Interspecific interactions between saprotrophic basidiomycetes: effects on ligninolytic enzyme activity, gene expression and metabolite production

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Abstract

Interspecific antagonism leads to morphological, biochemical and transcriptional changes in the competing mycelia. Several approaches were used to study interactions: enzyme assays, staining, expression of ligninolytic genes, and analysis of volatile organic compounds (VOCs). Laccase and manganese peroxidase (MnP) activities were assayed, and transcript levels of laccase, Mn-repressed peroxidase (MRP), LiP and catalase measured by RT-PCR during interactions between *Trametes versicolor* and other wood decay fungi on agar plates. Increased laccase activity occurred in all interactions, irrespective of outcome, with expression of a variety of laccase isozymes during growth alone and interactions. The highest laccase and MnP activities were detected in the interaction zone, with minor changes occurring in other regions of interacting mycelia. MnP activity was detected but not expression of MnP genes; instead, activity of MRP could explain the observed activity. Whilst no LiP activity was detected in any pairing, low level expression of LiP genes was detected. Any increases in gene expression were probably transient, or missed at the time of sampling. No differences in combative ability or enzyme production were detected between homo- and heterokaryotic isolates of *T. versicolor*. VOC production was not linked to combative ability, although several constitutive and interaction-specific VOCs were potentially antagonistic. Other VOCs may be involved in the stimulation of laccase activity, or supporting peroxidase activity by the generation of H_2O_2.

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Publications

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Chapter 1

Introduction

Conservative estimates place the number of fungal species at 1.5 million (Hawksworth, 1991, 2001; Mueller and Schmit, 2007). Currently only 72,000 have been described, spread over seven phyla, although the number is increasing as new techniques are developed for detecting and profiling fungal diversity (Bridge and Spooner, 2001; Bridge et al., 2005; Hibbett et al., 2007). Fungi are spectacularly successful not only in number of species but also in terms of biomass, variety of habitats, and capacity for symbiosis with other microorganisms, plants and animals (Hawksworth, 1991). Their success reflects massive morphological and behavioural diversity, and developmental plasticity. The vast majority of fungi, the Eumycota, are believed to belong to a single phyletic series, with the main stream of evolution culminating in the Basidiomycota (Förster et al., 1990). The basidiomycetes comprise approximately one third of known fungal species and represent a diverse phylum comprising species with equally diverse ecological roles. These roles are of key importance in ecosystems; from the symbiosis with algae and cyanobacteria to form lichens, to the association of mycorrhizal fungi with plants, to the numerous plant, animal and fungal pathogens. Further basidiomycetes are vital to carbon and nutrient cycling, food webs, succession and biodiversity in every

ecosystem, via decomposition.

The greatest fungal diversity can be found in the soil environment and woodland ecosystems (Peay et al., 2008). Fungal hyphae often account for the greatest fraction of soil biomass (Wardle, 2002), with an estimated ton (dry weight) of basidiomycetous mycelium produced annually per hectare in a typical angiosperm forest (de Jong and Field, 1997). It has been estimated that only 17% of described fungal species can be grown in culture, so many species are not readily studied (Bridge and Spooner, 2001), and much of the diversity is cryptic and present in resting stages such as conidia, spores and inactive mycelium (Peay et al., 2008). Woodland ecosystems are heterogeneous in terms of the biotic and abiotic environment, both spatially and temporally (White et al., 1998; Boddy, 1999). Fungi have evolved and adapted to this variable environment, and niche specialisation has resulted in a range of morphologies, ecological strategies and habitats.

1.1 Decomposition in woodland ecosystems

Decomposition facilitates nutrient recycling by making resources in plant and animal material available for utilisation to a wide range of organisms; as such, it is a vital process. Many sources of carbon are easily metabolised, but more complex polymers (e.g. cellulose, lignin and chitin) are less easily decomposed and only utilised by specialists. The predominant carbon resource in woodlands is woody litter (Wells, 2002), which is resistant to decomposition and many organisms are unable to use it as a resource. The fungal biomass of woodland soils is dominated by two major ecological groups of basidiomycetes: the saprotrophic wood/litter decomposers and the biotrophic ectomycorrhizal fungi (Wells, 2002). Both groups play a major role in the acquisition, mobilisation and relocation of nutrients. Specialist saprotrophic basidiomycetes are the main agents of wood decomposition in terrestrial ecosystems (Boddy, 2001), breaking

down the more recalcitrant compounds into simpler forms, which are then available to other organisms. The importance of this role in nutrient mobilisation is shown by the 10-fold increase in soil microbial biomass (typically 0.1%) when saprotrophic fungi colonise the overlying litter (Lodge, 1993). Similarly, eliminating fungi results in a significant decrease in both carbon and nitrogen depletion from litter (Bridge et al., 1992).

Lignins are what makes wood resistant to decomposition. Few organisms are able to decompose lignin, and as such it is a rate-limiting step in carbon turnover. Lignins are amorphous, insoluble polymers formed by the oxidative coupling of coniferyl, sinapyl and coumaryl alcohols. The molecular composition of lignins varies hugely between plant species, and indeed within individual plants. The nature of the bonds between the phenolic subunits makes the polymer highly recalcitrant and resistant to hydrolysis. Fungi dominate the decomposition of lignified cellulose; the most common group are white rot fungi, which are able to penetrate the tissues of wood and digest lignin, cellulose and hemicellulose enzymatically, and leave wood with a characteristic bleached and soft appearance. Less common are the brown rot fungi, which are only able to digest cellulose and hemicellulose, while lignin is left intact or slightly modified, leaving wood brown and crumbly (Owens et al., 1994). Lignin is not directly used for nutrition by white rot fungi, but its decomposition exposes cellulose and hemicellulose which can be hydrolysed as energy sources (Doyle et al., 1998).

Fungal decomposition is facilitated by a suite of enzymes that act on wood constituents. As lignin is not susceptible to hydrolytic attack, white rot fungi produce phenoloxidases (laccases) and peroxidases which generate highly reactive, non-specific free radicals in order to oxidise the lignin polymer. These work in combination with enzymes that do not directly attack lignin but support peroxidase and laccase activity (e.g. superoxide dismutase and glyoxal oxidase), and with feedback-regulated enzymes which coordinate metabolic pathways during decomposition (Leonowicz et al., 2001). Low molecular

weight compounds and free radicals may also act as mobile factors that can permeate wood and initiate decay where high molecular weight enzymes cannot penetrate due to their size (Evans et al., 1994).

The environment within wood is heterogeneous, with water potential, gaseous regime and pH varying with the status of the wood (Boddy, 1999). For example, heartwood has a low nitrogen content relative to attached branches; and the CO_2 content of wood is generally high, with low levels of O_2. Also, wood contains numerous tannins, gums and resins which vary between species and between individuals of the same species (Griffith and Boddy, 1991; Boddy, 1999). Through the transition from living, to fallen, dead wood, these conditions change; dead wood dries rapidly, and compounds within the wood leach out or break down (Griffith and Boddy, 1991). The space and resources available change as decomposition progresses, favouring different species specialised to different niches (Hättenschwiler et al., 2005). Thus as conditions change, so does the fungal community.

1.2 The basidiomycete life cycle

The initial stages of colonisation for a wood decay fungus are arrival at the resource and establishment within it. Arrival can be as mycelium, or as spores, gaining access to woody tissues via bark discontinuities such as wounds or branch stubs, or from the development of latent propagules (Rayner and Boddy, 1988; Boddy, 2001). Exit from the resource can be achieved as mycelium, or, following the formation of reproductive structures, as spores. In most basidiomycetes sexual reproduction occurs via the fusion and nuclear exchange of sexually compatible mycelia (Burnett, 2003). A variety of terms have been used for mycelia that are unmated/mated: primary/secondary, monokaryotic/dikaryotic, homokaryotic/heterokaryotic. Monokaryotic and dikaryotic are appropriate only when it is known that there are only one or two nuclei per compart-

ment, whereas basidiomycetes may have several nuclei per compartment (Ainsworth, 1986). Homo-/heterokaryotic imply that either all nuclei are similar, or that there is more than one type of nucleus present (e.g. from different parents), so are more appropriate where the nuclear balance of a species is unknown.

The typical basidiomycete life cycle presents particular challenges at different stages: spore dispersal, deposition and germination; establishment of the homokaryon in the resource; contact with a sexually compatible conspecific; growth of the heterokaryon; fruit body formation and spore production (Figure 1.1). Once established within a substratum, the homokaryotic mycelium faces the same biotic and abiotic challenges as the heterokaryon; however, few studies have investigated differences in relative fitness between the stages. For a rare species, the duration of the homokaryotic phase may be considerable, as sexually compatible conspecifics are hard to find. Thus, in a rare species the homokaryotic phase must be particularly 'fit' to survive, and it has been shown that homokaryons of the rare species *Hericium coralloides* are more combative than the heterokaryons (Crockatt et al., 2008). However, for a common species the duration of the homokaryotic phase is likely to be shorter, but it is not known how this affects their fitness relative to that of the heterokaryons.

1.3 Fungal competition

Competition can be defined as 'the negative effects which one organism has upon another by consuming or controlling access to a resource that is limited in availability' (Keddy, 1989). For white rot fungi, nutrients are accessed by colonisation of solid resources, so competition for space and resources cannot be uncoupled into separate entities. Further to this, competition between white rot fungi can be described as 'interference competition', where access to a resource is influenced by the presence of a competitor, either through its behaviour or some sort of chemical mechanism

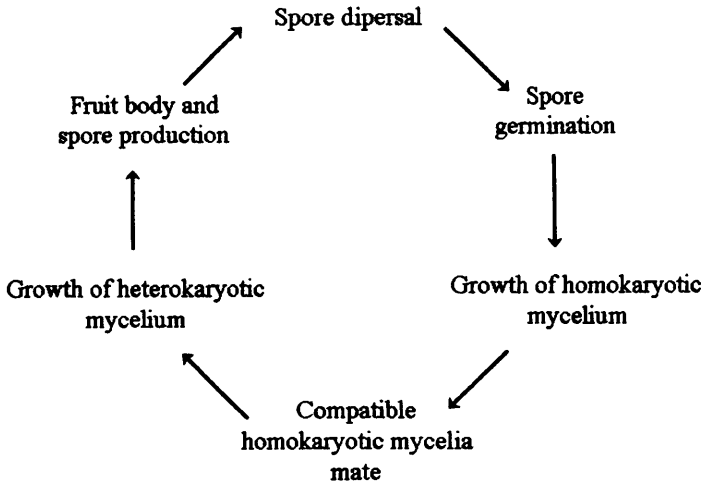

Figure 1.1: The lifecycle of a typical basidiomycete. Adapted from Crockatt (2008)

(Lockwood, 1992). Environmental conditions and host or substrate specificity set the fundamental niche of all fungi, but, as in many other organisms, competition appears to play a key role in determining the realised niche for many fungal species (Peay et al., 2008).

The abundance and diversity of fungi within woodland ecosystems means that contact or proximity between individuals is inevitable. Interactions can take the form of competition, neutralism and mutualism (Rayner and Webber, 1986). Whilst the whole spectrum of types of interaction can be observed in wood decay fungi, mutualistic and cooperative interactions are rare (Rayner and Boddy, 1988; Boddy, 2000). Antagonism is the most common type of interaction and has been reported in every fungal ecosystem, where it is an important factor in determining the organisation, composition and pattern of fungal colonisation (Gloer, 1995). The presence of other mycelia can delimit the domain occupied by an individual, and by active antagonism, nutrient limitation or other effects, these neighbours can restrict its potential growth. Antagonism is not restricted to interspecific interactions, as intraspecific interactions

between genetically distinct individuals (where there is no mating compatibility) can also exhibit combative responses (Rayner and Todd, 1979; Boddy, 2000). However, the processes governing intraspecific antagonism are likely to differ from those governing interactions between different species as they frequently involve genetically controlled incompatibility responses following hyphal fusion (Glass and Dementhon, 2006).

Fungal competition can be split into two main categories: primary resource capture and secondary resource capture (Boddy, 2000). Primary resource capture is the process by which 'pioneer' species gain access to a previously uncolonised resource. Primary colonisers may access live wood as pathogens, through wounds or via insect vectors, or some species may be present as latent propagules within the wood awaiting suitable conditions for growth (Boddy, 2001). Factors affecting the success of primary colonisers are the ability to reach and exploit resources first, which are determined by the efficiency of spore dispersal, spore germination, mycelial extension rate and decomposition ability (Rayner and Webber, 1986; Boddy, 2001). R-selected or ruderal species are favoured, which tend to have a transient existence during which the mycelium will only use those resources that are readily accessible. This allows rapid establishment, reproduction and dispersal. As the wood was previously uncolonised, there is no need for combat to gain access, so primary colonisers are often poor competitors (Holmer and Stenlid, 1997). However, often the primary colonisers may be able to hold or defend their territory quite well, but die back as as gradual changes in the resource occur which are detrimental to them (Griffith and Boddy, 1991).

As wood becomes increasingly colonised, primary colonisers will come into contact and other species may attempt to gain access. Combat between mycelia can take two forms: defence and secondary resource capture (Dowson et al., 1988). Defence strategies used by primary colonisers to resist replacement include production of pseudosclerotial plates and H_2O_2 (Griffith and Boddy, 1991; Silar, 2005). Conditions in the wood may change following primary colonisation, making it more favourable to secondary

colonisers (Griffith and Boddy, 1991). Secondary resource capture species need to either co-exist with primary colonisers, or displace them. Secondary resource capture species tend to have greater competitive ability, and are able to dominate a domain for longer and exploit it fully by utilising the more recalcitrant compounds within. They tend to be k-selected (Rayner and Webber, 1986; Boddy, 2001).

1.4 Mechanisms of combat

White rot fungi employ a variety of combative mechanisms, acting singly or in combination, depending on the species and conditions involved. Combat can begin before contact is established between competitors, either passively through changes in environment (e.g. accumulation of waste products, changes in pH), or actively through production of antibiotic compounds (Boddy, 2000; Wheatley, 2002). Chemical-mediated antagonism depends on the species involved, and implies recognition between species that elicits production of the antibiotic. Combat via contact can occur at the level of single hyphae, or between large regions of mycelia, or, infrequently, via parasitic associations (Boddy, 2000).

1.4.1 Mycoparasitism

Mycoparasitism occurs frequently in nature but is relatively rare among wood decay basidiomycetes. It is a species-specific interaction, where nutrients are obtained from the host species either biotrophically or necrotrophically (Jeffries, 1994). Mycoparasitism is thought to involve four sequential steps: chemotropism of parasitic hyphae towards the host, recognition of host (by lectin-carbohydrate binding), attachment and coiling of the mycoparasite hyphae around host hyphae, and penetration of the host by

production of hydrolytic enzymes (Steyaert et al., 2003; White and Traquair, 2006). Host penetration is followed by growth inside the host resulting in hyphal collapse. Known mycoparasites include *Trichoderma, Gliocladium* and *Pythium* spp., with *Trichoderma* spp. currently comprising one third of all fungal biocontrol preparations produced and sold for control of diseases of horticultural crops (Steyaert et al., 2003). However, parasitism is not the main strategy for nutrient acquisition of these species, rather it is a temporary strategy used to gain territory from a host (Boddy, 2000).

1.4.2 Hyphal interference

Hyphal interference occurs when a hypha makes contact with another hypha or a spore, and results in death of the contacted compartments following a sequence of cytoplasmic destruction (Boddy, 2000). Following non-self recognition, incompatibility systems trigger defence responses and release of non-enzymic diffusible metabolites (e.g. H_2O_2) which cause cell death and lysis of one or both of the compartments involved (Micali and Smith, 2003; Silar, 2005). Hyphal interference is fairly common among wood decay basidiomycetes; the most studied example is the interaction between *Phlebiopsis gigantea* and *Heterobasidion annosum*, where any hyphae of *H. annosum* that make contact with *P. gigantea* show rapid localised disruption of the cytoplasm and cell membrane (Adomas et al., 2006). This has applications in biocontrol of *H. annosum* (e.g Stenlid, 1994).

1.4.3 Gross mycelial contact

Gross mycelial contact occurs when whole colonies are involved in confrontations. This non-selective replacement occurs between a wide range of fungi and is very common in wood decay species. Though generally considered non-selective, perhaps this is

somewhat of a misnomer as a degree of selectivity can be inferred by substratum specificity, or where conditions generated by one fungus make replacement by a specific successor more likely. Certain secondary capture species have strong dependencies on specific preceding decay fungi, probably due to changes in abiotic conditions generated by the preceding fungus such as lignocellulose composition or water potential (Griffith and Boddy, 1991; Holmer and Stenlid, 1993, 1997).

The effects of gross mycelial contact are easily seen on agar culture during interactions, where the morphology of a colony changes dramatically in response to the presence of a combatant. Changes occur in the whole mycelium, but most dramatically in areas in direct contact with the competitor: the interaction zone. Common changes observed are pigment production, redistribution of mycelium, induction of sporulation and formation of hyphal assemblages (Rayner and Webber, 1986; Rayner and Boddy, 1988; Dowson et al., 1988). These changes may also occur in response to interactions with other microorganisms (e.g. *Bacillus subtilis*; Rayner et al., 1994).

Hyphal assemblages are aggregations of hyphae (thought to be non-assimilative) that can be divided into four groups based on function (Rayner et al., 1994). (1) Barrages, or defensive zones, consist of stationary assemblages of dense aerial and submerged hyphae and function as a barrier against invasion. They are typically produced by one or both of the competing mycelia. (2) Invasive mycelial replacement fronts have clearly defined margins and consist of regular arrays of diffuse or loosely associated hyphae. These can invade competitor mycelium in broad sections at the interaction interface. (3) Mycelial cords consist of semi-linear hyphal aggregations arising from localised foci at the interaction interface, which invade competitor mycelium more rapidly but more irregularly than replacement fronts. (4) Mycelial fans also arise from localised foci at the interaction interface, but the constituent hyphae are more diffuse and primarily radially oriented (Rayner et al., 1994; Boddy, 2000). The emergence of hyphal assemblages may differ along the length of the same interaction interface,

and can be regarded as indeterminate, feedback-regulated mycelial responses to local circumstances (Rayner et al., 1994). Their production determines interaction outcome.

1.5 Outcomes of interactions

There are a spectrum of potential outcomes, from intermingling of colonies to complete replacement of one competitor by another. Intermingling generally occurs during mycoparasitism, or when a mycelium of an isolate meets itself and there is self-recognition. Intermingling can also occur when mycelia of opposing colonies are sparse enough that hyphae can interdigitate without actually making contact, for example in conditions of poor nutrient availability (Rayner and Webber, 1986; Tordoff et al., 2006). The most common interaction outcomes are deadlock and replacement (Boddy, 2000). Deadlock occurs when neither individual is able to gain territory from the other; replacement occurs when one individual invades the domain of another and gains control of the territory and resources within. There is a continuum of outcomes between the two extremes of deadlock and total replacement, for example partial replacement followed by deadlock, or mutual partial replacement in different regions.

Interaction outcomes are affected by a range of biotic and abiotic factors. The outcome of an interaction is largely dependent on the combative ability of the species involved (Holmer and Stenlid, 1997), but the outcome may not always be consistent between different conditions, or between different genotypes (Boddy, 2000). The size of the territory occupied by mycelia affects the outcome of interactions (Shearer, 1995; Holmer and Stenlid, 1993, 1997), as does the spatial configuration of competitors, especially where there is contact with more than one competitor (White and Boddy, 1992; Kennedy et al., 2007). The state of decay of the substrate can also affect the outcome of an interaction (Wells, 2002). Interactions have been observed under a variety of experimental conditions including different gaseous regimes, pH, temperature,

water potentials and nutrient availability (Boddy and Rayner, 1983; Boddy et al., 1985; Griffith et al., 1994; Wald et al., 2004). Abiotic conditions were shown to affect the outcome of interactions, as different species may cope with different conditions better than others, or it may influence the ecological strategy a species adopts.

For all interactions there is a dynamic equilibrium in operation, and over time resources may become depleted and conditions may change, which in turn changes the status of the interaction. Outcomes are also influenced by the interactions between fungi and other organisms such as bacteria and invertebrates (Baldrian, 2006; Tordoff et al., 2006). Variation in outcomes under different abiotic conditions, and the biotic effects of other organisms on interactions, makes prediction of community change impossible (Boddy, 2000).

1.6 Studying interactions

Many previous studies have investigated interactions, confronting species using a variety of systems, for example inoculating onto agar, liquid broth, wood blocks, soil trays, logs, sawdust, microscope slides, three-dimensional columns and gradient plates (including Holmer and Stenlid, 1997; Donnelly and Boddy, 2001; Baldrian et al., 2005; Tordoff et al., 2006). Interactions are easily observed in agar culture, which allows controlled manipulation of environmental factors and allows easy separation of mycelium from the media for study. Although agar culture can offer valuable insight into the mechanisms involved in interactions, it is debatable whether interaction outcomes on agar are representative of those that occur in the field. Some studies indicate a strong correlation between the results of interactions on agar plates and those in wood, however, others do not (Rayner and Todd, 1979; Holmer and Stenlid, 1993; Owens et al., 1994; Wells, 2002). Fungal growth in wood is spatially restricted by the tracheid walls, a constraint that is not present in agar culture, which may explain some of the differ-

ences (Holmer and Stenlid, 1993). Ideally, a combination of methods is necessary for the full understanding of interactions.

1.7 Production of volatile and diffusible organic compounds

In some situations interactive events begin before mycelial contact is established, through the action of diffusible and/or volatile organic compounds (DOCs and VOCs). Chemical signalling is likely to play a major role in fungal recognition systems. Fungal metabolites have been shown to play antagonistic roles in the diseases of plants and insects, in animal poisonings, as well as in interspecific mycelial interactions (Gloer, 1995). They often have antibiotic properties that have been exploited in medicine and industry. Many fungi constitutively produce a profile of VOCs and DOCs, frequently species-specific and reproducible, which can be used as a 'fingerprint' for their identification (Woodward et al., 1993; Nilsson et al., 1996; Bruce et al., 2000; Heilmann-Clausen and Boddy, 2005). DOCs and VOCs may function (at least in part) to protect occupied territory from potential invaders. Some exert their effects indirectly, for example by lowering pH, whilst others can be considered to be antibiotics (Woodward and Boddy, 2008). Following detection of a competitor, the composition of the secondary metabolite profile may change (Griffith et al., 1994; Hynes et al., 2007), indicating specific functions during combat.

