Flor's gene-for-gene model on the flax–flax rust pathosystem.** It is now essential to determine to which extent this model can be confirmed in other plant–rust fungi interactions. With the availability of genome sequences for rust fungi, and adequate technologies to sequence genomes from large numbers of strains of known virulence, it should be possible to identify new putative rust avirulence genes. Constant attempts are made to develop tools for genetic transformation of rust fungi (Schillberg et al. 2000; Lawrence et al. 2010; Djulic et al. 2011). Hopefully, functional studies of rust avirulence genes should improve the understanding of their role in disease development and interaction with host plants. Importantly, a lot of plant–pathogen interactions follow a gene-for-gene model like that which applies for biotroph pathogens and to some necrotophs (Dangl and Jones 2001). **Evolution of the plant immune system may be driven by a constant adaptation to new virulence determinants produced by pathogens, and a zig-zag-like model of plant–pathogen co-evolution has been proposed that fits with Flor's theory** (Jones and Dangl 2006). However, deviations from the classic gene-for-gene model have now become very common, with numerous examples of recessive resistance, indirect interaction between the R and Avr gene products, and multiple Avr targets recognition in the host cell (Mukhtar et al. 2011).

B. General Recognition of Rust Pathogens

The ability of plants to successfully **perceive and identify** invading pathogens is crucial for appropriate initiation of the signaling process leading to execution of multicomponent

defense responses. Specific recognition of successful pathogens is thought to occur after the delivery of effector proteins into host cells, as opposed to the general recognition of PAMPs from non-specialized microbes (Jones and Dangl 2006; Boller and Felix 2009). For instance, **broadly recognized fungal PAMPs are derived from cell-wall components, such as chitin and its N-acetylchitooligosaccharides fragments**. These general components have been known for a long time to act as potent elicitor signals in several plant species (Shibuya and Minami 2001; Zipfel, 2009).

Plant receptors that resides at the plasma membrane, such as the *A. thaliana* CERK1, a lysin motif (LysM)-containing receptor-like kinase, and the rice CeBiP (a LysM glycoprotein), can detect by direct binding chitin, chitosan, or chito-oligomers (Kaku et al. 2006; Petutschnig et al. 2010). Rapid changes after PAMP perception are ion fluxes across the plasma membrane, including influx of Ca^{2+} into the cytosol, and the production of reactive oxygen species (ROS). A crucial role for protein phosphorylation through the activation of MAP kinase pathway similar to the one reported in flagellin studies is required for chitin signaling in plant innate immunity (Wan et al. 2004; Zipfel and Robatzek 2010; Boller and Felix 2009). Although extensive study of chitin synthase and chitin deacetylase expression were conducted in rust fungi (see previous section; Broeker et al. 2006, 2011), no data is available with regard to chitin detection and its role in the activation of basal plant defense responses.

However, **perception of rust pathogens in non-host species triggers inducible defense reactions that are probably brought about by the recognition of invariant PAMP**. For instance, the model plant *A. thaliana* is able to mount plant defense responses against several rust species which block fungal infection at the penetration stage and before haustorium formation (Mellersh and Heath 2003; Loehrer et al. 2008; Azinheira et al. 2010). Usually, attempted penetrations resulted in a HR of the related guard or epidermal cells, depending on the mode of penetration used. In situations where a few haustoria were even produced, they were subsequently curtailed by encasement in deposits containing callose (Mellersh and Heath 2003; Shafiei et al. 2007). Plant molecular response analysis showed that defense genes of the salicylic acid (SA)- and jasmonic acid (JA)-dependent resistance signaling pathways were activated after *P. triticina* (Shafiei et al. 2007) and *P. pachyrhizi* (Loehrer et al. 2008) challenge, suggesting involvement of particular recognition events in *A. thaliana* non-host resistance. Another example of non-host resistance involves rice *(Oryza sativa)* which is strikingly immune to rust fungi. Cereal rust species, including *Puccinia graminis f. sp. tritici, P. triticina, P. striiformis, and P. hordei*, are able to produce all the infection structures necessary for plant penetration (Ayliffe et al. 2011; Li et al. 2012). In some instances, haustoria were produced in large infection sites that colonized hundreds of mesophyll cells prior to fungal growth being suppressed by rice resistance responses that involved callose deposition, production of reactive oxygen species, and, occasionally, cell death (Ayliffe et al. 2011). At the proteome level, a series of proteins were up-regulated after infection, consistent with an active rice non-host resistance response (Li et al. 2012).

C. Specific Recognition of Rust Pathogens

In many plant–rust interactions studied, specific plant resistance is believed to occur after the rust fungus attempts to produce haustoria (Heath 1997). For instance, the cowpea rust fungus elicits a HR only after cell penetration in the resistant cultivar. **Purification and characterization of HR-inducing peptides from the cowpea rust fungus were the first demonstration that rust fungi produce race-specific elicitors outside of the host cell** (D'Silva and Heath 1997). It has now become clear that rust fungi secrete a vast array of effectors for establishing themselves inside the host plant, and that the secretomes of rust fungi include many virulence factors and avirulence products recognized by the host plant immune system (Ellis et al. 2009). For some rust pathogens, pioneer studies indicated that candidate effector genes are specifically expressed in haustoria (Dodds et al. 2004; Puthoff et al. 2008). For instance, **a series of haustorial Avr proteins from the flax rust fungus are recognized**

inside plant cells (Catanzariti et al. 2006; Rafiqi et al. 2010). Flax rust Avr proteins have no homology to any known protein (Catanzariti et al. 2006), but homologs were identified in the related species *M. larici-populina* (Duplessis et al. 2011a; Hacquard et al. 2012). **All the flax rust *Avr* gene variants studied so far encode small secreted proteins that are expressed in haustoria**. They are also characterized by **high levels of polymorphism associated with differences in recognition specificity** (Barrett et al. 2009; Ravensdale et al. 2011). In the same way, it was possible to identify a family of **rust-transferred proteins (RTPs)** conserved between some *Uromyces* spp. and some *Puccinia* spp. (Puthoff et al. 2008). The RTP1 protein that was first identified in *U. fabae* is transported from the haustoria into the host, where it accumulates in the nucleus (Kemen et al. 2005b). Several orthologs of RTP1 have been found in other rust species, including *M. larici-populina* (Duplessis et al. 2011a), *Puccinia striiformis* f. sp. *tritici* (Cantu et al. 2011), and *H. vastatrix* (Fernandez et al. 2012), suggesting a conserved role for these rust effectors. The host targets of these effectors and their function during infection are still not resolved.

In biotrophic fungi and oomycetes, effector proteins may be subdivided into two broad categories depending on whether they are secreted in the apoplast or delivered into the cytoplasm of the host cell (for review see Stergiopoulos and De Wit 2009). Interestingly, some rust effectors may intervene at early stages of fungal infection, through secretion outside spores or germinating hyphae. In the barley–stem rust pathosystem, recent studies indicated that within 5 min of avirulent *Puccinia graminis* f. sp. *tritici* spore exposition, phosphorylation of the resistance protein "Reaction to *Puccinia graminis* 1 (RPG1)" could be detected in the host (Nirmala et al. 2010). Two HR-inducing protein effectors isolated from ungerminated avirulent urediniospores interacted with RPG1 in yeast (Nirmala et al. 2011). These two rust effectors activate cooperatively the stem-rust resistance protein RPG1 long before haustoria formation, initiating HR and resistance to stem rust (Nirmala et al. 2011). These data show that some effectors may be already produced by urediniospores and recognized outside host cells. In the same way, molecular plant responses to rust pathogens are often detected when the first attempts at penetration occur (Ganesh et al. 2006; Fofana et al. 2007; van de Mortel et al. 2007; Ramiro et al. 2009). For instance, in the soybean–P. *pachyrhizi* interactions, the first burst of gene expression correlates with appressorium formation and penetration of epidermal cells, while the second burst of gene expression changes follows the onset of haustoria formation in both compatible and incompatible interactions (van de Mortel et al. 2007; Schneider et al. 2011; Diniz et al. 2012).

D. Specific Defense Reactions Against Rust Pathogens

Different resistance mechanisms against rust fungi may be operative at different stages of the infection process. Interestingly, there is no report for the existence of preformed defenses in rust-resistant host genotypes which could limit spore germination and appressorium formation, although several resistance mechanisms are induced after stomata or epidermal cell penetration. **For most rust pathogens studied, urediniospores usually germinate and penetrate stomata equally well on susceptible and resistant plants** (Heath 1974; Hu and Rijkenberg 1998; Laurans and Pilate 1999; Silva et al. 2002; Bozkurt et al. 2010). Specific host resistance responses to rust fungi are usually expressed by a HR, with localized cell death restricted to epidermal, stomatal, or mesophyll cells at the site of infection (Heath 1999). **HR cell death is the most common response of gene-for-gene interactions, and occurs at the site of successful recognition of avirulent pathogens** (Heath 1999). HR cell death exhibits all characteristics of plant cell necrosis, but may also be accompanied by the features of vacuolar cell death, thus constituting a separate cell-death modality (van Doorn et al. 2011). For instance, in coffee leaves infected by the leaf rust *H. vastatrix*, transmission electron microscope observation of host cells undergoing HR

revealed membrane breakdown at the level of the plasmalemma and in different organelles, namely chloroplasts and mitochondria as well as nuclei, with a change in the appearance of the chloroplast and the nucleus, and coagulation of cytoplasm (Silva et al. 2006). **Although the HR may be an effective defense against biotrophic pathogens, it is likely that this host response is only a single part of the overall defensive strategy of the plant.** In plant–rust interactions, **HR is usually associated with post-haustorial defense responses, including haustoria encasement** (Fig. 12.3). This host response may also be observed in compatible interactions, but later in the infection process and only in a small number of haustoria (Silva et al. 2002). In several plants resistant to rust and other obligate biotrophs, haustorium encasement has been regarded as one expression of incompatibility (Littlefield and Heath 1979; Cohen et al. 1990; Škalamera et al. 1997). **Callose, the major compound of haustorial encasement,** has been reported to be less permeable to small molecules than other cell-wall components (Heslop-Harrison 1966), and may therefore restrict the passage of nutrients to the fungus and consequently slow fungal growth (Silva et al. 2002).

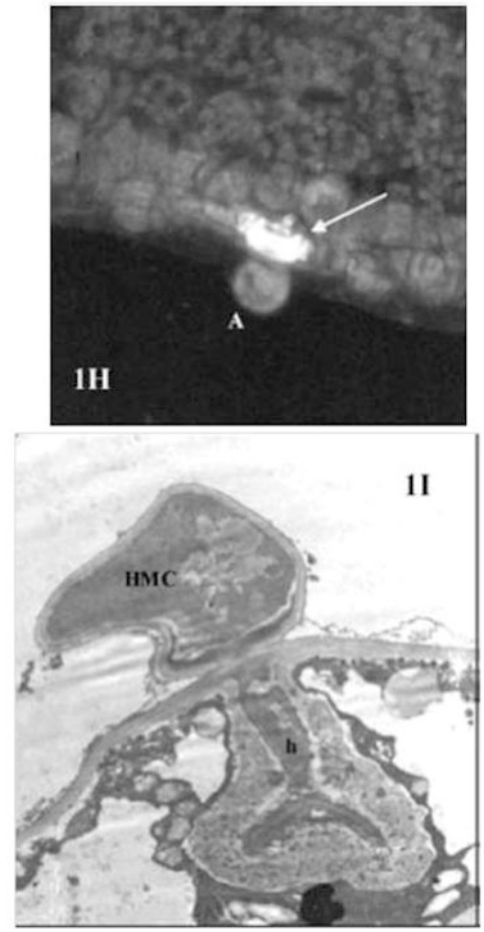

Fig. 12.3. Coffee–*Hemileia vastatrix* interaction. Resistance responses induced by the fungus. **1H** Light microscope observation, epifluorescence test using blue and u.v. light. Infection site showing an appressorium (*A*) over stomata associated with autofluorescence (death) of the guard and subsidiary cells (*arrow*), 48 h after the inoculation (× 400) **1I** Transmission electron microscope observations, uranyl acetate and lead citrate staining. Encased haustorium (*h*) in a subsidiary host dead cell, 3 days after inoculation. *HMC* = haustorial-mother cell (× 4,500) (From Silva et al. (2006))

V. Agricultural Applications

High crop yields and product quality are main objectives of modern agriculture to ensure food security. Genetic vulnerability to disease caused by microbial pathogens is one of the principal threats to be overcome. Bioinformatics analyses allow scientists to guide the identification of virulence factors expressed during microbial infection of plants and genes that are essential for the pathogen life cycle. In recent years, the knowledge of genome composition and gene function of phytopathogenic fungi has increased tremendously. Re-sequencing genomes of strains of a given species will give further insights into evolutionary processes and how new races/virulence arose from their ancestors. In addition, knowledge of pathogen genomes will help design diagnostic tools, and could be used in rapid-detection technologies. In addition, genomics research promises to deliver new targets that can be screened for antifungal drugs, and new insights for the generation of crops resistant against fungal invasion. Hereafter, we review new advances about genomics application for controlling rust diseases.

A. R Gene Management and Its Limitations

One important consideration in disease control programs is the selection and planting of cultivars that are resistant to pathogens. There are some disadvantages to the use of resistant cultivars for rust disease control. The greatest shortcoming is that resistance is not available for all races on all varieties. **Another disadvantage to resistance is that resistance may not be long-lasting when relying on only a few sources of resistance.** In particular, the failure of plant resistance to rust diseases may be a regular event, **causing a continual boom–bust cycle** where a variety of plants seemingly unaffected by rust in one year was annihilated in the next. In most cases, resistance failure is attributed to the development of new strains of the target pathogen that overcome the resistance genes of the previously resistant cultivar. **The stem-rust disease of wheat provides a good case study of this phenomenon.** Stem rust caused by *Puccinia graminis* f. sp. *tritici* is the most feared disease of wheat, with rust epidemics having been recorded for more than 3,000 years (Roelfs et al. 1992). Genetic resistance to stem rust has been a priority objective of wheat breeders for over 100 years. **The discovery and deployment of various major resistance genes has enabled effective protection** during the past 50 years, and has been instrumental in the green revolution that increased crop production in various developing countries in the 1960s and 1970s. However, since 1999, a new lineage of stem-rust races, labeled "Ug99", was observed, causing significant damage to the previously resistant wheat lines in Uganda. Since then, it has spread within North Africa and into the Middle East. An international initiative, The Borlaug Global Rust Initiative (BGRI), named after the wheat pathologist Nobel laureate Dr. Norman Borlaug (1914–2009), was created in 2005 with the final objective of containing the threat of wheat rusts in the world. This collaborative research effort has established that Ug99 defeats virtually every race-specific resistance gene used in commercial varieties grown throughout the world. Up to now, Ug99 remains a major threat for wheat culture in the world. **Several methods of using host resistance more effectively have been proposed, including pyramiding genes for resistance or using multilines or cultivar mixtures**. Each of them presents advantages and disadvantages (Roelfs et al. 1992), and may not be similarly applicable in annual or perennial crops. For example, many gene pyramids have been successful in wheat cultivars. The combined rust-resistance genes usually acted independently, exhibiting the infection type of the gene that conditions the lower infection type when present singly (Dyck and Kerber 1985; Roelfs 1988). Such an approach would require too much time for stacking resistance genes in perennial crops. In contrast, cultivation of mixtures of coffee genotypes has proven useful in controlling coffee leaf rust epidemics in Colombia, for example. The variety Colombia is a composite type variety (five generation progenies) of coffee, resulting from an initial cross between a rust-susceptible *Coffea arabica* variety and a natural (*C. arabica* x *Coffea canephora*) hybrid carrying several resistance genes to leaf rust (*H. vastatrix*) (Moreno-Ruiz and Castillo-Zapata 1990).

B. New Fungal Targets for Agrochemical Control

For the past 50 years, fungicides have played an important part in the increased productivity of crops, with improved disease control, crop yield, and quality. The antifungal agents currently available for crop protection may be harmful to human health and the environment. Hence, the use of agrochemicals is restricted, and European and other international directives aim at the regulation of plant-protection products to ensure their safety and effectiveness. In addition, as the use of fungicides has increased, so has the incidence of resistance to certain classes of these compounds. **There is now concern that control of some major diseases may be compromised by the emergence of pathogen strains resistant to more than a single chemical** (Lucas 2006). This situation strengthens the relevance of studies on the development of new methods of controlling rust fungi.

Potential drug targets may be identified from post-genomic analysis of pathogenic organisms. **Selecting new molecular targets by comparative genomics, homology modeling, and virtual screening of compounds is promising in the process of new drug discovery.** The increase in structural databases permits the satisfactory prediction of structures by theoretical methods, with advantages over more costly experimental methods. Molecular targets may be used to virtually screen chemical libraries, offering new perspectives on technological development and innovation of antifungal agents against pathogens. In parallel, linking bioactive compounds to their cellular targets is now facilitated by chemical–genomic profiling in yeast. Using collections of fission yeast strains expressing open reading frames (Winzeler et al. 1999), or combination of gene deletion mutants, and relating them to gene ontology annotations now enables high-throughput functional genomics analyses for establishing mechanistic links between drug sensitivity and particular biochemistry activities (Andrusiak et al. 2012). Arrayed clones of yeast genes or mutants are probed to identify the targets of small molecule antifungal agents and the molecular networks that they perturb.

Comparative genomics strategy has been used for identifying potential new drug targets, such as putative essential genes and/or those affecting the cell viability that are conserved in pathogenic organisms. For fungal species pathogenic to humans, *in silico* and manual mining provided a series of genes conserved in eight species and a set of four fungal targets were selected, including proteins conserved among fungi, but absent in the human genome (Abadio et al. 2011). These characteristics potentially minimize toxic side-effects exerted by pharmacological inhibition of the cellular targets. Other recent examples include the identification of new drug targets for human-pathogenic bacteria, nematodes, and parasites (White and Kell 2004; Kumar et al. 2007; Caffrey et al. 2009). **Targets of choice for drug development include enzymes, which activity may be blocked by chemicals. In particular, enzymes linked to amino-acid biosynthesis may be relevant in fungi.** These are not essential for cell growth on rich media, but are important in virulence. Abadio et al. (2011) focused on candidates that encode for a thioredoxin reductase, an enzyme that plays a critical role in maintaining the cell redox status, and two enzymes involved in membrane integrity maintenance and formation: a-1,2-mannosyltransferase involved in mannose residue addition to cell-wall compounds, and a $\Delta(24)$-sterol C-methyltransferase involved in the ergosterol/cholesterol biosynthesis pathway. The next step will be to virtually screen chemical libraries to identify molecules that could potentially interact with these essential proteins and block or decrease their activity. Undoubtedly, with the availability of genomic sequences, such studies will be now extended to plant pathogenic fungi for controlling important disease.

C. RNAi to Block Fungal Gene Expression

Techniques based on RNA interference (RNAi) to engineer resistance in crops against parasites have been developed (Runo et al. 2011). RNAi is a cellular process that occurs in many eukaryotes and functions to regulate gene expression through dsRNA-mediated sequence-specific degradation of RNA (Fire et al. 1998). RNAi may result in a drastic reduction in the relative amount of candidate gene transcripts. Targeted down-regulation of gene expression mediated by double-stranded RNA (dsRNA) is now widely used in reverse genetics for knocking down a gene function in several organisms, including fungi (Kemppainen et al. 2009; Wälti et al. 2006). **By using RNAi it is possible to target a specific, essential gene(s) in a parasite for 'silencing', and hence incapacitate it.** Alternatively, successful silencing of parasitic genes can be achieved by using a host crop stably transformed with a construct that carries either an antisense or a hairpin dsRNA targeting essential parasitic genes. Because specific dsRNA from the parasite gene are delivered to plant cells, the RNAi sequence is chosen in such a way that it shares no homology with the host's genome. Upon parasitism, the RNAi sequence must be delivered to the parasite, presumably by simply feeding the

dsRNA of the target gene, leading to suppression of the parasite growth. Engineering of host resistance by expressing dsRNA in plants has notably shown promise for both insect and nematode resistance (Huvenne and Smagghe 2010; Lilley et al. 2012). These parasites directly feed from the host cells, and are, as such, highly amenable to dsRNA uptake and RNAi-mediated gene suppression throughout their life cycle. **Targeted genes may include essential genes for the life cycle of the parasite, as well as genes described to have a virulence function into the host cell.** An outstanding example is the silencing of the parasitism gene *16D10* of the root-knot nematode *Meloidogyne incognita*, which conferred resistance of *Arabidopsis* plants to four *Meloidogyne* species by reducing (63–90 %) the numbers of galls and egg-laying females on the host plant (Huang et al. 2006).

The dsRNA can be processed by the plant RNAse III (Dicer) enzymes of the RNAi machinery into small interfering (si)RNA. It is not clear if the parasite takes up these plant-derived siRNA or the unprocessed dsRNA, which is subsequently processed into siRNA by fungal Dicer enzymes (Gheysen and Vanholme 2007). A number of novel approaches are being developed to assess whether fungi, including obligate biotrophs, possess functional RNA silencing machinery. Genome data mining of 54 fungal species for specific RNA silencing annotated domains for the three main RNAi enzymes, Argonaute (IPR003100), Dicer (IPR005034), and RdRp (IPR007855) showed that the number of RNA silencing gene homologs is quite variable among fungal species. Many fungi contain two to three copies of each gene, although some species contain only a single copy of each gene or are totally devoid of the RNA silencing machinery (Nunes et al. 2011). For rust fungi, a demonstration of successful RNAi in fungal cells comes from the work of Lawrence et al. (2010) who developed an elegant *Agrobacterium*-mediated transformation methodology for *M. lini*. By taking advantage of the gene-for-gene interaction, namely between *M. lini AvrL567* and the cognate flax receptor *L6*, **silencing of *AvrL567* gene expression with *A. tumefaciens* carrying a silencing cassette able to produce *AvrL567* siRNAs resulted in disease development in a flax line carrying the L6 receptor** (Lawrence et al. 2010). *M. lini* silenced cells, exhibiting knocked-down *AvrL567* transcript levels, thus escaped host recognition.

Techniques based on host-mediated RNAi to suppress rust gene expression have been recently tested in wheat (Yin et al. 2011). A series of constitutively and haustorially expressed genes from the stem rust *Puccinia striiformis* f. sp. *tritici* and stripe rust *P. graminis* f. sp. *tritici* were chosen as targets, but no data about the possible involvement of these genes in virulence was available. Virus-induced gene silencing (VIGS) through the *Barley stripe mosaic virus* system was used to produce dsRNA of target fungal gene fragments in wheat. The ability to detect suppression was associated with the expression patterns of the fungal genes. Only those transcripts with relatively high levels of expression in fungal haustoria were reduced in infected wheat leaves. Fungal transcript reduction in infected silenced plants varied from 0.3- to 0.6-fold as compared to inoculated control plants. For any of the five genes analyzed whose expression was constitutive, including β-tubulin, GAPDH, actin, and EF1, no silencing was apparent. One hypothesis provided by (Yin et al. 2011) is that delivering of dsRNA into fungal cells could only occur in those cells near to or in direct contact with the host-cell cytoplasm (e.g., haustorial cells), enabling successful silencing only of genes that are specifically or highly expressed in haustoria. For constitutively expressed genes, silencing in the haustorial cells may not affect the fungus if the neighboring cells can still provide either the protein or the metabolic product to the haustorial cell. However, although the average levels of expression in wheat-silenced plants were approximately 50 % of the controls, obvious reductions in rust development or sporulation were not observed for any of the seven genes tested. **Contrary to nematode and insect RNAi-based resistance strategies, an important consideration for engineering host resistance to biotrophic fungi would then be selection of fungal genes that are essential for pathogenicity.** An example comes from the barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei*, another obligate biotrophic fungus that produces haustoria inside host cells, and

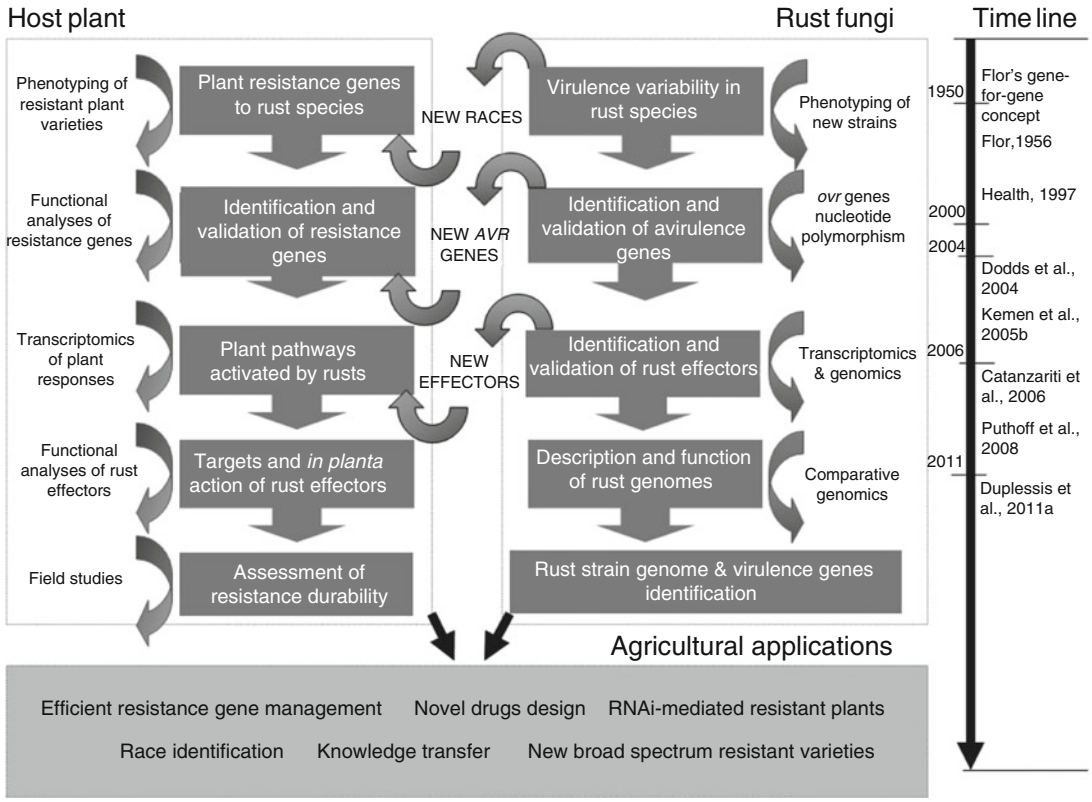

Fig. 12.4 Flowchart of research achievements on rust fungi, and future milestones for agricultural applications

where approx. 20 % of the 76 tested RNAi constructs induced a reduction in fungal haustorium formation (Nowara et al. 2010). The corresponding target genes had been preselected for their putative role during host infection based on their expression profiles (e.g., up-regulation in planta). In particular, about 50 % reduction of haustoria formed in the plant tissue was observed when two *Avr* genes were silenced. This study also provided evidence of RNA trafficking between host plant cells and powdery mildew at an early infection stage. **These results indicate that an in-planta RNAi approach could potentially be used to engineer durable resistance against rust fungi. Such a strategy might be used to control multiple races of a given rust species, because constructs can be designed such as those containing multiple stacked RNAi target sequences.** Overall, success of this host-derived resistance relies on the identification of a parasite target sequence that is essential for parasite virulence and absent from the host genome. However, another important aspect to consider is the persistence of the system, and to determine properly for how long such dsRNA delivery would remain effective in engineered plants.

