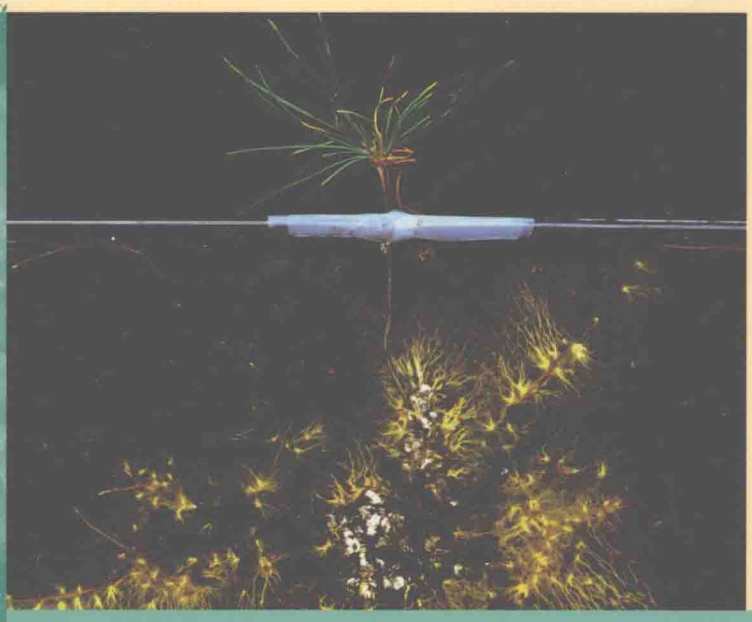
Stress in Yeasts and Filamentous Fungi



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STRESS IN YEASTS AND FILAMENTOUS FUNGI

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Preface

Yeasts and filamentous fungi need to cope with stress, in one form or another, whether growing in the laboratory or in the natural environment, whether victims or offenders in interactions with other organisms. Fungal research gives invaluable insight to the environmental impact of stress, and to the molecular mechanisms of action and response. These considerations define the ethos of this volume, which considers stress in this broad sense, within the context of mycology.

The chapters herein are contributed by invited speakers at the 2006 Annual Scientific Meeting of the British Mycological Society, Birmingham, UK. The meeting shared the same title as this volume, Stress in Yeasts and Filamentous Fungi, and shared a similar blend of content dedicated to both environmental and cell and molecular aspects of stress effects and responses in fungi. This volume draws on the expertise of leaders in the field from both the yeast and filamentous-fungal communities.

The stresses associated with organism–organism interactions (e.g. predation, competition, etc.) are highlighted in contributions from Naresh Magan, Peter Mills, Levente Kiss, Michael Shaw and their co-authors, which consider these effects at the whole organism level in fungi in their natural environments. The stress interface between different organisms is probed further in the context of fungal pathogenicity by Jan Quinn and Ken Haynes with their respective work on the human pathogen *Candida* spp., and with an oomycete agent of downy mildew by Jim Beynon.

There is increasing awareness that stress may arise under the comparatively controlled conditions of fungal culture in the laboratory, aspects of which are tackled in the chapter by Geoff Robson and co-authors. Stress under controlled conditions may be exploited for control of fungal growth, and Mehdi Mollapour with Peter Piper examine how yeasts respond to stress provoked by weak acids that may be used for food preservation. Stefan Hohmann describes a systems biology approach to modelling such stress responses in yeast.

Another area that continues to attract research attention is anthropogenic stress towards fungi in the environment, for example via metal pollutants generated through industrial activities. The impacts that such stressors may have on different organisms and communities in the wild are explained by Jan Colpaert (ectomycorrhizal fungi), William Purvis (lichens) and Roger Finlay (mycorrhizal fungi), with their co-authors. Effects of anthropogenic stress on fungi in the environment are underpinned by effects originating at the cellular and molecular level. Many such stressors act as pro-oxidants, and different aspects of the responses to oxidative stress exhibited by fungal cells are explored in the contributions by Chris Grant (protein synthesis response), Scott Moye-Rowley

(Yap1-mediate transcriptional response), Jesus Pla (signalling response) and Wilhelm Hansberg (cell differentiation response) and their co-authors.