Exposure to competitor DOCs has been shown to affect spore germination and foraging behaviour in fungi, to dramatically alter the mycelial morphology of *Hypholoma fasciculare*, and to increase ligninolytic enzyme production in *Marasmius pallescens* (Rayner et al., 1994; Heilmann-Clausen and Boddy, 2005; Ferreira-Gregorio et al., 2006). A variety of chemical classes have been reported as potential antagonistic DOCs, for example

aromatic compounds (Wheatley, 2002; Wald et al., 2004). DOCs may have an antagonistic effect on certain potential invaders, but they could also stimulate the growth of other species (Heilmann-Clausen and Boddy, 2005). Primary fungal colonisers have a marked influence on subsequent establishment of later fungal colonisers (Heilmann-Clausen and Boddy, 2005). These predecessor-successor relationships may be mediated by secondary metabolites or their by-products. Release of secondary metabolites by preceding species may inhibit the colonisation and establishment of most secondary colonisers, excepting the well-adapted natural successor (Holmer and Stenlid, 1997). This is believed to be supported by the fact that in interaction combinations that would not be found in nature (due to different host species etc.), less combative primary colonisers are often more successful than combative secondary colonisers (Holmer and Stenlid, 1997; Heilmann-Clausen and Christensen, 2003; Peiris et al., 2008).

DOCs have antagonistic potential in circumstances where they can accumulate or diffuse through aqueous media, but could only feasibly function over short distances. VOCs, however, can function in a much more heterogeneous environment, and have the potential to act as long range signals, inducing behavioural changes in potential competitors at a distance from the organism producing them. A variety of volatile chemicals are produced by fungi, which have a variety of potential ecological roles (Schoeman et al., 1996; Wheatley et al., 1997; Abraham, 2001), including acting as attractants to 'pollinators' and foraging insects (Schiestl et al., 2006; Steiner et al., 2007). They may affect fungal development and can also induce defence responses in plants (Mendgen et al., 2006; Splivallo et al., 2007). VOC profiles are affected by the substrate, both in terms of overall composition and also by specific components in the medium (Wheatley et al., 1997; Bruce et al., 2000; Wheatley, 2002); for example, the VOC profile of *Serpula lacrymans* is more complex when grown on pine shavings compared to growth on agar (Ewen et al., 2004). VOC production is also affected by temperature, pH and culture age (Tronsmo and Dennis, 1978; Chen et al., 1984; Nilsson et al., 1996; de Jong and Field, 1997; Jelen, 2002). During interactions between fungi

different VOC profiles have been detected compared to those produced by individual species grown alone (Hynes et al., 2007). The nature of the effects of VOCs on fungi at the molecular level is largely unknown. However, exposure to VOCs may change the profile of proteins produced, and thus the implication is that VOCs may have effects at the level of gene expression (Humphris et al., 2002; Myung et al., 2007).

1.8 Metabolic changes during interactions

Changes in production of metabolites have been shown to occur during interspecific interactions (including Rayner et al., 1994; Hynes et al., 2007; Eyre, 2007; Peiris et al., 2008), and there is considerable evidence of differential regulation of particular groups of enzymes (including Freitag and Morrell, 1992; Baldrian, 2004; Lindahl and Finlay, 2006). Huge metabolic changes occur in interacting mycelia, necessary for the production of antagonistic compounds, morphological changes, and re-assignment of nutrients (Boddy, 2000; Donnelly and Boddy, 2001). Many of these changes are classed as secondary metabolism. It has been proposed that interactions stimulate the synthesis of hydrophobic metabolites such as phenolics, which, after conversion to melanins, lead to the sealing-off of hyphal boundaries at the interaction interface (Rayner et al., 1994). These events would initiate secondary metabolism by disrupting resource supply, ultimately leading to the production of reactive oxygen species (ROS) through the disruption of oxidative phosphorylation (Rayner et al., 1994).

Increases in primary metabolism during interactions have been reported, as evidenced by increased CO_2 evolution and protein production (Owens, 1989; Freitag and Morrell, 1992). Most of the differentially regulated metabolites identified in *Stereum hirsutum* during interactions with *Coprinus disseminatus* or *Coprinus micaceous* were primary metabolites (Peiris et al., 2008); however, as the majority of the metabolites produced during these interactions were not identified, the induction of secondary metabolites

cannot be ruled out. Interestingly, there was up-regulation of metabolites and production of interaction-specific metabolites during interactions where *S. hirsutum* was replaced by *C. disseminatus*, but down-regulation of metabolites when *S. hirsutum* overgrew *C. micaceous* (Peiris et al., 2008). This suggests that the up-regulation of metabolites in *S. hirsutum* is connected to a defensive role, rather than an aggressive role.

Exposure of cultures of *H. fasciculare* and *Phlebia radiata* to DNP (2,4-dinitrophenol) had similar effects on development and metabolite profiles to the effects of interactions: generally suppressing metabolite release (Rayner et al., 1994; Griffith et al., 1994). DNP disrupts oxidative phosphorylation and is thought to be involved in the switch to secondary metabolism. However, whilst cultures of *S. hirsutum* exposed to DNP showed decreased growth rate, metabolite profiles of these cultures did not resemble those of interacting cultures (Peiris et al., 2008), implying that interaction responses in *S. hirsutum* result from detection of a competitor, rather than as a response to oxidative stress as observed in *H. fasciculare* and *P. radiata*.

1.9 Molecular studies of interactions

Little is known about the molecular basis of fungal interspecific interactions. Most studies of fungal gene expression have used model organisms such as *Aspergillus nidulans* and *Saccharomyces cerevisiae*, and what work has been done using basidiomycetes has focused on fruit body development, or different types of hyphal growth (e.g. Wessels, 1992; Nugent et al., 2004). Studies of fungal interactions have centred around their interaction with plants or with bacteria (e.g. Cramer and Lawrence, 2004; Schrey et al., 2005). Fungal-fungal interactions that have been investigated from a molecular perspective have had potential for biocontrol, for example the biocontrol of *Heterobasidion* species by *Physisporinus sanguinolentus* (mycoparasitic interaction) and

Phlebia gigantea (hyphal interference), and between *Trichoderma hamatum* and the phytopathogen *Sclerotinia sclerotiorum* (Iakovlev et al., 2004; Carpenter et al., 2005; Adomas et al., 2006). Eyre (2007) conducted the first study addressing changes in gene expression during interspecific basidiomycete interactions with different outcomes. Microarrays were used to detect changes in gene expression of *Trametes versicolor* during interactions where *T. versicolor* replaced, deadlocked with, or was replaced by the competitor.

A subtractive cDNA library was generated for *T. versicolor* during interaction with *Stereum gausapatum* (resulting in replacement of *S. gausapatum* by *T. versicolor*) compared to during growth alone. Of the ESTs (expressed sequence tags) identified as changing during this interaction, 24% were involved with metabolism and proteolysis, 10% were transcription-related, 5% related to signalling processes and 8% were involved in stress or protective responses (Eyre, 2007). The EST library was tested by microarrays using cDNA from *T. versicolor* during interactions with *S. gausapatum* (replacement by *T. versicolor*), with *Bjerkandera adusta* (deadlock), and with *Hypholoma fasciculare* (replacement by *H. fasciculare*). Interactions where *T. versicolor* was successful (i.e. where it replaced or deadlocked with its competitor) showed upregulation of genes involved in post-transcriptional regulation, cell wall biosynthesis, carbohydrate metabolism and starch breakdown (Eyre, 2007). Similar upregulation of proteins important in substrate utilisation and nutrient acquisition occurred in *P. gigantea* when it overgrew *Heterobasidion parviporum* (Adomas et al., 2006), although this interaction involves hyphal interference so comparisons to the non-selective combat occurring in *T. versicolor* interactions must be treated with caution. During interactions where *T. versicolor* was not successful and was replaced by the competitor, a smaller number of targets changed in expression, and the pattern of genes changing in expression was subtly different (Eyre, 2007).

Down-regulation of cell growth and division was common to all three interactions in-

volving *T. versicolor*, perhaps indicating a situation where hyphal extension is favoured over cell division (Eyre, 2007). Up-regulated in all three interactions were a transcript homologous to a ubiquitin-activated enzyme and two NADPH oxidases. Ubiquitin-mediated proteolysis is an important regulator in all eukaryotes for removing unwanted proteins (Aguilar and Wendland, 2003). Increased ubiquitin-mediated proteolysis has been implicated in other stress responses of *T. versicolor* such as nitrogen deprivation (Staszczak, 2008), and in other basidiomycete species in response to heat shock and UV (Higgins and Lilly, 1993; Taupp et al., 2008). NADPH oxidases are important in the generation of ROS, and increased production of ROS has been associated with interaction zones in several interactions (Tornberg and Olsson, 2002; Silar, 2005). Interestingly, one of the interaction-induced genes during mycoparasitism of *Heterobasidion annosum* by *P. sanguinolentus* was a protein involved in DNA repair and stress responses to ROS damage (RAD51; Iakovlev et al., 2004).

1.10 The role of ligninolytic enzymes during interactions

More than 100 different types of enzyme have been isolated from fungi, and isozymes vary in number and function between species (Baldrian et al., 2005; Luis et al., 2005). There is considerable evidence of different regulation of particular groups of extracellular enzymes during interactions, most notably the increases in ligninolytic enzyme activity (including Freitag and Morrell, 1992; Baldrian, 2004; Lindahl and Finlay, 2006; Ferreira-Gregorio et al., 2006). The primary function of these laccases and peroxidases is the non-specific oxidative attack of the lignin polymer (Leonowicz et al., 2001). Their function during interactions is unclear, but they have a variety of potential roles.

1.10.1 Laccase

Laccases (benzenediol:oxygen oxidoreductases) are multicopper oxidases, produced by certain Basidiomycota and Ascomycota (Baldrian, 2006), with high levels of production by white rot fungi. Laccases are also widely distributed among plants where they are involved in the synthesis of lignin and in the wounding response (Piontek et al., 2002). Laccase acts to oxidise a variety of hydrogen donors, including phenolic hydroxyl groups, phenolic methyl groups, polyphenols and aromatic diamines (Leonowicz et al., 2001; Mayer and Staples, 2002). It is difficult to define laccase by its reducing substrate due to the broad range of compounds, which varies from one laccase to another and overlaps with the substrate range of the monophenol monooxygenase tyrosine (although laccases cannot oxidise tyrosine; Baldrian, 2006). More detailed information on laccases can be found in Appendix A.1.

Laccase catalyzes the four electron reduction of O_2 to H_2O, coupled with four single-electron substrate oxidation steps (Thurston, 1994). Each one-electron oxidation generates a free radical, which is typically unstable and may undergo a second enzyme-catalyzed oxidation, or non-enzymic reactions such as hydration or disproportionation and/or polymerisation (Thurston, 1994). The overall reaction catalyzed by laccase is:

$$O_2 \; + \; 4AH \; \longrightarrow \; 2H_2O \; + 4 \cdot A$$

where AH is the reduced form of the substrate, and $\cdot$A is the oxidised form. Fungal laccases have a higher redox potential than other blue multicopper oxidases, and a large range in reduction potentials between different laccases. The reasons for this are not fully understood but are thought to be controlled by structural determinants (Jönsson et al., 1995).

1.10.2 Peroxidases

Peroxidases utilise hydrogen peroxide (H_2O_2) to catalyse the one-electron oxidation of a range of organic and inorganic compounds. The majority contain a heme (protoporphyrin) group in the catalytic centre and fall into two superfamilies based on differences in this group: the mammalian and plant peroxidase superfamilies. Fungal peroxidases are included in Class II of the plant peroxidase superfamily based on sequence homology (Welinder, 1992). The main peroxidases are lignin peroxidase (LiP) and manganese peroxidase (MnP), although others such as versatile peroxidase and haloperoxidases may play lesser roles (Conesa et al., 2002). LiP and MnP were first discovered in *Phanerochaete chrysosporium*, and have since been isolated from a wide range of white rot fungi, although not all (Conesa et al., 2002). More detailed information on peroxidases can be found in Appendix A.2.

Peroxidases catalyse a 2-substrate, 2-product reaction. There are several suggested mechanisms for peroxidase reactions, the most common of which is:

$$\text{Native peroxidase} + H_2O_2 \longrightarrow \text{Compound I} + H_2O$$
$$\text{Compound I} + AH \longrightarrow \text{Compound II} + \cdot A$$
$$\text{Compound II} + AH \longrightarrow \text{Native peroxidase} + \cdot A + H_2O$$

where AH is the reduced substrate, and $\cdot$A is the oxidised substrate, and Compounds I and II are the oxidised forms of peroxidase. The sum of the 3-step reaction is:

$$H_2O_2 + 2AH \longrightarrow 2H_2O + 2 \cdot A$$

Common to all peroxidases is the requirement for extracellular H_2O_2, the electron acceptor. Nearly all peroxidases also require mediators to function. Mediators are easily oxidisable, small-sized substrates that, once oxidised by peroxidase (or laccase),

mediate the 'indirect' oxidation of substrates (Tadesse et al., 2008). Ligninolytic peroxidases differ in terms of their reducing substrates and mediators. LiPs catalyse the oxidation of a wide variety of aromatic nonphenolic compounds (Martinez, 2002). The preferred substrate for LiP is the mediator veratryl alcohol (VA) which is secreted by the fungus. VA acts to reduce LiP-Compound II, which has a lower redox potential than LiP-Compound I, thus enabling enzyme turnover in the presence of recalcitrant substrates (Martinez, 2002). VA also acts to oxidise substrates that LiP cannot access due to its size (Conesa et al., 2002). Although LiP is able to directly oxidise lignin dimers, VA is required for depolymerisation of synthetic lignin (Dunford, 1999; Martinez, 2002).

MnP catalyses the manganese-mediated oxidation of aromatic phenolic compounds (Martinez, 2002). MnP is relatively specific for reducing substrates and only Mn^{2+} efficiently supports enzyme turnover (Conesa et al., 2002; Martinez, 2002). MnP-Compound I can be reduced by phenolic compounds and Mn^{2+}, but only Mn^{2+} can reduce MnP-Compound II (Martinez, 2002). MnP oxidises Mn^{2+} to Mn^{3+}, which is a strong oxidiser but unstable in aqueous media, so it is stabilised by chelation with organic acids such as oxalate and malonate, both of which are secreted by the fungus. The chelated Mn^{3+} acts as a diffusible oxidising agent (Conesa et al., 2002; Martinez, 2002).

Certain peroxidases defy easy classification. Versatile peroxidase (VP), isolated from several species including *Pleurotus* and *Bjerkandera* spp., functions to directly oxidise hydroquinones and substituted phenols that are not efficiently oxidised by LiP or MnP in the absence of VA or Mn^{2+} (Camarero et al., 1999; Ruiz-Dueñas et al., 2009). VP has structural features of both LiP and MnP, and is able to oxidise both Mn^{2+} and VA (Camarero et al., 1999). *T. versicolor* produces a type of VP that is repressed by Mn^{2+}, the so-called manganese-repressed peroxidase (MRP; Collins et al., 1999). These 'hybrid' enzymes demonstrate the close relationship between different types of

peroxidase (Hofrichter, 2002).

In most peroxidases, direct access to the heme is restricted, but the heme edge is available for substrate interactions (Conesa et al., 2002). An exposed tryptophan is a conserved residue present in all LiPs, and is an oxidation site for VA and other aromatic substrates. Replacement of this residue by site-directed mutagenesis resulted in loss of VA-oxidising ability (Doyle et al., 1998), and inclusion of the residue into MnP resulted in an enzyme which could oxidise VA (Timofeevski et al., 1999). In MnP there are three conserved acidic residues (two glutamic acids and one aspartic acid) which are involved in Mn^{2+}-oxidation, but not in the reaction with phenolic substrates of H_2O_2. Both the tryptophan residue and the three Mn^{2+}-binding residues are present in VPs (Ruiz-Dueñas et al., 2009).

1.10.3 Localisation of laccase and peroxidase activity

Laccases and peroxidases should occur exclusively extracellularly due to their high oxidative ability and location of substrates. However, intracellular laccases have been detected in several species (Dittmer et al., 1997; Schlosser et al., 1997; Velázquez-Cedeño et al., 2004; Baldrian et al., 2005), although in all cases the vast majority of activity was extracellular. A substantial part of *T. versicolor* and *P. ostreatus* laccase was found to be cell wall-associated, and some MnP may also be wall-associated as liquid cultures of *T. versicolor* have demonstrated MnP activity in bound fractions (Valášková and Baldrian, 2006). Differences in localisation are probably connected with physiological function and determine the range of substrates available to the enzymes (Baldrian, 2006).

1.10.4 Regulation of gene expression

Many external regulatory factors have been identified, with conditions for expression of different genes varying between isolates and species (Conesa et al., 2002; Chi et al., 2007). Laccase-encoding genes are disparately regulated at transcriptional levels, some constitutively expressed, and some inducible (Mäkelä et al., 2006). Nutrient status affects laccase and peroxidase production in some species, but whilst carbon and nitrogen limitation may trigger gene expression in some species, for others high carbon and nitrogen may act as triggers (Gold and Alic, 1993; Kaal et al., 1995; Schlosser et al., 1997; Galhaup et al., 2002; Mikiashvili et al., 2005). Phenolic compounds induce lignolytic gene expression in certain species, especially compounds related to lignin or lignin derivatives (Galhaup et al., 2002).

The presence of redox mediators can stimulate peroxidase production. VA stimulated production of both LiP and MnP in *Phlebia radiata, P. chrysosporium* and *T. versicolor* (Lundell et al., 1990; Rogalski et al., 1991; Niku-Paavola et al., 1990). Similarly, transcription of MnP genes is regulated by Mn^{2+}; in *P. chrysosporium* putative metal response elements (MREs) have been identified upstream of two out of three MnP genes, with addition of Mn^{2+} increasing expression of the MRE-linked genes only (Kersten and Cullen, 2007). Conversely, although MREs are not found upstream of MnP genes in *T. versicolor* or *Cerporiopsis subvermispora*, addition of Mn^{2+} does increase MnP production by these species (Johansson and Nyman, 1993; Kersten and Cullen, 2007). Copper has also been shown to regulate laccase transcription in a variety of species (including Collins and Dobson, 1997; Galhaup et al., 2002; Álvarez et al., 2009).

Stress is also an important regulatory factor, with putative xenobiotic response elements (XREs) occurring in the promoter regions of LiP and MnP genes of *P. chrysosporium* and *Pleurotus eryngii* (Fink-Boots et al., 1999; Kersten and Cullen, 2007). Lac-

case and peroxidase activity have been shown to increase in cultures following heat shock (Fink-Boots et al., 1999) and oxidative stress (Jaszek et al., 2006a), and activity of laccase but not peroxidase increased after heavy metal exposure (Baldrian et al., 2005).

1.10.5 Functions of ligninolytic enzymes

The highly oxidative, non-specific nature of ligninolytic enzymes, plus the variety of isozymes, enables a wide range of potential roles during fungal growth. Their primary role is in the decomposition of lignin; yet the actual role of laccase during this process is not completely understood. Inhibition of laccase in cultures of *T. versicolor* did not affect lignin breakdown (Evans, 1985), whereas it did in cultures of *Sporotrichium pulverulentum* and *Pycnoporus cinnabarinus* (Ander and Eriksson, 1976; Eggert et al., 1996; Baldrian, 2006). Laccase alone is incapable of decomposing lignin model compounds, but can do so in the presence of a mediator. The natural laccase mediators are unknown for most species (Eggert et al., 1996). Laccases may act to break down toxic compounds generated by other ligninolytic enzymes before they can enter the hyphae, and the reduced ligninolytic ability in laccase-deficient cultures may result from the toxic effects of these reactive species (Thurston, 1994; Solomon et al., 1996).

Laccase and peroxidases are produced in response to stress, so have potential defensive functions. Peroxidases and laccases initiate the polymerisation and cross-linking of melanins, both wall-bound and extracellular (i.e. synthesised completely apart from cell walls; Bell and Wheeler, 1986; Henson et al., 1999). Whilst not essential for growth and development, melanins enhance the survival and competitive abilities of species in certain environments. In contrast to the intracellular location of melanins in animal cells, fungal melanins occur either in cell walls or as extracellular polymers formed in the medium around cell walls. Melanins constitute appreciable amounts of the biomass

produced by saprotrophic fungi (Bell and Wheeler, 1986), and are formed from different biochemical pathways to melanins of animals and plants. Wall-bound melanins of basidiomycetes are derived from γ-glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol, whilst extracellular melanins are formed from various phenols (fungal, plant or agrochemical) and proteins, carbohydrates and lipids present in the fungal environment (Bell and Wheeler, 1986). Melanin confers structural strength, and protection from ROS, toxins and temperature extremes (Henson et al., 1999), and melanins from the cell walls of *Phellinus weirii* also have antibiotic properties (Haars and Hutterman, 1980). However it is thought the most important role of melanin is protecting against hydrolytic enzymes; generally, the ability of hydrolytic enzymes to degrade fungal walls is inversely correlated with the melanin content of the wall (Bloomfield, 1967). Pigmentation in fungal cultures is often the result of melanisation, and thus is also dependent on laccase and peroxidase activity (Leatham and Stahman, 1981; Solomon et al., 1996). The formation of extracellular pigments coupled to oxidative polymerisation of cell wall components strengthens cell-to-cell adhesion and confers rigidity to structures, crucial in fungal morphogenesis. Laccase and peroxidase activity are associated with these changes in morphogenesis, for example laccase activity increases during fruit body formation in *Schizophyllum commune* (de Vries et al., 1986).

1.11 Ecological implications of interspecific interactions

Ecosystems are complex networks of inter-relationships between many organisms and the environment on many different levels. Interactions between wood decay fungi and their environment influence the rate of decomposition, drive niche differentiation and act to maintain diversity and function. Fungal interactions are inevitable within decaying wood, and they determine the pattern of colonisation and develop-

ment of decay communities (Rayner and Todd, 1979). Since different species effect decomposition, mineralisation and nutrient translocation to different extents, interaction outcomes will impact directly onto these processes. Also, the interaction itself may affect these processes; interactions can affect the movement and partitioning of carbon within mycelial systems, and mineral nutrient uptake, movement and release (Holmer and Stenlid, 1997; Wells, 2002). Interspecific interactions are one of the main factors releasing nutrients to soil (along with grazing by invertebrates), for example interactions between *Phanerochaete velutina* and *H. fasciculare* resulted in significantly greater losses of ^{32}P to soil than self-pairings, with leakage not only occurring in the interaction zone but also elsewhere (J.M. Wells and L. Boddy, unpub.). During interactions, the decay rate may change, perhaps through increased activity of ligninolytic enzymes. Both increases and decreases in decay rate have been reported in different interactions (Boddy, 2001; Chi et al., 2007; Woodward and Boddy, 2008). Clearly, fungal interactions have a huge impact on the decomposition of woody debris.