VI. Conclusions

Fundamental understanding of basic biology, infection mechanisms, host–fungus interactions, genetic regulation, and evolution through comparative genomic studies of closely related rust species is now facilitated with the availability of rust genome sequences. In addition, transcriptomic studies on obligate plant parasites benefit greatly from data generated using next-generation sequencing, even in non-model fungal species. We summarized in Fig. 12.4 the research achievements on rust fungi, and the future milestones for agricultural applications.

More work is still required to further validate rust gene functions unraveled by these techniques, and to develop effective tools to control these fascinating plant pathogens.

References

Abadio AKR, Kioshima ES, Teixeira MM, Martins NF, Maigret B, Felipe MSS (2011) Comparative genomics allowed the identification of drug targets against human fungal pathogens. BMC Genomics 12:75

Agrios G (2005) Plant pathology, 5th edn. Elsevier/Academic, London/San Diego

Alexopoulos CJ, Mims CW, Blackwell M (1996) Introductory mycology, 4th edn. Wiley, New York

Allen EA, Hazen BE, Hoch HC, Kwon Y, Leinhos GME, Staples RC, Stumpf MA, Terhume BT (1991) Appressorium formation in response to topographical signals by 27 rust species. Phytopathology 81:323–331

Anderson PA, Lawrence GJ, Morrish BC, Ayliffe MA, Finnegan EJ, Ellis JG (1997) Inactivation of the flax rust resistance gene M associated with loss of a repeated unit within the leucine-rich repeat coding region. Plant Cell 9:641–651

Andrusiak K, Piotrowski JS, Boone C (2012) Chemical-genomic profiling: systematic analysis of the cellular targets of bioactive molecules. Bioorg Med Chem 20:1952–1960. doi:10.1016/j.bmc.2011.12.023

Ayliffe M, Devilla R, Mago R, White R, Talbot M, Pryor A, Leung H (2011) Nonhost resistance of rice to rust pathogens. Mol Plant Microbe Interact 24:1143–1155

Azinheira HG, Silva MC, Guerra-Guimarães L, Mendgen K, Rodrigues C, Ricardo CP (2001) Development of infection structures of *Hemileia vastatrix* on artificial membranes. In: 11th conference of the mediterranean phytopathological union, 17–20 Sept 2001, Évora, Andalus Academic Publishing, Redondo, pp 353–355

Azinheira HG, Silva MC, Talhinhas P, Medeira C, Maia I, Petitot A-S, Fernandez D (2010) *Arabidopsis thaliana* non-host resistance responses to the coffee leaf rust fungus (*Hemileia vastatrix*). Botany 88: 621–629

Baka ZA, Larous L, Losel DM (1995) Distribution of ATPase activity at the host-pathogen interfaces of rust infections. Physiol Mol Plant Pathol 47:67–82

Baker LG, Spaine P, Covert SF (2006) Effect of surface wettability on germination and gene expression in *Cronartium quercuum* f. sp. *fusiforme* basidiospores. Physiol Mol Plant Pathol 68:168–175

Barrett LG, Thrall PH, Dodds PN, van der Merwe M, Linde CC, Lawrence GJ, Burdon JJ (2009) Diversity and evolution of effector loci in natural populations of the plant pathogen *Melampsora lini*. Mol Biol Evol 26:2499–2513

Baxter L, Tripathy S, Ishaque N, Boot N, Cabral A, Kemen E, Thines M, Ah-Fong A, Anderson R, Badejoko W, Bittner-Eddy P, Boore JL, Chibucos MC, Coates M, Dehal P, Delehaunty K, Dong S, Downton P, Dumas B, Fabro G, Fronick C, Fuerstenberg SI, Fulton L, Gaulin E, Govers F, Hughes L, Humphray S, Jiang RH, Judelson H, Kamoun S, Kyung K, Meijer H, Minx P, Morris P, Nelson J, Phuntumart V, Qutob D, Rehmany A, Rougon-Cardoso A, Ryden P, Torto-Alalibo T, Studholme D, Wang Y, Win J, Wood J, Clifton SW, Rogers J, Van den Ackerveken G, Jones JD, McDowell JM, Beynon J, Tyler BM (2010) Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. Science 330:1549–1551

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60:379–406

Bozkurt TO, McGrann GRD, MacCormack R, Boyd LA, Akkaya MS (2010) Cellular and transcriptional responses of wheat during compatible and incompatible race-specific interactions with *Puccinia striiformis* f. sp. *tritici*. Mol Plant Pathol 11: 625–640

Broeker K, Bernard F, Moerschbacher BM (2006) An EST library from *Puccinia graminis* f. sp. *tritici* reveals genes potentially involved in fungal differentiation. FEMS Microbiol Lett 256:273–281

Broeker K, Fehser S, Tenberge KB, Moerschbacher BM (2011) Two class III chitin synthases specifically localized in appressoria and haustoria of *Puccinia graminis* f. sp. *tritici*. Physiol Mol Plant Pathol 76: 27–33

Caffrey CR, Rohwer A, Oellien F, Marhöfer RJ, Braschi S, Oliveira G, McKerrow JH, Selzer PM (2009) A comparative chemogenomics strategy to predict potential drug targets in the metazoan pathogen, *Schistosoma mansoni*. PLoS One 4:e4413

Cantu D, Govindarajulu M, Kozik A, Wang M, Chen X et al (2011) Next generation sequencing provides rapid access to the genome of *Puccinia striiformis* f. sp. *tritici*, the causal agent of wheat stripe rust. PLoS One 6:e24230. doi:doi:10.1371/journal.pone.0024230

Catanzariti A-M, Dodds PN, Lawrence GJ, Ayliffe MA, Ellis JG (2006) Haustorially-expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. Plant Cell 18:243–256

Chen ZJ, Nunes MA, Silva MC, Rodrigues CJ (2004) Appressorium turgor pressure of *Colletotrichum kahawae* might have a role in coffee cuticle penetration. Mycologia 96:1199–1208

Cohen Y, Eyal H, Hanania J (1990) Ultrastructure, autofluorescence, callose deposition and lignification in susceptible and resistant muskmelon leaves infected with the powdery mildew fungus *Sphaerotheca fuliginea*. Physiol Mol Plant Pathol 36:191–204

Cooper B, Neelam A, Campbell KB, Lee J, Liu G, Garrett WM, Scheffler B, Tucker ML (2007) Protein accumulation in the germinating *Uromyces appendiculatus* uredospore. Mol Plant Microbe Interact 20:857–866

Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826–833

de Bari HA (1863) Recherches sur le developpement de quelques champignons parasites. Ann Sci Nat Part Bot 20:5–148

Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD (2012) The top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol 13:414–430. doi:10.1111/j.1364-3703.2012.2011.00783.x

Diniz I, Talhinhas P, Azinheira HG, Várzea V, Medeira C, Maia I, Petitot A-S, Nicole M, Fernandez D, Silva MC (2012) Cellular and molecular analyses of coffee resistance to *Hemileia vastatrix* and nonhost resistance to *Uromyces vignae in* the resistance-donor genotype HDT832/2. Eur J Plant Pathol 133: 141–157. doi:10.1007/s10658-011-9925-9

Djulic A, Schmid A, Lenz H, Sharma P, Koch C, Wirsel SGR, Voegele RT (2011) Transient transformation of the obligate biotrophic rust fungus *Uromyces fabae* using biolistics. Fungal Biol 115:633–642

Dodds PN, Lawrence GJ, Catanzariti A-M, Ayliffe MA, Ellis JG (2004) The *Melampsora lini* AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell 16:755–768

Dodds PN, Lawrence GJ, Catanzariti AM, Teh T, Wang CIA, Ayliffe MA, Kobe B, Ellis JG (2006) Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. Proc Natl Acad Sci USA 103:8888–8893

Dracatos PM, Dumsday JL, Olle RS, Cogan NO, Dobrowolski MP, Fujimori M, Roderick H, Stewart AV, Smith KF, Forster JW (2006) Development and characterization of EST-SSR markers for the crown rust pathogen of ryegrass (*Puccinia coronata* f.sp. *lolii*). Genome 49:572–583

Duplessis S, Cuomo CA, Lin Y-C, Aerts A, Tisserant E, Veneault-Fourrey C, Joly DL, Hacquard S, Amselem J, Cantarel BL, Chiu R, Coutinho PM, Feau N, Field M, Frey P, Gelhaye E, Goldberg J, Grabherr MG, Kodira CD, Kohler A, Kües U, Lindquist EA, Lucas SM, Mago R, Mauceli E, Morin E, Murat C, Pangilinan JL, Park R, Pearson M, Quesneville H, Rouhier N, Sakthikumar S, Salamov AA, Schmutz J, Selles B, Shapiro H, Tanguay P, Tuskand GA, Henrissat B, Van de Peer Y, Rouzé P, Ellis JG, Dodds PD, Schein JE, Zhong S, Hamelin RC, Grigoriev IV, Szabo LJ, Martin F (2011a) Obligate biotrophy features unraveled by the genomic analysis of rust fungi. Proc Natl Acad Sci USA 108:9166–9171

Duplessis S, Hacquard S, Delaruelle C, Tisserant E, Frey P, Martin F, Kohler A (2011b) *Melampsora larici-populina* transcript profiling during germination and timecourse infection of poplar leaves reveals dynamic expression patterns associated with virulence and biotrophy. Mol Plant Microbe Interact 24:808–818

Duplessis S, Joly DL, Dodds PN (2012) Rust effectors. In: Martin F, Kamoun S (eds) Effectors in plant-microbe interactions. Wiley, New York

Dyck PL, Kerber ER (1985) Resistance of the race-specific type. In: Roelfs AP, Bushnell WR (eds) The cereal rusts, vol II, Diseases, distribution, epidemiology and control. Academic, Orlando

Ellis JG, Lawrence GJ, Luck JE, Dodds PN (1999) Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. Plant Cell 11:495–506

Ellis JG, Rafiqi M, Gan P, Chakrabarti A, Dodds PN (2009) Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens. Curr Opin Plant Biol 12: 399–405

Fasters MK, Daniels U, Moerchbacher BM (1993) A simple and reliable method for growing the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*, in liquid culture. Physiol Mol Plant Pathol 42:259–265

Fehser S, Beike U, Stöveken J, Pretorius ZA, van der Westhuizen AJ, Moerschbacher BM (2010) Histological and initial molecular analysis of Ug99, the sr31-breaking race of the wheat stem rust fungus. J Plant Pathol 92:709–720

Fernandez D, Tisserant E, Talhinhas P, Azinheira HG, Vieira A, Loureiro A, Poulain J, da Silva C, Silva MC, Duplessis S (2012) 454-pyrosequencing of *Coffea arabica* leaves infected by the rust fungus *Hemileia vastatrix* reveals in planta expressed pathogen secreted proteins and plant functions expressed in a late compatible plant-rust interaction. Mol Plant Pathol 13:17–37

Feuillet C, Travella S, Stein N, Albar L, Nublat A, Keller B (2003) Map-based isolation of the leaf rust disease resistance gene Lr10 from the hexaploid wheat (*Triticum aestivum* L.) genome. Proc Natl Acad Sci USA 100:15253–15258

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811

Flor HH (1956) The complementary genic systems in flax and flax rust. Adv Genet 8:29–54

Fofana B, Banks TW, McCallum B, Strelkov SE, Cloutier S (2007) Temporal gene expression profiling of the wheat leaf rust pathosystem using cDNA microarray reveals differences in compatible and incompatible defence pathways. Int J Plant Genom 2007:17542

Frey P, Pinon J (2004) Poplar rust: a model pathosystem. Biofutur 247:28–32

Ganesh D, Petitot A, Silva MC, Alary R, Lecouls AC, Fernandez D (2006) Monitoring of the early molecular resistance responses of coffee (*Coffea arabica* L.) to the rust fungus (*Hemileia vastatrix*) using real-time quantitative RT-PCR. Plant Sci 170:1045–1051

Gheysen G, Vanholme B (2007) RNAi from plants to nematodes. Tr Biotechnol 25:89–92

Goellner K, Loehrer M, Langenbach C, Conrath U, Koch E, Schaffrath U (2010) *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. Mol Plant Pathol 11:169–177

Haas BJ, Kamoun S, Zody MC, Jiang RH, Handsaker RE, Cano LM, Grabherr M, Kodira CD, Raffaele S, Torto-Alalibo T, Bozkurt TO, Ah-Fong AM, Alvarado L, Anderson VL, Armstrong MR, Avrova A, Baxter L, Beynon J, Boevink PC, Bollmann SR, Bos JI, Bulone V, Cai G, Cakir C, Carrington JC, Chawner M, Conti L, Costanzo S, Ewan R, Fahlgren N, Fischbach MA, Fugelstad J, Gilroy EM, Gnerre S, Green PJ, Grenville-Briggs LJ, Griffith J, Grünwald NJ, Horn K, Horner NR, Hu CH, Huitema E, Jeong DH, Jones AM, Jones JD, Jones RW, Karlsson EK, Kunjeti SG, Lamour K, Liu Z, Ma L, Maclean D, Chibucos MC, McDonald H, McWalters J, Meijer HJ, Morgan W, Morris PF, Munro CA, O'Neill K, Ospina-Giraldo M, Pinzón A, Pritchard L, Ramsahoye B, Ren Q, Restrepo S, Roy S, Sadanandom A, Savidor A, Schornack S, Schwartz DC, Schumann UD, Schwessinger B, Seyer L, Sharpe T, Silvar C, Song J, Studholme DJ, Sykes S, Thines M, van de Vondervoort PJ, Phuntumart V, Wawra S, Weide R, Win J, Young C, Zhou S, Fry W, Meyers BC, van West P, Ristaino J, Govers F, Birch PR, Whisson SC, Judelson HS, Nusbaum C (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature 461:393–398

Hacquard S, Delaruelle C, Legué V, Tisserant E, Kohler A, Frey P, Martin F, Duplessis S (2010) Laser Capture Microdissection of uredinia formed by *Melampsora larici-populina* revealed a transcriptional switch between biotrophy and sporulation. Mol Plant Microbe Interact 23:1275–1286

Hacquard S, Joly DL, Lin YC, Tisserant E, Feau N, Delaruelle C, Legue V, Kohler A, Tanguay P, Petre B, Frey P, Van de Peer Y, Rouze P, Martin F, Hamelin RC, Duplessis S (2012) A comprehensive analysis of genes encoding small secreted proteins identifies candidate effectors in *Melampsora larici-populina* (poplar leaf rust). Mol Plant Microbe Interact 25:279–293

Haerter AC, Voegele RT (2004) A novel ß-glucosidase in *Uromyces fabae*: feast or fight? Curr Genet 45: 96–103

Hahn M, Mendgen K (1992) Isolation by ConA binding of haustoria from different rust fungi and comparison of their surface qualities. Protoplasma 170:95–103

Hahn M, Mendgen K (1997) Characterization of in planta induced rust genes isolated from a haustorium-specific cDNA library. Mol Plant Microbe Interact 40:427–437

Harder DE, Chong J (1991) Rust haustoria. In: Mendgen K, Lesemann D-E (eds) Electron microcopy of plant pathogens. Springer, Berlin, pp 235–250

Harder DE, Mendgen K (1982) Filipin-sterol complexes in bean rust- and oat crown rust-fungal/plant interactions: freeze-etch electron microscopy *Uromyces appendiculatus*. Protoplasma 112:46–54

Heath MC (1974) Light and electron microscope studies of the interactions of host and nonhost plants with cowpea rust – *Uromyces phaseoli* var. *vignae*. Physiol Mol Plant Pathol 4:403–414

Heath MC (1990) Influence of carbohydrates on the induction of haustoria of the cowpea rust fungus in vitro. Exp Mycol 14:84–88

Heath MC (1997) Signalling between pathogenic rust fungi and resistant or susceptible host plants. Ann Bot 80:713–720

Heath MC (1999) The enigmatic hypersensitive response: induction, execution, and role. Physiol Mol Plant Pathol 55:1–3

Heath MC, Nimchuk ZL, Xu H (1997) Plant nuclear migrations as indicators of critical interactions between resistant or susceptible cowpea epidermal cells and invasion hyphae of the cowpea rust fungus. New Phytol 135:689–700

Heslop-Harrison J (1966) Cytoplasmic continuities during spore formation in flowering plants. Endeavour 25:65–72

Hoch HC, Staples RC, Whitehead B, Comeau J, Wolfe ED (1987) Signalling for growth orientation and differentiation by surface topography in *Uromyces*. Science 235:1659–1662

Howard RJ, Ferrari MA, Roach DH, Money NP (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. Proc Natl Acad Sci USA 88:11281–11284

Hu GG, Rijkenberg FHJ (1998) Scanning electron microscopy of early infection structure formation by *Puccinia recondita* f. sp. *tritici* on and in susceptible and resistant wheat lines. Mycol Res 102:391–399

Hu GG, Linning R, McCallum B, Banks T, Cloutier S, Butterfield Y, Liu J, Kirkpatrick R, Stott J, Yang G, Smailus D, Jones S, Marra M, Schein J, Bakkeren G (2007) Generation of a wheat leaf rust, *Puccinia triticina*, EST database from stage-specific cDNA libraries. Mol Plant Pathol 8:451–467

Huang L, Brooks SA, Li W, Fellers JP, Trick HN, Gill BS (2003) Map-based cloning of leaf rust resistance gene Lr21 from the large and polyploid genome of bread wheat. Genetics 164:655–664

Huang G, Allen R, Davis EL, Baum TJ, Hussey RS (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proc Natl Acad Sci USA 103:14302–14306

Huvenne H, Smagghe G (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 56:227–235

Jakupovic M, Heintz M, Reichmann P, Mendgen K, Hahn M (2006) Microarray analysis of expressed sequence tags from haustoria of the rust fungus *Uromyces fabae*. Fungal Genet Biol 43:8–19

Joly DL, Feau N, Tanguay P, Hamelin R (2010) Comparative analysis of secreted protein evolution using expressed sequence tags from four poplar leaf rusts (*Melampsora* spp.). BMC Genom 11:422

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Kaitera J, Nuorteva H (2010) Effects of Melampyrum extracts on the growth of axenic cultures of *Cronartium flaccidum* and *Peridermium pini*. Silva Fenn 44:197–202

Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K, Minami E, Shibuya N (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc Natl Acad Sci USA 103:11086–11091

Kämper J, Kahmann R, Bölker M, Ma LJ, Brefort T, Saville BJ, Banuett F, Kronstad JW, Gold SE, Müller O, Perlin MH, Wösten HA, de Vries R, Ruiz-Herrera J, Reynaga-Peña CG, Snetselaar K, McCann M, Pérez-Martín J, Feldbrügge M, Basse CW, Steinberg G, Ibeas JI, Holloman W, Guzman P, Farman M, Stajich JE, Sentandreu R, González-Prieto JM, Kennell JC, Molina L, Schirawski J, Mendoza-Mendoza A, Greilinger D, Münch K, Rössel N, Scherer M, Vranes M, Ladendorf O, Vincon V, Fuchs U, Sandrock B, Meng S, Ho EC, Cahill MJ, Boyce KJ, Klose J, Klosterman SJ, Deelstra HJ, Ortiz-Castellanos L, Li W, Sanchez-Alonso P, Schreier PH, Häuser-Hahn I, Vaupel M, Koopmann E, Friedrich G, Voss H, Schlüter T, Margolis J, Platt D, Swimmer C, Gnirke A, Chen F, Vysotskaia V, Mannhaupt G, Güldener U, Münsterkötter M, Haase D, Oesterheld M, Mewes HW, Mauceli EW, DeCaprio D, Wade CM, Butler J, Young S, Jaffe DB, Calvo S, Nusbaum C, Galagan J, Birren BW (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. Nature 444:97–101

Kemen E, Hahn M, Mendgen K, Struck C (2005a) Different resistance mechanisms of *Medicago truncatula* ecotypes against the rust fungus *Uromyces striatus*. Phytopathology 95:153–157

Kemen E, Kemen AC, Rafiqi M, Hempel U, Mendgen K, Hahn M, Voegele RT (2005b) Identification of a protein from rust fungi transferred from haustoria into infected plant cells. Mol Plant Microbe Interact 18:1130–1139

Kemen E, Gardiner A, Schultz-Larsen T, Kemen AC, Balmuth AL, Robert-Seilaniantz A, Bailey K, Holub E, Studholme DJ, Maclean D, Jones JD (2011) Gene gain and loss during evolution of obligate parasitism in the white rust pathogen of *Arabidopsis thaliana*. PLoS Biol 9:e1001094

Kemppainen M, Duplessis S, Martin F, Pardo AG (2009) RNA silencing in the model mycorrhizal fungus *Laccaria bicolor*: gene knock-down of nitrate reductase results in inhibition of symbiosis with Populus. Environ Microbiol 11:1878–1896

Kolmer JA (1996) Genetics of resistance to wheat leaf rust. Annu Rev Phytopathol 34:435–455

Kolmer JA, Ordonez ME, Groth JV (2009) The rust fungi. In: Encyclopedia of life sciences. Wiley, Chichester

Kumar S, Chaudhary K, Foster JM, Novelli JF, Zhang Y, Wang SL, Spiro D, Ghedin E, Carlow CKS (2007) Mining predicted essential genes of *Brugia malayi* for nematode drug targets. PLoS One 2:e1189

Kushalappa AC, Eskes AB (1989) Coffee rust: epidemiology, resistance, and management. CRC Press, Boca Raton

Laurans F, Pilate G (1999) Histological aspects of a hypersensitive response in poplar to *Melampsora larici-populina*. Phytopathology 89:233–238

Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG (1995) The L6 gene for flax rust resistance is related to the *Arabidopsis* bacterial-resistance gene RPS2 and the tobacco viral resistance gene-N. Plant Cell 7: 1195–1206

Lawrence GJ, Dodds PN, Ellis JG (2010) Transformation of the flax rust fungus, *Melampsora lini*: selection via silencing of an avirulence gene. Plant J 61: 364–369

Lebrun MH (2012) Interacting with plants: lessons from fungal genomes. In: 11th European conference on fungal genetics, Marburg, Mar 2012, abstract p 27

Li H, Goodwin P, Han Q, Huang L, Kang Z (2012) Microscopy and proteomic analysis of the non-host resistance of *Oryza sativa* to the wheat leaf rust fungus, *Puccinia triticina* f. sp. *tritici*. Plant Cell Rep 31:637–650

Lilley CJ, Davies LJ, Urwin PE (2012) RNA interference in plant parasitic nematodes: a summary of the current status. Parasitology 139:630–640

Ling P, Wang M, Chen X, Campbell KG (2007) Construction and characterization of a full-length cDNA library for the wheat stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*). BMC Genom 8: 145

Link TI, Voegele RT (2008) Secreted proteins of *Uromyces fabae*: similarities and stage specificity. Mol Plant Pathol 9:56–66

Link T, Lohaus G, Heiser I, Mendgen K, Hahn M, Voegele RT (2005) Characterization of a novel NADP+−dependent D-arabitol dehydrogenase from the plant pathogen *Uromyces fabae*. Biochem J 389:289–295

Littlefield LJ, Heath MC (1979) Ultrastructure of rust fungi. Academic, New York/San Francisco/London

Liu Z, Szabo LJ, Bushnell WR (1993) Molecular cloning and analysis of abundant and stage-specific mRNAs from *Puccinia graminis*. Mol Plant Microbe Interact 6:84–91