Finally, it is hoped that this volume will provide a valuable resource for those interested in the effects of stress on fungi, bringing together researchers who study these issues at different levels and who, consequently, may rarely gain exposure to each other's work. The content encompasses a breadth of scope from the bigger picture of stress effects on fungi in their natural habitats, down to the fascinating recent advances in our underlying molecular-level understanding. I hope that readers will benefit from seeing how their particular angle of interest fits within this broader view, while of course feeding their own particular interest and hopefully enjoying an all-round stimulating read!

Simon V. Avery
April 2007

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CHAPTER 1

Interactions Between *Agaricus bisporus* and the Pathogen *Verticillium fungicola*

Peter Mills, John Thomas, Martin Sergeant, Ana Costa, Patrick Collopy, Andy Bailey, Gary Foster and **Mike Challen**

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Abstract

The interaction between *Agaricus bisporus* and *Verticillium fungicola* may be the most economically significant interaction between two fungi. Work described in this chapter covers diverse aspects of the biology and molecular interaction of the host and pathogen and has established a robust baseline for future study of a pathogenic system involving two members of the same kingdom.

Pathogen variability has been assessed, and specific genotypes important to major mushroom-producing countries have been characterised. This has enabled representative isolates to be identified for use in this study.

A wide range of cell-wall-degrading enzymes from *V. fungicola* have been identified, and principal component analysis showed a complex correlation between enzyme production and symptom expression. It is likely that some, or indeed many of these enzymes play a critical role in the pathogenicity of *Verticillium*.

Although it is generally accepted that *A. bisporus* has a relatively narrow genetic base, our work, and that of other groups, has shown that variability in host tolerance to infection with *V. fungicola* exists to a large extent to justify a detailed study to exploit it. We have developed

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transformation technologies for the mushroom *A. bisporus* (Challen *et al.*, 2000; Leach *et al.*, 2004; Foster *et al.*, 2004b; Burns *et al.*, 2005, 2006), its pathogen *V. fungicola* (Amey *et al.*, 2002, 2003) and other pathogenic fungi (Rogers *et al.*, 2004; Gewiss-Mogensen *et al.*, 2006). Recent advances in gene suppression technologies for the host include deployment of anti-sense and RNAi hairpin constructs to down-regulate endogenous *A. bisporus* genes (Burns, 2004; Heneghan *et al.*, in press). Similarly gene knockout methodology has been established for the pathogen *V. fungicola* (Amey *et al.*, 2003; Foster *et al.*, 2004a).

An extensive range of genetic resources have been established, which include: *V. fungicola* pathogenicity simulated cDNA library (mushroom cell wall (MCW) agar), numerous *A. bisporus* fruiting cDNA libraries (macro-arrayed), host (*A. bisporus*)–pathogen (*V. fungicola*) lesions infection SSH libraries (forward and reverse subtractions for up- and down-regulated mushroom genes) and macro-arrayed cDNA infection library, genomic DNA libraries for *A. bisporus* and other homobasidiomycete mushrooms, EST fungal–fungal interaction database; transformation and expression vectors designed specifically for *Agaricus* and *Verticillium*, and vectors to expedite cloning of fragments for gene silencing experiments.

The resources now in place will enable us to determine, in a systematic manner, the impact of gene expression on the disease phenotype.

This work provides platform technologies for alternative methods of disease control, reduced pesticide use and sustainable crop production. Investigations into this model fungal–fungal interaction will also provide information on the research and development of fungal biological control agents of fungal plant pathogens.

1. INTRODUCTION

The homobasidiomycete fungus *Agaricus bisporus* (Lange) Imbach, the white button mushroom, is a high-value cultivated crop species with approximately 1.5 million tonnes p.a. cultivated for human consumption worldwide. Pathogen threats to *A. bisporus* include bacteria, viruses, mites, insects and fungi. The latter are the most detrimental and pose a significant threat to the industry.