1.12 Industrial applications of interactions

Interspecific interactions have been manipulated for several industrial purposes, and there is potential for development of further applications as the processes involved are more fully understood. Aggressive isolates have potential as biological control agents of nuisance wood decay fungi; probably the most successful example is the use of *P. gigantea* to protect felled pine and spruce stumps against the establishment of *H. annosum* (causative agent of root and butt rot; Stenlid, 1994). Similarly, antagonistic non-wood decay organisms, e.g. *Trichoderma* sp., have been used to inoculate wounds in trees (such as those caused by pruning) to prevent establishment of wood decay fungi (Rayner and Boddy, 1988; Score et al., 1997).

The high metabolic diversity, and the potential number of fungal species, mean that

there is special interest in fungal metabolites as starting materials for pharmaceuticals. Many metabolites produced by fungi are bioactive (Abraham, 2001). The increased production of secondary metabolites during interactions, or the increased diversity in metabolite production, could provide a route for elucidation of further compounds with pharmaceutical potential.

The increase in ligninolytic enzyme production that occurs in many interactions has potential to be used in industrial applications, mainly in biopulping and bioremediation. Ligninolytic enzymes are capable of *in vitro* transformation or mineralisation of a wide range of highly recalcitrant organopollutants including synthetic dyes, pesticides, munition wastes and fuel additives (Pointing et al., 2005). Enhanced degradation of pollutants during interactions has been reported during co-culture of white rot fungi and microorganisms, and there is potential for using this in bioremediation of contaminated soils (Baldrian, 2004). Production of laccase and peroxidases by certain white rot fungi, most notably *T. versicolor*, have been shown to bleach and delignify kraft pulp, a product of the common chemical delignification method used in paper-making (Paice et al., 1989), which is a more environmentally-friendly alternative to the current bleaching methods. Co-culturing of fungi can increase lignin decomposition, although it depends on the combination of species used (Chi et al., 2007). However, despite the potential of using fungi in these treatments, it takes much longer than the chemical methods (Addleman and Archibald, 1993), and the current level of understanding of the processes involved is too inadequate to be industrially viable.

1.13 Project objectives

Little is known about the chemical basis of interspecific interactions between saprotrophic basidiomycetes. The relationship between interaction outcome and production of enzymes and metabolites has not been explored. Nor have the differences in enzyme

production and combative ability between different isolates, at different stages of the fungal lifecycle. A better understanding of the chemicals and enzymes involved in interactions, and how they are regulated, may help lead to a better understanding of the fungi themselves. Previous work used a single species, *T. versicolor*, to investigate changes in gene expression where it replaced, deadlocked with, or was replaced by a competitor (Eyre, 2007). *T. versicolor* was suitable because it is a common species with intermediate combative ability (late primary to early secondary coloniser), and thus displays a range of interaction outcomes with other species. To this end, the objectives of this project are:

- Qualitatively (activity staining) and quantitatively (enzyme assays) measure changes in activity of laccase and peroxidases during interactions involving *T. versicolor* that display a range of outcomes

- Use semi-quantitative RT-PCR to compare laccase and peroxidase gene expression in *T. versicolor* mycelia during interactions with a range of outcomes

- Compare enzyme activity and gene expression in different regions of *T. versicolor* mycelia during interactions

- Use GC/MS (gas chromatography-mass spectrometry) to identify the profile of VOCs produced during interactions involving *T. versicolor* and make comparisons to self-pairing VOC profiles

- Determine whether VOC and DOC profiles are directly antagonistic, and/or whether they act as long-distance chemical signals, by exposing them to remote mycelia

- Compare combative ability and enzyme production between isolates of *T. versicolor*, and establish whether there are any differences in these characters between homokaryons and heterokaryons

Chapter 2

Ligninolytic enzyme activity during interspecific interactions

2.1 Introduction

There is considerable evidence for the up-regulation of ligninolytic enzymes during antagonistic interactions between wood decay species (Freitag and Morrell, 1992; White and Boddy, 1992; Iakovlev and Stenlid, 2000; Baldrian, 2004; Chi et al., 2007; Ferreira-Gregorio et al., 2006; Peiris, 2009). The extent of the increase in activity differs, depending on the innate enzyme producing ability of a species, and the combination of species interacting (Iakovlev and Stenlid, 2000; Chi et al., 2007). Within interacting mycelia, spatial patterns of activity are inconsistent between interaction combinations, although some general trends have been observed. For example, the interaction-induced increase in mycelial laccase decreased with distance from the interaction zone (Iakovlev and Stenlid, 2000). As yet, no study has fully investigated the links between ligninolytic enzyme activity during interactions and the outcomes of these interactions.

Though their primary role is in lignin degradation, the highly oxidative and non-specific nature of laccase and peroxidase permits a variety of other potential roles. Laccase and peroxidase activity have been shown to increase during interactions (White and Boddy, 1992; Iakovlev and Stenlid, 2000; Baldrian, 2004; Ferreira-Gregorio et al., 2006; Chi et al., 2007; Peiris, 2009), and also in fungal cultures exposed to abiotic stress such as temperature (Fink-Boots et al., 1999), heavy metals (Baldrian et al., 2005) and oxidative stress (Jaszek et al., 2006a). Activities of laccase and peroxidases are not necessarily coincident within interactions, indicating differences in their roles (White and Boddy, 1992).

Accumulation of ROS and H_2O_2 is associated with interaction zones, likened to the oxidative burst defence reaction of plant and animal cells (Tornberg and Olsson, 2002; Silar, 2005), and increased laccase and MnP production has been reported in fungal cultures in response to chemically-induced oxidative stress (Jaszek et al., 2006a,b). Peroxidases could mediate oxidative stress by removing H_2O_2; catalase levels are also known to increase in *T. versicolor* cultures during periods of oxidative stress (Jaszek et al., 2006a; Zhao et al., 2009), and production may increase during interactions for the same purpose. Laccase and peroxidases are involved in the generation of melanins which may protect hyphae from toxic compounds and ROS (Henson et al., 1999). Interestingly, peroxidases may also function aggressively to degrade the melanins of an opponent, as MnP effects degradation of both synthetic and natural melanins (Henson et al., 1999). Laccase may directly detoxify antagonistic compounds, as it is known to detoxify fungicides in yeast (Baldrian, 2006). Production of laccase and peroxidase may be stimulated by toxins themselves, as production has been shown to increase in cultures exposed to competitor DOCs (diffusible organic compounds; Ferreira-Gregorio et al., 2006; Peiris, 2009). Increased enzyme activity may also be a result of changes in morphology, as increases in laccase and peroxidases were detected during production of hyphal cords in *H. fasciculare*, and concurrent with the change from coenocytic to septate mycelium in *Phlebia rufa* and *P. radiata* which occurs prior to production of

barrages and replacement fronts (Griffith et al., 1994; Rayner et al., 1994).

Aside from a defensive function, increased laccase and peroxidase activity during interactions may imply increased acquisition of nutrients, and there is evidence of increased production of non-ligninolytic enzymes involved in wood decomposition. Activity of β-glucosidase, an exo-cleaving cellulase which primarily functions to hydrolyse cellobiose oligomers to glucose, increased during interactions between *T. versicolor* and *Trichoderma harzianum* (Freitag and Morrell, 1992). Acid phosphatase occurs in ascomycetes and basidiomycetes, and functions to release P_i from organophosphates. Acid phosphatase activity could function during interactions to release bound organophosphates from captured opponent mycelium (Reyes et al., 1990). Increased chitinase activity occurred during interactions involving *Hypholoma fasciculare, Resinicium bicolor* and *Coniophora arida* (Lindahl and Finlay, 2006). Chitinases function to degrade chitin, and the chitinous cell walls of previous colonisers would be an important nitrogen source for later colonisers (Patil et al., 2000). Other roles of chitinases during interactions include morphological changes, cell wall modifications, or permeabilisation of competitor cell walls to allow entry of toxic or antifungal compounds (Lindahl and Finlay, 2006).

Trametes versicolor is one of the most efficient lignin decomposers, due to its ability to produce high levels of ligninolytic enzymes during growth on natural or artificial media, including laccase, LiP, MnP and MRP (Mn-repressed peroxidase). Probably the majority of work done on *T. versicolor* enzyme production has aimed to assess the impact of culture conditions on enzyme activities, to take advantage of its ligninolytic abilities in industrial processes. Changes in ligninolytic enzyme activity occur during growth on several different substrates and nutrient regimes (White and Boddy, 1992; Archibald et al., 1997; Schlosser et al., 1997; Mikiashvili et al., 2005) and also during interactions with a range of species (Freitag and Morrell, 1992; White and Boddy, 1992; Baldrian, 2004).

With the exception of studies that use plate-based assays to screen for enzyme production, the majority of research into ligninolytic enzyme activity during interactions has been performed in liquid culture. Liquid culture is inappropriate for several reasons. There is diffuse mixing of mycelia rather than the delimited, defended territories observed in natural interactions. This makes it nearly impossible to deduce which combatant, if not both, is responsible for any enzyme activity. Also, most of the mycelial growth is submerged, which would affect the oxygen availability. Other studies have used wood shavings or soil, and although working with natural substrata is most ecologically relevant, agar culture has the advantage of being consistent in terms of nutrients, allows sampling of different regions within the mycelia of interacting fungi, and provides a much more realistic interface than in liquid culture. Interactions on agar plates have been used to qualitatively stain for laccase activity (White and Boddy, 1992; Iakovlev and Stenlid, 2000), and to quantify laccase activity during the interaction between *Heterobasidion annosum* and *Resinicium bicolor* (Iakovlev and Stenlid, 2000).

The aim of this study was to investigate changes in enzyme activity during interactions involving *T. versicolor* that result in a range of outcomes. The role of ligninolytic enzymes as a defence mechanism to biotic and abiotic stress leads to the hypothesis that ligninolytic enzyme production is highest in interactions where *T. versicolor* is replaced by the competitor, compared to where it is successful (replaces or deadlocks with the competitor), and that enzyme activity declines as distance from the interaction zone increases. Assaying the activity of chitinase, acid phosphatase and β-glucosidase during interactions assesses whether increased nutrient acquisition is occurring, which is a potential reason for the increased activity of ligninolytic enzymes. Cell-free competitor diffusibles were used to test whether the physical presence of the competitor is necessary to stimulate antagonistic responses in *T. versicolor*. The effects of enhancing MnP production on laccase activity and interaction outcomes were also tested.

2.2 Materials and Methods

2.2.1 Isolates and culture conditions

Strains of *Trametes versicolor* (TvD2) and six other wood decay fungi were obtained from the Cardiff University Fungal Ecology Group culture collections (Table 2.1). Isolates were chosen to display a range of outcomes during interactions with *T. versicolor* (Table 2.1). Cultures were maintained on 2% (w/v) malt agar (MA; 20 g Munton and Fison spray malt; 20 g Lab M agar No. 2; distilled water) in 9 cm plastic non-vented Petri dishes (Greiner Bio-one, Austria), incubated upside down at 20°C in the dark. For each interspecific pairing 6 mm diameter plugs were removed from the margins of actively growing colonies using a No. 3 cork borer and inoculated 30 mm apart on 2% MA in 9 cm non-vented Petri dishes. Slower-growing species were inoculated earlier to ensure that competing mycelia met in the centre of the plate.

Table 2.1: Competitor isolates and the outcome of their interaction with *T. versicolor* D2 on 2% MA.

Ecological role[a]	Species	Strain	Outcome of interaction with *T. versicolor*
Primary coloniser	*Stereum gausapatum*	Sg1	Replacement by *T. versicolor*
	Daldinia concentrica	Dc290495	Replacement by *T. versicolor*
Early secondary coloniser	*Bjerkandera adusta*	BaMA313	Deadlock/partial replacement of *T. versicolor*
Heart rotter	*Fomes fomentarius*	Ff1	Deadlock
Late secondary coloniser	*Hypholoma fasciculare*	GTWV2	Replacement by *H. fasciculare* GTWV2
		DD2	Replacement by *H. fasciculare* DD2

[a], Rayner and Boddy (1988)

2.2.2 Localisation of enzyme activity by staining

Interspecific interactions involving *T. versicolor* vs. *S. gausapatum*, *H. fasciculare* GTWV2 or *B. adusta*, and self-pairings, were set up in triplicate. Localisation of lac-

case activity was determined in 9 cm Petri dishes of 2% MA plus 250 mg l^{-1} ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), which is oxidised to form a green/violet product. MnP activity was localised by growth on 2% MA plus 0.5 mM manganese chloride ($MnCl_2 \cdot 4H_2O$), which is oxidised to form a brown precipitate (Steffen et al., 2000). LiP activity was localised by growth on 2% MA plus 0.02% (w/v) Azure B which is decolorised by LiP oxidation (Pointing et al., 2005). Photographs were taken with a Nikon Coolpix camera 2 d, 8 d 1 month and 2 months after establishment of mycelial contact, and for ABTS and Azure B photographs were taken on a lightbox in a dark room. Interaction outcomes were assessed visually after 2 months.

General peroxidase activity and superoxide accumulation were visualised at three times, following methods adapted from Silar (2005). 2.5 mM diaminobenzidine (DAB) in 0.1 M potassium phosphate buffer at pH 6.9 was used to stain for peroxidase activity (in the presence of H_2O_2). Superoxide was localised using 2.5 mM nitroblue tetrazolium (NBT) in 5 mM (*n*-morpholino) propane sulfonate-NaOH (MOPS) at pH 7.6. Interactions at 2, 5 and 8 d post-contact on 2% MA in 9 cm Petri dishes were flooded with either stain, and incubated with gentle rotation for 30 min. The stain was drained off and the development of coloured precipitate recorded after 4 h. Areas with general peroxidase activity developed a red precipitate, areas with superoxide accumulation developed a purple precipitate. Control plates were supplemented with buffer only.

2.2.3 Sample preparation for enzyme assays

Interspecific interactions between *T. versicolor* and the six combatant species (Table 2.1), plus all relevant self-pairing controls, were set up as previously described on 16 ml 2% MA in 9 cm non-vented Petri dishes. Material was removed for extraction at 2 d and

8 d after establishment of mycelial contact for combinations involving *S. gausapatum*, *B. adusta* and *H. fasciculare* - 8 d interactions showed the greatest difference in activity between treatments so material was removed at 8 d only for other interactions. Six plugs (5 mm diam.) were removed with a no. 2 cork borer from each of four regions of the interacting mycelia: the interaction zone itself (IZ); just behind the interaction zone in *T. versicolor* (A); 30-40 mm from the interaction zone within the *T. versicolor* mycelium (B); and just behind the interaction zone in the competitors' mycelium (C; Figure 2.1). Total area of mycelium extracted was 118 mm^2 per region (approx. 200 mg wet weight). For self-pairings of combatant species, plugs were removed from the interaction zone only. Interactions were also set up on 16 ml 2% MA enriched with 0.5 mM $MnCl_2 \cdot 4.H_2O$, and plugs were removed from 8 d-old interactions.

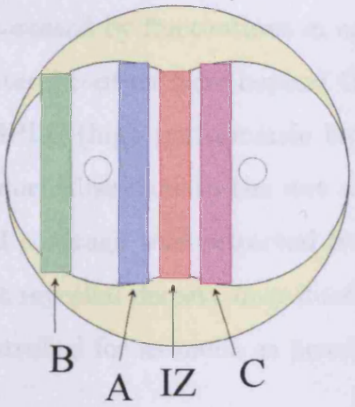

Figure 2.1: Sampling of interacting mycelia for enzyme extraction. *T. versicolor* is on the left. Six agar plugs were removed from each of four different regions. Region 'IZ' corresponds to the interaction zone; 'A' is just behind the interaction zone in the *T. versicolor* mycelium; 'B' is further back in the *T. versicolor* mycelium; 'C' is just behind the interaction zone in the mycelium of the competitor.

Plugs were cut in half and transferred to a 1.5 ml Eppendorf tube, to which 1 ml deionised water was added. Tubes were shaken gently at 4°C overnight, then 0.8 ml

of extract removed and centrifuged (8,000 g) for 10 min at 4°C to pellet any debris. Extracts were kept at 4°C and assays performed immediately. Three replicate plates for each region, isolate and time-point were used. Data were normalised to the wet weight of agar plus mycelium extracted for each sample.

Normalisation of the data proved tricky. Mycelium is so fine that it is hard to quantify areas of high density, which occur at interaction zones. Liquid grown cultures tend to have enough protein content in extracts to be quantified, whilst studies that use natural substrates tend to normalise to the dry weight of substrate. Here, the protein contents of extracts were below the level of detection of a Bradford assay, and total protein extraction of the mycelial plugs was unsuccessful, probably because the plugs were mostly agar. The dry weight of agar plus mycelium (i.e. the plugs) for each extract was measured, but were too variable to be explained by differences in mycelial coverage, and were probably caused by fluctuations in agar content. Measurements of fungal biomass such as ergosterol content were beyond the scope of this experiment as they require serial runs of HPLC (high performance liquid chromatography). It was decided that it was best to normalise data to the wet weight of the sample, ensuring that a fixed area of mycelial coverage was extracted from each region. It should be taken into consideration that mycelial density may fluctuate in different regions of an interaction, but this was controlled for as much as possible.

2.2.4 Assays

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of ABTS in citrate-phosphate buffer (100 mM citrate, 200 mM phosphate, pH 5.0), following Bourbonnais and Paice (1990). 50 μl extracts were added to wells of a well Bioscreen C plate (100-well plate; Oy Growth Curves Ab Ltd., Finland) and 150 μl citrate-phosphate buffer added to all sample-containing wells using a multichannel pipette.

50 μl 0.08% (w/v) ABTS was added to wells using a multichannel pipette and the plate was immediately transferred to a Bioscreen C plate reader (Oy Growth Curves Ab Ltd., Finland). Formation of a green coloration was followed spectrophotometrically at 420 nm. Activity was calculated using the molar extinction coefficient of ABTS (36,000 M cm^{-3}). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of product per min.

Activity of MnP (EC 1.11.1.13) was assayed according to Ngo and Lenhoff (1980) in succinate-lactate buffer (100 mM, pH 4.5). MBTH (3-methyl-2-benzothiazoline-hydrazone hydrochloride) and DMAB (3-(dimethyl amino)-benzoic acid) were oxidatively coupled by MnP action, and formation of a purple indamine dye followed spectrophometrically at 590 nm. 50 μl extracts were added to wells of a Bioscreen II plate, 200 μl substrate solution was added using a multichannel pipette, and plates were immediately transferred to a Bioscreen C plate reader. The substrate solution comprised 25 mM DMAB and 1 mM MBTH in 100 mM succinate-lactate buffer with 2 mM $MnSO_4$ and 5 mM hydrogen peroxide. The results were corrected by activity of samples: (1) without manganese, where manganese sulphate was replaced by 2 mM EDTA (ethylene diamine tetraacetate) to chelate Mn present in the extract, and (2) in the absence of H_2O_2 to allow detection of activity of oxidases but not peroxidases.

Lignin peroxidase (EC 1.11.1.14) activity was measured according to Collins et al. (1996) by monitoring the oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde in 250 mM tartaric acid buffer (pH 2.5) containing 5 mM H_2O_2. 130 μl sample was mixed with 50 μl 10 mM veratryl alcohol, 50 μl tartaric acid and 20 μl H_2O_2, and transferred to a quartz cuvette. The change in absorbance at 310 nm was followed using a Helios UV-vis spectrophotometer (Tecan, USA) and readings taken every 30 s for 5 min.

The activity of β-glucosidase (EC 3.2.1.21) was assayed in microplates using *p*-nitrophenyl-

β-D-glucoside (pNPG; Glycosynth, UK) as described previously (Valášková et al., 2007). 40 μl sample was pipetted into 96-well plates (Nunc, Nalgene) in duplicate. Reaction mixture was added to both aliquots at the same time and plates incubated at 37°C. The reaction mixture consisted of 160 μl 1.2 M pNPG in 50 mM sodium acetate buffer (pH 5.0). Activity was measured by stopping the reaction with 100 μl 0.5 M sodium carbonate after 2 min in one replicate and 120 min in the other, and absorbance was measured at 405 nm using a Dynex MRX plate reader (Dynex Technologies Ltd., Sussex, UK). Enzyme activity was calculated using the molar extinction coefficient of *p*-nitrophenol (11,600 M cm^{-3}). One unit of enzyme activity (U) was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenyl per minute. Activity of phosphomonoesterase (acid phosphatase; EC 3.1.3.2) and 1,4-N-acetylglucosaminidase (chitinase; EC 3.2.1.50) were assayed using *p*-nitrophenyl phosphate (pNPP; Glycosynth, UK) and *p*-nitrophenyl N-acetyl-β-D-glucosaminide (pNPN; Glycosynth, UK), respectively, using the same method.

Catalase activity was assayed by measuring decomposition of H_2O_2 (Beers and Sizer, 1952; Cakmak et al., 1993). The substrate solution comprised 0.035% (v/v) H_2O_2 in 50 mM potassium phosphate monobasic buffer (pH 7.0). The absorbance at 240 nm of the substrate solution was measured in a quartz cuvette in a Helios UV-vis spectrophotometer (Tecan, USA), using 50 mM potassium phosphate buffer as a blank; if the A_{240} of the substrate solution was outside 0.520-0.550 absorbance units, H_2O_2 was added to increase the absorbance or buffer added to decrease the absorbance. 0.1 ml enzyme extract was added to 2.9 ml substrate solution and mixed. The time required for the absorbance to decrease from 0.450 to 0.400 absorbance units was measured and used to calculate catalase activity. This method was modified to deal with pigmented solutions; enzyme extract and buffer were mixed and used to blank the spectrophotometer, H_2O_2 added and the time taken for the A_{240} to decrease from 0.450 to 0.400 measured.

2.2.5 Effect of interaction diffusibles

Interspecific interactions involving *T. versicolor* vs. *S. gausapatum*, *B. adusta* or *H. fasciculare* GTWV2, and self-pairings of these fungi and *F. fomentarius*, were set up in Reacsyn™ vessels (Biodiversity, Enfield, UK). Reacsyn™ vessels are compartmentalised polypropylene bottles designed to allow sampling of culture medium and headspace volatiles (Figure 2.2). Plugs (6 mm diam.) from agar cultures were made with a no. 3 cork borer and placed 30 mm apart on a filter paper support, resting on 35 ml 2% malt broth (20 g l^{-1} Lab M malt extract; distilled water). The mycelia colonise horizontally across the paper support and also penetrate into the broth. This has the advantage that mycelia have non-submerged discrete domains rather than the interspersal of mycelia that occurs in liquid culture. *H. fasciculare* was inoculated 2 days before *T. versicolor* to ensure mycelia were of similar size on contact. Vessels were incubated at 20°C in the dark. Mycelia met approximately 3 days post-inoculation, and were left to interact for a further 7 days. Uninoculated vessels containing broth only were used as control treatments.