Loehrer M, Langenbach C, Goellner K, Conrath U, Schaffrath U (2008) Characterization of nonhost resistance of Arabidopsis to the Asian Soybean Rust. Mol Plant Microbe Interact 21:1421–1430

Lucas JA (2006) Avoiding and managing fungicide resistance. Arable crop protection in the balance: profit and the environment. London: Home Grown Cereals Authority (HGCA) conference

Ma J, Huang X, Wang X, Chen X, Qu Z, Huang L, Kang Z (2009) Identification of expressed genes during compatible interaction between stripe rust (*Puccinia striiformis*) and wheat using a cDNA library. BMC Genom 10:586

Martin F, Kamoun S (2012) Effectors in plant-microbe interactions. Wiley, Chichester, UK, pp 444

Mellersh DG, Heath MC (2003) An investigation into the involvement of defense signaling pathways in components of the nonhost resistance of *Arabidopsis thaliana* to rust fungi also reveals a model system for studying rust fungal compatibility. Mol Plant Microbe Interact 16:398–404

Mendgen K, Hahn M (2002) Plant infection and the establishment of fungal biotrophy. Trends Plant Sci 7:352–356

Moreno-Ruiz G, Castillo-Zapata J (1990) The variety Colombia: a variety of coffee with resistance to rust (*Hemileia vastatrix* Berk. & Br.). Cenicafé Chinchiná-Caldas-Colombia Techn Bull 9:1–27

Mukhtar MS, Carvunis AR, Dreze M, Epple P, Steinbrenner J, Moore J, Tasan M, Galli M, Hao T, Nishimura MT, Pevzner SJ, Donovan SE, Ghamsari L, Santhanam B, Romero V, Poulin MM, Gebreab F, Gutierrez BJ, Tam S, Monachello D, Boxem M, Harbort CJ, McDonald N, Gai L, Chen H, He Y, EU Effectoromics Consortium, Vandenhaute J, Roth FP, Hill DE, Ecker JR, Vidal M, Beynon J, Braun P, Dangl JL (2011) Independently evolved virulence effectors converge onto hubs in a plant immune system network. Science 333:596–601

Nirmala J, Drader T, Chen X, Steffenson B, Kleinhofs A (2010) Stem rust spores elicit rapid RPG1 phosphorylation. Mol Plant Microbe Interact 23:1635–1642

Nirmala J, Drader T, Lawrence PK, Yin C, Hulbert S, Steber CM, Steffenson BJ, Szabo LJ, von Wettstein D, Kleinhofs A (2011) Concerted action of two avirulent spore effectors activates Reaction to *Puccinia graminis* 1 (Rpg1)-mediated cereal stem rust resistance. Proc Natl Acad Sci USA 108:14676–14681

Nowara D, Gay A, Lacomme C, Shaw J, Ridout C, Douchkov D, Hensel G, Kumlehn J, Schweizer P (2010) HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. Plant Cell 22:3130–3141

Nunes CC, Sailsbery JK, Dean RA (2011) Characterization and application of small RNAs and RNA silencing mechanisms in fungi. Fungal Biol Rev 25:172–180

O'Connell RJ, Panstruga R (2006) Tête-à-tête inside a plant cell: establishing compatibility between plants and biotrophic fungi and oomycetes. New Phytol 171:699–718

Perfect SE, Green JR (2001) Infection structures of biotrophic and hemibiotrophic fungal plant pathogens. Mol Plant Pathol 2:101–108

Petutschnig EK, Jones AM, Serazetdinova L, Lipka U, Lipka V (2010) The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. J Biol Chem 285:28902–28911

Posada-Buitrago ML, Frederick RD (2005) Expressed sequence tag analysis of the soybean rust pathogen *Phakopsora pachyrhizi*. Fungal Genet Biol 42:949–962

Puthoff DP, Neelam A, Ehrenfried ML, Scheffler BE, Ballard L, Song Q, Campbell KB, Cooper B, Tucker ML (2008) Analysis of expressed sequence tags from *Uromyces appendiculatus* hyphae and haustoria and their comparison to sequences from other rust fungi. Phytopathology 98:1126–1135

Raffaele S, Kamoun S (2012) Genome evolution in filamentous plant pathogens: why biger can be better. Nature Rev Microbiol 10:417–430. doi:10.1038/nrmicro2790

Raffaele S, Win J, Cano LM, Kamoun S (2010) Analyses of genome architecture and gene expression reveal novel candidate virulence factors in the secretome of Phytophthora infestans. BMC Genomics 11:637

Rafiqi M, Gan PHP, Ravensdale M, Lawrence GJ, Ellis JG, Jones DA, Hardham AR, Dodds PN (2010) Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. Plant Cell 22:2017–2032

Ramiro D, Escoute J, Petitot A-S, Nicole M, Maluf M, Fernandez D (2009) Biphasic haustorial differentiation of the orange rust *Hemileia vastatrix* race II associated with differential defense responses in resistant coffee cultivar. Plant Pathol 58:944–955

Ravensdale M, Nemri A, Thrall PH, Burdon JJ, Ellis JG, Dodds PN (2011) Co-evolution of host resistance and pathogen effector genes in flax rust disease. Mol Plant Pathol 12:93–102

Rinaldi C, Kohler A, Frey P, Duchaussoy F, Ningre N, Couloux A, Wincker P, Le Thiec D, Fluch S, Martin F, Duplessis S (2007) Transcript profiling of poplar leaves upon infection with compatible and

incompatible strains of the foliar rust *Melampsora larici-populina*. Plant Physiol 144:347–366
Roelfs AP (1988) Genetic control of phenotypes in wheat stem rust. Annu Rev Phytopathol 26:351–367
Roelfs AP, Singh RP, Saari EE (1992) Rust diseases of wheat: concepts and methods of disease management. CIMMYT, Mexico
Rouxel T, Grandaubert J, Hane JK, Hoede C, van de Wouw AP, Couloux A, Dominguez V, Anthouard V, Bally P, Bourras S, Cozijnsen AJ, Ciuffetti LM, Degrave A, Dilmaghani A, Duret L, Fudal I, Goodwin SB, Gout L, Glaser N, Linglin J, Kema GH, Lapalu N, Lawrence CB, May K, Meyer M, Ollivier B, Poulain J, Schoch CL, Simon A, Spatafora JW, Stachowiak A, Turgeon BG, Tyler BM, Vincent D, Weissenbach J, Amselem J, Quesneville H, Oliver RP, Wincker P, Balesdent MH, Howlett BJ (2011) Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by repeat-induced point mutations. Nat Commun 2:202. doi:10.1038/ncomms1189
Runo SS, Alakonya AA, Machuka JJ, Sinha NN (2011) RNA interference as a resistance mechanism against crop parasites in Africa: a Trojan horse approach. Pest Manag Sci 67:129–136
Saunders DGO, Win J, Cano LM, Szabo LJ, Kamoun S, Raffaele S (2012) Using hierarchical clustering of secreted protein families to classify and rank candidate effectors of rust fungi. PLoS One 7: e29847
Savile DBO (1984) Taxonomy of the cereal rust fungi. In: Bushnell WR, Roelfs AP (eds) The cereal rusts, vol I, Origins, specificity, structure, and physiology. Academic, Orlando, pp 89–122
Schillberg S, Tiburzy R, Fischer R (2000) Transient transformation of the rust fungus *Puccinia graminis* f. sp. *tritici*. Mol Gen Genet 262:911–915
Schirawski J, Mannhaupt G, Münch K, Brefort T, Schipper K, Doehlemann G, Di Stasio M, Rössel N, Mendoza-Mendoza A, Pester D, Müller O, Winterberg B, Meyer E, Ghareeb H, Wollenberg T, Münsterkötter M, Wong P, Walter M, Stukenbrock E, Güldener U, Kahmann R (2010) Pathogenicity determinants in smut fungi revealed by genome comparison. Science 330:1546–1548
Schneider KT, van de Mortel M, Bancroft TJ, Braun E, Nettleton D, Nelson RT, Frederick RD, Baum TJ, Graham MA, Whitham SA (2011) Biphasic gene expression changes elicited by *Phakopsora pachyrhizi* in soybean correlate with fungal penetration and haustoria formation. Plant Physiol 157:355–371
Shafiei R, Hang C, Kang JG, Loake GJ (2007) Identification of loci controlling non-host disease resistance in Arabidopsis against the leaf rust pathogen *Puccinia triticina*. Mol Plant Pathol 8:773–784
Shibuya N, Minami E (2001) Oligosaccharide signalling for defence responses in plant. Physiol Mol Plant Pathol 59:223–233
Silva MC, Nicole M, Guerra-Guimarães L, Rodrigues CJ Jr (2002) Hypersensitive cell death and post-haustorial defense responses arrest the orange rust (*Hemileia vastatrix*) growth in resistant coffee leaves. Physiol Mol Plant Pathol 60:169–183
Silva MC, Várzea V, Guimarães LG, Azinheira HG, Fernandez D, Petitot A, Bertrand B, Lashermes P, Nicole M (2006) Coffee resistance to the main diseases: leaf rust and coffee berry disease. Braz J Plant Physiol 18:119–147
Singh RP, Hodson DP, Huerta-Espino J, Jin Y, Bhavani S, Njau P, Herrera-Foessel S, Singh PK, Singh S, Govindan V (2011) The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. Annu Rev Phytopathol 49:465–481
Škalamera D, Jibodh S, Heath MC (1997) Callose deposition during the interaction between cowpea (*Vigna unguiculata*) and the monokaryotic stage of the cowpea rust fungus (*Uromyces vignae*). New Phytol 136:511–524
Sohn J, Voegele RT, Mendgen K, Hahn M (2000) High level activation of vitamin b1 biosynthesis genes in haustoria of the rust fungus *Uromyces fabae*. Mol Plant Microbe Interact 13:629–636
Spanu PD (2012) The genomics of obligate (and nonobligate) biotrophs. Annu Rev Phytopathol 50:91–109. doi:10.1146/annurev-phyto-081211-173024
Spanu PD, Abbott JC, Amselem J, Burgis TA, Soanes DM, Stüber K, Loren V, van Themaat E, Brown JK, Butcher SA, Gurr SJ, Lebrun MH, Ridout CJ, Schulze-Lefert P, Talbot NJ, Ahmadinejad N, Ametz C, Barton GR, Benjdia M, Bidzinski P, Bindschedler LV, Both M, Brewer MT, Cadle-Davidson L, Cadle-Davidson MM, Collemare J, Cramer R, Frenkel O, Godfrey D, Harriman J, Hoede C, King BC, Klages S, Kleemann J, Knoll D, Koti PS, Kreplak J, López-Ruiz FJ, Lu X, Maekawa T, Mahanil S, Micali C, Milgroom MG, Montana G, Noir S, O'Connell RJ, Oberhaensli S, Parlange F, Pedersen C, Quesneville H, Reinhardt R, Rott M, Sacristán S, Schmidt SM, Schön M, Skamnioti P, Sommer H, Stephens A, Takahara H, Thordal-Christensen H, Vigouroux M, Wessling R, Wicker T, Panstruga R (2010) Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. Science 330:1543–1546
Staples RC, Grambow HJ, Hoch HC, Wynn WK (1983) Contact with membrane grooves induces wheat stem rust uredospore germlings to differentiate appressoria but not vesicles. Phytopathology 73: 1436–1439
Stergiopoulos I, de Wit PJGM (2009) Fungal effector proteins. Ann Rev Phytopathol 47:233–263
Struck C, Hahn M, Mendgen K (1996) Plasma membrane H+−ATPase activity in spores, germ tubes, and haustoria of the rust fungus *Uromyces viciae-fabae*. Fungal Genet Biol 20:30–35

Struck C, Siebels C, Rommel O, Wernitz M, Hahn M (1998) The plasma membrane H+−ATPase from the biotrophic fungus *Uromyces fabae*: molecular characterisation of the gene (PMA1) and functional expression of the enzyme in yeast. Mol Plant Microbe Interact 11:458–465

Struck C, Ernst M, Hahn M (2002) Characterization of a developmentally regulated amino acid transporter (AAT1p) of the rust fungus *Uromyces fabae*. Mol Plant Pathol 3:23–30

Struck C, Mueller E, Martin H, Lohaus G (2004) The *Uromyces fabae* UfAAT3 gene encodes a general amino acid permease that prefers uptake of in planta scarce amino acids. Mol Plant Pathol 5: 183–189

Thara VK, Fellers JP, Zhou J-M (2003) planta induced genes of *Puccinia triticina*. Mol Plant Pathol 4:51–56

Tremblay A, Li S, Scheffler BE, Matthews BF (2008) Laser capture microdissection and expressed sequence tag analysis of uredinia formed by *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. Physiol Mol Plant Pathol 73:163–174

van de Mortel M, Recknor JC, Graham MA, Nettleton D, Dittman JD, Nelson RT, Godoy CV, Abdelnoor RV, Almeida ÁMR, Baum TJ, Whitham SA (2007) Distinct biphasic mRNA changes in response to Asian soybean rust infection. Mol Plant Microbe Interact 20:887–899

van Doorn WG, Beers EP, Dangl JL, Franklin-Tong VE, Gallois P, Hara-Nishimura I, Jones AM, Kawai-Yamada M, Lam E, Mundy J, Mur LA, Petersen M, Smertenko A, Taliansky M, Van Breusegem F, Wolpert T, Woltering E, Zhivotovsky B, Bozhkov PV (2011) Morphological classification of plant cell deaths. Cell Death Differ 18:1241–1246

Vaz Patto MC, Niks R (2001) Leaf wax layer may prevent appressorium differentiation but does not influence orientation of the leaf rust fungus *Puccinia hordei* on *Hordeum chilense* leaves. Eur J Plant Pathol 107:795–803

Vieira A, Talhinhas P, Loureiro A, Thürich J, Duplessis S, Fernandez D, Silva MC, Paulo OS, Azinheira HG (2012) Expression profiling of genes involved in the biotrophic colonisation of *Coffea arabica* leaves by *Hemileia vastatrix*. Eur J Plant Pathol 133:261–277. doi:10.1007/s10658-011-9864-5

Voegele RT, Mendgen K (2011) Nutrient uptake in rust fungi: how sweet is parasitic life? Euphytica 179:41–55

Voegele RT, Struck C, Hahn M, Mendgen K (2001) The role of haustoria in sugar supply during infection of *Vicia faba* by the rust fungus *Uromyces fabae*. Proc Natl Acad Sci USA 98:8133–8138

Voegele RT, Hahn M, Mendgen K (2009) The Uredinales: cytology, biochemistry, and molecular biology. In: Deising HB (ed) The mycota: plant relationships, 2nd edn. Spinger, London/San Diego, pp 69–98

Waller JM, Bigger M, Hillocks RJ (2007) Coffee pests, diseases and their management. CABI, Oxfordshire

Wälti MA, Villalba C, Buser RM, Grünler A, Aebi M, Künzler M (2006) Targeted gene silencing in the model mushroom *Coprinopsis cinerea* (*Coprinus cinereus*) by expression of homologous hairpin RNAs. Eukaryot Cell 5:732–744. doi:10.1128/EC.5.4.732-744.2006

Wan J, Zhang S, Stacey G (2004) Activation of a mitogen-activated protein kinase pathway in Arabidopsis by chitin. Mol Plant Pathol 5:125–135

Warren JM, Covert SF (2004) Differential expression of pine and *Cronartium quercuum* f. sp. *fusiforme* genes in fusiform rust galls. Appl Environ Microbiol 70:441–451

White TA, Kell DB (2004) Comparative genomic assessment of novel broad spectrum targets for antibacterial drugs. Comp Funct Genomics 5: 304–327

Williams PG (1984) Obligate parasitism and axenic culture. In: Bushnell WR, Roelfs AP (eds) The cereal rusts, vol I, Origins, specificity, structure, and physiology. Academic, Orlando, pp 399–430

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre A, Bangham R, Benito R, Boeke JE (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285:901–906

Xu J, Linning R, Fellers J, Dickinson M, Zhu W, Antonov I, Joly DL, Donaldson ME, Eilam T, Anikster Y, Banks T, Munro S, Mayo M, Wynhoven B, Ali J, Moore R, McCallum B, Borodovsky M, Saville B, Bakkeren G (2011) Gene discovery in EST sequences from the wheat leaf rust fungus *Puccinia triticina* sexual spores, asexual spores and haustoria, compared to other rust and corn smut fungi. BMC Genom 12:161

Yin C, Chen X, Wang X, Han Q, Kang Z, Hulbert SH (2009) Generation and analysis of expression sequence tags from haustoria of the wheat stripe rust fungus *Puccinia striiformis* f. sp. *tritici*. BMC Genom 10:626

Yin C, Jurgenson JE, Hulbert SH (2011) Development of a host-induced RNAi system in the wheat stripe rust fungus *Puccinia striiformis* f. sp. *tritici*. Mol Plant Microbe Interact 24:554–561

Zhang Y, Qu Z, Zheng W, Liu B, Wang X, Xue X, Xu L, Huang L, Han Q, Zhao J, Kang Z (2008) Stage-specific gene expression during urediniospore germination in *Puccinia striiformis* f. sp. *tritici*. BMC Genom 9:203

Zipfel C (2009) Early molecular events in PAMP-triggered immunity. Curr Opin Plant Biol 12:414–420

Zipfel C, Robatzek S (2010) Pathogen-associated molecular pattern-triggered immunity: veni, vidi. . .? Plant Physiol 154:551–554

13 The Biotrophy–Necrotrophy Switch in Fungal Pathogenesis

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CONTENTS

I. Introduction: Fungal Pathogenic Lifestyles

Fungal plant pathogens cause a wide array of disease symptoms, ranging from severe necroses of the attacked host organ, occurring soon after infection, to greening of the plant tissue surrounding the infection site, leading to longevity of the infected plant tissue, and long-lasting production of spores. According to their lifestyles, pathogens can thus be classified as **necrotrophic or biotrophic, depending on whether they kill the host prior to or immediately after invasion**, or keep the plant actively alive and retrieve carbon sources over a long time (Horbach et al. 2011; Behr et al. 2010). Typical examples of **necrotrophs are those fungi that produce and secrete toxins** at the infection site of attempted invasion, and toxin formation may even be morphogenetically controlled. Analysis of spore germination fluids by plasma desorption mass spectrometry has shown that highly virulent isolates of the maize leaf blight fungus *Cochliobolus carbonum* produce the host-selective HC toxin exactly at a time coincident with maturation of appressoria, i.e., before entering the host tissue. On surfaces that did not stimulate appressorium formation, toxin formation was not detected (Weiergang et al. 1996). In contrast to necrotrophs, **biotrophs manipulate their host tissue** by various means, e.g., effector-mediated down-regulation of plant-defense responses, modification of microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) exposed on the surface of invading hyphae, and/or by remodeling of the phytohormone balance at the infection site, as indicated by the occurrence of green islands (Mendgen and Hahn 2002; Maor and Shirasu 2005; Behr et al. 2010, 2012).

The hallmark shared by biotrophs such as rust fungi and powdery mildews is a highly specialized nutrition cell called the **haustorium**. While powdery mildews differentiate haustoria from appressoria formed on the plant surface, the vast majority of urediniospores of rust species differentiate complex infection structures in order to invade their host tissue through stomatal pores, and develop intercellular infection hyphae from which haustorial mother cells are separated by a septum, and only these cells penetrate the host cell wall to give rise to a haustorium.

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Transmission electron microscopy was used to examine details of the host–pathogen interface in daylily leaf cells infected by the rust fungus *Puccinia hemerocallidis* (Mims et al. 2002). Samples were prepared by high-pressure freezing, followed by freeze substitution. The outstanding preservation of ultrastructural details afforded by this fixation protocol greatly facilitated the study of this host–pathogen interface. The extrahaustorial membrane that separated each dikaryotic haustorium from the cytoplasm of its host cell was well-preserved and appeared almost completely smooth. Large aggregations of tubular cytoplasmic elements were present near haustoria in infected host cells. Many of these tubular elements were found to be continuous with the extrahaustorial membrane. Distinctive tubular–vesicular complexes previously reported only in cryofixed rust haustoria were also found in the haustoria of *P. hemerocallidis*. These structural details suggest that haustoria and infected host cells are metabolically extremely active, and that an extensive exchange of molecules, e.g., sugars and proteins, occurs across the bifacial extrahaustorial matrix surrounding the haustoria of rust fungi. (Mendgen and Deising 1993)

One may speculate that **biotrophy had been established as the ancient lifestyle of fungal pathogens, and that necrotrophy represents a more recent evolutionary achievement**. Indeed, Lower Devonian fungi that lived ca. 400 million years ago, as identified in samples collected at the Rhynie Chert, had already established biotrophic parasitic interactions with fungi (Hass et al. 1994) and plants (Taylor et al. 1992), or symbiotic interactions (Taylor et al. 1995). Interestingly, intermediate forms of parasitism exist: ***hemibiotrophs*** establish an initial biotrophic interaction characterized by voluminous hyphae surrounded by intact host plasma membranes and viable host cells. Subsequently, upon perception of a hitherto unknown signal, thin fast-growing and highly destructive hyphae are formed that kill their host. ***Heminecrotrophy*** may also represent an intermediate nutritional strategy. The only example of this lifestyle, however, is provided by the Asian soybean rust fungus *Phakopsora pachyrrhizi*, which kills the first infected host cell and subsequently establishes a biotrophic relationship with other host cells. Thus, hemibiotrophy, and possibly heminecrotrophy may represent evolutionary intermediates reflecting the transition between biotrophy and necrotrophy, and vice versa.

A detailed analysis, using methods established in phytopathology, molecular biology, and cellular biology, will help to unravel factors required to establish pathogenic interactions, and may make it possible to develop novel strategies to control plant diseases.

II. The Challenges of the Initial Biotrophic Phase of Hemibiotrophs

Microbes, including plant pathogenic fungi, expose a large variety of MAMPs/PAMPs. MAMPs/PAMPs are invariable molecules, as modulation of their structure would cause non-functionality of the molecule and failure of the establishment of a parasitic interaction, possibly even non-viability. A wealth of literature provides examples of such molecules, many of which are fungal structural cell-wall components such as chitin or branched or linear β-1,3-glucans (Fliegmann et al. 2004; Klarzynski et al. 2000; Barber et al. 1989; Shetty et al. 2009; Vander et al. 1998; Felix et al. 1993). Indeed, structural cell-wall components are both invariable and indispensable, as indicated by studies with chitin and β-1,3-glucan synthase inhibitors belonging to the classes of Nikkomycins or Echinocandins. All these studies showed that **interference with cell-wall integrity causes drastically reduced growth rates, failure to differentiate infection structures, and loss of pathogenicity** (Georgopapadakou 2001; Kondoh et al. 2005; Werner et al. 2007; Serfling et al. 2007). Thus, conceivably, pattern-recognition receptors (PRRs) localized in the plasma membrane of the plant show specificity for invariable structures of attacking pathogens, and this mode of pathogen recognition reveals striking similarities between plants and animals (Nürnberger et al. 2004; Postel and Kemmerling 2009). Invariable structures of pathogens are either surface-exposed, such as chitin and β-1,3-glucan, or secreted by the invading pathogen, such as cell-wall-degrading enzymes such as xylanases (Postel et al. 2009). Importantly, infection structures of biotrophs and hemibiotrophs, i.e., haustoria or infection vesicles and primary hyphae, invaginate the plasma membrane of their host plant upon invasion, and grow in a bifacial matrix in intimate vicinity to the host plasma membrane

containing PRRs. Furthermore, **plants secrete enzymes degrading the cell walls of the pathogens** into this narrow bifacial matrix, creating a detrimental environment for invading hyphae. Chitinases generate elicitor-active chitin fragments, which lead to pathogen perception. For example, β-1,4-*N*-acetyl glucosamine oligomers are recognized by PRRs such as the two LysM receptors of rice, i.e., CEBiP and OsCERK1, which cooperatively mediate chitin elicitor signaling and immunity (Shimizu et al. 2010). In tomato, sub-nanomolar concentrations of *N*-acetylchitooligosaccharides are sufficient to induce defense responses (Felix et al. 1993). Likewise, **β-1,3-glucanases generate branched or linear β-1,3-glucan activating PAMP/MAMP-triggered immunity (PTI/MTI)**, and failure in establishment of a compatible parasitic interaction (Klarzynski et al. 2000; Fliegmann et al. 2004; Shetty et al. 2009).

In Fig. 13.1, plant defense, i. e., papillae formation and accumulation of fluorescent compounds as a response to invading hyphae of *C. graminicola*, has been made visible by confocal microscopy.

Compatible pathogens must have evolved **mechanisms to evade PAMP/MAMP recognition** and/or to block signal transduction activating PTI/MTI. Evasion of PAMP/MAMP recognition may be achieved by modifying the surface of biotrophic hyphae and/or sequestration of elicitor-active cell-wall fragments. An alternative or additional strategy for hindering activation of defense relies on secretion of effector molecules, which are taken up by the host plant and interfere with signal transduction required for activation of defense responses.