Dry bubble, caused by *Verticillium fungicola* (Preuss) Hassebrauk, is the most common and serious fungal disease of *A. bisporus*. Symptoms vary (Figure 1) and may depend on a number of factors such as developmental stage at the time of infection and genetic variability of the host and include bubble, cap spotting and stipe blowout (Fletcher *et al.*, 1994). The least severe symptom is cap spotting (lesions) where *V. fungicola* appears to infect only the surface of the fruiting body causing brown lesions to appear (Dragt *et al.*, 1996). 'Stipe blow-out' is a more extreme symptom where the *A. bisporus* stipe swells and peels; it is thought this symptom is due to infection earlier in mushroom development than in the case of cap spotting (Ware, 1933). Stipe blowout occurs experimentally when high levels of *V. fungicola* are used to inoculate *A. bisporus*, at the primordial stage prior to major differentiation of the fruiting body (North and Wuest, 1993). In the most

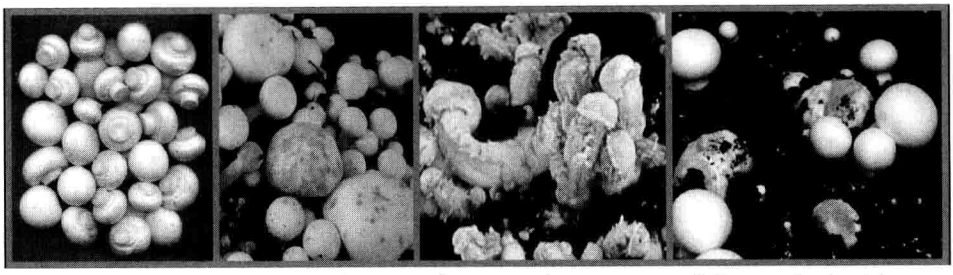

Figure 1 *Verticillium fungicola* infections of *Agaricus bisporus*. From left to right: healthy mushrooms, cap lesions, stipe blowout and dry bubble.

severe symptom, from which the disease derives its common name 'dry bubble', mycelia of *V. fungicola* and *A. bisporus* appear to grow together causing a 'mushroom mass' with little or no differentiation (Jeffries and Young, 1994). This only occurs when the early initials of *A. bisporus* (mushroom pins) are inoculated (North and Wuest, 1993). Hitherto this complex infection has been poorly characterised. Appressoria, penetration and intracellular growth of *Verticillium* hyphae in *A. bisporus* fruit bodies can be seen using light microscopy and TEM (Dragt *et al.*, 1996). EM evidence for *V. fungicola* action on host *A. bisporus* cell walls *in vitro*, and the production of hydrolytic enzymes in *V. fungicola* cultures grown on various carbon sources has also been demonstrated (Calonje *et al.*, 1997, 2000).

None of the commercially available *A. bisporus* spawns are resistant to this pathogen. *Verticillium* is currently controlled by the C14 demethylation inhibitor of egosterol biosynthesis, prochloraz-manganese, marketed as 'Sporgon'. That only a single fungicide control chemical is available is a very high-risk situation. If resistance to the fungicide were to become widespread (resistant strains have already been reported; Grogan and Gaze, 2000; Grogan *et al.*, 2000) or if fungicide control was to become unavailable for any reason, for example more stringent pesticide safety regulations, then the industry would be faced with an acute problem. In Holland, for example, there are increasing pressures against the use of 'Sporgon' because of perceived residue problems. Outbreaks of *Verticillium* can be devastating. The current 'boom and bust' situation exactly parallels the position in the 1970s where benomyl-type fungicides were used to control *Verticillium* and the pathogen soon developed resistance resulting in a major problem for the industry. The mechanisms of infection of *Agaricus* by fungal pathogens are poorly understood.

Advances in fungal–fungal interactions are significant in the context of mushroom (*A. bisporus*) pathology where *V. fungicola* is the single most geographically significant mycoparasite; it also serves as a relevant mycoparasitic model for the study of other mycoparasitic diseases (Williams *et al.*, 2003) and several biocontrol agents (e.g. *Coniothyrium*, *Trichoderma* and *Sporidesmium*) used against soil-borne fungal pathogens. However, unlike many of these systems where isolation of host or pathogen RNA may be limiting, the *Agaricus–Verticillium* interaction permits easy isolation of both host (basidiomycete) and

pathogen (ascomycete) sequences from a live interaction, minimises problems with cross-hybridisations and facilitates likely provenance of isolated sequences.