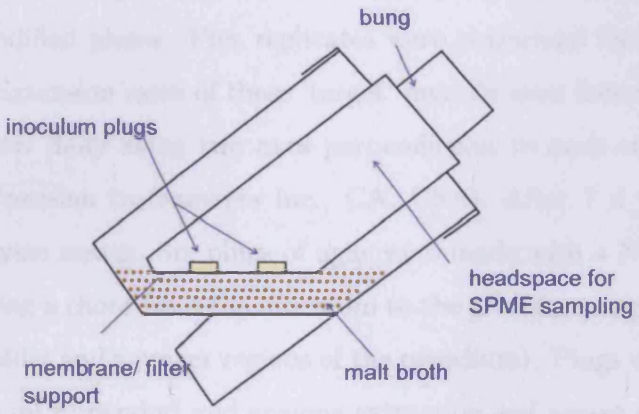

Figure 2.2: A Reacsyn™ vessel.

Culture broths from duplicate interactions were combined and paper-filtered (grade 541, Whatman International, Kent, UK) to remove large pieces of mycelium, then filter-sterilised (Minisart 0.20 μm pore size syringe filter, Sartorius Stedim Biotech., France). Culture extracts were stored at 4°C and used on the same day as extraction to minimise effects of deterioration. 1 ml culture extract was pipetted onto the surface of a 9 cm Petri dish containing 16 ml 2% MA. A sterile glass spreader was used to ensure even coverage of the agar surface, and plates were left open in a laminar flow hood until surface liquid had been absorbed/evaporated. To assess the effect of H_2O_2 on extension and enzyme production, 0.576 ml 30% H_2O_2 was added to 30 ml 2% malt broth. This was filtered, filter-sterilised and added to agar plates in the same way as broth containing diffusibles, giving an end concentration of 10 mM H_2O_2 in each 16 ml agar plate. This concentration was chosen because it is known to be inhibitory but not fungistatic to *T. versicolor* (Dr J Hunt, pers. comm.).

Effects of diffusibles on extension rate and enzyme production

Plugs of *T. versicolor*, *S. gausapatum*, *B. adusta* and *H. fasciculare* were inoculated centrally onto modified plates. Five replicates were performed for each species with each treatment. Extension rates of these 'target' mycelia were determined by measuring colony diameter daily along two axes perpendicular to each other using Vernier calipers (Swiss Precision Instruments Inc., CA, USA). After 7 d *T. versicolor* was harvested for enzyme assays. Six plugs of agar were made with a No. 2 cork borer (5 mm diameter) along a chord from the inoculum to the growing margin (i.e. plugs were taken from both older and younger regions of the mycelium). Plugs were transferred to a pre-weighed 1.5 ml Eppendorf and enzyme extraction and assays were performed as previously described. The analysis was performed in two batches, and enzyme activity of *T. versicolor* growing alone on unmodified agar was used to normalise the data.

2.2.6 Statistical analysis

Statistical comparisons of enzyme activity and extension rates were performed using one-way ANOVA, where data met the required assumptions: that residuals are normally distributed (Anderson-Darling test) with equal variances between groups (Levene's test). Significant results were further tested using the Tukey-Kramer *a posteriori* test to determine significant differences between means. If data did not meet the assumptions of ANOVA, Kruskal-Wallis tests in combination with *post hoc* Mann-Whitney U-tests were used. Two-sample t-tests (or Mann-Whitney U-tests if data were non-normally distributed) were used to compare enzyme activity in different regions of interactions with the equivalent regions in *T. versicolor* or competitor self-pairings. All tests were performed in Minitab (v. 15, Minitab Ltd., Coventry, UK).

2.3 Results

2.3.1 General observations on morphology during interspecific interactions

In interspecific interactions between *T. versicolor* and *S. gausapatum, B. adusta* and *H. fasciculare*, mycelial morphology changed following contact. Mycelia met after 3 d in agar culture, or 5 d after inoculation into Reacsyn^{TM} vessels. Mycelial barrages formed at the interaction zone in *T. versicolor* vs. *S. gausapatum* (TvSg, resulting in replacement by *T. versicolor*) and *T. versicolor* vs. *B. adusta* (TvBa, resulting in deadlock) 1 d after mycelia had met, but later in *T. versicolor* vs. *H. fasciculare* GTWV2 (TvHf, resulting in replacement of *T. versicolor*). A bright yellow-orange pigment was present in the agar in the interaction zone in TvSg just after contact,

but no pigmentation developed in TvBa, and did not occur until later stages in TvHf (Figure 2.3). Morphology was generally qualitatively consistent between replicates.

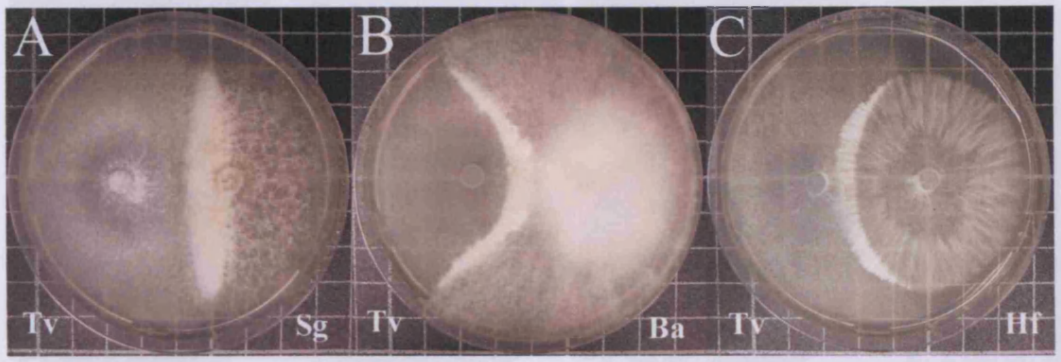

Figure 2.3: Interactions involving *T. versicolor* (left) against: A, *S. gausapatum*; B, *B. adusta*; C, *H. fasciculare*. Taken 4 d after mycelia had met.

2.3.2 Staining

Strong laccase activity was associated with interaction zones in all three pairings (Figure 2.4; Appendix Figure B.1), irrespective of outcome. However there was clearing at the interaction zone in TvSg and TvBa, but not in TvHf (Figure 2.4 A, F, K). Clearing indicates a greater laccase production in these interactions (with further oxidation of ABTS to a colourless derivative) compared to the interaction with *H. fasciculare* where *T. versicolor* is replaced. Laccase activity was also associated with the growing margins of *T. versicolor* cultures; older regions of the mycelium were initially stained purple (results not shown) but by 5 d the areas around the inocula were cleared again indicating high levels of laccase activity (Figure 2.4 A, F, K).

MnP activity occurred in older regions of *T. versicolor* mycelia, forming a diffuse zone around the inoculum (Figure 2.4 B, G, L; Appendix Figure B.2). There was distinct association of MnP activity along the length of the interaction zone in TvSg and TvBa

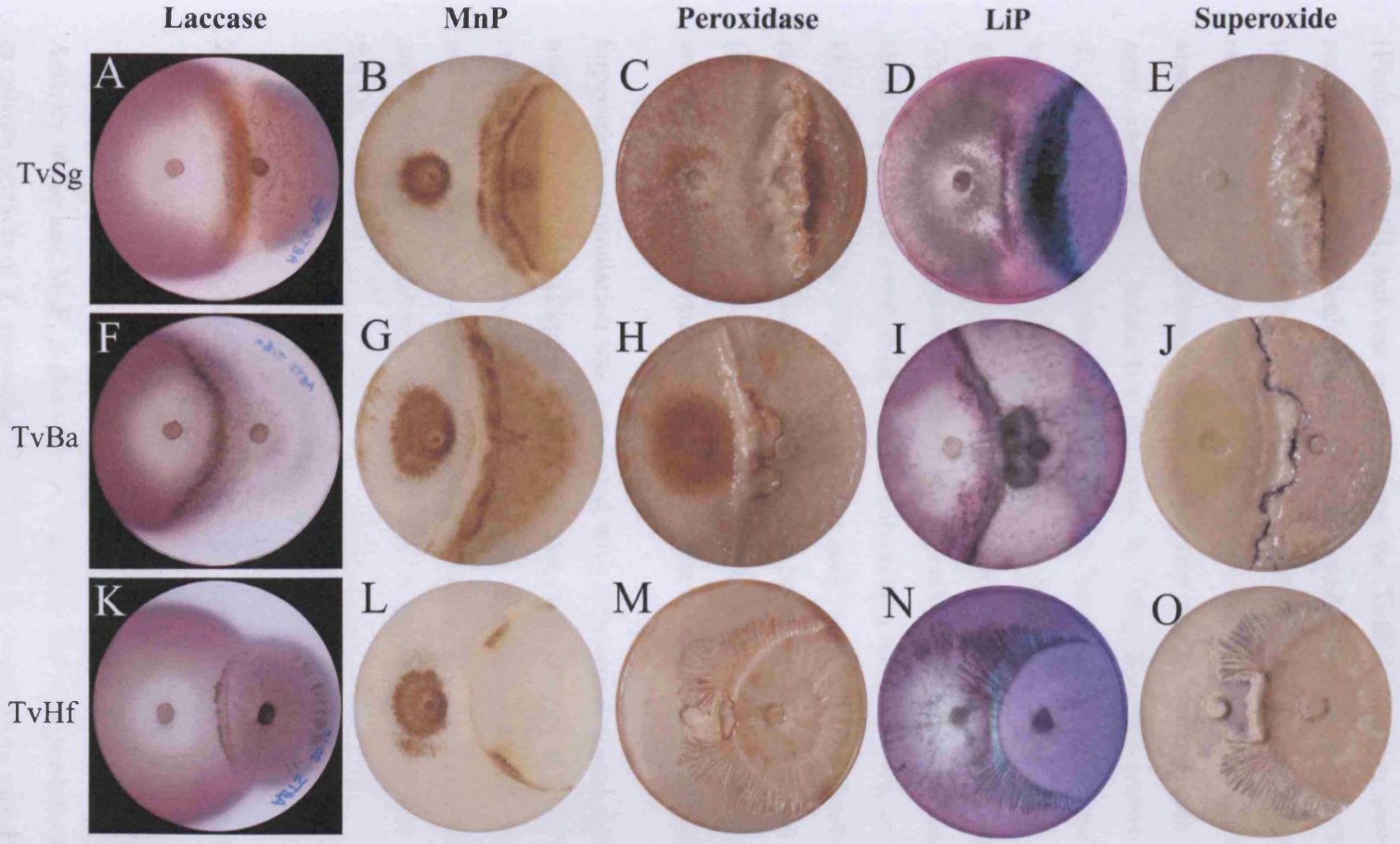

Figure 2.4: Localisation of laccase, MnP, general peroxidase, LiP and superoxide by staining interactions involving *T. versicolor* on 2% MA. *T. versicolor* is on the left in all pairings. A-E, vs. *S. gausapatum*; F-J, vs. *B. adusta*; K-O, vs. *H. fasciculare* GTWV2. A, F and K, laccase activity generates a purple stain, 2 d-old interactions; B, G and L, MnP activity results in deposition of a brown precipitate, 8 d-old interactions; C, H and M, peroxidase reacts with DAB to form a red-brown precipitate, 8 d-old interactions; D, I and N, LiP activity causes decolorisation of the blue Azure B dye, 8 d-old interactions; E, J and O, superoxide reacts with NBT to form a purple precipitate, 8 d-old interactions.

(Figure 2.4 B, G), but was patchy along the TvHf interaction zone with activity in regions where *H. fasciculare* invasive hyphal cords began to develop (Figure 2.4 L). The presence of Mn^{2+} affected the interaction outcome of TvHf, which resulted in deadlock, rather than the replacement of *T. versicolor* by *H. fasciculare* that occurred in the absence of Mn^{2+}. General peroxidase activity was associated with interaction zones again irrespective of interaction outcome. In TvSg there was activity at the replacing *T. versicolor* front, at the TvBa interaction barrage, and the invasive *H. fasciculare* hyphal cords in TvHf (Figure 2.4 C, H, M; Appendix Figure B.4). There was also general peroxidase activity around the inoculum in TvBa at 8 d but not in TvSg or TvHf. LiP activity occurred within the mycelia of *T. versicolor* and *B. adusta*, but there was no clear association with the interaction zones of TvSg or TvBa (Figure 2.4 D, I, N), although in TvSg decolorisation may be masked by dense mycelium at the interaction zone as a thin zone of activity can be seen in earlier stages of the interaction (Figure Appendix B.3). LiP activity occurs where *H. fasciculare* invasive hyphal cords are overgrowing *T. versicolor* mycelium at the interaction zone (Figure 2.4 N).

Superoxide accumulation was associated with interaction zones, again irrespective of interaction outcome (Figure 2.4 E, J, O; Appendix Figure B.5). The pattern of accumulation at interaction zones was roughly similar to general peroxidase activity, although there was no superoxide accumulation around the inoculum in TvBa. Some accumulation of superoxide occurred in the *T. versicolor* mycelium in TvHf (Figure 2.4 O).

2.3.3 Enzyme activity of *T. versicolor* self-pairings on 2% MA

Activity of laccase, MnP, β-glucosidase, chitinase and acid phosphatase were detected in culture extracts of *T. versicolor*; production by competitors varied (Table 2.2). LiP,

catalase and Mn-independent peroxidase activity were not detected either 2 or 8 d after contact in *T. versicolor* self-pairings or during interactions (Table 2.2). There were no significant differences ($P > 0.05$) in laccase activity in any region of *T. versicolor* self-pairings at 2 or 8 d after contact (Table 2.2), but for the other enzymes detected there were significant differences ($P \leq 0.05$) in activity levels in different regions of the *T. versicolor* self-pairing.

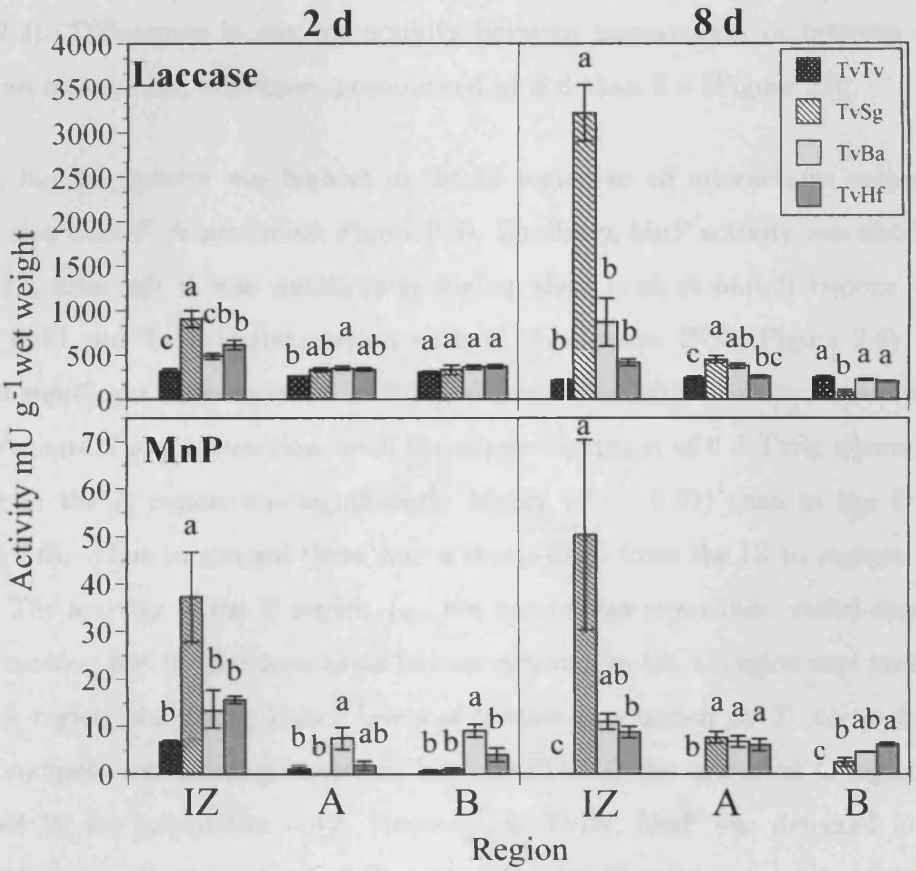

Figure 2.5: Activity of laccase and MnP in different regions of 2 and 8 d-old *T. versicolor* self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *B. adusta* (TvBa) or *H. fasciculare* GTWV2 (TvHf). Bars are mean ± SEM. Statistical comparisons are made separately for each enzyme and time, within a region different letters indicate a significant difference ($P \leq 0.05$).

2.3.4 Enzyme activity

Differences in enzyme activity between regions was distinct, with highly localised production at the interaction zone. For example, 3219 mU g^{-1} laccase activity was detected in IZ (interaction zone) extracts of 8 d TvSg, whilst in the A region (immediately behind the IZ in *T. versicolor*) there was only 450 mU g^{-1} activity, and only 0.2 mU g^{-1} activity in the C region (adjacent to the interaction zone in the competitor mycelium; Table 2.2). Differences in enzyme activity between interactions, or between regions within an interaction, were more pronounced at 8 d than 2 d (Figure 2.5).

At 8 d, laccase activity was highest in the IZ region in all interactions except TvFf (interaction with *F. fomentarius*; Figure 2.6). Similarly, MnP activity was also highest in the IZ, although it was significantly higher than both A and B regions only in TvDc, TvFf and TvHf-D (interaction with *H. fasciculare* DD3; Figure 2.6). There were no significant differences ($P > 0.05$) in laccase or MnP activity between the A and B regions of any interaction, with the single exception of 8 d TvSg where laccase activity in the A region was significantly higher ($P \leq 0.01$) than in the B region (Figure 2.6). Thus in general there was a sharp drop from the IZ to regions behind the IZ. The activity in the C region, i.e. the competitor mycelium, varied depending on the species, but in all interactions laccase activity in the C region was lower than in the A region, indicating higher levels of laccase production by *T. versicolor* than by the competitors. In all interactions bar one (TvDc) the extracted C regions were colonised by the competitor only. However, in TvDc, MnP was detected in the C region which implies overgrowth of *D. concentrica* by *T. versicolor*; as an ascomycete, *D. concentrica* would not produce peroxidases capable of oxidising Mn^{2+}. This was not visually discernible when extractions were performed.

For all enzymes detected, there was significantly ($P \leq 0.05$) higher enzyme activity in interactions than in self-pairings of at least one of the competitors (Table 2.2).

Table 2.2: Extracellular enzyme activity of *T. versicolor* and competitors in 8 d-old interactions.

Enzyme	Region	Activity mU g^{-1} wet weight												
		TvTv	SgSg	TvSg	DcDc	TvDc	BaBa	TvBa	FfFf	TvFf	HfHf	TvHf	Hf-DHf-D	TvHf-D
Laccase	IZ	216.0	27.3	3218.9*†	0.0	877.8*†	1.0	871.6*†	0.7	141.8†	31.4	417.1*†	23.3	1095.4*†
	A	238.3	-	449.8*	-	263.4	-	376.6*	-	149.1	-	253.6	-	260.0
	B	243.7	-	74.5*	-	189.7	-	222.2	-	215.5	-	202.7	-	226.9
	C	-	-	0.2 †	-	221.7 †*a*	-	5.9	-	3.3 †	-	122.6 †	-	173.1 †
MnP	IZ	0.0	2.7	33.6*†	0.0	10.4*†	3.9	11.0*†	0.0	5.9*†	8.9	8.7*	6.1	22.4*†
	A	0.0	-	7.7*	-	0.9	-	6.8*	-	0.0	-	6.1*	-	1.7*
	B	0.0	-	2.6*	-	0.0	-	4.7*	-	0.0	-	6.3*	-	2.2*
	C	-	-	6.0	-	3.8 †*a*	-	6.0	-	0.2	-	6.6	-	9.3
β-glucosidase	IZ	5.6	12.9	11.7*	15.7	4.9 †	4.6	37.7*†	2.8	2.0*	6.2	3.4 †	1.9	2.8 †
	A	7.8	-	4.5*	-	6.4*	-	6.0	-	4.4*	-	10.5	-	8.3
	B	11.1	-	1.3*	-	6.0	-	8.9	-	7.4*	-	8.3	-	8.2
	C	-	-	3.9 †	-	7.3 †*a*	-	2.4	-	4.1	-	7.3	-	3.6 †
Chitinase	IZ	0.4	13.3	3.7*†	49.1	1.0 †	3.8	10.0*	9.4	1.1 †	0.6	1.5*†	0.5	1.1
	A	0.5	-	0.8	-	0.9	-	0.8	-	0.4	-	1.7*	-	0.7
	B	1.0	-	0.3*	-	0.8	-	0.8	-	0.7	-	1.2	-	0.7
	C	-	-	4.7 †	-	19.5 †*a*	-	1.0 †	-	10.4	-	0.1	-	0.1 †
Acid Phosphatase	IZ	2.1	9.4	19.5*†	53.2	8.4*†	0.4	10.0*†	3.2	4.4*	3.9	2.9 †	5.2	3.6 †
	A	2.8	-	2.0	-	3.7	-	1.8	-	3.2	-	2.9	-	3.1
	B	4.3	-	0.9*	-	3.0	-	1.5*	-	4.6	-	2.1*	-	3.5
	C	-	-	10.5	-	44.7 *a*	-	0.4	-	3.8	-	6.0 †	-	6.9 †

Values given are means of three replicates. *, significant difference ($P \leq 0.05$) in activity compared with the corresponding region in the *T. versicolor* self-pairing (TvTv); †, significant difference ($P \leq 0.05$) in activity compared with the competitor self-pairing; −, samples not taken from this region; *a*, competitor probably overgrown by *T. versicolor*; IZ, interaction zone; A, the region adjacent to the interaction zone within *T. versicolor* mycelium; B, the region further back within *T. versicolor* mycelium; C, the region adjacent to the interaction zone within competitor mycelium.