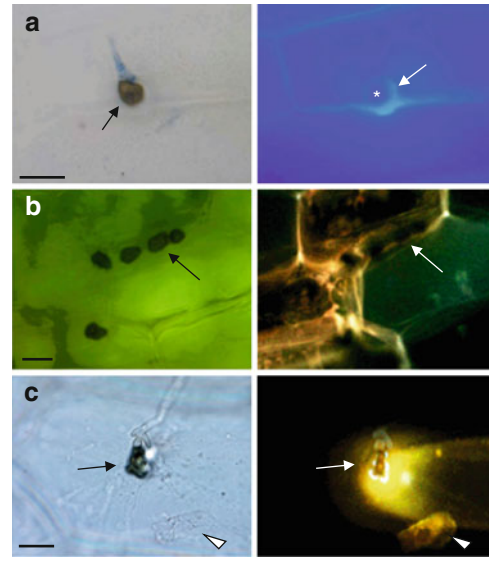

Fig. 13.1. Visualization of infection-inducible defense responses. (**a**) Infection of maize leaves with *C. graminicola*. Fluorescent papillae (*white arrow*) which appear frequently at the site of attempted penetration become visible under UV light as indicated by a bright halo (*white arrow*) surrounding the appressorium (*black arrow*, *asterisk*). Cell walls of attacked maize epidermis cells (**b**) show a bright fluorescence, whereas onion cells (**c**) respond with a localized accumulation of fluorescent compounds at the site of penetration. *Arrows* point at appressoria, *arrowheads* mark the nucleus. Bars = 10 μm. Micrographs: Alexander Mickel (**a**), Ralf Horbach (**b**, **c**)

1. Coping with Plant Defense

A. Masking the Surface of Invading Biotrophic Hyphae

Biotrophic pathogens depend on rigorous prevention of plant defense, and must therefore evolve mechanism(s) to protect their cell walls from degradation by cell-wall-degrading enzymes of the plant, i.e., chitinases or β-1,3-glucanases, and generation of elicitor-active chitin fragments. Chitin fragments are significantly more potent elicitors of plant defense than chitosan fragments of comparable size, as indicated by thorough comparative biochemical studies in wheat (Vander et al. 1998), and **de-acetylation of chitin**, yielding chitosan, might contribute to prevention of defense.

Interestingly, epi-fluorescence microscopy with the fluorescence-tagged chitin-specific lectin wheat germ agglutinin revealed that surfaces of infection structures of two rust fungi, the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* and the broad bean rust fungus *Uromyces fabae*, and of the causal agent of maize anthracnose, *Colletotrichum graminicola*, formed on the plant cuticle expose chitin, whereas surfaces of structures formed after invading the host do not (El Gueddari et al. 2002). Intriguingly, exactly when the *U. fabae* starts invading its host leaf through the stomatal pore, the activity of chitin deacetylases, i.e.,

enzymes, which convert chitin to chitosan, increases massively, and five isoforms were detected in intercellular washing fluids by substrate-inclusion SDS-PAGE (Deising et al. 1995). Polyclonal antibodies specifically recognizing chitosan indicated that chitosan is indeed exposed on the surfaces of cell walls of *in-planta* differentiated infection hyphae of both rust fungi and of *C. graminicola* (El Gueddari et al. 2002). These findings strongly suggest that surface modification of the fungal cell walls by chitin de-acetylation may contribute to protection of cell walls, and to avoiding the generation of an auto-catalytic defense response system in the infected host tissue.

Alternative or additional ways of interfering with chitin-triggered immunity are used by the rice blast fungus *M. oryzae*, a hemibiotroph, and by the obligate biotroph and tomato pathogen *Cladosporium fulvum*. Both fungi secrete **chitin-binding proteins** during *in-planta* development (de Jonge et al. 2010; Mentlak et al. 2012). In *M. oryzae*-infected rice leaves, the **LysM** protein Slp1 accumulates at the interface between the fungal cell wall, and the rice plasma membrane is able to bind to chitin, and to suppress chitin-induced rapid first-line plant immune responses such as generation of reactive oxygen species (Mentlak et al. 2012). In *C. fulvum*, two chitin-binding proteins have been characterized in detail. The effector protein (see below) Avr4 fully protects fungal cell walls against hydrolysis by secreted chitinases of plants. In contrast, however, another chitin-binding protein of this fungus, Ecp6, failed to protect hyphae against enzymatic hydrolysis by chitinases.

In tomato and tobacco cell suspensions, chitin-triggered immunity is associated with pH shifts of the culture medium. Treatment of the cells with nanomolar concentrations of *N*-acetylglucosamine hexamers caused medium alkalinization, but addition of the LysM-containing protein Ecp6 indeed attenuated this response. In contrast, Avr4 did not affect chitin-induced alkalinization of the culture medium (de Jonge et al. 2010). These experiments suggested that Ecp6 did not provide protection from hydrolysis of fungal cell walls by plant chitinases, but, like Slp1 of *M. oryzae*, sequestered chitin fragments, blocked PAMP recognition, and interfered with PAMP-triggered immunity. De Jonge and Thomma (2009) surveyed sequence data of 70 fungal species, and reported that putatively secreted LysM-containing proteins are widespread in the fungal kingdom, and present in both mammalian and plant pathogens. Thus, fungal LysM proteins may be regarded as general extinguishers of host immunity.

B. Fungal Effectors

According to Kamoun (2009), effectors may be defined as products of genes residing in the genomes of pathogens, but function at the interface with the host or even inside the plant cell, altering host cell structure and function, thereby facilitating infection or triggering defense responses. Genome sequencing has revealed that **the effector secretome of pathogenic oomycetes is extremely complex, with perhaps several hundred proteins dedicated to manipulating host cell structure and function** (Kamoun 2006). According to the definition above, effectors either exhibit a function in virulence (e.g., toxins; Walton 1996) or represent avirulence factors or elicitors. Depending on their target site in the host, effectors can be classified into **apoplastic and cytoplasmic effectors**.

Examples of apoplastic effectors are proteinase such as EPI1, EPI10, EPIC1, and EPIC2B of the hemibiotroph and causal agent of potato late blight, *Phytophthora infestans*, targeting apoplastic proteases of the host, and Avr9 of the biotroph *C. fulvum*. Other apoplastic effectors function as *endo*-β-1,3-glucanases inhibitors, e.g., GIP1 and GIP2 of *Phytophthora sojae*, or exhibit chitin-binding activities such as Avr4 of *C. fulvum* or Slp1 of *M. oryzae*. (see above; for review, see Kamoun 2009)

Obviously, these effectors function in counter-defense by protecting the cell walls of infectious hypha of the invading pathogen from degradation. Cytoplasmic effectors, in contrast, are secreted by the pathogen and delivered into the host cell. In *P. infestans*, several cytoplasmic

effectors, exhibiting a characteristic structure, have been identified. In addition to a C-terminal effector domain and a N-terminal signal peptide, cytoplasmic effectors of *Phytophthora* have a **RXLR motif** residing close to the N-terminus (Haas et al. 2009), and RXLR-like motifs are thought to be required for uptake of effector proteins into the host cell (Whisson et al. 2007; Kamoun 2006). However, although evidence has been presented that RXLR-like motifs and that phospholipids are required for uptake of fungal/oomycete effectors (Yaeno et al. 2011), the signal and the mechanism mediating uptake of effectors is at present not clear in fungal pathogens. Importantly, in *P. infestans*, genes encoding effectors such as AVR1, AVR2, AVR3a, AVR4, AVRblb1, AVRblb2, and AVRvnt1, all of which exhibit a N-terminal RXLR motif, are strongly expressed during the biotrophic phase of pathogenesis, and expression is down-regulated when the pathogen enters necrotrophy (Vleeshouwers et al. 2011), strongly suggesting that **effectors are required for avoiding defense during biotrophic development.**, Effectors have also been analyzed in detail in fungal hemibiotrophs. Deep sequencing of the transcriptome of the Arabidopsis pathogen *C. higginsianum* indicated that most effector genes are host-induced, and that consecutive waves of distinct effector suites are secreted at different stages of pathogenesis. Some of these effectors suppress, whereas others induce cell death, strongly suggesting that hemibiotrophy in this fungus is orchestrated by coordinated expression of antagonizing effectors (Kleemann et al. 2012). In the closely related pathogen *C. graminicola*, a functional screen for secreted proteins, including putative effectors, has been performed in yeast. This so-called **yeast secretion signal trap** (YSST) screen yielded several candidate effector genes, several of which showed infection stage-specific expression (Krijger et al. 2008). However, targeted deletion of a large number of putative effectors has not led to the identification of either an apoplastic or a cytoplasmic effector (F. Weihmann, J.-J. Krijger, E. Oliveira-Garcia, H.B. Deising, and S.G.R. Wirsel, unpublished data).

Comparative genome-wide expression profiling indicated significant differences in the infection between *C. higginsianum* and *C. graminicola*. While in the genome of *C. graminicola* only 177 genes encoding candidate secreted effectors lacking homology to proteins outside the genus *Colletotrichum* were found, 365 of such proteins, i.e., more than twice as many, were found in the *C. higginsianum* genome (O'Connell et al. 2012). It would be interesting to study in detail cell-wall modifications, including secretion of chitin-sequestering proteins and appositions of masking proteins such as hydrophobins (Wessels 1996) and other proteins in both fungi. Such studies might indicate that *C. graminicola* rather relies on avoiding PAMP exposition by masking its surface, whereas *C. higginsianum* prevents initiation of plant defense by massive secretion of effectors. Such studies may indicate differences in modes of infection and nutritional strategies, even within the same genus, which are governed by the differences of the host plant, and reveal the enormous capacity of pathogens to adapt to the requirements of its host.

2. Uptake of Nutrients from the Apoplast

Pathogens, upon entry into their host plant, face very different nutritional scenarios, depending on their lifestyle. Among different classes of nutrients, carbohydrates play a primary role in the development of a pathogen, and sugar uptake for reproduction can be regarded as the primary goal of the pathogen. Biotrophic hyphae are encased by the plant plasma membrane, creating a **bifacial matrix** with an extremely low volume, and one may assume that extracellular/apoplastic sugars are quickly consumed. Thus, on the plant side the key questions address mechanisms leading to maintenance of stable extracellular sugar concentrations during the biotrophic interaction, sufficient to support fungal development, and on the fungal side mechanisms of **highly efficient sugar uptake into the pathogen** must be addressed.

Efflux of sugars from plants is essential for several aspects of elementary importance, including inter-cellular and inter-organismic exchange of carbon and energy. Despite the importance of the mechanism of **sugar efflux**, and despite known solute efflux from the host plant at the interfaces between hosts and

microorganisms (Patrick 1989), the identity of sugar efflux transporters has remained elusive for a long time. Recently, Chen et al. (2010) used a FRET-based glucose sensor system to isolate the first sugar efflux transporter from plants. These transporters were designated as **SWEET transporters**, and families of genes encoding SWEET transporters have several members.

In Arabidopsis, 17 SWEET genes exist, and, interestingly, several members of the SWEET gene family are induced by bacterial and fungal pathogens. Pathogenic bacteria such as *Pseudomonas* or *Xanthomonas* live in the intercellular space of their host plants, and are referred to as biotrophic pathogens (Bonas et al. 2000). In Arabidopsis, *Pseudomonas syringae* pv. *tomato* strain DC3000 massively induced the transcript levels of seven SWEET transporter genes. Another set of SWEET transporters was induced by the fungal powdery mildew of *A. thaliana*, *Golovinomyces chicoracearum*, which is also a biotroph: these observations clearly support the broad validity of the concept that pathogens hijack the sugar export system of plants to support their own development and propagation in the intercellular space (Chen et al. 2010; Sonnewald 2011). In rice, the SWEET transporter gene *OsSWEET11* underlies the dominant allele (*Xa13*) of the recessive resistance gene *xa13*, and susceptibility alleles of *xa13* confer disease susceptibility. Both mutations in the promoter region and RNA interference of *OsSWEET11* confer resistance to *X. oryzae* pv. *oryzae* (Chen et al. 2010, and references therein), since extracellular sugar concentrations are probably not increased in promoter mutants and RNAi lines.

In order to compromise plant defense responses and to maintain the biotrophic interaction, a bacterial type III secretion system is required to inject effector cocktails into the host cell. One of these effectors, PthXo1, is a **TAL (transcriptional activator-like) effector**. Importantly, PthXo1 directly interacts with the promoter of *OsSWEET11*, as indicated by chromatin immuno-precipitation and co-expression in *Nicotiana benthamiana*, and manipulates sugar export in order to support pathogen development (Chen et al. 2010). At present, it is unclear whether the manipulation of sugar efflux transporters is characteristic for biotrophs, as some increase in SWEET gene transcripts of Arabidopsis has also been observed after inoculation with the necrotroph *Botrytis cinerea* (Chen et al. 2010).

Sugar uptake is mediated by plasma membrane-localized **carbohydrate transporters** as identified from two pathogenic biotrophic basidiomycetes, the broad bean rust fungus *Uromyces fabae* and the smut fungus *Ustilago maydis*, the symbiotic, mycorrhiza-forming glomeromycete *Geosiphon pyriformis*, and from the root-colonizing ascomycete *Metarhizium robertsii*, which antagonizes plant pathogens and herbivores (Voegele et al. 2001; Wahl et al. 2010; Schüssler et al. 2006; Fang et al. 2010). The transporters UfHXT1 of *U. fabae* and GpMST1 of *G. pyriformis* are classic monosaccharide transporters that reconstitute the growth defect and/or reconstitute the lost transport activities of hexose uptake-deficient baker's yeast mutants (Voegele et al. 2001; Schüssler et al. 2006), and the gene identified in *M. robertsii* encodes the oligosaccharide transporter MRT with specificity for sucrose and galactosides such as raffinose, stachyose, and verbascose, as suggested by growth assays (Fang et al. 2010). The *MRT* gene is expressed in germlings of *M. robertsii* growing in the vicinity of grass roots, and Δ*mrt* mutants show reduced growth in the rhizosphere. However, disruption of the gene did not affect virulence to insects, suggesting that redundant transporters with overlapping substrate specificities exist in this fungus (Fang et al. 2010). Interestingly, the transporter UmSRT1 of *U. maydis* is absolutely specific for sucrose, and the gene encoding this transporter is specifically expressed in fungal hyphae growing *in planta*. Importantly, *U. maydis* mutants lacking the *UmSRT1* gene are strongly reduced in virulence on maize (Wahl et al. 2010), highlighting the fact that efficient **carbohydrate and energy transfer from the host into the pathogen is essential for pathogenicity**.

In silico analyses revealed that more than 80 sugar transporters exist in the genome of the hemibiotroph *C. graminicola* (Lingner 2012). Five hexose transporter genes of this fungus, *HXT1* to *HXT5*, and the melibiose transporter gene *MBT1*, have been cloned, the proteins have been functionally characterized, and their expression has been studied during vegetative and pathogenic development (Lingner et al. 2011a, b).

Interestingly, transcript abundances of *HXT1* and *HXT3* are transiently up-regulated during biotrophy, and *HXT2* and *HXT5* are expressed exclusively during necrotrophic development. *HXT4* appears to be constitutively expressed. Detailed biochemical analyses characterized HXT1 to HXT3 as high-affinity/low-capacity transporters, with specificity to different sugars such as glucose, mannose, fructose, galactose, and xylose. HXT5 is a low-affinity/high-capacity hexose transporter with narrow substrate specificity for glucose and mannose. HXT4 has only minor transport activity, and may function as a sugar sensor. Functional characterization of the MBT1 protein in baker's yeast expressing the *MBT1* cDNA showed that α-D-galactopyranosyl sugars such as melibiose, galactinol, and raffinose are substrates of MBT1, with melibiose most probably being the preferred substrate. This gene is expressed during biotrophic and necrotrophic development. (Lingner et al. 2011a, b)

These studies clearly indicate that fungi have large sets of sugar transporters, presumably allowing the pathogens to grow under the conditions they meet in their host plants. In hemibiotrophs, the sugar composition in their environment may vary with their lifestyle, as they first, during biotrophic development, will utilize sugars present in the apoplast, probably primarily the disaccharide sucrose and the hexoses glucose and fructose, whereas a broad spectrum of sugars will probably be present during necrotrophic development, when the fungus secretes a large array of plant cell-wall-degrading enzymes (O'Connell et al. 2012; Krijger et al. 2008). Thus, pathogenic fungi are not only able to manipulate the host metabolism and **re-direct carbon flow to the site of infection** (Voll et al. 2011), but also to link the expression profile of the sugar uptake transporter genes to their developmental and nutritional requirements.

3. Initiation of Necrotrophy

Plant infection by hemibiotrophic fungi follows a general scheme which is characterized by successive invasion and nutritional strategies. Initial establishment of a stable host–pathogen interaction in the early stage of infection is followed by a massive disease outbreak. Intercellular hyphal growth during the biotrophic phase and nutrient uptake from the apoplast without affecting the integrity of the host plasma membrane is facilitated by subtle molecular mechanisms evolved to avoid or minimize recognition by the plant defense (see section II.1). In contrast, **nectrotrophic development is characterized by the differentiation of destructive secondary hyphae dedicated to ramify within the host tissue, killing host cells and gaining nutrients** for further spread of infection and hence propagation. Although several fungal species belonging to different genera have been classified as hemibiotrophs, detailed studies that may justify this classification are rare. Moreover, current literature reveals a remarkable variability in defining hemibiotrophy. The causative of potato late blight *Phytophthora infestans*, for example, has been described as a hemibiotrophic, biotrophic, and necrotrophic pathogen. A comprehensive summary of the requirements to classify biotrophs and necrotrophs can be found in Oliver and Ipcho (2004).

The concept of hemibiotrophy requires a certain amount of flexibility, since **at least three unrelated infection strategies are pursued by hemibiotrophs**. First, a temporally separate and non-overlapping differentiation of biotrophic and necrotrophic infection structures is symptomatic for some members of the genus *Colletotrichum*. The second mode of infection is characterized by an extended asymptotic phase of 4–14 days and applies to plant pathogens such as *Mycosphaerella graminicola* and *Cladosporium fulvum*. Infection hyphae of these species do not differ during biotrophic or necrotrophic *in-planta* development (Oliver and Ipcho 2004). Neither of these criteria appear to apply to the rice blast pathogen *Magnaporthe oryzae*. Disease development in rice leaves is distinguished by a progressive zone where each successive plant cell invasion is biotrophic, but invaded cells are no longer viable by the time the fungus moves into the next cell (Kankanala et al. 2007). A consequence of the variability of hemibiotrophic lifestyles is the difficulty in comprehensively defining the time of transition from biotrophy to necrotrophy for the different types of hemibiotrophic interactions. Furthermore, it is still controversial whether hemibiotrophy can be

regarded in some cases as a temporal delay of the infection progress rather than as a programmed development. Reorganization from penetration to invasive growth or active plant defense mechanisms may result in slower growth at the early stage of infection. With the focus on phytopathogenic ascomycetes, this review summarizes the current understanding of the modifications at the transcriptional, physiological, and morphological level that are associated with the transition from the biotrophic to the necrotrophic lifestyle.

A. External Signals that Trigger the Switch to Necrotrophy

The most intriguing enigma yet to be solved is certainly the nature of the signal(s) controlling the **initiation of necrotrophy** in pathosystems featuring a distinct biotrophic and necrotrophic phase such as *Colletotrichum*–host systems. A promising approach to understanding the requirements that are necessary for the differentiation of highly destructive infection hyphae would be a close examination of **changes in metabolite levels** within host cells harboring biotrophic fungal structures. Depletion of certain nutrients, i.e., sugars, sugar alcohols, or amino acids, might lead to carbon or nitrogen starvation, which triggers changes in gene expression and hence invasive growth.

This concept may be supported by a targeted deletion strain of *Colletotrichum gloeosporioides* that lacks glycerol-3-phosphate dehydrogenase. Severe conidiation and rhythmic growth defects of the mutant strain could be restored by adding glycerol or glycerol-3-phosphat, but no other carbon sources. Surprisingly, pathogenic development of the deletion strain was not affected. These findings indirectly suggest that glycerol is taken up by the fungus during the biotrophic phase.

Moreover, analysis of the glycerol content of leaf discs surrounding the infection sites revealed a time-dependent decline within 72 h of biotrophic development (Wei et al. 2004). It is tempting to hypothesize that the duration of biotrophy is controlled by the concentration of **available glycerol**. Experimental manipulation of the host metabolism aiming at the reduction of free glycerol would therefore be helpful to answer the question whether a certain threshold level of glycerol may induce the switch from biotrophy to necrotrophy.

Similarly, **nitrogen starvation** may constitute a cue regulating genes that are required for pathogenicity. During growth on its host tomato, the apoplast-colonizing fungal pathogen and biotroph *Cladosporium fulvum* secretes several effector proteins, some of which have proven indispensable for full virulence. The expression of the Avr9 gene encoding one of these effector proteins is strongly induced during infection and in vitro, under conditions of nitrogen depletion that are encountered in the host (van den Ackerveken et al. 1994). Therefore, nitrogen limitation, especially at the early stage of infection, was considered to be the signal for the fungus to induce further virulent genes (Snoeijers et al. 2000; Divon et al. 2006). However, the nitrogen response regulator Nrf1 was shown to regulate only Avr9 expression during infection of the host, whereas none of the other known effectors is significantly controlled by this transcription factor *in planta*. Since deletion of Nrf1 but not of Avr9 significantly reduced virulence of *C. fulvum,* the authors reasoned that Nrf1 controls, in addition to Avr9, unidentified effector genes that are required for full virulence of *C. fulvum* (Thomma et al. 2006).

In an attempt to identify control factors of fungal virulence genes, Pellier and co-workers (2003) deleted the *Colletotrichum lindemuthianum clnr1* gene, a key regulator in nitrogen metabolism. *Clnr1*-mutants were unable to use a wide array of nitrogen sources, and symptom development was drastically reduced, with only few anthracnose lesions that occurred rarely on whole plantlets. Cytological analysis revealed that the mutants were not impaired in their ability to penetrate host cuticles or differentiate biotrophic hyphae; however, no necrotrophic development of fungal infection structures has been observed. Notably, deletion of the α-aminoadipate reductase gene AAR1 in *Colletotrichum graminicola*, a single copy gene that catalyzes a key step in the biosynthesis of lysine, led to **biotrophy arrest** as described for *clnr1*-mutants of *C. lindemuthianum* (Horbach

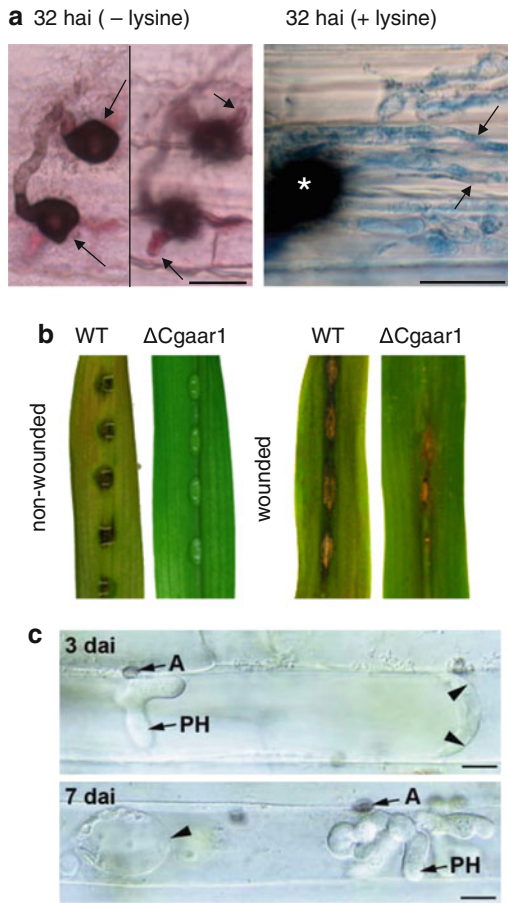

Fig. 13.2. Colletotrichum mutants impaired in the switch from biotrophy to necrotrophy. (**a**) Micrographs of intact leaves inoculated with the Δaar1 strain of *C. graminicola* formed melanized appressoria (32 h after infection, *hai*; −lysine, *left image*, *arrows*) and primary infection hyphae (32 hai, −lysine, *right image*, *arrows*), which were unable to form secondary hyphae and to switch to necrotrophic development. Addition of lysine restored the ability to switch to necrotrophy, as indicated by the occurrence of thin intracellular secondary hyphae (32 hai, +lysine, *arrows*; *asterisk* marks appressorium). Sample Δaar1, 32 hai, −lysine was acid fuchsin-stained, sample Δaar1, 32 hai, +lysine was aniline blue stained. (**b**) Plant infection assays showed that the wild-type, but not the lysine-deficient Δaar1 strain, caused anthracnose disease symptoms on non-wounded maize leaves. Symptom development was restored on wounded leaves. (**c**) Infection of *Arabidopsis* with ATMT-derived mutant *path-36 of C. higginsianum*. The mutant formed normal melanized appressoria (*A*) with penetration pores and successfully established biotrophic primary hyphae (*PH*) in host epidermal cells, but necrotrophic secondary hyphae were rarely produced. In order to test for viability of penetrated epidermal cells, Arabidopsis seedlings were inoculated with mutant *path-36* and hypocotyls were plasmolyzed with 0.85 M KNO3. At 3 days after infection (*dai*), biotrophic primary hyphae (*PH*) were visible beneath appressoria (*A*) and the penetrated epidermal cell remained alive, as shown by its ability to plasmolyze normally (*arrowheads* indicate the plasma membrane). At 7 dai, the primary hyphae were larger but secondary hyphae were not present, and the penetrated host cells were still alive. Bars = 10 μm (Pictures were taken from Horbach et al. (2009) The Plant Cell 21:3379–96, www.plantcell.org and Huser et al. (2009) Mol Plant Microbe Interact 22:143–156. Copyright American Society of Plant Biologists

et al. 2009). This result clearly showed that lysine biosynthesis by the fungus is still required during biotrophic *in planta* development (see Fig. 13.2).