The fungal kingdom encompasses a diverse range of organisms occupying numerous ecological niches with various lifestyles, from litter decomposers to human pathogens. Although there have been significant efforts to characterise interactions of the pathogenic fungi with plants and humans, studies of fungal pathogens of other fungi remain in their infancy.

In order to understand the underlying mechanisms involved in the interaction between *Verticillium* and *Agaricus* a number of experimental components were required. These included a measure of pathogen variability, an understanding of the possible mechanism of pathogenicity, pathogen and host transformation technologies and a measure of host resistance. Collectively these components were put in place at the Universities of Warwick and Bristol and have allowed a detailed study of the molecular interaction of the two component fungi.

2. PATHOGEN VARIABILITY

V. fungicola is classified into three varieties based on conidiophore morphology and maximum growth temperatures. *V. fungicola* var. *fungicola* is thought to be the primary cause of *Verticillium* disease in Europe, whereas *V. fungicola* var. *aleophilum* is thought to cause brown spotting and has been associated more commonly with *Agaricus bitorquis* and higher growing temperatures. A third variety *V. fungicola* var. *flavidum*, isolated from wild agarics appears to be weakly pathogenic to cultivated *Agaricus* species. To complicate the situation further *V. psalliotae* has been reported in the literature as a minor pathogen of mushrooms causing brown cap spotting. Further information on *Verticillium* varieties is available in Collopy *et al.* (2001). It is also interesting to note that *V. fungicola* var. *aleophilum* can be more aggressive to some strains of *A. bisporus* than var. *fungicola* (Largeteau *et al.*, 2005).

In an attempt to clarify the causal agent of disease in the UK isolates were collected from UK farms and also from other major mushroom-producing countries including France, Spain, Germany, Hungary, The Netherlands, New Zealand and the USA. Using a range of routine molecular tests including ribosomal DNA RFLP, the diversity of this group of isolates was assessed. Results indicated that all European isolates formed a single group and could be distinguished from American and New Zealand isolates. Mitochondrial DNA (mtDNA) RFLPs revealed further genetic variation within the three major groups. European isolates possessed four haplotypes and were quite distinct from the New Zealand and American isolates, which shared a common mtDNA RFLP pattern. Sequencing of the internal transcribed spacer (ITS) 1 region of the rDNA confirmed the genetic uniformity of the European isolates. Isolates from America and New Zealand possessed nearly identical ITS 1 sequence whilst having a 3.5% divergence from European isolates.

The outcome of the analyses of the variability of pathogenic *Verticillium* isolates from mushrooms has shown that there are four distinct taxa within

V. fungicola. The European isolates relate to the *ex*-type isolate of *V. fungicola* var. *fungicola*. The isolates from New Zealand are divided into two taxa of which one taxon conforms to the *ex*-type isolate of *V. fungicola* var. *aleophilum*; the fourth taxon comprised isolates from the USA. The taxon containing the European isolates is genotypically very different from the other three taxa.

The significance of these data is that there are considerable genetic differences in mushroom pathogenic *Verticillia* across the world but that geographical regions generally possess only one major genotype. This contradicts the previously accepted view that all varieties of *Verticillium* may be present in all regions.

It is also worth noting that the full sequence of the nitrate reductase gene has been obtained for *V. fungicola* (Amey *et al.*, unpublished data). The central portion of this gene was amplified and sequenced from a number of *V. fungicola* isolates and related fungi, and the resulting phylogenies were compared to those obtained from analysis of the rDNA ITS regions for these fungi. Both nitrate reductase and ITS analyses provided additional evidence that suggests the mushroom pathogenic *Verticillium* species are related more to other chitinolytic fungi such as the insect pathogens *Verticillium lecanii* and *Beauveria bassiana* than to the plant pathogenic *Verticillia*. These data suggest that *V. fungicola* could be reclassified separately from other *Verticillium* species. Although there were high similarities with the insect pathogenic *Beauveria* species it was clear that sufficient differences were evident at the genetic and biological levels for *V. fungicola* to be placed in a form genus of its own. A new genus called *Lecanicillium* has been proposed to include the majority of entomogenous and fungicolous taxa (Zare and Gams, 2001).