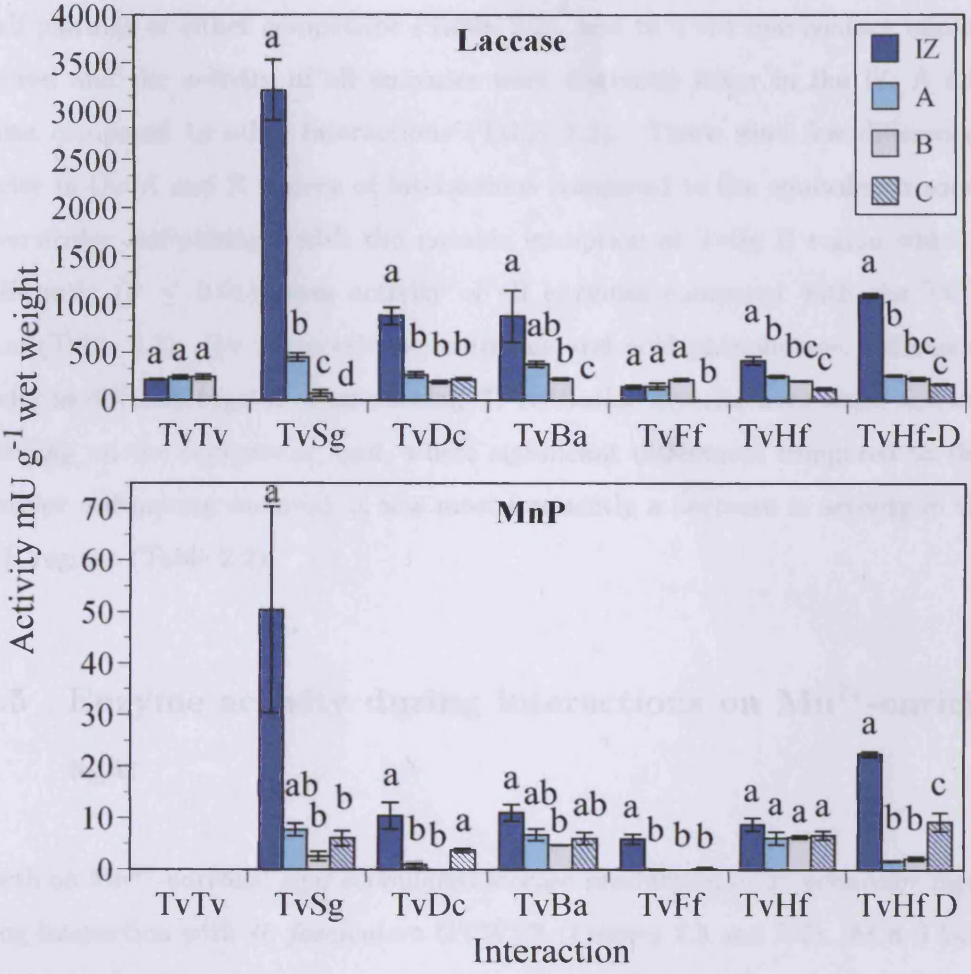

Figure 2.6: Activity of laccase and MnP in different regions of 8 d *T. versicolor* self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *D. concentrica* (TvDc), *B. adusta* (TvBa), *F. fomentarius* (TvFf), *H. fasciculare* GTWV2 (TvHf) or *H. fasciculare* DD2 (TvHf-D) . Bars are mean ± SEM. Statistical comparisons are made separately for each enzyme and interaction, within a pairing different letters indicate a significant difference ($P \leq 0.05$) between regions.

In fact laccase and MnP activities were significantly ($P \leq 0.05$) higher at the IZ than in self-pairings of either competitor in TvSg, TvDc, TvBa and TvHf-D (Table 2.2). However in TvHf only laccase activity was significantly ($P \leq 0.05$) higher than in self-pairings of either competitor (Table 2.2), and in TvFf non-contact inhibition occurred and the activity of all enzymes were distinctly lower in the IZ, A and C regions compared to other interactions (Table 2.2). There were few differences in activity in the A and B regions of interactions compared to the equivalent regions in *T. versicolor* self-pairings, with the notable exception of TvSg B region which had significantly ($P \leq 0.01$) lower activity of all enzymes compared with the TvTv B region (Table 2.2). For β-glucosidase, chitinase and acid phosphatase, differences in activity in different regions of interacting *T. versicolor* mycelia were small and varied depending on the competitor, and, where significant differences compared to the *T. versicolor* self-pairing occurred, it was most frequently a decrease in activity in the A and B regions (Table 2.2).

2.3.5 Enzyme activity during interactions on Mn^{2+}-enriched agar

Growth on Mn^{2+}-enriched agar stimulated laccase production in *T. versicolor* mycelia during interaction with *H. fasciculare* GTWV2 (Figures 2.5 and 2.7). At 8 d laccase activities in the IZ and A regions of TvHf were significantly ($P \leq 0.05$) higher than the equivalent regions in TvTv on Mn^{2+}-enriched agar, which did not occur during growth on 2% MA (Figures 2.5 and 2.7). However, *H. fasciculare* laccase activity appeared to be repressed on the Mn^{2+}-enriched agar since there were no changes in laccase activity in the TvHf C region relative to *H. fasciculare* self-pairings, despite a significant ($P \leq 0.05$) increase during interactions on non-enriched agar (Figure 2.8). There were no significant differences ($P > 0.05$) between changes in laccase or MnP

activity relative to self-pairings in the TvBa or TvSg C regions when grown on enriched or non-enriched agar (Figure 2.8). Growth on Mn^{2+}-enriched agar stimulated MnP activity in the *T. versicolor* self-pairing where it was detectable in all three regions. It also affected the pattern of MnP activity at the IZ, which was similar to the pattern of laccase activity: all interactions had significantly ($P \leq 0.05$) higher activity than TvTv, with TvSg > TvHf > TvBa (Figure 2.7). Similar patterns of activity between laccase and MnP did not occur in the A and B regions.

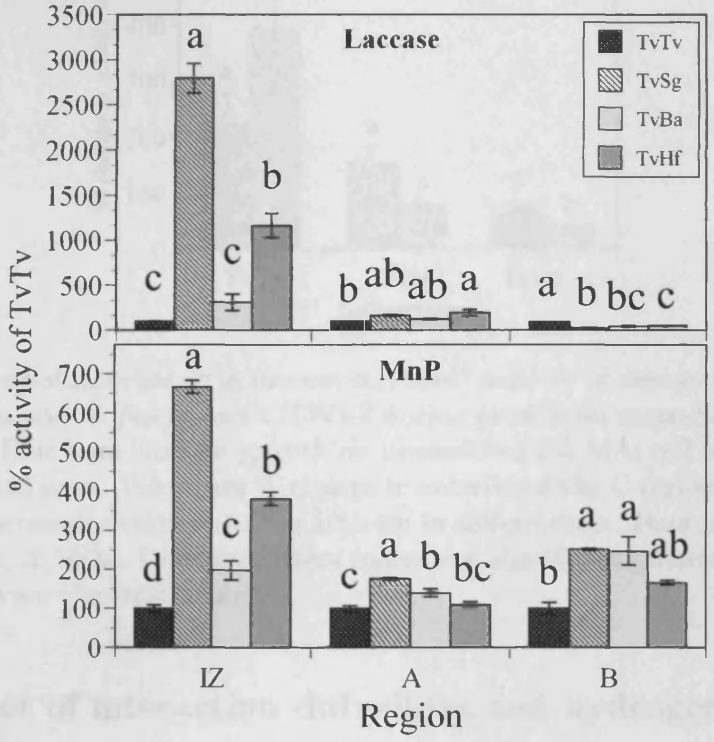

Figure 2.7: Activity of laccase and MnP on Mn^{2+}-enriched agar, in different regions of 8 d *T. versicolor* self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *B. adusta* (TvBa) or *H. fasciculare* GTWV2 (TvHf). Bars are mean ± SEM. Statistical comparisons are made separately for each enzyme, within a region different letters indicate a significant difference ($P \leq 0.05$).

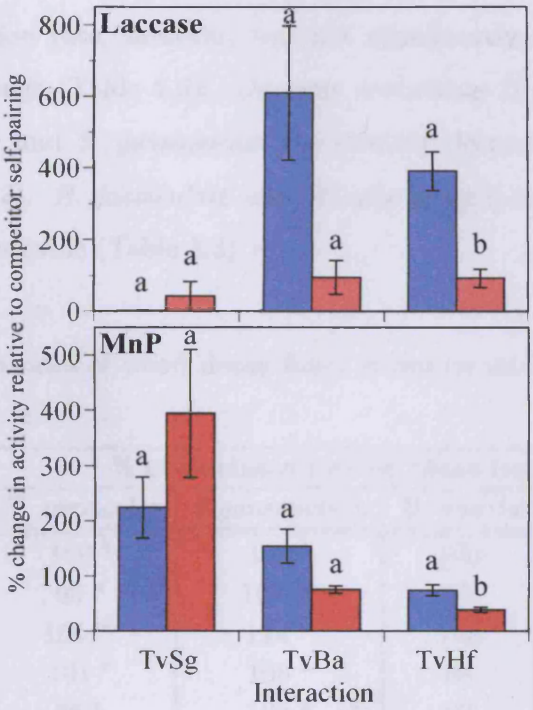

Figure 2.8: Percentage change in laccase and MnP activity of antagonists *S. gausapatum*, *B. adusta* and *H. fasciculare* GTWV2 during growth on unmodified and Mn^{2+}-enriched agar. Blue bars indicate growth on unmodified 2% MA; red bars are growth on Mn^{2+}-enriched agar. Values are % change in activity of the C region of 8 d interactions with *T. versicolor* relative to the activity in self-pairings. Bars are the mean of three replicates, ± SEM. Different letters indicate a significant difference ($P \leq 0.05$) in activity between the treatments.

2.3.6 Effect of interaction diffusibles and hydrogen peroxide

Extension rate of *T. versicolor* decreased significantly ($P \leq 0.05$) and *S. gausapatum* increased significantly ($P \leq 0.05$) when grown on agar to which diffusibles from their self-pairings had been added compared to agar with no diffusibles (Table 2.3). *T. versicolor* extension rate was stimulated by diffusibles from the *S. gausapatum* self-pairing, and repressed by diffusibles from *B. adusta* and *H. fasciculare* self-pairings. Diffusibles from the *F. fomentarius* self-pairing inhibited extension of *T. versicolor*, *S.*

gausapatum and *B. adusta*, as did diffusibles from the interaction TvHf (Table 2.3). *H. fasciculare* extension rate, however, was not significantly affected by diffusibles from any of the pairings (Table 2.3). On agar containing 10 mM H_2O_2, extension rates of *T. versicolor* and *S. gausapatum* significantly decreased, by 30% and 50% respectively (Table 2.3). *H. fasciculare* and *B. adusta* were completely inhibited on the agar containing peroxide (Table 2.3).

Table 2.3: Extension rates of wood decay fungi grown on interaction diffusibles and H_2O_2.

Agar modification	% of extension rate on blank treatment			
	T. versicolor	*S.gausapatum*	*B. adusta*	*H. fasciculare*
Blank	100 ^{b}	100 ^{b}	100	100
TvTv	90 ^{a}	107 ^{ab}	87	91
SgSg	105 ^{ab}	124 ^{a}	103	111
TvSg	101 ^{b}	106 ^{b}	88	83
BaBa	86 ^{a}	103 ^{b}	97	88
TvBa	101 ^{b}	107 ^{b}	99	98
HfHf	98	103 ^{b}	69 ^{ab}	98
TvHf	81 ^{a}	72 ^{ab}	70 ^{ab}	100
FfFf	64 ^{ab}	50 ^{ab}	75 ^{ab}	83
Peroxide	70 ^{ab}	50 ^{ab}	0 ^{ab}	0 ^{ab}

Values given are means of 5 replicates. ^{a}, significant difference ($P \leq 0.05$) in extension rate compared to growth on agar modified with 'blank' diffusibles (i.e. uncolonised malt broth); ^{b}, significant difference ($P \leq 0.05$) in extension rate compared to growth on agar modified with diffusibles from the relevant self-pairing.

T. versicolor demonstrated significantly ($P \leq 0.05$) lower laccase activity on agar containing diffusibles from interactions TvSg, TvBa, TvHf and *H. fasciculare* self-pairings compared to growth normal 2% MA (Figure 2.9). There was significantly ($P \leq 0.05$) lower activity of chitinase, acid phosphatase and MnP in cultures grown on agar containing TvTv self-pairing diffusibles compared to growth on normal 2% MA. Residual laccase activity was found on uninoculated agar plates modified with diffusibles, with the exception of diffusibles from self-pairings of *S. gausapatum*, *B. adusta* and *F. fo-*

mentarius (Figure 2.9). The most striking differences in activity were the increases in MnP during growth on diffusibles from TvHf and the *S. gausapatum* self-pairing. Interestingly, MnP activity was significantly ($P \leq 0.05$) decreased during growth on TvTv diffusibles compared to growth on normal 2% MA. Chitinase and acid phosphatase activity were significantly ($P \leq 0.05$) lower during growth on diffusibles from TvBa and the self-pairings of *T. versicolor*, *S. gausapatum*, *B.adusta* and *H. fasciculare* compared to growth on normal 2% MA. β-glucosidase activity was decreased by diffusibles from TvSg only. Addition of H_2O_2 to the substrate did not cause any differences in enzyme activity (Figure 2.9), but there was detection of chitinase activity in the uninoculated H_2O_2-agar blank which implies either contamination of the samples or that the H_2O_2 interfered with the chitinase assay.

2.4 Discussion

2.4.1 Ligninolytic enzyme activity increased during interactions but was not linked to interaction outcome

Of the enzymes assayed, laccase showed the largest increase in activity during interactions: 14-fold that of self-pairings in the TvSg interaction zone at 8 d post-inoculation. This large laccase response agrees with previous findings for *T. versicolor* during interactions (White and Boddy, 1992; Baldrian, 2004, 2006). As has been previously reported, enzyme activity was higher at the interaction zone than in other regions of the interaction (Iakovlev and Stenlid, 2000). However, the extent of this increase varied because of production by the competitor and/or modulation of the *T. versicolor* response based on the identity of the competitor. Different levels of enzymatic response to different competitors has been reported for a variety of interactions, including those involving *Pleurotus ostreatus* and *Stereum hirsutum* (Iakovlev and Stenlid, 2000; Chi

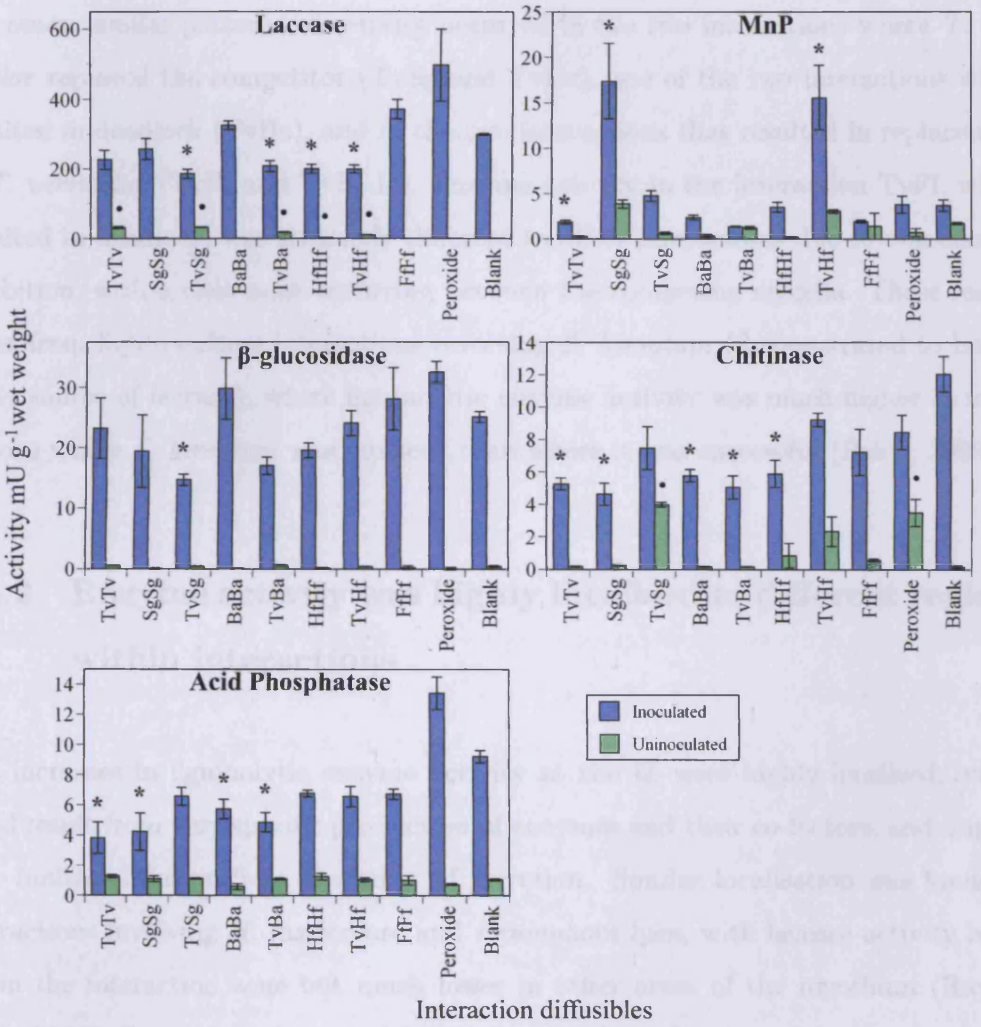

Figure 2.9: Activity of selected enzymes secreted by *T. versicolor* during growth on interaction diffusibles or H_2O_2. Blue bars, activity of *T. versicolor* mycelia growing on the modified agar; green bars, activity of uninoculated modified agar; *, significantly different ($P \leq 0.05$) to activity of *T. versicolor* growing on blank agar (i.e. no diffusibles); •, significantly different ($P \leq 0.05$) to activity of blank agar.

et al., 2007; Peiris, 2009).

There were no clear patterns of laccase and MnP activity in interactions with different outcomes; similar patterns of activity occurred in the two interactions where *T. versicolor* replaced the competitor (TvSg and TvDc), one of the two interactions which resulted in deadlock (TvBa), and in the two interactions that resulted in replacement of *T. versicolor* (TvHf and TvHf-D). Enzyme activity in the interaction TvFf, which resulted in deadlock, was strikingly different to other interactions due to non-contact inhibition, with a clear zone occurring between the competing mycelia. These results differ from liquid culture interactions involving *S. hirsutum* (demonstrated to be the likely source of laccase), where ligninolytic enzyme activity was much higher in interactions where *S. hirsutum* was replaced than where it was successful (Peiris, 2009).

2.4.2 Enzyme activity was highly localised to different regions within interactions

The increases in ligninolytic enzyme activity at the IZ were highly localised, which could result from very specific production of enzymes and their co-factors, and implies very limited diffusion from the point of secretion. Similar localisation was found in interactions involving *H. fasciculare* and *Peniophora lycii*, with laccase activity highest in the interaction zone but much lower in other areas of the mycelium (Rayner et al., 1994). Staining for laccase, MnP and peroxidase also showed localised activity, although the laccase staining indicates more diffuse localisation than did the assays, most probably due to the diffusion of the reaction products. The stark contrasts in activity between different regions could be caused by secretion of enzyme inhibitors by one or both of the antagonists, which has been suggested for interactions between *T. versicolor* and *Trichoderma harzianum* (Freitag and Morrell, 1992).

2.4.3 Minor changes in enzyme activity were found in the A and B regions of interactions

Minor changes in enzyme activity occurred in the A and B regions of interacting *T. versicolor* mycelia compared to the *T. versicolor* self-pairing. Qualitative changes in laccase activity have been reported in regions of interacting mycelia away from the interaction zone, but these changes were not large (Iakovlev and Stenlid, 2000). Here, the notable change was the decrease in laccase activity in the B region of TvSg. This may be because resources were being prioritised to the replacing front at the interaction zone, and away from regions further back in the *T. versicolor* mycelium. An overall pattern for laccase and MnP activity can be expressed as IZ > A $\geq$ B (i.e. activity in the IZ was greater than in A or B), but this pattern did not consistently occur for β-glucosidase, chitinase and acid phosphatase. Whilst some significant increases in activity did occur at the IZ, the decreases in activity in A and B regions suggest that increased nutrient acquisition does not occur during interactions, perhaps resulting from the rich substrate: other regions do not need to support the interaction zone because nutrients are not limited. Similarly, activity of chitinase was not enhanced during interactions involving *S. hirsutum* on potato dextrose agar (Peiris, 2009). The chitinase assay used here is specific for exo-cleaving chitinases, which have a purely nutritional role, whereas endo-cleaving chitinases could function antagonistically and have been shown to increase in activity in some interactions (Lindahl and Finlay, 2006). Activity of β-glucosidase and chitinase in culture extracts may be underestimated because significant amounts of the exo-cleaving enzymes of *T. versicolor* are mycelium-associated to provide mono- and disaccharides for uptake into hyphae (Valášková and Baldrian, 2006). Thus although no increases in secreted enzyme activity were detected during interactions, increased production of enzymes involved in nutrient acquisition cannot be excluded.

2.4.4 The lack of LiP and catalase activity may be due to assay sensitivity or intracellular localisation

The lack of detectable LiP activity may have been due to low enzyme production, or due to low sensitivity of the assay. LiP activity at interaction zones was indicated by staining, but this could be attributable to low activity accumulating over time that was not detected by the assay. LiP has not previously been identified in the response to biotic stresses such as interactions (Baldrian, 2004; Peiris, 2009). Catalase activity was not detected in any region of any interaction, probably due to intracellular localisation of the enzyme, or again low sensitivity of the assay. Thus, extracellular H_2O_2 levels are likely to be regulated by other mechanisms. Whole mycelial extracts, rather than culture extracts, would be better suited to detect changes in catalase activity during interactions.