It should be taken into account that the deficient synthesis of amino acids may be responsible for the observed *clnr1*-phenotype, rather than a biased regulatory cross-talk.

Taken together, these data provide strong evidence that **fungal pathogenicity genes are controlled by nitrogen response regulators.** Sensing of the nitrogen status may be one of the general schemes used by phytopathogenic fungi to transmit specific environmental signals to the gene regulatory network (López-Berges et al. 2010).

Several studies support the concept of *in-planta* threshold levels of certain metabolites as determinants of the biotrophy–necrotrophy switch. Detailed analysis of promising target compounds will certainly contribute to shedding light on the nature of the signal that initiates the transition. A general problem in **studying metabolite profiles in host–parasite interactions**, however, arises from the quality of the sample material. Inoculated leafs usually consist of a small population of infected cells that are clearly outnumbered by the majority of non-infected cells, especially during the early stage of infection. In some cases, e.g., the interaction of mildews with their hosts, the infection is restricted to a single type of cells, whereas the other leaf tissues are not directly affected. Traditional analysis of metabolites using whole leaves may therefore lead to biased results due to interfering metabolite profiles. The same holds true for samples with fungal structures belonging to different developmental stages as a consequence of the asynchronous differentiation of infection structures. To avoid this, **analysis of single cells** will be advantageous; however, studying the metabolic state of a single cell is challenging because of the low amounts of compounds therein. Recent advances in metabolite detection including techniques that combine mass spectrometry and imaging systems, e.g., laser ablation electrospray ionization–mass spectrometry (LAESI), which do not depend on extensive sample preparation will substantially contribute to a deeper insight into the subcellular processes of host–pathogen interactions (Shrestha and Vertes 2009).

An additional difficulty in analyzing biotrophic hyphae is that they are only in contact with apoplastic metabolites present in the bifacial matrix surrounding them. The use of FRET sensors (Chen et al. 2010) has been a major methodological breakthrough, and might also be feasible for analyzing compounds other than glucose.

B. Transcriptional Changes on the Threshold to Necrotrophy

The transition from biotrophy to necrotrophy is associated with significant changes in hyphal morphology, enzyme secretion, and nutrient uptake (see above). Since most cellular processes are subject to transcriptional regulation, extensive changes in transcript levels of genes involved in disease outbreak, i.e., genes encoding **cell-wall-degrading enzymes, transporters or toxin-producing enzymes can be regarded as indicators for the onset of necrotrophic development.** This concept may be supported by the discovery of the CLTA1 gene of the bean anthracnose fungus *Colletotrichum lindemuthianum*. CLTA1 encodes a GAL4-like transcriptional activator belonging to the fungal zinc cluster ($Zn[II]_2Cys_6$) family, and proved to be indispensable for pathogenicity. Targeted inactivation of CLTA1 led to strains that were unable to differentiate secondary hyphae, which are indicative of necrotrophic growth. Infection assays with mutant strains and susceptible cultivars resulted in local necrotic spots reminiscent of hypersensitive lesions, which may be explained by a defect in the suppression of defense responses leading to cell death, or the inability of the fungus to activate its developmental program within the plant (Dufresne et al. 2000). Unfortunately, no further studies have been conducted in order to unveil the targets of CLTA1. Screening a mutant collection of the crucifere pathogen *Colletotrichum higginsianum* generated by *Agrobacterium tumefaciens* mediated transformation (ATMT) Huser et al. (2009) identified five mutants that successfully established intracellular biotrophy, but then became

arrested at the primary hyphae stage and only rarely entered the necrotrophic phase or formed secondary hyphae. Sequence information could be retrieved for two mutants, both of which harbored T-DNA integrations in a gene with high homology to conserved importin-β 2 proteins, known to mediate the nuclear import of pre-mRNA processing proteins. Since vegetative growth and differentiation of functional appressoria and biotrophic hyphae of these mutants were not affected, these results may be indicative of mRNA processing by proteins specific for genes transcribed at the beginning of the necrotrophic stage. Misregulation of pre-mRNA processing through the loss of such an important protein could profoundly affect fungal gene expression and thus pathogenicity.

Large-scale approaches designed to detect changes across the entire transcriptome have become valuable tools for the identification of genetic key players acting at the transition between the biotrophic and the necrotrophic phase.

Searching for candidate genes in *Colletotrichum truncatum*, Bhadauria et al. (2011) sequenced 5,000 ESTs from mRNA isolated from infected *Lens culinaris* leaflet tissues displaying the biotrophy–necrotrophy switch. Sequence analysis of 122 tentative unigenes featuring N-terminal signal peptides revealed four potential groups: hydrolases, cell-envelope-associated proteins, effectors and others. Based on the *in-planta* transcript profiling of 11 unigenes harboring a N-terminal signal peptide, four genes were exclusively accumulated during the necrotrophic phase of the infection process. The predicted proteins encoded by these genes share the characteristics of small cysteine-rich effectors known from other fungal host–pathogen interactions (Bolton et al. 2008).

Microarray analysis of stage-specific cDNA libraries is a powerful approach in order to detect candidate genes with relevance for invasive growth of hemibiotrophs (Voll et al. 2011). However, the identification of fungal genes that are differentially expressed during plant infection is problematic because of the low ratio of fungal to plant RNA. A solution offers the application of fluorescence-activated cell sorting (FACS). Intact biotrophic hyphae from arabidopsis leaves inoculated with *Colletotrichum higginsianum* were selectively stained with fluoresceine diacetate (FDA) and subsequently pre-enriched using a Percoll gradient. Fungal target structures were significantly enriched from <0.01 % in the leaf homogenate to 94 % purity after cell sorting. (Takahara et al. 2009)

Similar approaches based on the immunopurification of fungal material using a monoclonal antibody which specifically binds to surface glycoproteins of infection hyphae of *Colletotrichum lindemuthianum* (Pain et al. 1994a), or affinity chromatography with Concanavalin A, a lectin that binds to carbohydrates displayed at the surface of haustoria of rust fungi (Hahn and Mendgen 1992; Catanzariti et al. 2006), have been successfully employed.

Another method for sampling of a fungal plant–pathogen transcriptome during the infection process is laser microdissection (LM). Tang et al. (2006) developed a protocol for microwave-accelerated acetone fixation that preserved tissue histology, RNA integrity, and GFP fluorescence expressed by the fungus, in order to facilitate the identification of fungal *in-planta* structures for LM sampling. RNA of individual maize stalk cells associated with *Colletotrichum graminicola* hyphae at an early stage of infection was of sufficient quality for global expression profiling using a fungal microarray. Comparing replicated LM samples with samples from in-vitro-germinated conidia, the authors identified 437 and 370 *C. graminicola* genes showing significant up- or downregulation at the onset of necrotrophy respectively.

Vargas et al. (2012) performed transcriptional profiling on infected tissue at early stages of the *C. graminicola*–maize interaction, and compared biotrophic and necrotrophic transcription profiles. Based on sequencing results of two suppression subtractive hybridization (SSH) libraries that represent the interaction transcriptome at 48 and 72 h post inoculation (hpi), more than 200 genes were found to be differentially expressed. Among them, ten sequences of fungal origin had homologs annotated in the pathogen–host interaction database (PHI-database) as pathogenicity factors whereas 16 genes encode hypothetical proteins conserved in pathogenic fungi. The candidate effector metalloprotease fungalysin showed a maximum expression level at the switch of lifestyles at 60 hpi. Fungalysin belongs to a family of Zn-dependent proteases that seem to be part of the host invasion mechanism by *Aspergillus fumigatus* and *Micrococcus canis* (Brouta et al. 2002; Jousson et al. 2004; Rosenblum et al. 2008; Mathy et al. 2010). Notably, a set of putative respiration-related genes showed a similar expression profile, suggesting a respiratory burst during the infection of maize.

The yeast secretion signal trap system has been used to identify genes encoding secreted proteins of the maize pathogen *C. graminicola* (Krijger et al. 2008). Of 103 identified unigenes, 50 showed significant similarities to genes with a reported function, 25 sequences were similar to genes without a known function, and 28 sequences had no similarity to entries in the databases. Macroarray hybridization and quantitative reverse transcriptase polymerase chain reactions confirmed

that only some genes were constantly expressed, whereas a larger set showed peaks of transcript abundances at specific phases of pathogenesis. The *in-planta* expression profiles of 24 clones produced consistent signals. Among these, five clones showed maxima of transcript abundance at the necrotrophic stage. Sequence comparison revealed similarity to a fungal serine protease, a class II hydrophobin, a glucanase, and the cell-wall glycoprotein cap22. Thus, the YSST screen has made it possible to identify a number of candidate genes that might specifically contribute to necrotrophic development. However, the role of individual genes played at different stages of pathogenesis needs to be analyzed by targeted deletion of the candidate genes, followed by infection assays in intact maize leaves.

Kleemann et al. (2012) employed a RNAseq deep sequencing strategy to identify stage-specific effectors of the hemibiotroph *Colletotrichum higginsianum* that are involved in maintaining the viability of the host during biotrophy, and effectors that elicit host death for necrotrophic development. Two of these putative effectors, ChNLP1 encoding a necrosis- and ethylene-inducing peptide 1-like protein (NLP) and *ChToxB*, a homolog of a gene involved in synthesis of the host-selective toxin ToxB from *Pyrenophora tritici-repentis*, were expressed specifically at the switch to necrotrophy, suggesting that their toxic products contribute to the termination of the biotrophic phase. ChNLP1 was demonstrated to be a potent inducer of cell death in *Nicotiana benthamiana*, whereas the functional role of ChToxB is still elusive.

Using RNAseq, O'Connell et al. (2012) found a vast array of lytic enzymes of *C. higginsianum* to be induced at the transition to necrotrophy, including 44 putative secreted proteases and 146 enzymes assumed to be involved in degradation of plant polysaccharides. Concomitantly, numerous genes encoding plasma membrane transporters that may function as uptake systems for the products of this degradative activity are also induced.

Next-generation sequencing has the potential to become a standard tool in whole transcriptome analysis, since costs have decreased drastically in recent years.

C. Physiological Switch

Initiation of necrotrophic development is associated with massive colonization of host tissues by fast-growing hyphae. In contrast to the symptomless biotrophic phase, decay of host tissue now becomes visible, starting with chlorotic and necrotic spots that expand as the infection advances. During biotrophic growth, hemibiotrophic pathogens apply strategies to evade recognition by the plant defense system, e.g., masking of fungal structures, avoiding release of elicitors, or actively suppressing signal transduction that triggers host defense reactions by secreted effectors (see section "Coping with Plant Defense"). Necrotrophic strategies, in contrast, aim at breaking plant defense by rapid killing of host cells. As a consequence, virtually all plant material, if degradable, becomes available as nutrient source, especially high molecular weight compounds such as cell-wall polysaccharides, nucleic acids, and proteins. Extracellular degradation of these molecules requires secretion of hydrolytic enzymes. The **onset of necrotrophic development should therefore be accompanied by massive degradation of plant material and drastically increased activity of lytic enzymes** such as CWDEs, peptidases and nucleases.

In line with this is a study by Bowling et al. (2010) that provides indirect evidence for release of **fungal CWDEs** by necrotrophic hyphae of *Colletotrichum gloeosporioides* invading stems of *Sesbania exaltata*. Using a battery of antibody probes that recognize components of the host cell wall, and immunogold-silver cytochemistry to visualize these probes, the authors provide evidence for a massive loss of specific plant cell wall polysaccharides in the region surrounding the primary fungal infection site, which is characterized by voluminous biotrophic hyphae, i.e., rhamnogalacturon-1 and esterified and de-esterified homogalacturon-reactive epitopes. Despite the loss of so much homogalacturonan at the periphery of the lesions, the tissue at the center of the lesion was labeled strongly with these same antibodies, indicating the retention of pectins during the initial biotrophic stage at the center of the lesion, and their loss in the more peripheral areas of the lesions characterized by necrotrophic hyphae.

Similar results come from a detailed study of the *Colletotrichum lindemuthianum* endopolygalacturonase genes *CLPG1* and *CLPG2*. While *CLPG2* is exclusively expressed in appressoria, high expression of *CLPG1* was detected only during necrotrophic growth of *C. lindemuthianum*. Pectin degradation was not detected around the infection peg by immunostaining using the monoclonal antibody JIM7, specific for

methyl-esterified galacturonan. However, extensive pectin dissolution was observed during the development of secondary hyphae, which is in agreement with the results by Bowling et al. (Herbert et al. 2004). Since pectinases determine plant cell wall porosity and possibly elasticity (Baron-Epel et al. 1988), fungal pectinases could play a role in facilitating spread of fungal infection from cell to cell during invasive growth.

Indirect evidence for increased protein secretion during necrotrophic growth comes from a restriction-enzyme-mediated insertional (REMI) mutant screen of *C. graminicola* performed to identify genes that are required for pathogenicity of both stalks and leaves. One of the candidate strains, the nonpathogenic CPR1 mutant, contained a plasmid integration in the 3' untranslated region of the gene, which resulted in a significant reduction of transcript levels in comparison to the wild-type. The predicted polypeptide encoded by this gene is similar to a family of proteins that comprise one subunit of the eukaryotic microsomal signal peptidase. The CPR1 gene product may be essential for viability as targeted deletion failed. Microscopic examination of the REMI mutant on maize leaves revealed full penetration competence and normal colonization of host cells during the initial biotrophic phases of the disease, but it appeared to be unable to switch to a necrotrophic mode of growth. The authors conclude that the CPR1 REMI mutant may be unable to secrete sufficient amounts of degradative enzymes to support that transition. (Thon et al. 2002)

Another study by Remy et al. (2009) links primary metabolism with necrotrophic development. The ATMT-derived mutant m186 of *Leptosphaeria maculans* was found to be specifically blocked at the invasive growth phase after an unaffected initial penetration stage, and is unable to switch to the necrotrophic lifestyle. Insertion of the T-DNA lead to overexpression of the *Lmepi*1 gene predicted to encode an UDP-glucose-4-epimerase, which is involved in galactose metabolism. The mutant was significantly affected in its capacity to grow on xylan as the sole carbon source, whereas growth on pectin was not perturbed. In addition, m186 exhibited a significant reduction in polygalacturonase and xylanase activities when challenged with plant cell wall extract during in-vitro growth.

The importance of CWDEs during necrotrophic development is also highlighted by the fact that **the genomes of the hemibiotrophic *M. oryzae* and the necrotrophic *Fusarium graminearum* harbor 138 and 103 genes encoding CWDEs respectively**. In comparison, the genome of the biotrophic corn smut fungus *Ustilago maydis* is poorly equipped with genes encoding CWDEs, containing only 33 such hydrolytic enzymes (Kämper et al. 2006; Horbach et al. 2011). Although CWDEs produce fragments resulting from cell-wall degradation that effectively elicit defense responses, their large number and redundancy strongly argues that cell-wall-degrading enzymes are indispensable for full virulence of plant pathogenic fungi (Walton 1994).

Reports on fungal metabolites with a putative role in the transition from biotrophy to necrotrophy are rare. Only recently, a study of maize anthracnose disease caused by *Colletotrichum graminicola* presented evidence of an infection-related role of fungus-derived **superoxide anions**. The staining protocol yielded positive reactions in tips of hyphae approaching the border of the plant cells, and preparing to cross into the neighboring cell, which is regarded as the initiation of necrotrophy. Staining was also observed in fungal hyphae beginning to colonize a second cell, where the presence of superoxide was restricted to the hyphal tips in contact with the plant cell border. In contrast, hyphae growing in vitro under saprophytic conditions did not show a specific and confined pattern of superoxide production. These results suggest that a localized oxidative burst may be part of the strategy of the fast-growing fungal hyphae to pass through plant cell walls and/or membranes (Vargas et al. 2012).

C. acutatum, as well as other species of *Colletotrichum*, produces ammonia, causing local pH increase during the shift to necrotrophy (Prusky et al. 2001). Modification of pH at the infection site may facilitate host colonization because **ammonia secretion**, and the resulting pH increase leads to higher secretion of pectate lyase (PL), a key virulence factor in disease development (Yakoby et al. 2000, 2001). The finding that the *C. gloeosporioides* pelB gene-disrupted mutant produces amounts of

ammonia similar to the wild-type suggests that ammonia accumulation is independent of pelB expression. By secreting ammonia, the fungus ensures that PL is produced under optimal pH conditions, because the protein has an apparent pH optimum of 8.9 (Wattad et al. 1994). Similarly, O'Connell et al. (2012) found that necrotrophy in *C. higginsianum* is associated with local alkalinization of *Arabidopsis* tissue, probably resulting from fungal ammonia secretion. However, tissue alkalinization was less pronounced in maize colonized by *C. graminicola* at this stage. Therefore, it is suggested that local alkalinization as a result of ammonia is a virulence factor that mediates the initiation of necrotrophic development in some host–pathogen interactions.

D. Changes in Morphology

Production of dimorphic infectious mycelia, with voluminous biotrophic hyphae and **narrow necrotrophic hyphae**, is a characteristic feature of hemibiotrophs, e.g., *Magnaporthe oryzae* and several *Colletotrichum* species (Wharton et al. 2001; O'Connell et al. 1993; Latunde-Dada et al. 1996; Heath et al. 1990). Larger surface area, volume, and thinner walls of invasive hyphae would be advantageous for nutrient uptake and secretion of diverse compounds. **Large-diameter biotrophic hyphae**, on the other hand, would minimize the contact area with the plant plasma membrane, which might help to avoid recognition by the host, and thick cell walls provide protection from lytic enzymes.

For *Colletotrichum lindemuthianum*, diameters of primary hyphae were found to be 4–5 μm with cell walls of 100–130 nm thickness, whereas secondary hyphae were 1.5–3.5 μm in diameter with very thin walls of 25–40 nm. WGA labeling revealed no differences in the chitin content of either primary or secondary hyphae. In contrast, immunostaining using a panel of antibodies directed against glycoproteins was positive for conidia, germ-tubes, appressoria, primary hyphae, and mycelium grown in vitro, but not for secondary hyphae. These results suggest that the fungal cell surface becomes modified during necrotrophic growth, with none of the glycoproteins associated with earlier stages of the infection process being produced. (Pain et al. 1994b; Perfect et al. 2001)

III. Conclusions

Not only analyses of transcript abundances by microarray or RNA deep sequencing studies, but, more importantly, detailed knowledge of the function of candidate genes essential for fungal viability of pathogenicity is required to develop novel concepts for controlling fungal-diseases. This is true for virtually all taxonomical and lifestyle groups of pathogens. However, although biotrophic plant pathogenic fungi are of enormous economical significance, functional genetics is extremely difficult in obligate biotrophs. Over a number of years, pathogens such as *M. oryzae* and the two *Colletotrichum* species, *C. graminicola* and *C. higginsianum*, have been studied in detail and developed into model pathogens. The work with these fungi has several advantages, including the possibility to study biotrophy and necrotrophy in the same genetic background. For example, the identification of genes required for the establishment of compatible host-pathogen interactions, including the discovery of chemical compounds with inhibitory activity against the products of these genes, might make it possible to control biotrophs and also hemibiotrophs at the early stage of pathogenesis, i.e., when biotrophic hyphae are formed. Furthermore, several genes required for the biotrophy–necrotrophy switch have already been identified, and these genes or their products could be targets, particularly in hemibiotrophs, as in these fungi only the necrotrophic phase causes major tissue damage. We have begun to analyze fungal mechanisms to activate the biotrophy–necrotrophy switch, with special emphasis on the contribution of secondary metabolism. Identifying secondary metabolites of hemibiotrophs and understanding the regulation of the genes responsible for their synthesis will not only help to understand the transition between lifestyles,

but also may result in identifying novel lead structures for chemical disease control.

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References

Barber MS, Bertram RE, Ride JP (1989) Chitin oligosaccharides elicit lignification in wounded wheat leaves. Physiol Mol Plant Pathol 34(1):3–12

Baron-Epel O, Gharyal PK, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. Planta 175:389–395

Behr M, Humbeck K, Hause G, Deising HB, Wirsel SGR (2010) The hemibiotroph *Colletotrichum graminicola* locally induces photosynthetically active green islands but globally accelerates senescence on aging maize leaves. Mol Plant Microbe Interact 23:879–892

Behr M, Motyka V, Weihmann F, Malbeck J, Deising HB, Wirsel SGR (2012) Remodeling of cytokinin metabolism at infection sites of *Colletotrichum graminicola* on maize leaves. Mol Plant Microbe Interact 25(8):1073–1082

Bhadauria V, Banniza S, Vandenberg A, Selvaraj G, Wei Y (2011) EST mining identifies proteins putatively secreted by the anthracnose pathogen *Colletotrichum truncatum*. BMC Genomics 12:327

Bolton MD, Van Esse HP, Vossen JH, De Jonge R, Stergiopoulos I, Stulemeijer IJE, Van Den Berg G, Borrás-Hidalgo O, Dekker HL, De Koster CG, De Wit PJGM, Joosten MHAJ, Thomma BPHJ (2008) The novel Cladosporium fulvum lysin motif effector Ecp6 is a virulence factor with orthologs in other fungal species. Mol Microbiol 69:119–136

Bonas U, Van den Ackerveken G, Büttner D, Hahn K, Marois E, Nennstiel D, Noël L, Rossier O, Szurek B (2000) How the bacterial plant pathogen *Xanthomonas campestris* pv. *vesicatoria* conquers the host. Mol Plant Pathol 1:73–76

Bowling AJ, Vaughn KC, Hoagland RE, Stetina K, Douglas Boyette C (2010) Immunohistochemical investigation of the necrotrophic phase of the fungus Colletotrichum gloeosporioides in the biocontrol of hemp sesbania (*Sesbania exaltata*; Papilionaceae). Am J Bot 97:1915–1925

Brouta F, Descamps F, Monod M, Vermout S, Losson B, Mignon B (2002) Secreted metalloprotease gene family of *Microsporum canis*. Infect Immun 70: 5676–5683

Catanzariti AM, Dodds PN, Lawrence GJ, Ayliffe MA, Ellis JG (2006) Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. Plant Cell 18:243–256

Chen LQ, Hou BH, Lalonde S, Takanaga H, Hartung ML, Qu XQ, Guo WJ, Kim JG, Underwood W, Chaudhuri B, Chermak D, Antony G, White FF, Somerville SC, Mudgett MB, Frommer WB (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. Nature 468:527–532

de Jonge R, Thomma BPHJ (2009) Fungal LysM effectors: extinguishers of host immunity? Trends Microbiol 17:151–157

de Jonge R, van Esse HP, Kombrink A, Shinya T, Desaki Y, Bours R, van der Krol S, Shibuya N, Joosten MH, Thomma BP (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science 329:953–955

Deising H, Rauscher M, Haug M, Heiler S (1995) Differentiation and cell wall-degrading enzymes in the obligately biotrophic rust fungus *Uromyces viciae-fabae*. Can J Bot 73:624–631

Divon HH, Ziv C, Davydov O, Yarden O, Fluhr R (2006) The global nitrogen regulator, FNR1, regulates fungal nutrition-genes and fitness during Fusarium oxysporum pathogenesis. Mol Plant Pathol 7:485–497

Divon HH, Fluhr R (2007) Nutrition acquisition strategies during fungal infection of plants. FEMS Microbiol Lett 26:65–74

Dufresne M, Perfect S, Pellier AL, Bailey JA, Langin T (2000) A GAL4-like protein is involved in the switch between biotrophic and necrotrophic phases of the infection process of *Colletotrichum lindemuthianum* on common bean. Plant Cell 12:1579–1590

El Gueddari NE, Rauchhaus U, Moerschbacher BM, Deising HB (2002) Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi. New Phytol 156:103–112

Fang W, St. Leger RJ (2010) Mrt, a gene unique to fungi, encodes an oligosaccharide transporter and facilitates rhizosphere competency in Metarhizium robertsii. Plant Physiol 154:1549e–1557e

Felix G, Regenass M, Boller T (1993) Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. Plant J 4:307–316

Fliegmann J, Mithöfer A, Wanner G, Ebel J (2004) An ancient enzyme domain hidden in the putative beta-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. J Biol Chem 279(2):1132–1140

Georgopapadakou NH (2001) Update on antifungals targeted to the cell wall: focus on beta-1,3-glucan synthase inhibitors. Expert Opin Investig Drugs 10:269–280

Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature 461:393–398

Hahn M, Mendgen K (1992) Isolation by ConA binding of haustoria from different rust fungi and comparison of their surface qualities. Protoplasma 170:95–103

Hass H, Taylor TN, Remy W (1994) Fungi from the Lower Devonian Rhynie Chert: mycoparasitism. Am J Bot 81:29–37

Heath MC, Valent B, Howard RJ, Chumley FG (1990) Interactions of two strains of *Magnaporthe grisea* with rice, goosegrass, and weeping lovegrass. Can J Bot 68:1627–1637

Herbert C, O' Connell R, Gaqulin E, Salesses V, Esquerre-Tugaye MT, Dumas B (2004) Production of a cell wall-associated endopolygalacturonase during bean infection. Fungal Genet Biol 41:140–147

Horbach R, Graf A, Weihmann F, Antelo L, Mathea S, Liermann JC, Opatz T, Thines E, Aguirre J, Deising HB (2009) Sfp-type 4′-phosphopantetheinyl transferase is indispensable for fungal pathogenicity. Plant Cell 21:3379–3396

Horbach R, Navarro-Quesada AR, Knogge W, Deising HB (2011) When and how to kill a plant cell: infection strategies of plant pathogenic fungi. J Plant Physiol 168:51–62