3. EXTRA-CELLULAR ENZYME PRODUCTION

Although *Verticillium* species cause a range of symptoms on *A. bisporus* the infection process is not fully understood because of the complexity, involving the presence of *Verticillium* spores and the subsequent interaction between mycelia of the pathogen and host (Calonje *et al.*, 1997).

Kalberer (1984) assayed *Verticillium* proteolytic enzymes, but was unable to prove their role in the infection process of mushrooms. Scanning electron microscopy (North and Wuest, 1993) and *in vitro* studies (Matthews, 1983) found interhyphal penetration by *V. fungicola* var. *fungicola* into mushroom fruiting body tissue, indicating that hydrolytic enzymes were involved in the mycoparasitism by *Verticillium*. Dragt *et al.* (1996) using light microscopy and TEM showed the formation of appressoria, penetration and intracellular growth of *Verticillium* hyphae in the *A. bisporus* fruiting body hyphae. The thin wall of mycoparasitised hyphal cells compared with the electron-dense wall of healthy mushroom cells again supported the view that wall-lytic enzymes played a role in the infection process.

In an attempt to correlate enzyme production by *V. fungicola* and symptom expression, a study was undertaken on 17 isolates (Table 1) selected on the basis of differences in pathology demonstrated in cropping trials or on the basis of

Table 1 *Verticillium* isolates studied

Warwick HRI No.	Designated species	Geographic origin
5-3	*V. fungicola* var. *Fungicola* (ICMP3343)	New Zealand
7-3	*V. Fungicola* var. *aleophilum*^{T} (CBS357.80)	Netherlands
7-4	*V. fungicola* var. *fungicola*^{T} (CBS440.34)	UK
14-4	*V. fungicola* var. *fungicola*	USA
19-4	*V. fungicola* var. *fungicola*	Taiwan
19-5	*V. fungicola* var. *fungicola*	South Africa
21-3	*V. fungicola* var. *fungicola*	Spain
26-10	*V. fungicola* var. *fungicola*	Netherlands
55-3	*V. fungicola* var. *fungicola*	USA
55-12	*V. fungicola* var. *fungicola*	USA
115-1	*V. fungicola* var. *fungicola*	UK
124-1	*V. fungicola* var. *fungicola*	Eire
150-1	*V. fungicola* var. *fungicola*	UK
157-1	*V. lecanii*	UK
170-3	*V. psalliotae* (IMI246435)	UK
171-1	*V. fungicola* var. *fungicola*	Brazil
174-1	*V. fungicola* var. *fungicola* (TMI65020)	Japan

Note: T, type strain.

molecular typing described above. Cultures were grown on minimal synthetic media supplemented with MCW material. Culture filtrates were assayed for enzymes likely to be involved in the breakdown of host cell walls.

A wide range of enzymes were produced. Lipase production from 17 *Verticillium* spp. was initially determined by a clearing zone assay on agar containing Tween 80. All isolates showed lipase activity. Esterase (C4 degradation) activities of *Verticillium* spp. were relatively uniform, with the notable exceptions of three *V. fungicola* var. *fungicola* isolates (two from the USA and one from South Africa) and one *V. psalliotae* isolate. There was generally an inverse relationship between *Verticillium* spp. enzymic activities and the carbon chain length of 4-*p*-nitrophenyl fatty acid substrates. Only 50% of *V. fungicola* var. *fungicola* isolates showed significant stearase (C18) activity.

Most *Verticillium* spp. produced high levels of 1,3-β-glucanase, though there was considerable variation between isolates with three European isolates *V. fungicola* var. *fungicola* showing much lower levels of activity.

Proteinase activities of *V. fungicola* var. *fungicola* 7-4^T was assayed using a synthetic peptide substrate. A pH profile of *Verticillium* serine proteinase activities (assayed using suc-ala-ala-pro-phe-*p*N substrate) showed it to be an alkaline proteinase, having activity optima between pH 7 and 10. This enzyme activity was inhibited by PMSF and partially inhibited by EDTA but not iodoacetamide or pepstatin, thus confirming it to be a serine proteinase. Three aminopeptidase activities were found using glu-*p*-NA, arg-*p*-NA and leu-*p*-NA substrates; an isolate of *V. psalliotae* produced the highest activities of these enzymes.

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