2.4.5 Addition of Mn^{2+} to the medium increased ligninolytic enzyme production

Mn^{2+} is known to regulate MnP and MRP (Mn-repressed peroxidase) in *T. versicolor*, and it also alters the production of laccase (Johansson et al., 2002; Mikiashvili et al., 2005). Due to the abundance of Mn^{2+} in the substrate, production of MnP would be expected. On Mn^{2+}-enriched agar, there was a similar pattern of MnP and laccase activity at the interaction zone, implying similar regulation of these enzymes in this region when there is ample Mn^{2+}. At the TvSg interaction zone, laccase activity relative to TvTv was much higher during growth on Mn^{2+}-enriched agar compared to growth on 2% MA, but activity levels were similar in TvBa and TvHf relative to TvTv on both substrates. This may be caused by different susceptibility of the competitor to Mn^{2+}, or stimulation of laccase production by increased levels of quinone products of

MnP activity, to function in their detoxification similar to the suggested role of laccase during lignin decomposition (Thurston, 1994; Solomon et al., 1996). The differences in laccase and MnP activity in the A and B regions of interactions during growth on Mn^{2+}-enriched agar suggests that whilst the roles of MnP and laccase may be similar at the interaction zone, roles in other areas of the mycelium are not. Abiotic conditions have previously been shown to alter interaction outcomes (Rayner and Boddy, 1988; Boddy, 2000). Here, Mn^{2+} levels changed the outcome of interactions with *H. fasciculare* from replacement of *T. versicolor* to deadlock. This may have been caused by increased MnP production by *T. versicolor*, or the effects of Mn^{2+} on other factors.

2.4.6 DOCs affect extension rate but not ligninolytic enzyme production

The reduction of *T. versicolor* extension rate during growth on diffusibles from self-pairings of *H. fasciculare* GTWV2 and *F. fomentarius* implies constitutive production of antagonistic DOCs by these isolates. The reduction in *T. versicolor* extension rate to DOCs from interactions involving *H. fasciculare* may be a response to constitutive or interaction-specific DOCs. There was difficulty deciding on a suitable control: addition of plain malt broth (the 'blank' control) to agar plates would have increased the nutrient content of the plates more than those which had culture extracts added to them, which would have been depleted in nutrients by the previous colonisers. Also, using self-pairings as controls does not take into account any effects 'self' DOCs may have. Whichever control is used, laccase was not upregulated by DOCs from any self-pairing or interaction, and relative to the 'blank' control there was inhibition of laccase in response to DOCs from three interactions and the *H. fasciculare* self-pairing. Similarly, cell-free filtrates (DOCs) and sterilised culture homogenates of *T. harzianum* failed to increase laccase activity in *T. versicolor* (Baldrian, 2004). However, MnP

activity was stimulated by diffusibles from *S. gausapatum* and interactions involving *H. fasciculare.*

It might be expected that chitinase and acid phosphatase activities would increase during growth on fungal diffusibles, which would likely contain wall fragments which could be degraded as nitrogen and phosphate sources. However, the reverse occurred in cultures grown on certain interaction diffusibles, and those grown on *T. versicolor* self-pairing diffusibles, with decreased levels of chitinase and acid phosphatase compared to the blank control. This may have been a result of nutrient depletion in liquid cultures compared to the broth control; because the response was the same to 'self' diffusibles it is unlikely to be a result of toxicity or enzyme inhibition.

2.4.7 H_2O_2-inhibition of growth is not linked to changes in enzyme activity

The extension rate of *T. versicolor* was considerably inhibited by growth on agar containing H_2O_2. The accumulation of H_2O_2 and superoxide is known to occur during interactions (Silar, 2005). Despite this, no significant changes in enzyme activity were detected. Laccase activity was increased relative to controls during growth on 10 mM H_2O_2, although this was not supported by the statistics. MnP activity was not affected by growth on 10 mM H_2O_2, which is surprising as peroxidases could function to remove excess H_2O_2, thus reducing its potential to cause damage through oxidative stress. The increase in laccase activity could not directly mediate H_2O_2 stress in this way, but may be up-regulated to detoxify the quinone products of increased peroxidase activity (Thurston, 1994; Solomon et al., 1996). Although H_2O_2 can generate oxidative stress, it plays such a key role in ligninolysis that white rot fungi generate and regulate levels of it as part of normal growth. Addition of exogenous H_2O_2 to cultures of *T. versicolor* has previously been found to enhance activity of ligninolytic enzymes, although not as

effectively as the prooxidant paraquat (Zhao et al., 2009). H_2O_2 had more of an effect on the expression of intracellular antioxidant enzymes (such as catalase and superoxide dismutase), which were enhanced rapidly after addition of the H_2O_2 (Zhao et al., 2009). It would appear that addition of prooxidants such as paraquat or menadione to fungal cultures is an effective way of increasing ligninolytic enzyme activity, and are more suitable to test the involvement of ligninolytic enzymes in mediating oxidative stress (Jaszek et al., 2006a,b; Zhao et al., 2009).

Chapter 3

Expression of genes encoding ligninolytic enzymes during interactions

3.1 Introduction

Although several studies have used enzyme assays to analyse the changes in ligninolytic enzyme activity during interactions, very few have looked at changes in their gene expression. There have been several studies examining global changes in gene expression during interactions using microarrays, detecting differences in expression of genes involved with stress responses and ROS, but not of ligninolytic enzymes (Iakovlev et al., 2004; Adomas et al., 2006; Eyre, 2007). Although expression of laccase genes during interactions involving *S. hirsutum* compared to growth alone has been analysed (Peiris, 2009), changes in expression of both laccase and peroxidase genes during interactions with a variety of outcomes have not yet been assessed.

Laccase and peroxidase sequences derived from basidiomycetes are highly conserved, with, on average, 58% and 54% similarity respectively (Conesa et al., 2002; Necochea et al., 2005). The copy number of laccase genes varies between species (Eggert et al., 1996), and laccase genes from different species may display a higher degree of homology than two laccase genes from the same species, probably due to a conserved function (Jönsson et al., 1995). Laccases and peroxidases are often secreted as multiple isozymes; the recently sequenced *P. chrysosporium* genome contains large and complex families of structurally related peroxidase genes, and large gene families have also been found in *Pleurotus* and *Ceriporiopsis* spp. (Camarero et al., 1999; Kersten and Cullen, 2007). The physiological significance of this multiplicity is unclear, perhaps conferring adaptability to different environmental conditions, or merely reflecting redundancy (Kersten and Cullen, 2007).

Genes encoding ligninolytic enzymes of *Trametes versicolor* have been extensively studied, with forty sequences for *T. versicolor* laccases and peroxidases accessioned in GenBank (The National Centre for Biotechnology Information, NCBI, MD, USA: http://www.ncbi.nlm.nih.gov). Two databases of short sequences are also freely available online: the Concordia database (http://fungalgenomics.concordia.ca/fungi/Tver.php), and the EST (expressed sequence tag) library generated by Eyre (2007). *T. versicolor* produces high quantities of laccase and peroxidases, in multiple isozymes; in one study, 21 peroxidases were detected in carbon-limited cultures (Johansson and Nyman, 1993). This multiplicity has led to some confusion, as much of the previous work isolated laccase and peroxidase isozymes based on their chromatographic elution profiles, which introduces problems as sequences that are not closely related often share similar isoelectric points (Necochea et al., 2005). The situation is further complicated by the appearance of several isoforms of a particular isozyme due to differences in post-translational modification (e.g. glycosylation patterns; Necochea et al., 2005). This has led to an over-estimation of the number of laccase and peroxidase isozymes (Necochea et al., 2005).

Laccases in *T. versicolor* are encoded by at least four genes. There are twenty *T. versicolor* laccase sequences available from GenBank, sixteen of which cover the whole coding region of the gene. On the basis of amino acid sequence similarity, the sequences can be assigned to four groups: α, β, δ and γ (Figure 3.1; Necochea et al., 2005). High homology between sequences within these groups implies that these are closely related genes, perhaps allelic variants (Necochea et al., 2005). Laccase genes contain 10 introns, more or less uniformly distributed throughout the gene, ranging in size from 48 to 64 bp (Jönsson et al., 1995; Ong et al., 1997).

T. versicolor LiP and MnP are encoded by multigene families of clustered and unclustered genes, again producing a variety of isozymes (Johansson and Nyman, 1995). Both LiP and MnP genes contain 5-6 introns, most of which occur in conserved locations (Jönsson et al., 1995). There appears to be production of a greater number of LiP isoforms compared to MnP, with only 5 MnP isoforms detected in *T. versicolor* cultures compared to 16 LiPs (Johansson and Nyman, 1993). There are 9 LiP sequences and 9 MnP sequences accessioned in GenBank, and two almost identical sequences for Mn-repressed peroxidase (MRP). Identity between the amino acid sequences is approximately 80% between LiP isozymes, whilst MnP are more heterogeneous at 70% (Johansson and Nyman, 1993). Based on amino acid sequence similarity, three variants of LiP and three of MnP can be designated (Figure 3.2). Although the two MRP nucleotide sequences differ slightly, the resulting amino acid sequence is identical. The LiP and MnP sequences of *T. versicolor* are more similar to LiPs and MnPs from other species than to each other (Conesa et al., 2002; Martinez, 2002).

The conserved features of peroxidase amino acid sequences, such as the cysteine residues involved in formation of disulfide bridges, are found in all LiP, MnP and MRP sequences from *T. versicolor*. Different types of peroxidase have different residues essential for their activity (Conesa et al., 2002; Martinez, 2002). An annotated alignment of the available peroxidase amino acid sequences is given in Appendix Figure C.1. All LiP

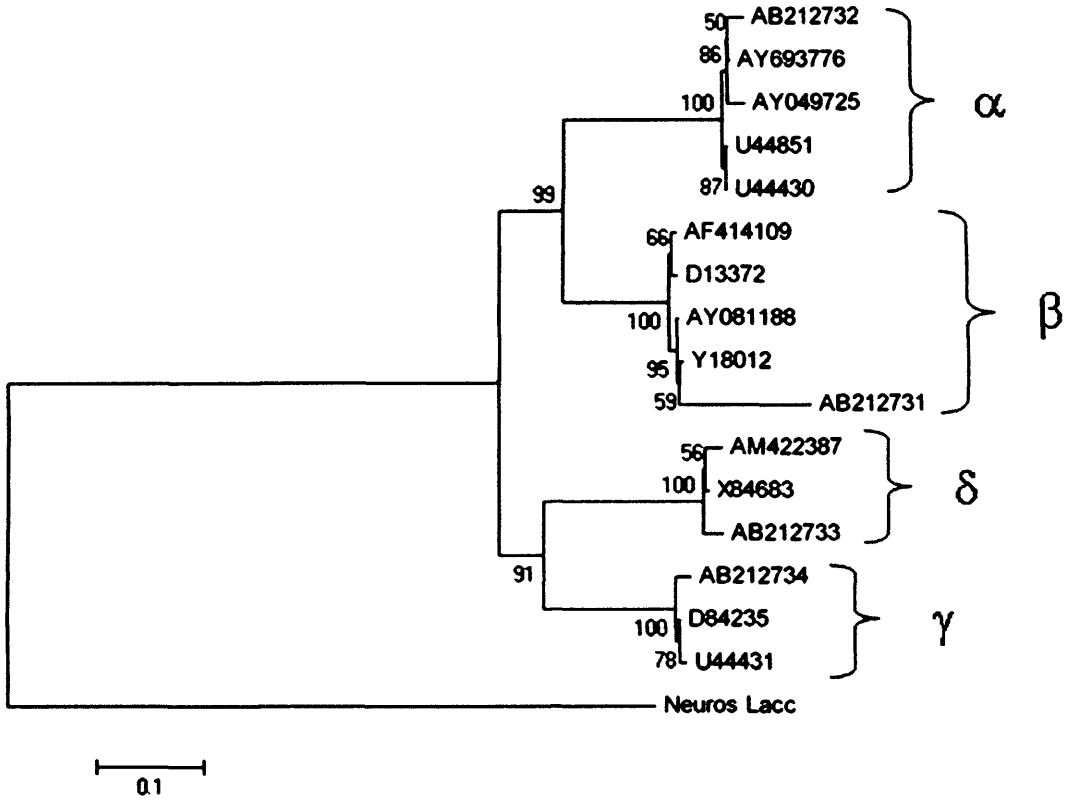

Figure 3.1: Neighbour joining reconstruction of the phylogenetic relationships among *T. versicolor* laccase amino acid sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree (sum of branch length = 1.89) is shown, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were calculated using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data (complete deletion option). There were a total of 493 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). The laccase amino acid sequences are identified by their GenBank accession code. α, β, δ and γ correspond to groups based on a phylogenetic classification, following Necochea et al. (2005). 'Neuros Lacc' is the *Neurospora crassa* laccase sequence (GenBank accession M18334), used as an outgroup sequence to root the tree.

sequences contain the exposed tryptophan (Trp) residue necessary for oxidation of the mediator veratryl alcohol (VA). All MnP sequences contain three acidic residues which are involved in Mn^{2+}-binding (two glutamic acids, one aspartic acid; Figure 3.2). However, there are a couple of exceptions. LiP7 (accession Z30667) is closely related to other LiP isozymes based on sequence homology, but as well as the VA-oxidation Trp residue, it also contains the three Mn^{2+}-binding residues, so could potentially function like LiP or MnP (Figure 3.2). MRP sequences also have the VA-oxidation Trp residue and the three Mn^{2+}-binding residues, but there is no close sequence homology to either LiP or MnP (Figure 3.2). The exact role MRP plays is unclear, but it has been suggested that the Mn^{2+}-binding residues could function to bind alternative substrates, such as aromatic amines, when levels of Mn^{2+} or VA are depleted (Collins et al., 1999).

The previous chapter examined the activity of ligninolytic enzymes during interactions involving *T. versicolor*, both spatially and temporally within the interacting mycelia. This chapter aims to link these changes in activity with changes in expression of genes encoding ligninolytic enzymes using semi-quantitative RT-PCR. Analysis of ligninolytic gene expression in different regions of interacting mycelia had not previously been performed, nor the relationship between ligninolytic gene expression in interactions with different outcomes. In accordance with assay results, an up-regulation of laccase and MnP transcripts during interactions was expected, but no upregulation of MRP or LiP transcripts. Similar to assay results, the largest up-regulation relative to *T. versicolor* self-pairings was expected to occur in the interaction zone. RT-PCR was also used to analyse the expression of a sequence with homology to catalase which was identified as changing in expression during interactions involving *T. versicolor* (Eyre, 2007). PCR products from different primer sets were cloned and sequenced to determine primer specificity and prevalence of any particular transcripts. As the multiplicity of isozymes has been attributed to different roles in combating stress, it is expected that different transcripts would be expressed during interactions compared to during growth alone.

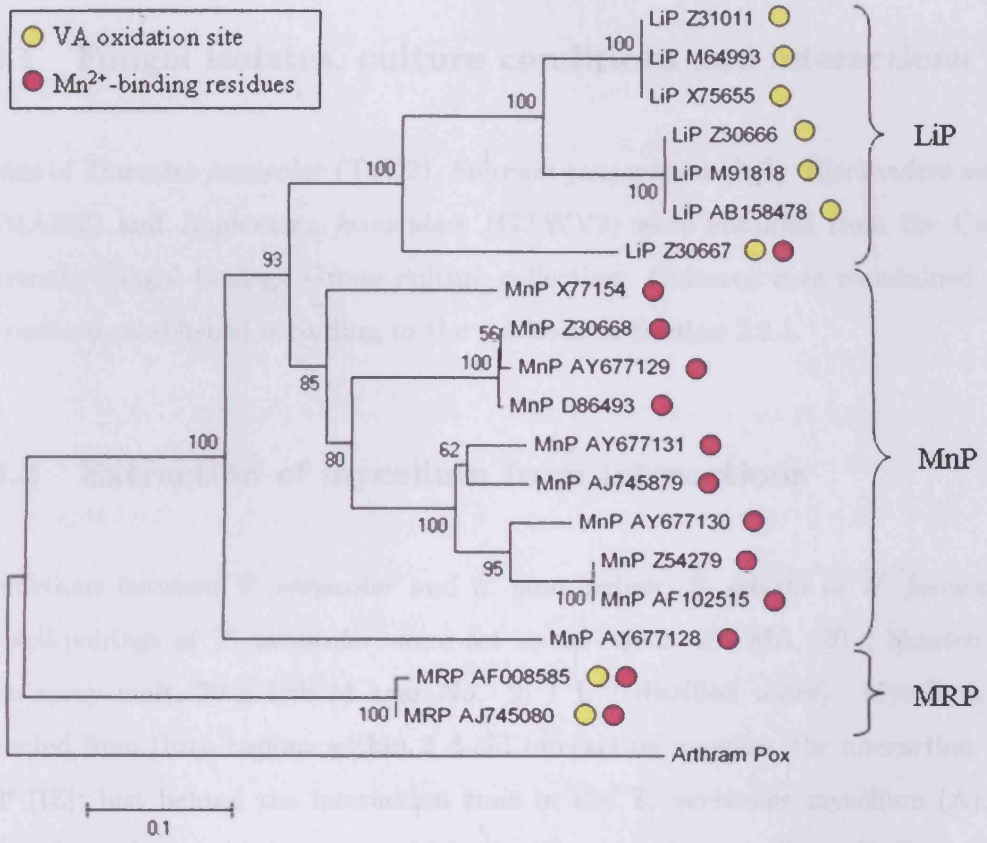

Figure 3.2: Neighbour joining reconstruction of the phylogenetic relationships among *T. versicolor* peroxidase amino acid sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree (sum of branch length = 2.15) is shown, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were calculated using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data (complete deletion option). There were a total of 359 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). The peroxidase amino acid sequences are identified as LiP, MnP or MRP and by GenBank accession code. Pink dots next to sequence names indicate presence of the three residues involved in Mn^{2+} binding, yellow dots indicate presence of the Trp residue involved in VA oxidation, based on the sequence alignment given in Figure C.1. 'Arthram Pox' is the *Arthromyces ramosus* peroxidase sequence (accession D63792), used as an outgroup sequence to root the tree.

3.2 Materials and Methods

3.2.1 Fungal isolates, culture conditions and interactions

Strains of *Trametes versicolor* (TvD2), *Stereum gausapatum* (Sg1), *Bjerkandera adusta* (BaMA313) and *Hypholoma fasciculare* (GTWV2) were obtained from the Cardiff University Fungal Ecology Group culture collection. Cultures were maintained, and interactions established according to the methods in Section 2.2.1.

3.2.2 Extraction of mycelium from interactions

Interactions between *T. versicolor* and *S. gausapatum*, *B. adusta* or *H. fasciculare*, and self-pairings of *T. versicolor*, were set up on 'thick' 2% MA (20 g Munton and Fison spray malt; 20 g Lab M agar No. 2; 1 L^{-1} distilled water). Mycelium was extracted from three regions within 2 d-old interacting mycelia: the interaction zone itself (IZ); just behind the interaction zone in the *T. versicolor* mycelium (A); 30-40 mm from the interaction zone within the *T. versicolor* mycelium (B; Figure 2.1). Mycelium was scraped off the surface of the agar using a sterile spatula and kept on ice before flash-freezing in liquid nitrogen. Extracted mycelium was stored at −80°C until used. One region only was extracted from a plate. Mycelium collected from 25 plates was combined to make a single biological replicate; two biological replicates were used. Total mycelial growth of 2 d-old cultures of *S. gausapatum*, *B. adusta* and *H. fasciculare* were also collected, four replicates for each species. Extraction of mycelia from 8 d-old interactions was also performed.

3.2.3 Extraction of RNA

Mycelium was ground to a fine powder in liquid nitrogen in a sterile, chilled pestle and mortar. 2 ml of Tri-Reagent (Sigma) was added and grinding continued to form a smooth paste. Equal amounts were transferred to two 1.5 ml Eppendorf tubes, vortexed, and allowed to stand at room temperature for 5 min. Tubes were then centrifuged at 10,000 *g* at 4°C for 10 min. The clear supernatants were transferred to two fresh Eppendorf tubes, 0.2 ml chloroform added to each tube, vortexed, and left to stand at room temperature for 5 min. Tubes were then centrifuged at 10,000 *g* at 4°C for 15 min. The aqueous (top) layers were transferred to fresh Eppendorf tubes, making sure not to disturb the other layers. Isopropanol (0.5 ml) was added to each tube, mixed by hand, and left to stand at room temperature for 10 min. Tubes were then centrifuged at 10,000 *g* at 4°C for 10 min. The supernatants were removed, taking care not to disturb the pellets. Ethanol (1 ml 75%) was added and tubes vortexed for 15 s, then centrifuged at 10,000 *g* at 4°C for 10 min. The supernatant was discarded and the pellets allowed to air-dry in a laminar flow hood (10-30 min). Pellets were resuspended in 50 μl sterile distilled water and the contents of the two tubes combined. Samples were stored at −80°C.

Gel electrophoresis was performed on 5 μl samples to check for the presence of RNA, using a gel tank and tray that had been soaked in 0.1 M NaOH for 10 min and rinsed copiously in distilled H_2O. RNA concentration was quantified using a Nanodrop spectrophotometer (Thermo Scientific, USA).

3.2.4 DNase treatment of RNA

DNase treatment was performed to remove any residual DNA from the RNA samples. The DNase digestion was set up as follows: $2 - 16$ μl RNA solution (equivalent to

2 μg RNA), 2 μl RQ1 DNase 10× buffer, 2 μl RQ1 DNase (Promega), made up to 20 μl with sterile distilled water. Reactions were incubated at 37°C for 30 min, 2 μl RQ1 DNase Stop solution was added, then incubated at 65°C for 10 min to inactivate the DNase. The success of DNase treatment was confirmed by PCR using the treated RNA, where a lack of PCR product indicated successful DNase treatment.

3.2.5 Generation of cDNA by reverse transcription

DNase-treated RNA (19 μl) was transferred to a fresh PCR tube. Oligo$(dT)_{15}$ (500 μg/ml; deoxy polyT primer which anneals to the polyA tail of mRNA; Promega) was added (1 μl) and tubes incubated at 70°C for 10 min, then cooled on ice for 10 min. 6 μl of 5× first strand buffer, 0.2 μl 0.1 M DTT (dithiothreitol), and 1 μl 10 mM dNTPs were added and tubes incubated at 42°C for 2 min. 1 μl Superscript II reverse transcriptase (Promega) was added and tubes incubated at 42°C for a further 50 min, followed by 15 min at 70°C. The resulting single strand DNA was stored at −20°C. Presence of cDNA was checked by PCR.

3.2.6 Extraction of DNA

Mycelium of a 7 d-old culture was scraped from a plate with a sterile spatula and transferred to a 1.5 ml Eppendorf tube. Dried skimmed milk (300 μl, 4% (w/v)) and 300 μl extraction buffer (200 mM Tris·HCl, 250 mM NaCl, 25 mM EDTA and 0.5% SDS at pH 8.5) were added and the mixture ground by hand with a plastic grinder. Tubes were vortexed for 15 s, shaken for 30 min at 37°C, then centrifuged at 11,000 *g* for 5 min. The supernatant was transferred to a fresh 1.5 ml Eppendorf and half the supernatant volume of 3 M sodium acetate added. Tubes were incubated at −20°C for 10 min, then centrifuged at 11,000 *g* for 5 min. The supernatant was transferred

to a 14 ml Falcon tube.