Huser A, Takahara H, Schmalenbach W, O'Connell R (2009) Discovery of pathogenicity genes in the crucifer anthracnose fungus *Colletotrichum higginsianum*, using random insertional mutagenesis. Mol Plant Microbe Interact 22:143–156

Jousson O, Léchenne B, Bontems O, Capoccia S, Mignon B, Barblan J, Quadroni M, Monod M (2004) Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. Microbiol 150:301–310

Kamoun S (2006) A catalogue of the effector secretome of plant pathogenic oomycetes. Annu Rev Phytopathol 44:41–60

Kamoun S (2009) The secretome of plant-associated fungi and oomycetes. In: Deising H (ed) Plant relationships, vol V, 2nd edn, The Mycota. Springer, Berlin/Heidelberg

Kämper J, Kahmann R, Bölker M, Ma LJ, Brefort T, Saville BJ et al (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. Nature 444:97–101

Kankanala P, Czymmek K, Valent B (2007) Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. Plant Cell 19:706–724

Klarzynski O, Plesse B, Joubert JM, Yvin JC, Kopp M, Kloareg B, Fritig B (2000) Linear beta-1,3 glucans are elicitors of defense responses in tobacco. Plant Physiol 124(3):1027–1038

Kleemann J, Rincon-Rivera LJ, Takahara H, Neumann U, van Themaat EVL, van der Does HC, Hacquard S, Stüber K, Will I, Schmalenbach W, Schmelzer E, O'Connell RJ (2012) Sequential delivery of host-induced virulence effectors by Appressoria and intracellular hyphae of the phytopathogen *Colletotrichum higginsianum*. PLoS Pathog 8(4): e1002643

Kondoh O, Inagaki Y, Fukuda H, Mizuguchi E, Ohya Y, Arisawa M, Nobuo Shimma N, Aoki Y, Sakaitani M, Watanabe T (2005) Piperazine propanol derivative as a novel antifungal targeting 1,3-β-D-glucan synthase. Biol Pharm Bull 28:2138–2141

Krijger J-J, Horbach R, Behr M, Schweizer P, Deising HB, Wirsel SGR (2008) The yeast signal sequence trap identifies secreted proteins of the hemibiotrophic corn pathogen *Colletotrichum graminicola*. Mol Plant Microbe Interact 21:1325–1336

Latunde-Dada AO, O'Connell RJ, Nash C, Pring RJ, Lucas JA, Bailey JA (1996) Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum* O'Gara) from cowpea (*Vigna unguiculata* (L.) Walp.). Mycol Res 100:1133–1141

Lingner U (2012) Stoffaustausch bei der Interaktion zwischen Pflanze und Pilz: Identifizierung von Zuckertransportern und deren Charakterisierung. In: Naturwissenschaftliche Fakultät. Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen-Nuremberg, p 131

Lingner U, Muench S, Deising HB, Sauer N (2011a) Hexose transporters of a hemibiotrophic plant pathogen: functional variations and regulatory differences at different stages of infection. J Biol Chem 286:20913–20922

Lingner U, Münch S, Sode B, Deising HB, Sauer N (2011b) Functional characterization of a eukaryotic melibiose transporter. Plant Physiol 156:1565–1576

López-Berges MS, Rispail N, Prados-Rosales RC, Di Pietro A (2010) A nitrogen response pathway regulates virulence functions in *Fusarium oxysporum* via the protein kinase TOR and the bZIP protein MeaB. Plant Cell 22:2459–2475

Maor R, Shirasu K (2005) The arms race continues: battle strategies between plants and fungal pathogens. Curr Opinion Microbiol 8(4):399–404

Mathy A, Baldo A, Schoofs L, Cambier L, Defaweux V, Tabart J, Maréchal F, Symoens F, Mignon B (2010) Fungalysin and dipeptidyl-peptidase gene transcription in *Microsporum canis* strains isolated from symptomatic and asymptomatic cats. Vet Microbiol 146:179–182

Mendgen K, Deising H (1993) Infection structures of fungal plant pathogens – a cytological and physiological evaluation. New Phytol 124:193–213

Mendgen K, Hahn M (2002) Plant infection and establishment of fungal biotrophy. Trends Plant Sci 7 (8):352–356

Mentlak TA, Kombrink A, Shinya T, Ryder LS, Otomo I, Saitoh H, Terauchi R, Nishizawa Y, Shibuya N, Thomma BP, Talbot NJ (2012) Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. Plant Cell 24:322–335

Mims CW, Rodriguez-Lother C, Richardson EA (2002) Ultrastructure of the host-pathogen interface in daylily leaves infected by the rust fungus *Puccinia hemerocallidis*. Protoplasma 219(3–4):221–226

Nürnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev 198:249–266

O'Connell RJ, Uronu AB, Waksman G, Nash C, Keon JPR, Bailey JA (1993) Hemibiotrophic infection of *Pisum sativum* by *Colletotrichum truncatum*. Plant Pathol 42:774–783

O'Connell RJ, Thon MR, Hacquard S et al (2012) Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. Nature Genet 44:1060–1065

Oliver RP, Ipcho SV (2004) Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens. Mol Plant Pathol 5(4):347–352

Pain NA, Green JR, Gammie F, O'Connell RJ (1994a) Immunomagnetic isolation of viable intracellular hyphae of *Colletotrichum lindemuthianum* from infected bean leaves using a monoclonal antibody. New Phytol 127:223–232

Pain NA, O'Connell RJ, Mendgen K, Green JR (1994b) Identification of glycoproteins specific to biotrophic intracellular hyphae formed in the *Colletotrichum*–bean interaction. New Phytol 127:233–242

Patrick JW (1989) Solute *efflux* at plant/microorganism interfaces. Aust J Plant Physiol 16:53–68

Pellier AL, Lauge R, Veneault-Fourrey C, Langin T (2003) CLNR1, the AREA/NIT2-like global nitrogen regulator of the plant fungal pathogen *Colletotrichum lindemuthianum* is required for the infection cycle. Mol Microbiol 48:639–655

Perfect SE, Green JR, O'Connell RJ (2001) Surface characteristics of necrotrophic secondary hyphae produced by the bean anthracnose fungus, *Colletotrichum lindemuthianum*. Eur J Plant Pathol 107:813–819

Postel S, Kemmerling B (2009) Plant systems for recognition of pathogen-associated molecular patterns. Semin Cell Dev Biol 20:1025–1031

Prusky D, McEvoy JL, Leverentz B, Conway WS (2001) Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. Mol Plant Microbe Interact 14:1105–1113

Remy E, Meyer M, Blaise F, Simon UK, Kuhn D, Balesdent MH, Rouxel T (2009) A key enzyme of the Leloir pathway is involved in pathogenicity of *Leptosphaeria maculans* towards oilseed rape. Mol Plant Microbe Interact 22:725–736

Rosenblum EB, Stajich JE, Maddox N, Eisen MB (2008) Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. Proc Natl Acad Sci USA 105:17034–17039

Schüßler A, Martin H, Cohen D, Fitz M, Wipf D (2006) Characterization of a carbohydrate transporter from symbiotic glomeromycotan fungi. Nature 444:933–936

Serfling A, Wohlrab J, Deising H (2007) Treatment of a clinically relevant plant-pathogenic fungus with an agricultural azole causes cross-resistance to medical azoles and potentiates caspofungin efficacy. Antimicrob Agents Chemother 51(10):' 3672–3676

Shetty NP, Jensen JD, Knudsen A, Finnie C, Geshi N, Blennow A, Collinge DB, Jørgensen HJ (2009) Effects of beta-1,3-glucan from *Septoria tritici* on structural defence responses in wheat. J Exp Bot 60 (15):4287–4300

Shimizu T, Nakano T, Takamizawa D, Desaki Y, Ishii-Minami N, Nishizawa Y, Minami E, Okada K, Yamane H, Kaku H, Shibuya N (2010) Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. Plant J 64:204–214

Shrestha B, Vertes A (2009) In situ metabolic profiling of single cells by laser ablation electrospray ionization mass spectrometry. Anal Chem 81: 8265–8271

Snoeijers SS, Pérez-García A, Joosten MHAJ, de Wit PJGM (2000) The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. Eur J Plant Pathol 106:493–506

Sonnewald U (2011) SWEETS – The missing sugar efflux carriers. Front Plant Sci 2, article 7

Takahara H, Dolf A, Endl E, O'Connell R (2009) Flow cytometric purification of *Colletotrichum higginsianum* biotrophic hyphae from Arabidopsis leaves for stage-specific transcriptome analysis. Plant J 59:672–683

Tang W, Coughlan S, Crane E, Beatty M, Duvick J (2006) The application of laser microdissection to *in planta* gene expression profiling of the maize anthracnose stalk rot fungus *Colletotrichum graminicola*. Mol Plant Microbe Interact 19:1240–1250

Taylor TN, Remy W, Hass H (1992) Parasitism in a 400-million-year-old green alga. Nature 357:493–494

Taylor TN, Remy W, Hass H, Kerp H (1995) Fossil arbuscular-mycorrhiza from the Early Devonian. Mycologia 87:560–573

Thomma BPHJ, Bolton MD, Clergeot PH, de Wit PJGM (2006) Nitrogen controls in planta expression

of *Cladosporium fulvum* Avr9 but no other effector genes. Mol Plant Pathol 7:125–130

Thon MR, Nuckles EM, Takach JE, Vaillancourt LJ (2002) CPR1: a gene encoding a putative signal peptidase that functions in pathogenicity of *Colletotrichum graminicola* to maize. Mol Plant Microbe Interact 15:120–128

van den Ackerveken GFJM, Dunn RM, Cozijnsen AJ, Vossen JPMJ, van den Broek HWJ, de Wit PJGM (1994) Nitrogen limitation induces expression of the avirulence gene Avr9 in the tomato pathogen *Cladosporium fulvum*. Mol Gen Genet 243:277–285

Vander P, Varum KM, Domard A, El-Gueddari NE, Moerschbacher BM (1998) Comparison of the ability of partially *N*acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. Plant Physiol 118:1353–1359

Vargas WA, Sanz Martín JM, Rech GE, Rivera LP, Benito EP, Díaz-Mínguez JM, Thon MR, Sukno SA (2012) Plant defense mechanisms are activated during biotrophic and necrotrophic development of *Colletotricum graminicola* in maize. Plant Physiol 158:1342–1358

Vleeshouwers VG, Raffaele S, Vossen JH, Champouret N, Oliva R, Segretin ME, Rietman H, Cano LM, Lokossou A, Kessel G, Pel MA, Kamoun S (2011) Understanding and exploiting late blight resistance in the age of effectors. Annu Rev Phytopathol 49:507–531

Voegele RT, Struck C, Hahn M, Mendgen K (2001) The role of haustoria in sugar supply during infection of broad bean by the rust fungus *Uromyces fabae*. Proc Natl Acad Sci USA 98:8133–8138

Voll LM, Horst RJ, Voitsik A-M, Zajic D, Samans B, Pons-Kühnemann J, Doehlemann G, Münch S, Wahl R, Molitor A, Hofmann J, Schmiedl A, Waller F, Deising HB, Kahmann R, Kämper J, Kogel K-H, Sonnewald U (2011) Common motifs in the response of cereal primary metabolism to fungal pathogens are not based on similar transcriptional reprogramming. Front Plant Sci 2:39

Wahl R, Wippel K, Goos S, Kämper J, Sauer N (2010) A novel high-affinity sucrose transporter is required for virulence of the plant pathogen *Ustilago maydis*. PLoS Biol 8(2):e1000303

Walton JD (1994) Deconstructing the cell wall. Plant Physiol 104:1113–1118

Walton JD (1996) Host-selective toxins: agents of compatibility. Plant Cell 8:1723–1733

Wattad C, Dinoor A, Prusky D (1994) Purification of pectate lyase produced by *Colletotrichum gloeosporioides* and its inhibition by epicatechin: a possible factor involved in the resistance of unripe avocado fruits to anthracnose. Mol Plant Microbe Interact 7:293–297

Wei Y, Shen W, Dauk M, Wang F, Selvaraj G, Zou J (2004) Targeted gene disruption of glycerol-3-phosphate dehydrogenase in *Colletotrichum gloeosporioides* reveals evidence that glycerol is a significant transferred nutrient from host plant to fungal pathogen. J Biol Chem 279:429–435

Weiergang I, Dunkle LD, Wood KV, Nicholson RL (1996) Morphogenic regulation of pathotoxin synthesis in *Cochliobolus carbonum*. Fungal Genet Biol 20:74–78

Werner S, Sugui JA, Steinberg G, Deising HB (2007) A chitin synthase with a myosin-like motor domain is essential for hyphal growth, appressorium differentiation and pathogenicity of the maize anthracnose fungus *Colletotrichum graminicola*. Mol Plant Microbe Interact 20:1555–1567

Wessels JGH (1996) Fungal hydrophobins: proteins that function at an interface. Trends Plant Sci 1:9–15

Wharton PS, Julian AM, O'Connell RJ (2001) Ultrastructure of the infection of *Sorghum bicolor* by *Colletotrichum sublineolum*. Phytopathology 91:148–158

Whisson SC, Boevink PC, Moleleki L, Avrova AO, Morales JG, Gilroy EM, Armstrong MR, Grouffaud S, van West P, Chapman S et al (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450:115–118

Yaeno T, Li H, Chaparro-Garcia A, Schornack S, Koshiba S, Watanabe S et al (2011) Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. Proc Natl Acad Sci USA 108:14682–14687

Yakoby N, Kobiler I, Dinoor A, Prusky D (2000) pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. Appl Environ Microbiol 66: 1026–1030

Yakoby N, Beno-Moualem D, Keen NT, Dinoor A, Pines O, Prusky D (2001) *Colletotrichum gloeosporioides pel*B is an important virulence factor in avocado fruit–fungus interaction. Mol Plant Microbe Interact 14:988–995

14 Pectin as a Barrier and Nutrient Source for Fungal Plant Pathogens

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CONTENTS

I. Introduction

Many fungi feed in or on plant tissues, either as **saprotrophs**, endophytes, symbionts, or pathogens. **Saprotrophs** grow on dead plant tissue, and participate in its biological decomposition and recycling. The other three types of fungi, however, proliferate in or on living plants, and often have intricate interactions with their host. These fungi must, in many cases, actively pass the plant surface through the cuticle and/or the **cell wall**, which collectively form a physical and chemical **barrier** between the environment and the internal tissues of the plant. **Cell walls** not only provide plant tissue strength and structure, but also protect against microbial invasion. Plants therefore invest substantial resources in constructing the **cell wall** and maintaining its integrity. **Cell-wall** material makes up 50–80 % of the total plant dry weight, and the vast majority of **cell-wall** polymers consist of carbohydrates. The large amount of carbon deposited in **cell walls**, on the other hand, offers opportunities for fungi to utilize **plant cell walls** as a **nutrient** resource. Regardless of their trophic lifestyle in an ecosystem (**saprotrophic**, endophytic, symbiotic, or pathogenic), many fungi indeed have the genetic potential to grow on **plant cell wall** carbohydrates as a **saprotroph** (Aro et al. 2005). There are only very few fungi which are known to have an extremely small number of genes encoding carbohydrate-degrading enzymes, including the **biotroph** *Ustilago maydis* and the obligate powdery mildew pathogen *Blumeria graminis* (Kämper et al. 2006; Spanu et al. 2010).

This chapter discusses the opposite roles of **plant cell walls**, both as a **barrier** for penetration and as a **nutrient** source for invading fungi. We will discuss the chemical structures of plant cell wall polysaccharides, the cell-wall-associated resistance mechanisms that plants display against pathogens, and the microbial enzymes that are involved in cell-wall decomposition. We

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will then focus on the **plant cell-wall-degrading enzymes** of pathogenic fungi, and illustrate with case studies how the grey mould ***Botrytis cinerea*** decomposes **pectins** deposited in **plant cell walls**, and utilizes their breakdown products as **nutrients**.

II. Structure of Plant Cell Walls

Plant **cell walls** are highly dynamic in chemistry and architecture. Their structure and composition vary between plant species, and depend on the type of cell they surround, the stage of differentiation of the cell, and the developmental stage of the plant itself. Plant **cell walls** consist mainly of polysaccharides which, together with lignin and proteins, form a complex three-dimensional network. The main components of plant **cell wall** polysaccharides are cellulose, hemicellulose, and **pectin**. **Cellulose** accounts for 20–30 % of the dry mass of most primary **cell walls**. It consists of β-1,4-linked D-glucose residues that form unbranched polymeric chains, which are associated by strong hydrogen bonds into crystalline cellulose microfibrils (Nishiyama et al. 2002). Cellulose microfibrils interact with hemicelluloses by hydrogen bonds and the cellulose–hemicellulose complex is physically entangled with **pectins** (Cosgrove 2001). Both hemicellulose and **pectin** are branched polysaccharides of varying composition.

Hemicelluloses are relatively complex polysaccharides, which have β-1,4-linked backbones with an equatorial configuration, including xyloglucans, xylans, mannans, and glucomannans, and β-(1,3;1,4)-glucans. Xyloglucan is present both in dicot and monocot **cell walls**, but it is more abundant in the walls of dicots (~20 %) than in those of monocots (~2 %). Xyloglucan consists of β-1,4-linked D-glucose residues, where D-xylose is α-1,6-linked to D-glucose chains and can be substituted at O-2 with β-D-galactose or α-L-arabinose. Xylans constitute the major hemicellulose in the primary **cell walls** of monocots. They are a diverse group of polysaccharides with a backbone of β-1,4-linked xylose residues, which can be substituted with α-1,2-linked glucuronosyl and 4-O-methyl glucuronosyl residues. Acetylation of xylose residues may occur at the O-2 and/or O-3 positions. The backbone of mannans consists of β-1,4-linked D-mannose residues, whereas the backbone of glucomannans consists of glucose and mannose in a non-alternating pattern. β-1,3;1,4-glucans consist of β-1,4-glucans with interspersed single β-1,3-linkages. (Caffall and Mohnen 2009; Scheller and Ulvskov 2010)

Pectins are the structurally most complex polysaccharides in nature. **Pectin** is the collective name for a series of polymers that are rich in **D-galacturonic acid**, including homogalacturonan (HG), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), and xylogalacturonan (XGA) (Fig. 14.1a). The most abundant type of **pectin** is HG, which comprises over 60 % of total **pectin** in plant **cell walls**. HG is a linear polymer of α-1,4-linked **D-galacturonic acid**, which can be modified to different degrees by methyl-esterification at the C-6 carboxyl group and acetylation at O-2 or O-3. RGI, comprising 20–35 % of **pectin**, has a different backbone, which consists of repeating units of α-1,4-D-galacturonic acid-α-1,2-L-rhamnose. The L-rhamnose residues in the backbone can be modified with side chains consisting of β-1,4-galactan, branched arabinan, and/or arabinogalactan. The structure of the side chains of RGI greatly varies among plants. RGII consists of an HG backbone, which can be substituted at O-2 or O-3 with different side chains. These side chains are composed of 12 different types of sugars in over 20 different linkages. Although RGII is the most structurally complex **pectin**, its structure is remarkably conserved among vascular plants. XGA also consists of an HG backbone, which can be substituted at O-3 with β-1,4-linked xylose residues (Mohnen 2008; Caffall and Mohnen 2009).

Currently, there are two models that describe how pectic polysaccharides are linked: the 'smooth and hairy region' model and the 'rhamnogalacturonan backbone' model (Fig. 14.1b). In the first model, **pectin** is composed of hairy regions, consisting of RGI decorated with neutral sugar side chains, which are interspersed with smooth regions of HG. The second model describes HG as a side chain of RGI, similar to the neutral sugar side chains (Schols et al. 2009).

III. Cell Wall-Associated Resistance to Plant Pathogens

Plants protect their **cell walls** from penetration by pathogens in several ways: (i) inhibition of **plant**

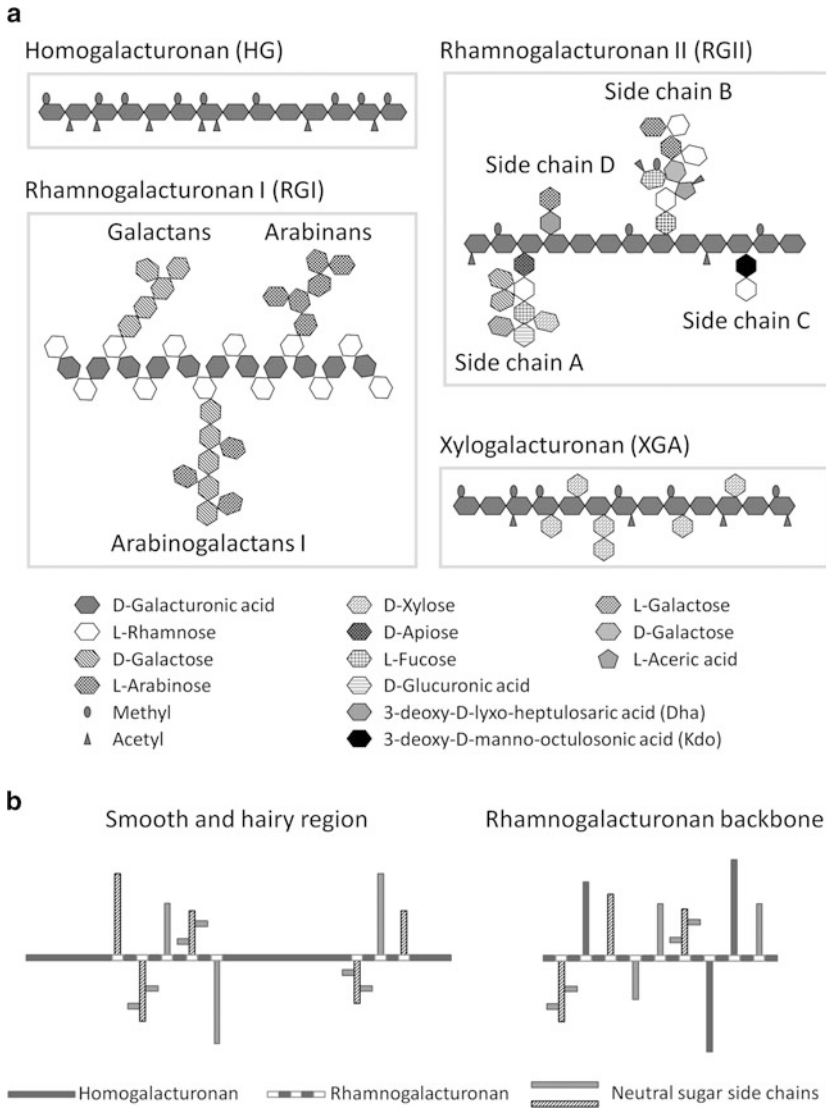

Fig. 14.1. Schematic structure of pectin components in plant cell walls (**a**) (Adapted from Mohnen (2008)); and alternative models for the organization of the pectin network (**b**) (Adapted from Schols et al. (2009))

cell-wall-degrading enzymes, (ii) remodelling of **cell walls** at the site of attempted penetration, or (iii) perception of **cell-wall**-derived molecules (damage-associated molecular patterns) followed by triggering of an immune response. We will focus on the first two aspects, and consider the third aspect to be beyond the scope of this chapter.

A. Inhibition of Cell-Wall-Degrading Enzymes

Polygalacturonase-inhibiting proteins (PGIPs) are extracellular leucine-rich repeat (eLRR) proteins that physically interact with, and thereby inhibit, polygalacturonases (PGs) produced by fungi, bacteria, and even insects (De Lorenzo et al.

2001; Federici et al. 2006; Juge 2006). Secreted PGs are important virulence factors in several fungal pathogens, including *Aspergillus flavus* (Shieh et al. 1997), ***Botrytis cinerea*** (ten Have et al. 1998), *Alternaria citri* (Isshiki et al. 2001), and *Claviceps purpurea* (Oeser et al. 2002). Fungal PGs are able to decompose **pectin** and thus cause cell-wall degradation and tissue maceration (see below). PGIPs are widely distributed in the plant kingdom, in plants such as apple, bean, grape, pepper, raspberry, soybean, tomato, leek, and *Arabidopsis thaliana* (De Lorenzo et al. 2001). The potential of PGIPs to limit host tissue colonization by fungi has been shown using overexpression and gene silencing. Specifically, the overexpression of a pear *PGIP* in tomato or grape plants, of bean *PvPGIP2* in tobacco, and of *AtPGIP1* and *AtPGIP2* in *A. thaliana* reduced ***B. cinerea*** infection (Powell et al. 2000; Ferrari et al. 2003; Aguero et al. 2005; Manfredini et al. 2005). *A. thaliana* plants in which the *AtPGIP1* gene was silenced showed reduced PGIP accumulation and enhanced susceptibility to ***B. cinerea*** (Ferrari et al. 2006). Furthermore, overexpression of *Phaseolus vulgaris PvPGIP2* in wheat reduced the symptoms caused by *Fusarium moniliforme* and *Bipolaris sorokiniana* infection (Janni et al. 2008).

PGIPs are encoded by small gene families. The isoforms within a single plant species may exhibit differential in-vitro inhibitory activities towards PGs from different fungi. The activity of several PGIP isoforms from bean and *Arabidopsis* was tested in vitro towards PGs from saprotrophic and plant pathogenic fungi (Ferrari et al. 2003), leading to hypotheses about differential inhibition and specificity in PG–PGIP interactions (Federici et al. 2006). It should, however, be noted that the in-vitro interaction between PGs and PGIPs may not be representative for the situation in planta. Joubert et al. (2007) reported that the grapevine protein VvPGIP1 was able to inhibit the activity of ***B. cinerea*** BcPG2 in planta, even though the two proteins were not able to interact in vitro. Thus the failure of a given PGIP to inhibit a particular PG in vitro may not be informative about the potential of this PGIP to interact with the PG in planta and thereby confer (partial) disease resistance.