DNA was cleaned using the QIAquick PCR purification kit (Qiagen), as follows: 5 volumes of buffer PB for each volume of DNA extract were added to Falcon tubes. 0.8 ml aliquots of this mixture were pipetted onto QIAprep columns in 2 ml collection tubes and centrifuged at 11,000 *g* for 1 min. The flowthrough was discarded. Buffer PE (wash buffer; 0.75 ml) was added to the columns and left to stand for 5 min, then centrifuged at 11,000 *g* for 5 min. The flowthrough was discarded and columns centrifuged again at 11,000 *g* for 1 min to remove all traces of buffer PE. Columns were placed in clean Eppendorf tubes and 30 μl of sterile distilled water was applied to the centre of the column membrane. Columns were left to stand for 5 min, then centrifuged at 11,000 *g* for 1 min. The flowthrough – the DNA solution – was stored at −20°C.

3.2.7 Primer design

T. versicolor laccase and peroxidase sequences were downloaded from GenBank (The National Centre for Biotechnology Information, NCBI, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov; Tables C.1, C.2 and C.3). Both genomic and cDNA sequences were obtained where possible, and primers were designed to cDNA sequences. The sequences from each group were aligned and analysed using BioEdit (http://www.mbio.ncsu.edu/BioEdit) and CLC Workbench (http://www.clcbio.com). Primers were designed to subgroup level (i.e. LiP, MnP and MRP) rather than to individual sequences, using the programs Primer 3 (http://frodo.wi.mit.edu/) and OligoAnalyzer (http://www.idtdna.com/analyzer). It was possible to design primers capable of differentiating between two subgroups within MnP. Primers were also designed to an EST with homology to catalase, previously identified as changing in expression during interactions involving *T. versicolor* (Eyre, 2007), and designated Cat-F/R. All primers

were tested on genomic DNA and cDNA from *T. versicolor, S. gausapatum, B. adusta* and *H. fasciculare*. Primer sequences can be found in Table 3.1.

3.2.8 Semi-quantitative RT-PCR

Individual reactions were set up as follows: 18.9 μl sterile distilled water, 0.5 μl 10 mM dNTPs, 2.5 μl 10× PCR buffer (Qiagen), 0.125 μl Hotstar Taq DNA polymerase (Qiagen), 1 μl 10 mM forward primer, 1 μl 10 mM reverse primer, 1 μl cDNA sample. Reactions were cycled in a Techne Flexigene thermocycler (Bibby Scientific, Staffordshire, UK) using the program: 94°C for 15 min; [94°C for 1 min, T_m for 1 min, 72°C for 1 min] × 30-35 cycles; 72°C for 6 min. The T_m used for Lacc and Cat was 55°C, for MRP, LiP, MnP and glyceraldehyde 3-phosphate dehydrogenase (GPD) it was 50°C, for 18S it was 48°C.

At least three replicates were performed for each primer set with each cDNA sample to eliminate equipment variability. PCR products were run on a 1.5% agarose gel containing ethidium bromide and visualised under UV light, and products were quantified using the Gene Genius bioimaging system and GeneSnap software (Syngene, Synoptics Ltd., Cambridge, UK).

PCR reactions with GPD/TvGPD primers were cycled as above, and product quantification from the target used to normalise results for the other primer sets. Cycle number was optimised and limited for each primer set and cDNA batch combination, by running dilution series of the cDNA and checking for linear responses. This ensured that reactions were in the exponential phase at a particular cycle number, allowing product quantification to be considered semi-quantitative with respect to transcript abundance. This method has previously been used successfully for a range of experimental systems (including Orchard et al., 2005; Parfitt et al., 2005; Wagstaff et al., 2005; Price et al.,

Table 3.1: Primer details

		Primer name	Oligo 5'-3'	no. bases	% GC	Tm °C	Product length	Ref
18S rRNA		EF4	GGA AGG GRT GTA TTT ATT AG	20	38	46	563	(Smit et al., 1999)
		fung5	GTA AAA GTC CTG GTT CCC C	19	53	52.8		
Laccase		LaccF	CTT CAA CGG CAC CAA CTT CTT	21	48	55.9	376	
		LaccR	GAA GTC GAT GTG GCA GTG GAG	21	57	57.9		
Lignin		LiP-F	ACG AAA TCT TGA CCG TGT G	19	47	51.1	353	
peroxidase		LiP-R	GAG CGA GTT GAT GTC CTG	18	56	52.9		
Manganese	1	MnP-F	CAT CTC TCC TTC CAT CGC CTC	21	57	57.1	211	
peroxidase		MnP-R	GGG GCA GTT GCT GAC ACC	18	67	59.4		
	2	MnP2-F	CAT CTC TCG CAA GGC CAA CAA	21	50	58	207	
		MnP2-R	GCC AGC GAA CTG AAT GAA GTC	21	52	56.5		
	3	JNC867mnp1	CCA TCC CTC AAA ACC TCA GG	20	55	55.4	1435	Johannson et al.
		JNC899mnp1	ACT CCC ACC TCA AGC TTA AG	20	50	54.2		(2002)
	4	JNC866mnp2	CGT CTA TCC TCC TCC TCA TA	20	50	52.2	1132	Johannson et al.
		JNC908mnp2	CTA CAG CGT CAT TTA CGA CG	20	50	53.3		(2002)
Manganese-repressed		MRP-F	CAG GAC AAG GTT GAC GAC T	19	52	54.5	171	
peroxidase		MRP-R	GGA CTG AAG ACG GAA CTC	18	56	52.6		
Catalase		Cat-F	AAC ATC CTC GAC CTG ACG AA	20	55	60.3	196	Eyre (2007)
		Cat-R	GAG AAG AGA CGC GAC TGG AG	20	60	59.7		
GPD - general		GPD-F	GTT CAA GTA CGA CTC CGT CCA	21	52	56.5	185	
		GPD-R	ACT TTT CGG TGG TGG TGA AG	20	50	55.4		
GPD - Tv-specific		TvGPD-F	ACC GCA TAC ATC CTA ATC TCG	21	48	54.2	450	
		GPD-R	ACT TTT CGG TGG TGG TGA AG	20	50	55.4		
M13		M13-F	TCA CAC AGG AAA CAG CTA TGA C	22	45.5	54.8	variable	Promega
		M13-R	GTT TTC CCA GTC ACG AC	17	52.9	50.6		

2008).

3.2.9 Normalisation of data

Normalisation was performed to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GPD). Two sets of GPD primers were designed based on a partial *T. versicolor* sequence (GenBank accession AY081189). The set GPD-F/R are not specific to *T. versicolor* and can be used as general fungal primers. *T. versicolor*-specific GPD primers, TvGPD-F/R, were designed by siting the forward primer in the 5' UTR of the gene. Samples from the A and B regions, where there was no competitor mycelium, were normalised using GPD primers. However, in the IZ region extraction of competitor mycelium could not be avoided, so samples were normalised using the *T. versicolor*-specific primers TvGPD. For comparison, normalisation of cDNA samples from the A and B regions was also performed with general fungal primers for 18S rRNA.

Normalisation to GPD transcript levels rather than to 18S rRNA levels was chosen for a variety of reasons. Firstly, IZ cDNA was normalised with TvGPD, so normalisation of the A and B regions to GPD was more consistent. Secondly, oligo(dT) was used in reverse transcription reactions, so it is more accurate to compare efficiency of cDNA generation with another mRNA sequence (GPD) rather than an rRNA sequence (18S). In practice, however, due to the AT richness of rRNA sufficient 18S rRNA would also be retrotranscribed and can thus be used for normalisation, an approach which has been used successfully in other systems (e.g., Orchard et al., 2005; Price et al., 2008). Finally, the two biological replicates used for each region of each interaction are more consistent with each other when GPD is used, with no significant differences between 80% replicate pairs when normalised with GPD, but only 70% when normalised with 18S. Graphs comparing gene expression between the biological replicates for GPD and

18S are presented in Appendix Figures C.2 and C.3. Data from the two biological replicates were combined, even where they disagreed, which is a conservative approach as this makes us less likely to see significant differences in expression compared to TvTv. For comparison to results normalised with GPD (Figure 3.4), results normalised with 18S are presented in Appendix Figure C.4.

3.2.10 Statistical analysis

Gene expression relative to other interactions in each region for each primer set were compared using one-way ANOVA, where data met the required assumptions: that residuals are normally distributed (Anderson-Darling test) with equal variances between groups (Levene's test). Significant results were further explored using the Tukey-Kramer *a posteriori* test to determine significant differences between means. If data did not meet the assumptions of ANOVA, Kruskal-Wallis tests in combination with *post hoc* Mann-Whitney U-tests were used. All tests were performed in Minitab (v.15).

3.2.11 Purification of PCR products

PCR reactions were carried out using cDNA from the A region in *T. versicolor* self-pairings and from the interaction between *T. versicolor* and *S. gausapatum*. The primers used were Lacc, LiP, MRP, Cat and GPD. PCR products were run on an agarose gel and visualised on a UV light box. Bands were excised using a razor blade and the agarose slices transferred to 1.5 ml Eppendorf tubes, and weighed.

A QIAquick Gel Extraction Kit (Qiagen) was used to extract the PCR products. Three volumes of buffer QG were added to one volume of gel (where 100 mg $\sim$ 100 μl). Tubes were incubated at 50°C for 10 min, or until the gel slice had completely

dissolved. One gel volume of isopropanol was added to the sample and mixed well, applied to a QIAquick spin column in a 2 ml collection tube, and centrifuged for 1 min at 11,000 *g*. The flowthrough was discarded. Buffer PE (wash buffer; 0.75 ml) was added to the column, and centrifuged for 1 min (performed twice). The flowthrough was discarded and the column centrifuged again for 1 min at 11,000 *g* to remove all traces of buffer PE. The column was placed into a fresh 1.5 ml Eppendorf tube. DNA was eluted by adding 30 μl buffer EB (10 mM Tris·Cl, pH 8.5) to the centre of the column membrane, letting it stand for 1 min, then centrifuging for 1 min. The eluted DNA was checked by running 5 μl on an agarose gel.

3.2.12 Ligation into pGEMT-Easy vector

For each PCR product of interest, a ligation was set up, comprising 1 μl (50-100 ng) of plasmid pGEM T-Easy (Promega), 1 μl 10× ligation buffer, 1 μl T4 DNA ligase (Promega) and 7 μl of the purified PCR product. Ligations were incubated at 4°C overnight.

3.2.13 Transformation of *E.coli* DH5α competent cells

Cells of competent *Escherichia coli* DH5α were prepared in house and kept frozen at −80°C in 100 μl aliquots in 1.5 ml Eppendorf tubes. One aliquot was thawed on ice, 2 μl of DNA ligation added and mixed thoroughly. The tube was left on ice for 20 min. Cells were heat shocked by placing the tube in a water bath at 42°C for 45 s exactly, then the tube transferred back to ice for 2 min. 900 μl of SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose) was added, and tubes incubated at 37°C with gentle shaking (100 rpm) for approximately 1 hr.

Cells were plated onto LB solid medium (Luria-Bertani medium; 1.0 g tryptone, 0.5 g yeast extract, 1.0 g NaCl, 1.5 g agar, 100 ml distilled water) containing 100 μg/ml ampicillin in 9 cm non-vented Petri dishes. Plates were incubated at 37°C overnight (for a maximum of 18 hrs) and transferred to 4°C.

Colony PCR was performed to identify positive clones following transformation. Single, well-separated colonies were transferred to 1.5 ml Eppendorf tubes containing 200 μl LB liquid medium (as before, excluding agar) plus 100 μg/ml ampicillin. Ten colonies were chosen at random for transferral. Tubes were incubated at 37°C for $3-4$ hrs with gentle shaking (100 rpm). Tubes were checked for bacterial growth by odour. 1 μl of the bacterial cultures were used in PCR reactions using M13-F/R primers (specific to either side of the pGEM-T ligation site). The rest of the cultures were stored at room temperature. PCR products were electrophoresed and positive cultures identified based on size of bands. Overnight cultures containing 10 μl bacterial culture in 3 ml LB liquid medium were set up for eight of the positive transformations per treatment.

3.2.14 Plasmid DNA purification

Plasmid DNA was extracted and purified using a QIAprep Spin Miniprep Kit (Qiagen). The overnight cultures were centrifuged to pellet bacterial cells, the supernatant discarded, and cells resuspended in 250 μl Buffer P1 containing RNase A. 250 μl Buffer P2 was added (lysis buffer) and mixed by inverting tubes $4-6$ times. This lysis reaction should not be allowed to proceed for more than 5 min. 350 μl Buffer N3 was added and mixed by inverting tubes $4-6$ times. Tubes were centrifuged for 10 min at 11,000 *g*. The supernatants were decanted into QIAprep spin columns, which were centrifuged for 45 s at 11,000 *g*. Flowthrough was discarded. The column was washed by adding 0.75 ml Buffer PE and centrifuging for 45 s at 11,000 *g* for 45 s. Flowthrough was discarded and this step repeated. Tubes were centrifuged for an additional 60 s

to remove any residual wash buffer. The spin columns were placed into clean 1.5 ml Eppendorf tubes, and 50 μl Buffer EB (Tris·Cl, pH 8.5) added to spin column membranes. Columns were left to stand for 1 min, and centrifuged for 1 min. Flowthrough – the purified plasmid DNA – was stored at −20°C until used.

3.2.15 Sequencing of plasmid DNA

Purified plasmid DNA was checked by agarose gel electrophoresis. DNA concentration and quantified using a Nanodrop spectrophotometer (Thermo Scientific, USA). Samples were submitted to the Cardiff University Sequencing Service as 10 μl aliquots. Sequencing was performed using M13-R primers. For each treatment, eight samples were submitted for sequencing except for GPD where only two samples were submitted.

3.3 Results

3.3.1 RNA extraction

RNA was successfully extracted from 2 d *T. versicolor* mycelia during interactions. However, by 8 d the *T. versicolor* mycelium had thickened, forming a thick layer which adhered to the agar and was very difficult to remove, especially in the A region. As a result there was a high agar content in all extracted mycelium at 8 d which interfered with RNA extraction, meaning that only small amounts, if any, RNA could be extracted. Alternative extraction methods were attempted (including RNeasy Plant Mini Kit, Qiagen, UK) but with no success. Thus semi-quantitative RT-PCR could only be performed for 2 d samples.

3.3.2 Primer testing

All primers were tested on genomic DNA and cDNA of *T. versicolor* and the three competitors during growth alone. The primers Lacc, LiP, MRP and Cat all amplified sequences from genomic DNA and cDNA of *T. versicolor* (Table 3.2). Cat primers also amplified sequences from *B. adusta* genomic DNA and cDNA, and MnP amplified sequences from *B. adusta* genomic DNA only (Table 3.2). Cat amplified sequences from *S. gausapatum* genomic DNA but not cDNA (Table 3.2). With the exception of Cat and MnP, the primers of interest were specific to *T. versicolor* even in the presence of cDNA from a competitor (Figure 3.3A). GPD and 18S amplified sequences from all species used, whilst TvGPD was *T. versicolor*-specific (Figure 3.3A).

Table 3.2: Primer specificity in *T. versicolor* and three competitor species

Primer pair	*T. versicolor*		*S. gausapatum*		*B. adusta*		*H. fasciculare*	
	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA
Lacc-F/R	+	+	-	-	-	-	-	-
LiP-F/R	+	+	-	-	-	-	-	-
MnP-F/R	+	-	-	-	+	-	-	-
MnP2-F/R	-	-	-	-	-	-	-	-
MRP-F/R	+	+	-	-	-	-	-	-
Cat-F/R	+	+	+	-	+	+	-	-
GPD-F/R	+	+	+	+	+	+	+	+
TvGPD-F/R	+	+	-	-	-	-	-	-

gDNA, genomic DNA; +, primers will amplify from this type of DNA sequence; -, primers will not amplify from this type of DNA sequence.

No MnP transcripts were detected at 2 d using MnP-F/R or MnP2-F/R primers. When tested on *T. versicolor* genomic DNA and cDNA, MnP-F/R amplified sequences from genomic DNA but not cDNA (Figure 3.3B). MnP2-F/R did not amplify sequences from either genomic DNA or cDNA (Figure 3.3). Two other sets of primers specific for *T. versicolor* MnP genes were obtained from Johansson et al. (2002), but these

also did not detect expression in *T. versicolor* cDNA (results not shown) indicating that expression was absent or at undetectably low levels. Primers were also tested on cDNA from interacting cultures in case expression was interaction-specific, but there was still no response.

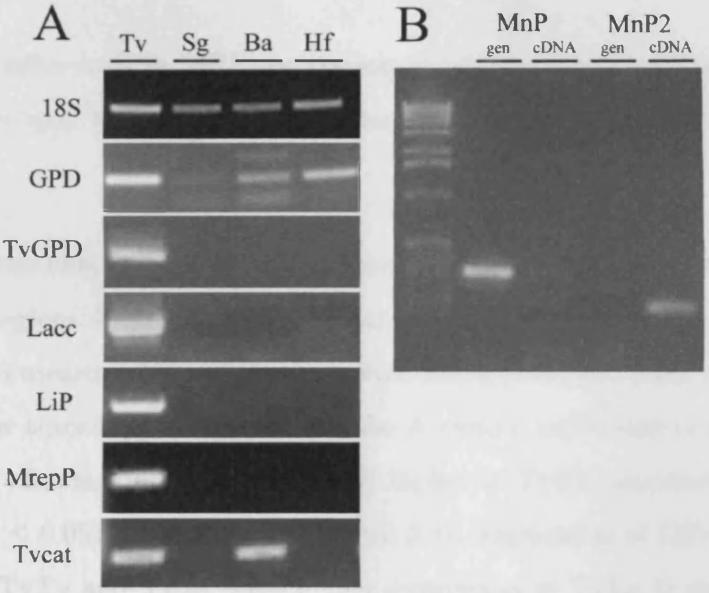

Figure 3.3: Testing specificity of primers of interest. **A**: Specificity of primers on cDNA from cultures of *T. versicolor* (Tv), *S. gausapatum* (Sg), *B. adusta* (Ba) and *H. fasciculare* (Hf) during growth alone. **B**: Testing of MnP-F/R and MnP2-F/R primers on genomic (gen) and cDNA of *T. versicolor*. The band in the far right lane is from primer-dimers. Details of all primers used are given in Table 3.1.

3.3.3 Semi-quantitative RT-PCR: expression of laccase, MRP, LiP and catalase genes

Laccase gene expression was detected in all regions of all the interactions and the TvTv self-pairing (Figure 3.4). There was significantly ($P \leq 0.01$) higher laccase expression

in the IZ region of TvHf (interaction with *H. fasciculare*) compared to TvTv IZ, and this higher expression was maintained in the A region but not in the B region. In the B region, there were more differences in expression, with TvBa (interaction with *B. adusta*) significantly ($P \leq 0.05$) higher than TvTv B, and TvSg (interaction with *S. gausapatum*) significantly ($P \leq 0.05$) lower than TvTv B.

No significant differences in MRP expression were detected in any region, although transcript levels were higher in the IZ regions of TvSg and TvHf compared to TvTv IZ (Figure 3.4).

In contrast to the enzyme assays (see Chapter 2), there was a detectable level of LiP transcripts in regions A and B though not at the IZ. However, a higher cycle number was required to measure LiP PCRs than with the laccase and MRP primers, which may suggest low transcript abundance. In the A region, expression of LiP was similar in TvTv and TvBa, significantly ($P \leq 0.05$) higher in TvHf compared to TvTv, and significantly ($P \leq 0.05$) lower in TvSg (Figure 3.4). Expression of LiP in the B region was similar in TvTv and TvSg, with higher expression in TvBa B and TvHf B, but these differences were not significant ($P > 0.05$; Figure 3.4).

Catalase expression was similar in the IZ and A regions. Expression in TvSg and TvHf was significantly ($P \leq 0.05$) lower than in TvTv in both IZ and A regions (Figure 3.4). Expression in TvBa was lower than in TvTv in both IZ and A region, but this was only significant ($P \leq 0.05$) in the A region: caution must be taken when interpreting transcript abundance in the TvBa IZ region as the Cat-F/R primers also amplify sequences from *B. adusta* cDNA. In the B region, TvSg catalase was still significantly ($P \leq 0.05$) lower than in TvTv B, but there was significantly ($P \leq 0.05$) higher expression in TvBa B compared to TvTv B (Figure 3.4).

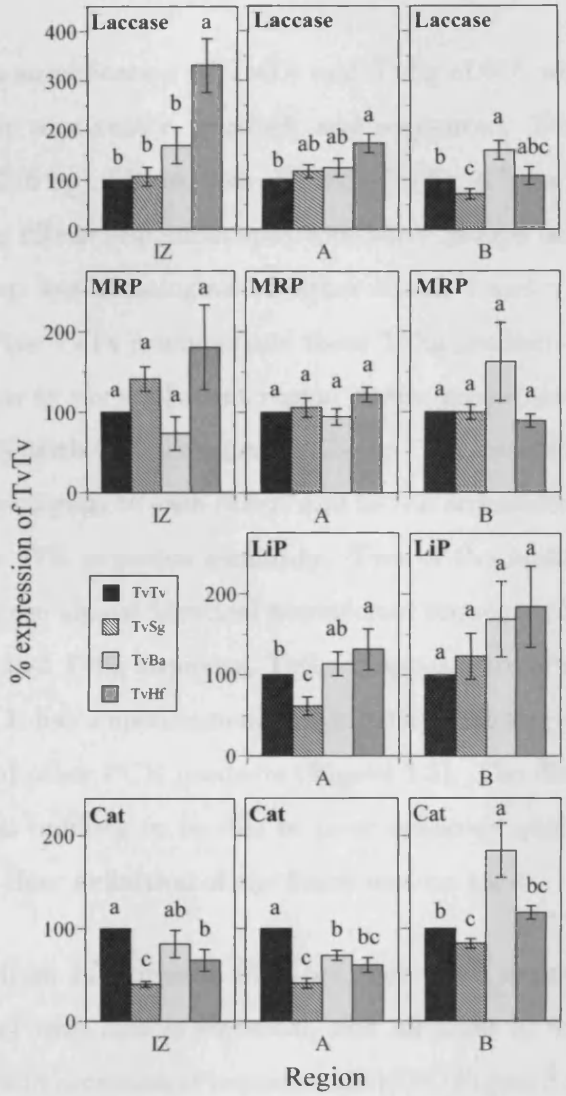

Figure 3.4: Expression of laccase, MRP, LiP and catalase (Cat) gene by *T. versicolor* mycelia during self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *B. adusta* (TvBa) or *H. fasciculare* (TvHf) at 2 d. Bars are the mean data for two biological replicates, each consisting of three technical replicates, ± SEM. Data are expressed as % transcript abundance relative to TvTv cDNA samples from each region. Statistical comparisons were made separately for each enzyme, within a region different letters indicate a significant difference ($P \leq 0.05$).