In addition to inhibitors of PGs, plants can also produce inhibitors of pectin methylesterases (PMEIs). The inhibition of PME activity results in a markedly reduced demethylation of pectin, which impacts on cell-wall properties and tissue texture (Jolie et al. 2010). Overexpression of a PMEI gene from kiwifruit in wheat was shown to increase resistance to fungal pathogens (Volpi et al. 2011). There is, however, an important conceptual difference between PGIPs and PMEIs. PGIPs only inhibit PGs of microbes and insects that attack the plant, but fail to inhibit endogenous plant PGs. On the contrary, PMEIs are active only against endogenous plant PMEs, and presumably inactive against non-plant PMEs (Jolie et al. 2010).

B. Remodelling of Cell Walls at the Site of Attempted Penetration

Callose, the major constituent of papillae, is an important factor contributing to the resistance of plants against penetration and invasion by pathogenic fungi. Callose is present at low levels throughout a plant, especially in the sieve plates of phloem cells and in plasmodesmata. In response to biotic stress, plant cells rapidly synthesize callose in the vicinity of the site of pathogen penetration (Jacobs et al. 2003; Nishimura et al. 2003; Adie et al. 2007; Flors et al. 2008; Garcia-Andrade et al. 2011). The *A. thaliana* callose synthase GSL5/PMR4 is required for pathogen-induced callose deposition (Jacobs et al. 2003; Nishimura et al. 2003). *gsl5/pmr4* mutant plants lack callose and show enhanced susceptibility to the **necrotrophic** pathogens *Alternaria brassicicola*, *Plectosphaerella cucumerina* and *Pythium irregulare* (Adie et al. 2007; Flors et al. 2008; Garcia-Andrade et al. 2011). By contrast, *gsl5/pmr4* mutant plants show increased resistance to **biotrophic** pathogens *Erysiphe cichoracearum*, *Golovinomyces orontii* and *Hyaloperonospora parasitica*, because of a hyperstimulation of salicylic acid-dependent defense pathways, which remains to be understood (Jacobs et al. 2003; Nishimura et al. 2003).

Reactive oxygen species (ROS) are well-known as signal molecules triggering plant defense response (Lamb and Dixon 1997). H_2O_2 is required for peroxidase-dependent lignification and for protein cross-linking in the **cell wall** (Hückelhoven 2007). Rapid oxidative cross-linking of proline-rich proteins in the **cell wall** strengthens the wall, and thereby makes it more resistant to **cell-wall-degrading enzymes** (Bradley et al. 1992; Brisson et al. 1994).

IV. Plant Cell Wall Polysaccharide Degradation

The plant cuticle and **cell wall** are the first **barriers** to pathogen invasion. Fungal plant pathogens secrete a series of enzymes to decompose **plant cell wall** polysaccharides in order to facilitate the penetration, the subsequent maceration, and the acquisition of carbon from decomposed plant tissues. Generally, these polysaccharide-degrading enzymes can be divided into two classes: exo-acting enzymes and endo-acting enzymes. Exo-acting enzymes can be specific for the reducing end or the non-reducing end of polysaccharides. They release monomeric or dimeric glycosyl moieties during each catalytic event, providing the fungus with low molecular mass compounds that can be easily taken up. Endo-acting enzymes cleave polysaccharides randomly within the chain, resulting in a rapid decrease in the average chain length. The cleavage products, however, are generally too large to serve as **nutrients** for the fungus. It is commonly observed that any particular polysaccharide is degraded by a combination of endo-acting and exo-acting enzymes, acting synergistically on the substrate. The plant cell-wall-degrading enzymes that are secreted by fungi are all **carbohydrate-active enzymes** (CAZymes, www.cazy.org, Cantarel et al. 2009). Fungal cellulases, hemicellulases, and pectinases can be assigned to CAZy families of glycoside hydrolases (GH), carbohydrate esterases (CE), and polysaccharide lyases (PL).

A. Cellulose Degradation

Three classes of enzymes, all belonging to GH families 6 and 7, are involved in cellulose degradation: β-1,4-endoglucanases, cellobiohydrolases, and β-glucosidases. The β-1,4-endoglucanases hydrolyse the internal bonds to disrupt the crystalline cellulose microfibrils and expose individual cellulose polysaccharide chains. Cellobiohydrolases cleave two glucose units from the ends of the exposed chains, resulting in the release of the disaccharide cellobiose, which is subsequently hydrolysed by β-glucosidases into individual D-glucose monomers.

B. Hemicellulose Degradation

Hemicellulose consists of a group of relatively complex branched polysaccharides. The various backbones of hemicelluloses are hydrolysed by the corresponding set of GH family enzymes. Specifically, xyloglucan is decomposed by a combination of β-1,4-endoglucanases and β-glucosidases, xylan is decomposed by a combination of β-1,4-endoxylanases and β-xylosidases, and, mannan and galactomannan are decomposed by a combination of β-1,4-endomannanases and β-mannosidases. Various side chains of hemicelluloses are cleaved by different enzymes, which belong to GH and CE families. For example, the α-linked D-xylose, D-galactose, D-glucuronic acid and L-arabinose residues are cleaved by α-xylosidases, α-galactosidases, α-glucuronidases, or α-arabinofuranosidases, respectively, whereas acetyl residues are cleaved by acetyl xylan esterases.

C. Pectin Degradation

Two types of backbones are present in **pectin**: the backbone of HG (smooth region) consisting of α-1,4-linked **D-galacturonic acid**, and the backbone of RGI (hairy region) consisting of alternating α-1,4 linked **D-galacturonic acid** and α-1,2-linked rhamnose residues. Enzymes involved in degradation of the **pectin** backbone belong to the GH and PL families. Smooth regions can be hydrolysed by **endo-polygalacturonases (endo-PGs)**, exo-polygalacturonases (exo-PGs), pectin lyases, and pectate lyases. Endo-PGs hydrolyse the (preferentially unmethylated) backbone of HG, releasing monomeric and/or oligomeric galacturonosyl fragments, whereas exo-PGs exclusively cleave at the non-reducing end of HG strands, thereby releasing D-galacturonic acid monomers. Pectin lyases and pectate lyases both cleave alternating α-1,4 linked **D-galacturonic acid** linkages via β-elimination, resulting in a novel reducing end. Pectin lyases prefer substrates with a high degree of methylesterification, whereas pectate lyases prefer substrates with a low degree of methylesterification, and require Ca^{2+}-ions for catalysis. The hairy regions of RGI can be hydrolysed by rhamnogalacturonan hydrolases and rhamnogalacturonan lyases, of which the former enzymes specifically cleave non-esterified galacturonosyl-rhamnosyl linkages.

Various substituents occur on the backbone of **pectins**: therefore, diverse enzymes are involved in **pectin** side chain decomposition. Some of these enzymes cleave the entire side chains from the backbone, whereas others cleave the internal or terminal linkages of side chains. In addition, some of the enzymes not only act on **pectin** side chains, but also on hemicelluloses. Specifically, α-arabinofuranosidases release L-arabinose, from both xylan and arabinoxylan, and from side chains of rhamnogalacturonan; endo/exo-arabinanases hydrolyse α-1,5 linked arabinose residues from the arabinan side chains of **pectins**; β-galactosidases release terminal D-galactose residues from the galactan side chains of **pectins**; and β-xylosidases can hydrolyse β-1,4-linked xylose residues, both from xyloglucan and from side chains of xylogalacturonan. Finally, pectin acetylesterases and pectin methylesterases remove the acetyl and methyl residues which are present in the smooth regions of **pectins**.

V. Cell-Wall-Degrading Enzymes in Plant Pathogenic Fungi: A Case Study of *Botrytis cinerea*

A. CAZymes in Genomes of Plant Pathogenic Fungi

The number of genome sequences of fungi that have been released has rapidly grown in recent years (Martin et al. 2011). This provides opportunities to examine the repertoire of plant cell-wall-degrading enzymes secreted by plant pathogenic fungi and to explore correlations between the **CAZyme** content in the genome, the **CAZyme** distribution over different enzyme families (GH, CE, PL), and the host range of the fungal pathogen.

Plant pathogens with different lifestyles appear to have different repertoires of the **CAZymes** that are involved in degrading plant cell walls. **Necrotrophs** and **hemi-biotrophs** (in the necrotrophic infection phase) secrete large amounts of **cell-wall-degrading enzymes** for host tissue decomposition and nutrient acquisition. The genomes of two closely related necrotrophs, ***Botrytis cinerea*** and *Sclerotinia sclerotiorum*, encode respectively 118 and 106 **CAZymes** associated with **plant cell wall** degradation (Amselem et al. 2011). These numbers are very similar to that in the **saprotroph** *Aspergillus niger*, but are lower than in other **necrotrophs** (*Phaeosphaeria nodorum* and *Pyrenophora teres f. teres*) and in the **hemi-biotrophs** *Fusarium graminearum* and *Magnaporthe oryzae* (Amselem et al. 2011). In contrast, many **biotrophic** pathogens and symbionts have a markedly lower content of **CAZymes** for **cell-wall** degradation in their genome (e.g., Baxter et al. 2010; Martin et al. 2010; Duplessis et al. 2011), presumably to reduce the damage to the host and avoid the plant defense responses triggered by the release of **cell-wall** fragments. The most extreme examples to date are the genomes of *Blumeria graminis* and *Ustilago maydis*, which contain only 10 and 33 genes encoding **plant cell-wall-degrading enzymes** (Kämper et al. 2006; Spanu et al. 2010).

Plant pathogens with distinct host preference seem to use different approaches to decompose plant tissues. **CAZyme** analyses show that not only the contents, but also the distribution of **CAZymes** differ among plant pathogens. ***B. cinerea*** and *S. sclerotiorum* can both infect a wide range of dicot host plants, and prefer to infect soft plant tissues that are rich in **pectin**, such as flowers and fruits. This is reflected by the observation that both fungi grow better (in vitro) on pectic substrates than on xylan and **cellulose**. Their genomes contain larger proportions of **CAZymes** involved in decomposition of **pectin** (37 % and 31 %) and lower amounts of **CAZymes** involved in decomposition of cellulose (18 % and 20 %) and hemicellulose (40 % and 41 %). In contrast, *P. nodorum*, *P. teres f. teres*, and *M. oryzae* are pathogens of wheat, barley, and rice, all of which belong to commelinoid monocots that contain less **pectin** in the cell wall. **CAZyme** analyses show that their genomes contain smaller proportions of **CAZymes** involved in decomposition of **pectin** (18 %, 17 %, and 12 %) and noticeably higher amounts of CAZymes involved in decomposition of cellulose (47 %, 38 %, and 37 %) and hemicellulose (66 %, 55 %, and 68 %), as compared to *B. cinerea* and *S. sclerotiorum* (Amselem et al. 2011).

B. Secretomes of Plant Pathogenic Fungi

Releasing **nutrients** from plant cell wall polysaccharides requires the secretion of an arsenal of plant **cell-wall-degrading enzymes** that act in synergy to decompose this complex structure. In culture filtrates of *Fusarium graminearum*, grown in medium containing hop **cell wall** material as sole carbon source, Phalip et al. (2009) detected 17 different GH activities, which could collectively hydrolyse crude plant material, with monosaccharide yields approaching 50 % of the total sugars released by acid hydrolysis.

Proteomics techniques are increasingly applied to study the **secretomes** of fungal pathogens, either in vitro, or during their interaction with plants. Besides being useful for annotating genomes, **secretome** analyses may enable identification of pathogen effectors (reviewed by Koeck et al. 2011) as well as (abundant) **plant cell-wall-degrading enzymes**. The latter information may provide leads to unravel the mechanisms that fungal pathogens utilize to decompose **plant cell**

wall polysaccharides and acquire **nutrients** from their host. Several studies, including those in ***B. cinerea*** discussed below in detail, have revealed that **plant cell-wall-degrading enzymes** are abundant in fungal **secretomes**. In *S. sclerotiorum* culture filtrates, 18 secreted proteins were identified, and nine of them were **plant cell-wall-degrading enzymes** (Yajima and Kav 2006). Studies on *F. graminearum* grown in medium containing various polysaccharide supplements, or in medium containing wheat or barley flour, resulted in the identification of 120 and 69 proteins, in which glycoside hydrolases represented approximately 25 % of all identified proteins (Paper et al. 2007; Yang et al. 2012). In *M. oryzae*, 85 proteins were identified, and 19 of them were annotated as plant cell wall hydrolases (Wang et al. 2011).

The **secretome** of ***B. cinerea*** has been analyzed in different culture conditions (Shah et al. 2009a, b; Espino et al. 2010; Fernandez-Acero et al. 2010). One study aimed to compare proteins secreted upon culturing ***B. cinerea*** in presence of extracts of red tomato, ripe strawberry, and *Arabidopsis* leaves. Overall, 89 ***B. cinerea*** proteins were identified by LC-MS/MS, of which 60 contained a signal peptide in the (predicted) protein sequence. 30 of these 60 proteins are involved in carbohydrate metabolism and transport, and these proteins were more abundant in cultures grown in the presence of tomato and strawberry extract, as compared to cultures containing *Arabidopsis* leaf extract (Shah et al. 2009b). The second study aimed to compare ***B. cinerea*** proteins induced by **pectins** with different degrees of methyl esterification. A total of 126 secreted proteins were identified in cultures containing highly or partially esterified **pectin**, or sucrose. The abundance of proteins with functions in **pectin** degradation was similar in both **pectin**-containing media, but higher than in sucrose-containing medium (Shah et al. 2009a). In a similar study (Fernandez-Acero et al. 2010), proteins were sampled from ***B. cinerea*** cultures grown in the presence of either glucose, starch, cellulose, **pectin**, or tomato cell walls and submitted to two-dimensional gel electrophoresis. Fifty-seven unique proteins were identified, of which more than 50 % are involved in plant cell wall polysaccharide decomposition (Fernandez-Acero et al. 2010). Finally, Espino et al. (2010) focused on the early **secretome** of ***B. cinerea***, because the early stages of development in planta are crucial in the establishment of a successful infection. Conidia were inoculated in minimal medium, supplemented with extracts of tomato, strawberry, or kiwifruit, and proteins were sampled after 16 h. A total of 105 proteins were identified, of which 36 are involved in **plant cell wall** polysaccharide degradation; proteins involved in **pectin** degradation were highly abundant (Espino et al. 2010). The lists of proteins identified in these studies show substantial overlap, as the methodology used was often comparable. Other culture methods, more sensitive protein identification methods, and more reliable gene models will be required to generate a more comprehensive list of proteins identified as being secreted by ***B. cinerea***.

VI. The Contribution of Cell-Wall-Degrading Enzymes to Virulence of *Botrytis cinerea*

Botrytis cinerea is able to infect over 200 host plant species and different tissue types: stems, leaves, flowers, and fruit. The pathogen can cause a variety of symptoms ranging from dry, necrotic areas to water-soaked, fully macerated lesions. The ability to cause disease on such different tissues and plant species suggests that ***B. cinerea*** has a large weaponry to kill and invade its hosts (Choquer et al. 2007). The ultimate purpose of a **necrotrophic** pathogen is not to kill its host, but to decompose the plant tissue and utilize host-derived **nutrients** for its own growth. As discussed above, ***B. cinerea*** secretes a spectrum of cell-wall-decomposing enzymes (including pectinases, cellulases, and hemicellulases) to facilitate plant tissue colonization and release carbohydrates for consumption (van Kan 2006;

Williamson et al. 2007). We will here discuss the relevance of various types of enzymes for virulence of *B. cinerea*, as studied by functional analyses using targeted knockout mutants.

A. Pectinases

B. cinerea often penetrates host leaf tissue at the anticlinal cell wall, and subsequently grows into and through the middle lamella, which consists mostly of low-methylesterified **pectin**. Effective **pectin** degradation thus is important for virulence of ***B. cinerea***. Several **pectinases** have been found to be abundant throughout the infection process, including pectin and pectate lyases, pectin methylesterases (PMEs), exo-polygalacturonases (exo-PGs), and **endo-polygalacturonases** (endo-PGs) (Rha et al. 2001; ten Have et al. 2001; Cabanne and Doneche 2002; Kars and van Kan 2004; Kars et al. 2005b). In particular, the roles of endo-PGs and PMEs in virulence have been intensively investigated.

1. Endo-polygalacturonases

The ***B. cinerea*** genome contains six genes encoding endo-PGs (Wubben et al. 1999). All gene family members are differentially expressed in vitro and in planta (Wubben et al. 2000; ten Have et al. 2001). Four regulatory mechanisms were proposed based on in vitro analysis: basal, constitutive expression was observed for *Bcpg*1, expression of *Bcpg*3 was induced by low ambient pH, irrespective of the carbon source present, and expression of *Bcpg*4 and *Bcpg*6 was induced by **D-galacturonic acid**; catabolite repression by glucose was observed for *Bcpg*4 only. Other monosaccharides present in cell-wall polymers, such as rhamnose, arabinose, and galactose, did not notably regulate the expression of *Bcpg* genes. Regulation of the expression of *Bcpg*2 and *Bcpg*5 remained unclear (Wubben et al. 2000).

Altogether, this endo-PG gene family equips the fungus with a flexible **pectin**-degrading machinery, which provides a potential advantage for a fungus with such a broad range of hosts and tissue types. All gene family members display various expression patterns during infection, depending on the stage of infection and on the host (tomato, broad bean, tobacco, and *Arabidopsis thaliana* leaves; apple and courgette fruits) (ten Have et al. 2001; Zhang and van Kan, unpublished). *Bcpg*1 is expressed in all tissues tested, although differences in transcript levels occur. *Bcpg*2 is expressed early in the infection of tomato, broad bean, and courgette, but not in tobacco, *A. thaliana*, and apple. *Bcpg*3 and *Bcpg*5 are mainly expressed in apple fruit tissue, in agreement with the in-vitro inducibility of *Bcpg*3 at low pH and of *Bcpg*5 by apple **pectin**. *Bcpg*4 and *Bcpg*6 are mostly expressed in late stages of infection, when extensive tissue maceration occurs, in agreement with in-vitro inducibility of *Bcpg*4 and *Bcpg*6 by **D-galacturonic acid**.

The endo-PG family members not only display diversity in their expression patterns but also in enzymatic characteristics. Five BcPGs were produced in *Pichia pastoris*, and their biochemical properties were analysed (Kars et al. 2005b). All enzymes display optimal activity at low ambient pH, which is consistent with the acidification of the environment during the early stages of colonization by ***B. cinerea*** (Verhoeff et al. 1988; Billon-Grand et al. 2012). BcPG1, BcPG2 and BcPG4 prefer the non-methylesterified substrate polygalacturonic acid (PGA) to **pectin**; however, they show differences in substrate affinities and hydrolysis rates. BcPG3 and BcPG6 have been shown to behave as processive endo-hydrolases, releasing monomers of D-galacturonate instead of oligomers (Kars et al. 2005b).

The function of endo-PG gene family members in virulence has been studied by deleting each single gene in ***B. cinerea***. BcPG1 seems to be universally required for full virulence. Knockout mutants in the *Bcpg*1 gene were reduced in virulence on tomato leaves, tomato fruit, and apple fruit (ten Have et al. 1998), as well as on leaves of broad bean (ten Have and van Kan, unpublished), *Nicotiana benthamiana*, and *Arabidopsis thaliana* (Zhang and van Kan, unpublished). This can be explained by the constitutive expression of *Bcpg*1 in planta (ten Have et al. 2001), and the observation that the BcPG1 protein is abundant in ***B. cinerea*** **secretomes** in different media (Shah et al. 2009b; Espino et al. 2010). Mutants in the *Bcpg*2 gene were reduced in virulence on

tomato as well as broad bean leaves (Kars et al. 2005b), but not on *N. benthamiana* leaves and *A. thaliana* leaves (Zhang and van Kan, unpublished). This is consistent with the observation that expression of *Bcpg*2 can be detected in tomato leaves but not in *N. benthamiana* and *A. thaliana* leaves (see above). Individual knockout mutants in *Bcpg*3, *Bcpg*4, *Bcpg*5, and *Bcpg*6 have been generated; however, all of them displayed similar virulence to that of the wild-type strain on tomato, broad bean and *N. benthamiana* leaves (Joubert et al. 2007; Zhang and van Kan, unpublished). The targeted mutation studies have indicated that none of the single *Bcpg* genes is absolutely essential for penetration and host colonization. This is probably because of the overlapping activities of the individual enzymes, resulting in functional redundancy. Silencing or deletion of multiple *Bcpg* genes should be performed to investigate functional overlap among BcPG isozymes in more detail.

2. Pectin Methylesterases

The degree of methylation (DM) of **pectin** in **plant cell walls** can range from 13 % to approximately 80 % (Voragen et al. 1986). Pectin methylesterases (PMEs) catalyse the hydrolysis of methyl esters, releasing methanol and pectate. Pectate is a preferred substrate for many of the BcPGs (Kars et al. 2005a). This predicts that PMEs are important for virulence on plant tissues with high DM **pectin** (such as in leaves), but not on tissues with low DM pectin (such as in fruit). However, the role of PMEs in the virulence of ***B. cinerea*** is controversial. The phenotype of a *Bcpme*1 knockout mutant in one strain of ***B. cinerea*** supported this hypothesis (Valette-Collet et al. 2003). However, results in a different strain with single and double knockout mutants in two *Bcpme* genes, including the same *Bcpme*1 gene, did not support this hypothesis (Kars et al. 2005a). In addition, the *Bcpme* mutants and the wild-type strain displayed better growth on 75 % methylesterified pectin than on non-methylesterified polygalacturonic acid, suggesting that **pectin** demethylation by PMEs is not important for depolymerization in vivo (Kars et al. 2005a). The profuse growth of ***B. cinerea*** on high DM **pectin** suggests that the biochemical properties of endo-PGs determined in vitro may not reflect their behaviour in vivo, or that accessory enzymes participate in the **pectin** degradation mediated by endo-PGs.

B. Other Cell-Wall-Degrading Enzymes

Other cell-wall-degrading enzymes produced by *B. cinerea*, such as cellulases and hemicellulases, have also been studied. Deletion of a cellulase gene *Bccel*5A, encoding an endo-β-1,4-glucanase, did not affect virulence (Espino et al. 2005), whereas the deletion of a hemicellulase gene *Bcxyn*11A, encoding an endo- β-1,4-xylanase, delayed lesion formation and reduced lesion size by more than 70 % (Brito et al. 2006). The contribution of the *Bcxyn*11A gene in virulence was, however, not dependent on xylanase enzyme activity, but rather required the necrosis-inducing elicitor activity of the xylanase protein (Noda et al. 2010).

C. Role of the D-galacturonic Acid Catabolic Pathway in Virulence of *B. cinerea*

The monosaccharide **D-galacturonic** acid is the ultimate hydrolytic product released by the joint action of endo-PGs and exo-PGs. In view of the large amount of carbon deposited in host cell walls, and the high proportion of pectin in the wall, **D-galacturonic acid** may constitute an important carbon supply for nutrition of *B. cinerea* when it colonizes and grows inside a host. As in many other ascomycetes (Martens-Uzunova and Schaap 2008), the **D-galacturonic acid catabolic pathway** in *B. cinerea* consists of three catalytic steps converting **D-galacturonic acid** to pyruvate and L-glyceraldehyde. The pathway involves two non-homologous galacturonate reductase genes (*Bcgar*1 and *Bcgar*2), a galactonate dehydratase gene (*Bclgd*1), and a 2-keto-3-deoxy-L-galactonate aldolase gene (*Bclga*1) (Zhang et al. 2011). The transcript levels of all these genes were induced substantially when the fungus was cultured in media containing **D-galacturonic acid**, pectate, or **pectin** as the sole carbon source. Deletion of these four

genes in *B. cinerea*, either separately or in combinations, affected growth on **D-galacturonic** acid or pectic substrates (pectate, apple pectin, and citrus pectin) as the sole carbon source. The extent of growth reduction of the mutants on pectic substrates was correlated with the proportion of **D-galacturonic acid** content in the substrate. Growth of the mutants on apple pectin (containing only 61 % D-galacturonic acid) was better than on citrus pectin (containing 78 % **D-galacturonic acid**), while growth on sodium pectate (containing >99 % **D-galacturonic acid**) was negligible (Zhang et al. 2011).

The deletion of these four genes in ***B. cinerea*** did not affect virulence on tomato leaves, apples, and peppers (Zhang et al. 2011), but reduced virulence on *N. benthamiana* and *A. thaliana* leaves (Zhang and van Kan 2013). The extent of reduction in virulence of mutants in the **D-galacturonic acid catabolic pathway** was correlated with the content of **D-galacturonic acid** in the **cell wall** of the host plant tested. This suggested that **D-galacturonic acid** released from **pectin** in plant cell walls makes up an important part of the nutrition of ***B. cinerea***. However, more detailed studies revealed that the ***B. cinerea*** mutants were retarded in growth as a result of inhibitory activity by catabolic pathway intermediates that accumulate in the **D-galacturonic acid** catabolic mutants (Zhang and van Kan 2013). Collectively, the functional analyses on the *Bcpg* genes and the **D-galacturonic acid** catabolic pathway genes indicate that ***B. cinerea*** secretes endo-PGs primarily for the purpose of **pectin** decomposition, which facilitates the penetration and colonization of host tissues. The utilization of the **pectin** breakdown products contributes little to the increase in fungal growth and biomass production.

D. Transcription Factors Regulating Cell-Wall-Degrading Enzymes in *B. cinerea*

As outlined above, *B. cinerea* produces a spectrum of **cell-wall-degrading enzymes** for the decomposition of host cell wall polysaccharides and the consumption of the monosaccharides that are released. The full consumption of available carbon sources requires a continuous action of the appropriate depolymerizing enzymes, monosaccharide **transporters**, and catabolic enzymes. The expression of the genes encoding these enzymes is likely to be co-regulated. Indeed, several genes involved in **pectin** decomposition and **D-galacturonic acid** catabolism are inducible in vitro by **D-galacturonic acid** (Wubben et al. 2000; Zhang et al. 2011), as is the expression of a putative **D-galacturonic acid transporter** (Zhang and van Kan, unpublished). Furthermore, most of these genes are expressed at higher levels during infection in the stage of lesion expansion, when host tissue decomposition occurs at high rates (Wubben et al. 2000; Zhang and van Kan 2013). These observations are collectively indicative of a continuous release, uptake, and consumption of **D-galacturonic acid** during infection. The co-expression pattern of genes suggests the presence of common sequence elements in the promoter regions, which mediate their regulation by (a) common **transcription factor(s)**.

A conserved sequence motif is indeed present in the promoter of several pectinolytic genes in ***B. cinerea*** (Zhang and van Kan, unpublished). Experiments are in progress to characterize the regulatory elements and identify **D-galacturonic acid**-responsive **transcription factor(s)** that operate in regulating the pectinolytic complex of *B. cinerea*. Two potential mechanisms of gene activation may be considered (Fig. 14.2b, c). Firstly, the **transcription factor** regulating pectinolytic genes might be present in an inactive form in *B. cinerea* in the absence of D-galacturonic acid, either in the cytoplasm or in the nucleus. The extracellular decomposition of pectin and subsequent uptake of **D-galacturonic acid** monosaccharides into the fungal cytoplasm leads to elevated levels of **D-galacturonic acid** in the cytoplasm. The **D-galacturonic acid** might, directly or indirectly, activate the transcriptional regulator, leading to its ability to promote transcription of pectinolytic genes (Fig. 14.2b). An alternative mechanism that might be operating is that the **transcription factor** which regulates the co-expression of pectinolytic genes in *B. cinerea* is not expressed in the absence of D-galacturonic acid. The presence of **D-galacturonic acid**, either at the hyphal periphery or at low levels inside the cytoplasm might, through an unknown regulatory mechanism,

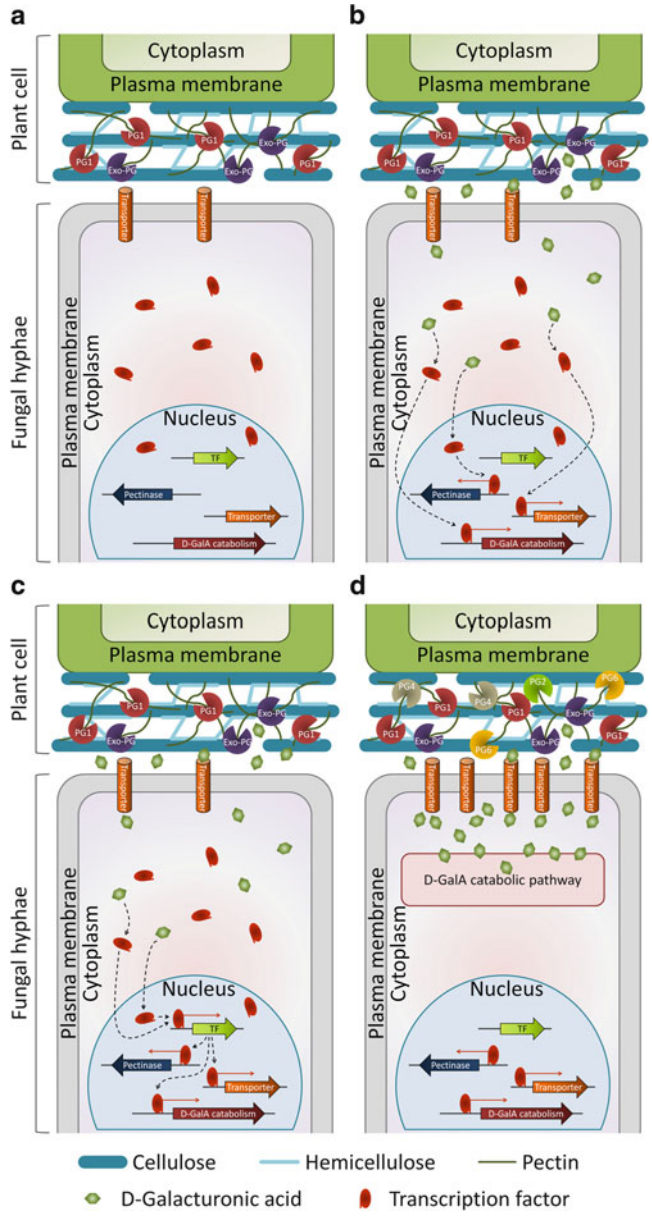

Fig. 14.2. Schematic illustration of pectin decomposition and D-galacturonic acid consumption in *B. cinerea*. Initial pectin decomposition by combinations of endo-PG (*PG1*) and exo-PG and uptake of D-galacturonic acid by plasma membrane-associated transporters (**a**). The elevated levels of D-galacturonic acid in the cytoplasm either directly activate the transcription factor(s) which could be present in the cytoplasm or the nucleus (**b**), or first induce the expression of the D-galacturonic acid-responsive transcription factor(s) (*TF*) (**c**). Subsequently, the activated transcription factor(s) co-regulate the expression of genes encoding additional pectinases (e.g. *PG2*, *PG4*, and *PG6*), additional transporter proteins, and D-galacturonic acid (*D-GalA*) catabolic enzymes (**d**)

lead to expression of the **D-galacturonic acid**-responsive **transcription factor(s)** in an active form (Fig. 14.2c). Both regulatory mechanisms are, at this point in time, hypothetical and will require further study.

VII. Conclusions and Perspectives

Plant **cell walls** are complex chemical structures. **Many fungi, including plant pathogens, have the genetic potential to decompose cell walls by means of combinations of enzymes (CAZymes)**. The degradation of plant **cell wall** polysaccharides provides monosaccharides that fungi can exploit as **nutrients** for growth. Genome sequences of plant pathogenic fungi will provide further insights into the cell-wall-decomposing potential of fungi. Some of these enzymes may have potential for industrial application, to decompose organic matter and release useful breakdown products. ***B. cinerea*** is not only a pathogenic fungus of great economic relevance (Dean et al. 2012), but it also serves as a good case to illustrate various aspects of plant **cell wall** decomposition by (pathogenic) fungi.

Figure 14.2 presents a schematic illustration of our current view of the different steps of pectin decomposition and monosaccharide consumption by *B. cinerea*. One endo-PG (BcPG1) and an (as yet uncharacterized) exo-PG are constitutively expressed and secreted, and they serve as pioneers to explore the environment (Fig. 14.2a). When a pectic substrate is present in the vicinity of hyphae, it is cleaved by the concerted action of the hydrolases. This will release monosaccharides that are taken up into the hyphae by a (possibly ligand-specific) monosaccharide **transporter** protein, expressed at low basal level. The accumulation of the monosaccharide in the fungal cytoplasm is evidence of the presence of the substrate, and it acts as a signal to boost the decomposing machinery for this particular polysaccharide. The presence of the monosaccharide activates a transcriptional regulator (Fig. 14.2b, c), which induces the coordinated transcription of three distinct groups of genes, encoding additional secreted depolymerizing enzymes, additional **transporter** proteins and monosaccharide catabolic enzymes, which collectively facilitate the complete degradation and consumption of pectin (Fig. 14.2d).

We propose that many conceptual features and steps that are illustrated here for decomposition of **pectin** by ***B. cinerea*** (the degradation by combinations of endo- and exo-hydrolases, uptake of monosaccharides by plasmamembrane **transporters**, the entry of the monosaccharide into the catabolic pathway, the co-regulation of genes involved in degradation of one particular type of cell-wall polysaccharide) may also be operational in other fungi, and for other plant **cell wall** polysaccharides as well. Different levels of complexity may be observed, and connections are likely to exist between pathways involved in decomposing the different types of plant **cell wall** polysaccharides. We propose that insights into the components and the regulation of the plant **cell-wall**-decomposing machinery in ***B. cinerea*** may provide new leads for designing a rational control strategy for this devastating pathogen.

References

Adie BA, Perez-Perez J, Perez-Perez MM, Godoy M, Sanchez-Serrano JJ, Schmelz EA, Solano R (2007) ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. Plant Cell 19: 1665–1681

Aguero CB, Uratsu SL, Greve C, Powell AL, Labavitch JM, Meredith CP, Dandekar AM (2005) Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera L.* expressing the pear *PGIP* gene. Mol Plant Pathol 6:43–51

Amselem J, Cuomo CA, van Kan JAL, Viaud M, Benito EP, Couloux A, Coutinho PM, de Vries RP, Dyer PS, Fillinger S, Fournier E, Gout L, Hahn M, Kohn L, Lapalu N, Plummer KM, Pradier JM, Quevillon E, Sharon A, Simon A, ten Have A, Tudzynski B, Tudzynski P, Wincker P, Andrew M, Anthouard V, Beever RE, Beffa R, Benoit I, Bouzid O, Brault B, Chen Z, Choquer M, Collemare J, Cotton P, Danchin EG, Da Silva C, Gautier A, Giraud C, Giraud T, Gonzalez C, Grossetete S, Guldener U, Henrissat B, Howlett BJ, Kodira C, Kretschmer M, Lappartient A, Leroch M, Levis C, Mauceli E, Neuveglise C, Oeser B, Pearson M, Poulain J, Poussereau N, Quesneville H, Rascle C, Schumacher J, Segurens B, Sexton A, Silva E, Sirven C, Soanes DM, Talbot NJ, Templeton M, Yandava C, Yarden O, Zeng Q, Rollins JA, Lebrun MH, Dickman M (2011) Genomic

analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genet 7:e1002230

Aro N, Pakula T, Penttila M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol Rev 29:719–739

Baxter L, Tripathy S, Ishaque N, Boot N, Cabral A, Kemen E, Thines M, Ah-Fong A, Anderson R, Badejoko W, Bittner-Eddy P, Boore JL, Chibucos MC, Coates M, Dehal P, Delehaunty K, Dong S, Downton P, Dumas B, Fabro G, Fronick C, Fuerstenberg SI, Fulton L, Gaulin E, Govers F, Hughes L, Humphray S, Jiang RH, Judelson H, Kamoun S, Kyung K, Meijer H, Minx P, Morris P, Nelson J, Phuntumart V, Qutob D, Rehmany A, Rougon-Cardoso A, Ryden P, Torto-Alalibo T, Studholme D, Wang Y, Win J, Wood J, Clifton SW, Rogers J, Van den Ackerveken G, Jones JD, McDowell JM, Beynon J, Tyler BM (2010) Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. Science 330:1549–1551

Billon-Grand G, Rascle C, Droux M, Rollins JA, Poussereau N (2012) pH modulation differs during sunflower cotyledon colonization by the two closely related necrotrophic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Mol Plant Pathol 13:568–578

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell 70:21–30

Brisson LF, Tenhaken R, Lamb C (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell 6:1703–1712

Brito N, Espino JJ, Gonzalez C (2006) The endo-beta-1,4-xylanase xyn11A is required for virulence in *Botrytis cinerea*. Mol Plant Microbe Interact 19: 25–32

Cabanne C, Doneche B (2002) Purification and characterization of two isozymes of polygalacturonase from *Botrytis cinerea*. Effect of calcium ions on polygalacturonase activity. Microbiol Res 157:183–189

Caffall KH, Mohnen D (2009) The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr Res 344:1879–1900

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37: D233–D238

Choquer M, Fournier E, Kunz C, Levis C, Pradier JM, Simon A, Viaud M (2007) *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. FEMS Microbiol Lett 277: 1–10

Cosgrove DJ (2001) Plant cell walls: wall-associated kinases and cell expansion. Curr Biol 11:R558–R559

De Lorenzo G, D'Ovidio R, Cervone F (2001) The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu Rev Phytopathol 39:313–335

Dean R, van Kan JAL, Pretorius ZA, Hammond-Kosack KE, DI Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD (2012) The Top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol 13:414–430

Duplessis S, Hacquard S, Delaruelle C, Tisserant E, Frey P, Martin F, Kohler A (2011) *Melampsora larici-populina* transcript profiling during germination and timecourse infection of poplar leaves reveals dynamic expression patterns associated with virulence and biotrophy. Mol Plant Microbe Interact 24:808–818

Espino JJ, Brito N, Noda J, Gonzalez C (2005) *Botrytis cinerea* endo-beta-1,4-glucanase *Cel5A* is expressed during infection but is not required for pathogenesis. Physiol Mol Plant Pathol 66:213–221

Espino JJ, Gutierrez-Sanchez G, Brito N, Shah P, Orlando R, Gonzalez C (2010) The *Botrytis cinerea* early secretome. Proteomics 10:3020–3034

Federici L, Di Matteo A, Fernandez-Recio J, Tsernoglou D, Cervone F (2006) Polygalacturonase inhibiting proteins: players in plant innate immunity? Trends Plant Sci 11:65–70

Fernandez-Acero FJ, Colby T, Harzen A, Carbu M, Wieneke U, Cantoral JM, Schmidt J (2010) 2-DE proteomic approach to the Botrytis cinerea secretome induced with different carbon sources and plant-based elicitors. Proteomics 10:2270–2280

Ferrari S, Vairo D, Ausubel FM, Cervone F, De Lorenzo G (2003) Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell 15:93–106

Ferrari S, Galletti R, Vairo D, Cervone F, De Lorenzo G (2006) Antisense expression of the *Arabidopsis thaliana AtPGIP1* gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. Mol Plant Microbe Interact 19:931–936

Flors V, Ton J, van Doorn R, Jakab G, Garcia-Agustin P, Mauch-Mani B (2008) Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. Plant J 54:81–92

Garcia-Andrade J, Ramirez V, Flors V, Vera P (2011) *Arabidopsis* ocp3 mutant reveals a mechanism linking ABA and JA to pathogen-induced callose deposition. Plant J 67:783–794

Hückelhoven R (2007) Cell wall-associated mechanisms of disease resistance and susceptibility. Annu Rev Phytopathol 45:101–127

Isshiki A, Akimitsu K, Yamamoto M, Yamamoto H (2001) Endopolygalacturonase is essential for citrus

black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. Mol Plant Microbe Interact 14:749–757

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell 15:2503–2513

Janni M, Sella L, Favaron F, Blechl AE, De Lorenzo G, D'Ovidio R (2008) The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen *Bipolaris sorokiniana*. Mol Plant Microbe Interact 21:171–177

Jolie RP, Duvetter T, Van Loey AM, Hendrickx ME (2010) Pectin methylesterase and its proteinaceous inhibitor: a review. Carbohydr Res 345:2583–2595

Joubert DA, Kars I, Wagemakers L, Bergmann C, Kemp G, Vivier MA, van Kan JAL (2007) A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* leaves without any evidence for in vitro interaction. Mol Plant Microbe Interact 20: 392–402

Juge N (2006) Plant protein inhibitors of cell wall degrading enzymes. Trends Plant Sci 11:359–367

Kämper J, Kahmann R, Bolker M, Ma LJ, Brefort T, Saville BJ, Banuett F, Kronstad JW, Gold SE, Muller O, Perlin MH, Wosten HA, de Vries R, Ruiz-Herrera J, Reynaga-Pena CG, Snetselaar K, McCann M, Perez-Martin J, Feldbrugge M, Basse CW, Steinberg G, Ibeas JI, Holloman W, Guzman P, Farman M, Stajich JE, Sentandreu R, Gonzalez-Prieto JM, Kennell JC, Molina L, Schirawski J, Mendoza-Mendoza A, Greilinger D, Munch K, Rossel N, Scherer M, Vranes M, Ladendorf O, Vincon V, Fuchs U, Sandrock B, Meng S, Ho EC, Cahill MJ, Boyce KJ, Klose J, Klosterman SJ, Deelstra HJ, Ortiz-Castellanos L, Li W, Sanchez-Alonso P, Schreier PH, Hauser-Hahn I, Vaupel M, Koopmann E, Friedrich G, Voss H, Schluter T, Margolis J, Platt D, Swimmer C, Gnirke A, Chen F, Vysotskaia V, Mannhaupt G, Guldener U, Munsterkotter M, Haase D, Oesterheld M, Mewes HW, Mauceli EW, DeCaprio D, Wade CM, Butler J, Young S, Jaffe DB, Calvo S, Nusbaum C, Galagan J, Birren BW (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. Nature 444: 97–101

Kars I, van Kan JAL (2004) Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. In: Elad Y, Williamson B, Tudzynski P, Delen N (eds) *Botrytis*: biology, pathology and control. Kluwer, The Netherlands, pp 99–118

Kars I, McCalman M, Wagemakers L, VAN Kan JA (2005a) Functional analysis of Botrytis cinerea pectin methylesterase genes by PCR-based targeted mutagenesis: Bcpme1 and Bcpme2 are dispensable for virulence of strain B05.10. Mol Plant Pathol 6: 641–652

Kars I, Krooshof GH, Wagemakers L, Joosten R, Benen JA, van Kan JAL (2005b) Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J 43:213–225

Koeck M, Hardham AR, Dodds PN (2011) The role of effectors of biotrophic and hemibiotrophic fungi in infection. Cell Microbiol 13:1849–1857

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275

Manfredini C, Sicilia F, Ferrari S, Pontiggia D, Salvi G, Caprari C, Lorito M, De Lorenzo G (2005) Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol Mol Plant Pathol 67:108–115

Martens-Uzunova ES, Schaap PJ (2008) An evolutionary conserved d-galacturonic acid metabolic pathway operates across filamentous fungi capable of pectin degradation. Fungal Genet Biol 45:1449–1457

Martin F, Kohler A, Murat C, Balestrini R, Coutinho PM, Jaillon O, Montanini B, Morin E, Noel B, Percudani R, Porcel B, Rubini A, Amicucci A, Amselem J, Anthouard V, Arcioni S, Artiguenave F, Aury JM, Ballario P, Bolchi A, Brenna A, Brun A, Buee M, Cantarel B, Chevalier G, Couloux A, Da Silva C, Denoeud F, Duplessis S, Ghignone S, Hilselberger B, Iotti M, Marcais B, Mello A, Miranda M, Pacioni G, Quesneville H, Riccioni C, Ruotolo R, Splivallo R, Stocchi V, Tisserant E, Viscomi AR, Zambonelli A, Zampieri E, Henrissat B, Lebrun MH, Paolocci F, Bonfante P, Ottonello S, Wincker P (2010) Perigord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature 464:1033–1038

Martin F, Cullen D, Hibbett D, Pisabarro A, Spatafora JW, Baker SE, Grigoriev IV (2011) Sequencing the fungal tree of life. New Phytol 190:818–821

Mohnen D (2008) Pectin structure and biosynthesis. Curr Opin Plant Biol 11:266–277

Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science 301:969–972

Nishiyama Y, Langan P, Chanzy H (2002) Crystal structure and hydrogen-bonding system in cellulose Ibeta from synchrotron X-ray and neutron fiber diffraction. J Am Chem Soc 124:9074–9082

Noda J, Brito N, Gonzalez C (2010) The *Botrytis cinerea* xylanase Xyn11A contributes to virulence with its necrotizing activity, not with its catalytic activity. BMC Plant Biol 10:38

Oeser B, Heidrich PM, Muller U, Tudzynski P, Tenberge KB (2002) Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea/rye* interaction. Fungal Genet Biol 36:176–186

Paper JM, Scott-Craig JS, Adhikari ND, Cuomo CA, Walton JD (2007) Comparative proteomics of extracellular proteins in vitro and in planta from the pathogenic fungus *Fusarium graminearum*. Proteomics 7:3171–3183

Phalip V, Goubet F, Carapito R, Jeltsch JM (2009) Plant cell wall degradation with a powerful Fusarium graminearum enzymatic arsenal. J Microbiol Biotechnol 19:573–581

Powell AL, van Kan JAL, ten Have A, Visser J, Greve LC, Bennett AB, Labavitch JM (2000) Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol Plant Microbe Interact 13:942–950

Rha E, Park HJ, Kim MO, Chung YR, Lee CW, Kim JW (2001) Expression of exo-polygalacturonases in *Botrytis cinerea*. FEMS Microbiol Lett 201:105–109

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289

Schols HA, Coenen GJ, Voragen AGJ (2009) Revealing pectin's structure. In: Schols HA, Visser RGF, Voragen AGJ (eds) Pectins and pectinases. Wageningen Academic Publishers, The Netherlands, pp 19–34

Shah P, Gutierrez-Sanchez G, Orlando R, Bergmann C (2009a) A proteomic study of pectin-degrading enzymes secreted by *Botrytis cinerea* grown in liquid culture. Proteomics 9:3126–3135

Shah P, Atwood JA, Orlando R, El Mubarek H, Podila GK, Davis MR (2009b) Comparative proteomic analysis of *Botrytis cinerea* secretome. J Proteome Res 8: 1123–1130

Shieh MT, Brown RL, Whitehead MP, Cary JW, Cotty PJ, Cleveland TE, Dean RA (1997) Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl Environ Microbiol 63:3548–3552

Spanu PD, Abbott JC, Amselem J, Burgis TA, Soanes DM, Stuber K, Loren V, van Themaat E, Brown JK, Butcher SA, Gurr SJ, Lebrun MH, Ridout CJ, Schulze-Lefert P, Talbot NJ, Ahmadinejad N, Ametz C, Barton GR, Benjdia M, Bidzinski P, Bindschedler LV, Both M, Brewer MT, Cadle-Davidson L, Cadle-Davidson MM, Collemare J, Cramer R, Frenkel O, Godfrey D, Harriman J, Hoede C, King BC, Klages S, Kleemann J, Knoll D, Koti PS, Kreplak J, Lopez-Ruiz FJ, Lu X, Maekawa T, Mahanil S, Micali C, Milgroom MG, Montana G, Noir S, O'Connell RJ, Oberhaensli S, Parlange F, Pedersen C, Quesneville H, Reinhardt R, Rott M, Sacristan S, Schmidt SM, Schon M, Skamnioti P, Sommer H, Stephens A, Takahara H, Thordal-Christensen H, Vigouroux M, Wessling R, Wicker T, Panstruga R (2010) Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. Science 330:1543–1546

ten Have A, Mulder W, Visser J, van Kan JAL (1998) The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol Plant Microbe Interact 11:1009–1016

ten Have A, Breuil WO, Wubben JP, Visser J, van Kan JAL (2001) *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. Fungal Genet Biol 33:97–105

Valette-Collet O, Cimerman A, Reignault P, Levis C, Boccara M (2003) Disruption of *Botrytis cinerea* pectin methylesterase gene *Bcpme1* reduces virulence on several host plants. Mol Plant Microbe Interact 16:360–367

van Kan JAL (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci 11: 247–253

Verhoeff K, Leeman M, Vanpeer R, Posthuma L, Schot N, Vaneijk GW (1988) Changes in pH and the production of organic-acids during colonization of tomato petioles by *Botrytis cinerea*. J Phytopathol 122: 327–336

Volpi C, Janni M, Lionetti V, Bellincampi D, Favaron F, D'Ovidio R (2011) The ectopic expression of a pectin methyl esterase inhibitor increases pectin methyl esterification and limits fungal diseases in wheat. Mol Plant Microbe Interact 24:1012–1019

Voragen AGJ, Schols HA, Pilnik W (1986) Determination of the degree of methylation and acetylation of pectins by h.p.l.c. Food Hydrocoll 1:65–70

Wang Y, Wu J, Park ZY, Kim SG, Rakwal R, Agrawal GK, Kim ST, Kang KY (2011) Comparative secretome investigation of Magnaporthe oryzae proteins responsive to nitrogen starvation. J Proteome Res 10:3136–3148

Williamson B, Tudzynski B, Tudzynski P, van Kan JAL (2007) *Botrytis cinerea*: the cause of grey mould disease. Mol Plant Pathol 8:561–580

Wubben JP, Mulder W, ten Have A, van Kan JAL, Visser J (1999) Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. Appl Environ Microbiol 65:1596–1602

Wubben JP, ten Have A, van Kan JAL, Visser J (2000) Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. Curr Genet 37:152–157

Yajima W, Kav NN (2006) The proteome of the phytopathogenic fungus *Sclerotinia sclerotiorum*. Proteomics 6:5995–6007

Yang F, Jensen JD, Svensson B, Jorgensen HJ, Collinge DB, Finnie C (2012) Secretomics identifies *Fusarium graminearum* proteins involved in the interaction with barley and wheat. Mol Plant Pathol 13:445–453

Zhang L, van Kan JAL (2013) *Botrytis cinerea* mutants deficient in D-galacturonic acid catabolism have a perturbed virulence on *Nicotiana benthamiana* and *Arabidopsis*, but not on tomato. Mol Plant Pathol 14:19–29

Zhang L, Thiewes H, van Kan JAL (2011) The D-galacturonic acid catabolic pathway in *Botrytis cinerea*. Fungal Genet Biol 48:990–997

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