3.3.4 Sequencing of PCR products

PCR products from amplification of TvTv and TvSg cDNA were cloned into *E. coli* using pGEM-T Easy as a vector, purified, and sequenced. For PCR products from Lacc-F/R primers (376 bp), fifteen clones (7 from TvTv, 8 from TvSg) were sequenced (see Table 3.1). The fifteen sequences split into three groups on the basis of sequence similarity, each group was homologous to either the β, δ and γ groups of accessioned laccase sequences. Five TvTv products and three TvSg products were almost identical, and were homologous to the equivalent region in the accessioned sequence AB212734 (γ group; Figure 3.5), with 98% sequence similarity. Two products from TvTv and two from TvSg were homologous to each other, and to the accessioned sequence AB212733 (δ group; 3.5), with 97% sequence similarity. Two of the remaining TvSg sequences were homologous to the almost identical accessioned sequences Y18012 and AY081188 (β group; 3.5). The last TvSg sequence, TvSg 5, appears to form a separate branch to the α and β groups. It has a maximum of 81% identity with any other laccase sequence, both accessioned and other PCR products (Figure 3.5). The discrepancy in homology to other sequences is unlikely to be due to poor sequence quality, as examination of the sequence shows clear definition of the bases and no gaps.

For PCR products from LiP primers (353 bp), the seven sequenced samples (4 from TvTv, 3 from TvSg) were almost identical, and all show at least 98% homology to the equivalent region in accessioned sequence M91818 (Figure 3.6). For PCR products from MRP primers (171 bp) the six sequenced samples (2 from TvTv, 4 from TvSg) were homologous to the equivalent region in accessioned sequences AF008585 and AJ745080 (>98% sequence similarity). The seven Cat PCR products were identical to each other and to the contig sequence (TVC00832; Eyre, 2007) from which primers were designed. Similarly, the GPD PCR products were identical to the equivalent region in the *T. versicolor* GPD sequence (AY081189).

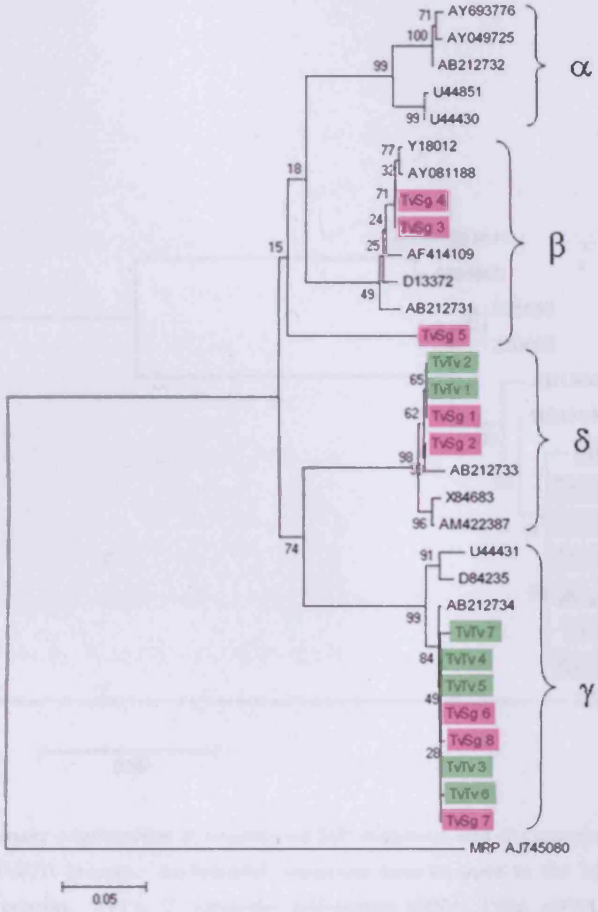

Figure 3.5: Evolutionary relationships between accessioned laccase sequences and PCR products of Lacc primers and *T. versicolor* cDNA from growth alone and during interactions with *S. gausapatum*. Accessioned laccase sequences were cropped to the length of the amplified product of PCR with Lacc-F/R primers. TvTv, *T. versicolor* self-pairing cDNA, replicates numbered 1-7, green; TvSg, cDNA from *T. versicolor* during interaction with *S. gausapatum*, replicates numbered 1-8, pink; cDNA samples from the 'A' region of interactions. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.95 is shown. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 314 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). α, β, δ and γ correspond to groups based on a phylogenetic classification, following Necochea et al. (2005). An MRP sequence was used as an outgroup sequence to root the tree.

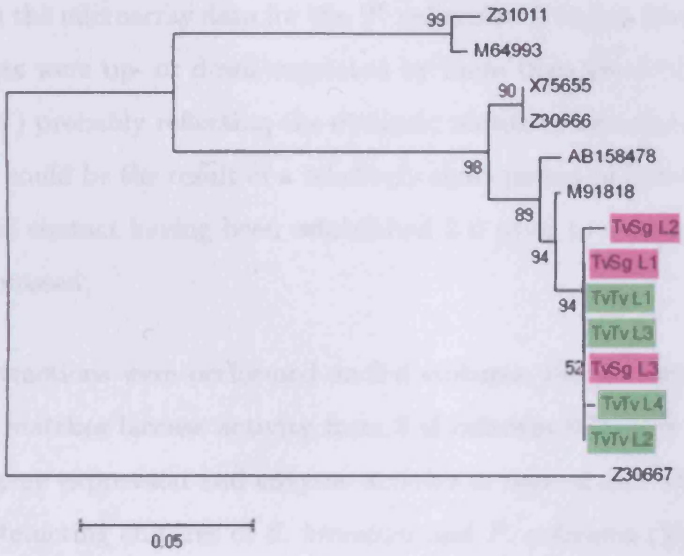

Figure 3.6: Evolutionary relationships of accessioned LiP sequences and the sequenced PCR products of TvTv and TvSg cDNA with LiP-F/R primers. Accessioned sequences were cropped to the length of the amplified PCR product with each set of primers. TvTv, *T. versicolor* self-pairing cDNA; TvSg, cDNA from *T. versicolor* during interaction with *S. gausapatum*; cDNA samples from the 'A' region of interactions. LiP sequenced product replicates are numbered L1-4 for each treatment. The tree was constructed using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.95 is shown. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 324 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

3.4 Discussion

Despite the large changes detected in enzyme activity between interacting *T. versicolor* mycelia and self-pairings (Figure 2.5), changes in gene expression were not dramatic. This agrees with the microarray data for the *T. versicolor* A region during interactions, where few targets were up- or down-regulated by more than two-fold during interactions (Eyre, 2007) probably reflecting the dynamic nature of mycelial responses. High enzyme activity could be the result of a relatively short period of increased expression, and, with hyphal contact having been established 2 d prior to sampling, this window may have been missed.

Whilst RNA extractions were performed on 2 d cultures, the pattern of laccase gene expression best matches laccase activity from 8 d cultures (Chapter 2, Figure 2.5) . A lag between gene expression and enzyme activity is normal and has been reported for laccase in interacting cultures of *S. hirsutum* and *P. ostreatus* (Velázquez-Cedeño et al., 2007; Peiris, 2009). In the B region, TvSg is down-regulated compared to TvTv, whereas TvBa and TvHf are the same or higher. In the A region, activity of laccase during the TvSg interaction at 8 d was increased compared to TvTv, and this is reflected in slightly higher expression of the transcripts (though not statistically significant), likewise the enzyme and transcript patterns for TvBa match, though again not fully supported by the statistics. The major difference is between activity in the A region of TvHf at 8 d, which is the same as TvTv, while the expression is elevated. The most striking difference is at the IZ: here the pattern of expression does not resemble activity at 2 or 8 d. Laccase activity increased relative to TvTv in TvSg and TvBa but not TvHf, whereas transcript levels of TvSg and TvBa are not different from TvTv but are higher in TvHf. However, at the IZ the analysis of the enzyme activity is complex due to potential contribution from both interacting species, whereas the transcript levels were specific to *T. versicolor*.

MnP gene expression was not detected during self-pairings or interactions, which conflicts with the MnP enzyme activity detected during assays (Chapter 2, Figure 2.5). The lack of MnP gene expression is supported by detection of MRP expression, indicating low levels of manganese in the agar which are known to repress transcription of MnP genes (Collins et al., 1999; Johansson et al., 2002). MRP enzymes can function like MnP and LiP, with active sites capable of oxidising veratryl alcohol (LiP-like) and Mn^{2+} (MnP-like), similar to the versatile peroxidase (VP) that occurs in a range of species (Ruiz-Dueñas et al., 2009). Whilst MRP has previously been isolated under conditions of Mn-repression (Collins et al., 1999), this transcriptional control does not exclude the ability of the enzyme to act like MnP when Mn^{2+} levels are rapidly increased, such as in the assay substrate solution. MRP enzymes could thus be responsible for the observed 'MnP' activity detected in assays. The LiP-like isozyme LiP7 (Z30667) could also function like both LiP and MnP (Johansson and Nyman, 1993), so could also contribute to the observed 'MnP' activity, but would not be detected by the primers used.

MRP transcript levels resembled more closely patterns of MnP activity at 2 d rather than 8 d. This might indicate a more rapid production of active secreted enzyme from transcripts for MRP compared to laccase. In the IZ, MnP activity was elevated in TvSg and TvHf interactions, and an increase was also seen in transcripts of MRP, although not statistically significant. The enzyme activity in TvBa at the IZ was less well correlated with the transcripts, which appeared repressed. In the A and B regions, TvSg and TvHf enzyme activities did not differ from TvTv and the same was true for transcript levels. The only significant change in enzyme activity compared to TvTv in the A and B regions was in the TvBa interaction, where activity was higher; transcripts were a little elevated in the B region though again not significantly.

Expression of LiP was not reflected in enzyme activity. LiP enzyme activity was not detected during growth alone or during interactions (Section 2.3.3), but LiP gene

expression was at measurable levels. Higher expression of LiP compared to TvTv occurred in TvHf in both A and B regions, and in TvBa in the B region (though not fully supported statistically). This might suggest a role for LiP during interactions. The lack of detectable activity was either attributable to low production (as suggested by the high cycle number necessary for PCR), or caused by lower sensitivity of the assay. Alternatively, there is some evidence that despite its extracellular role, LiP is stored intracellularly, which may explain why expression was detected from the whole mycelia whereas there was no detectable secreted activity (Garcia et al., 1987). LiP has not previously been identified in the response to biotic stresses such as interactions (Baldrian, 2004; Peiris, 2009).

Catalase gene expression was detected, but no extracellular activity was detected during growth alone of during interactions. Catalase is most likely an intracellular enzyme, with the primary function of controlling levels of H_2O_2. The pattern of catalase gene expression in different interactions was very similar in the interaction zone and A regions with lower catalase expression in all interactions compared to TvTv, although this was not significant for TvBa in the IZ perhaps due to amplification of sequences from *B. adusta*. Decreased catalase activity would result in increased H_2O_2 levels, which might stimulate peroxidase activity. For example, TvSg has the lowest levels of catalase expression but the highest levels of MnP activity. This would depend on export of the increased H_2O_2 from within hyphae, as increased intracellular H_2O_2 would lead to oxidative stress. The increase in catalase expression in the B region of TvBa could be a response to oxidative stress. These results for catalase expression disagree with the findings of Eyre (2007), where the gene was slightly up-regulated in the A region of all three interactions. This discrepancy could be attributable to differences in specificity between microarrays and RT-PCR, as microarray cDNA targets are likely to hybridise to several members of a gene family, whereas the PCR primers are much more specific.

Two normalisation methods were used to confirm gene expression patterns, because of the large differences compared to the patterns of enzyme activity. The use of housekeeping genes to quantify mRNA has, in certain cases, been shown to be somewhat unreliable, as although these genes are implicated in basal cell metabolism they may also participate in other functions and numerous studies have shown that their expression can vary in certain situations (Thellin et al., 1999; Stürzenbaum and Kille, 2001). Although GPD and 18S are both constitutively expressed during normal metabolism, fungal interactions have huge impacts on cellular processes so could alter the transcription of many genes. GPD is the most widely used control gene in RT-PCR (Stürzenbaum and Kille, 2001), and was also chosen to normalise the data because of the higher consistency between biological replicates. When normalised to GPD, the IZ and A regions of interactions are more similar to each other in laccase expression, but when normalised to 18S there is a significant increase in expression in the TvSg A region which resembles the assay results more closely (Appendix Figure C.4). Similarly, if 18S is used, a slight increase in catalase expression (though not significant) occurs in all interactions compared to TvTv, which agrees with the microarray data. Despite this, overall patterns of expression are similar when normalised to either GPD or 18S: there were no large differences in expression in different interactions compared to TvTv, in any part of the interacting *T. versicolor* mycelium. Whilst conditions were kept as consistent as possible for the generation of biological replicates (the same batch of agar was used, incubated at the same time, extraction at the same time), the developmental plasticity and rapid response of mycelia to change (Velázquez-Cedeño et al., 2007) could mean that uncontrollable factors during culture could result in differences between biological replicates.

Interactions have been reported to change the production of isozymes secreted by a fungus, for example the secretion of laccase isozymes by *P. ostreatus* was affected by interaction with *Trichoderma longibrachiatum* (Velázquez-Cedeño et al., 2004). Sequencing of PCR products showed that at least three laccase genes were expressed

during interactions, compared to only two during self-pairings; however, the sample size was too small and this would require further analysis. The expression of multiple isozymes is thought to involve adaptability to different environmental conditions (Baldrian, 2004). The expression of different isozymes with higher efficiency – or different post-translational modifications of these isozymes – could explain the discrepancy between laccase gene expression and activity during interactions. The sequenced laccase PCR products were homologous to sequences from either the β, δ or γ groups (Necochea et al., 2005), with the exception of a sequence from TvSg which was not closely related to any of the assigned groups, and may represent a new laccase gene. The expression of various isozymes during interactions with different outcomes would be really interesting to pursue, and is probably the next step for this research.

Chapter 4

Changes in volatile production during interspecific interactions

4.1 Introduction

Recognition as 'non-self' (where there is no mating compatibility) by basidiomycete mycelia triggers antagonistic responses including changes in colony morphology at the interaction zone and elsewhere, growth rate, and production and release of extracellular enzymes and secondary metabolites (including Rayner and Boddy, 1988; White and Boddy, 1992; Griffith et al., 1994; Score et al., 1997). Effects commonly occur following mycelial contact, but sometimes also prior to contact (Schoeman et al., 1996; Wheatley et al., 1997; Savoie et al., 2001; Humphris et al., 2002). The drivers of this 'distance antagonism' are volatile and diffusible secondary chemicals, which may continue to function following mycelial contact.

All basidiomycetes probably constitutively produce profiles of VOCs from mycelium and fruit bodies (Fäldt et al., 1999; Wu et al., 2005), and these have been used chemo-

taxonomically and in the detection of fungi within food and building timber (Nilsson et al., 1996). VOC profiles are reproducible but dependent on several factors including substratum composition (Wheatley et al., 1997; Bruce et al., 2000; Wheatley, 2002; Ewen et al., 2004), temperature (Tronsmo and Dennis, 1978; Jelen, 2002), pH (Chen et al., 1984) and growth stage (Nilsson et al., 1996; Fäldt et al., 1999; Jelen, 2002; Wu et al., 2005). The VOC profile of a fungus may also alter qualitatively and/or quantitatively during interactions, depending on the combination of species involved (Griffith et al., 1994; de Jong and Field, 1997; Hynes et al., 2007). However, it is not known how the profile alters during interactions with different outcomes. Basidiomycete VOC profiles comprise a diverse range of chemical classes, including alcohols, aldehydes, ketones, terpenes and aromatic compounds (Maga, 1981; Sunesson et al., 1995; Nilsson et al., 1996; Breheret et al., 1997; Wheatley et al., 1997; Rosecke et al., 2000; Humphris et al., 2001, 2002; Ewen et al., 2004). Each of these chemical classes contains compounds with known antifungal activity, perhaps most notably the sesquiterpenes. Many sesquiterpenes have antifungal properties and are involved in the defence responses of both plants and fungi (Stadler and Sterner, 1998; Fäldt et al., 1999; Abraham, 2001; Cheng et al., 2005). The antagonistic potential of the VOC profile of a fungus depends on the constituent chemicals, their relative amounts, and the susceptibility of the combatant. VOC-mediated effects during interactions are subtle, and are often manifested as changes in extension rate or enzyme activity (Strobel et al., 2001), and effects can be stimulatory as well as inhibitory (Dick and Hutchinson, 1966; Schoeman et al., 1996; Mackie and Wheatley, 1999).

VOC profiles of individual fungi can change over time, as can those produced during the time course of interactions (Griffith et al., 1994; Wu et al., 2005; Hynes et al., 2007). Following mycelial contact between the basidiomycetes *H. fasciculare* and *Resinicium bicolor*, 10 VOCs were identified that were not detected in single species controls (Hynes et al., 2007). The majority of the VOCs identified were sesquiterpenoids, perhaps further indicating a defensive role of VOCs, since many sesquiterpenoids are

known to have antifungal activity or roles in plant defence (Rostilien et al., 2000; Viiri et al., 2001; Hynes et al., 2007).

The aim of this work was to determine whether the VOC profile of a species changes when faced with different antagonists, during interactions with different outcomes. Effects of VOCs were examined by measuring extension rate of test fungi in agar plates taped above two interacting mycelia – *T. versicolor*, *B. adusta*, *H. fasciculare* and *S. gausapatum* in all combinations. VOC production was quantified and qualified, by solid-phase microextraction followed by analysis using gas chromatography-mass spectrometry (GC/MS), over the course of interactions with *T. versicolor* replacing *S. gausapatum*, deadlocking with *B. adusta* (partial replacement of *T. versicolor* followed by deadlock), and replaced by *H. fasciculare*. Determining which of the interacting pair produces which volatiles is, of course, not possible.

4.2 Materials and Methods

4.2.1 Fungal cultures

Strains of *T. versicolor* (TvD2). *S. gausapatum* (Sg1), *H. fasciculare* (HfGTWV2) and *B. adusta* (Bk1), from Cardiff University culture collection, were maintained on 0.5% (w/v) malt agar (MA; 5 g Munton and Fison spray malt, 15 g Lab M agar no. 2 per litre distilled water) in 9 cm non-vented Petri dishes, incubated upside down at 20°C in the dark.

4.2.2 Effect of VOCs produced during interactions on mycelial extension rate

Interactions between *T. versicolor* (Tv), *S. gausapatum* (Sg), *B. adusta* (Ba) and *H. fasciculare* (Hf) were set up on 0.5% MA in all combinations. Agar plugs (6 mm diam.), cut from the actively growing mycelia margin, were inoculated face down on the agar 30 mm away from a competitor. Fungi were inoculated at the same time except for *H. fasciculare* which was added 2 d before its competitor to ensure that mycelia were of similar size when they met. Five days after the mycelia had met, the Petri dish lid was discarded and the plate attached to a lidless dish containing 0.5% MA, onto which a single plug of a target species had been freshly inoculated. This method was used successfully in the past to examine effects of VOCs produced by single species (Dick and Hutchinson, 1966; Schoeman et al., 1996; Wheatley et al., 1997; Mumpuni et al., 1998; Mackie and Wheatley, 1999; Wheatley, 2002; Humphris et al., 2002; Stinson et al., 2003).

Dishes were attached using masking tape and incubated in the dark at 20°C. The dish bearing the interaction was always placed on the bottom (i.e. below the plate bearing the target species) to eliminate the possibility of diffusibles dripping down onto the target species. All species involved in the interactions were used as target species. Extension of the target species was measured daily, until growth was 10 mm from the edge of the plate, by taking two measurements of colony diameter at 90^{o} to each other. Controls consisted of (1) the target species inoculated onto a sterile agar plate; and (2) self-pairings of the target species. Five replicates were performed.

4.2.3 Interspecific interaction systems for VOC analysis

The method followed Hynes et al. (2007). Interactions took place in Reacsyn^{TM} fermentation vessels (Biodiversity, Enfield, UK), compartmentalised polypropylene bottles that allow sampling of both the headspace and the growth medium (Figure 2.2). Mycelia were grown on a Fluoropore^{TM} membrane (1 μm pore size; Millipore, Watford, Kent, UK) on top of a porous polypropylene/polyester support, which rested on the surface of 35 ml 0.5% malt extract broth (Lab M MC23 malt extract). The growth chamber was stoppered with a gas permeable polyurethane foam bung which was removed to aerate the chamber, and through which sampling apparatus was inserted.

T. versicolor was paired in triplicate with either *S. gausapatum*, *B. adusta* or *H. fasciculare* by inoculating 6 mm diam. agar plugs 20 mm apart on the membrane. Self-pairings were also made. *T. versicolor* and *S. gausapatum* were also inoculated as single plugs, in three replicates. A vessel containing only malt broth was included in the analysis to identify any non-fungal volatiles present. Vessels were incubated at 20°C in the dark, held in racking trays.

4.2.4 Sampling VOCs

Sampling times were guided by Hynes et al. (2007) and a preliminary experiment performed by C. Eyre. For the *T. versicolor* and *S. gausapatum* interaction (and all related control treatments), volatiles were sampled after 1, 3, 6, 11 and 19 d. Mycelial contact was made after 5 d. Sampling of this interaction at 1 d and 6 d was performed by C. Eyre, but all data have been included in this analysis for completeness. Based on the results of this experiment, sampling at 1 and 19 d were omitted for the treatments involving *B. adusta* and *H. fasciculare*.

Headspace VOCs were collected by solid-phase microextraction (SPME), a method previously used successfully with fungi (Nilsson et al., 1996; Fäldt et al., 1999; Jelen, 2003; Demyttenaere et al., 2004; Ewen et al., 2004; Hynes et al., 2007). Essentially, SPME involves the adsorption of volatile compounds to a thin layer of adsorbent material coated on a metal fibre, and the subsequent thermal desorption of these compounds into a gas chromatograph. A 100 μm PDMS fibre (polydimethylsiloxane; Supelco, Poole, Dorset, UK) was used at 20 $\pm$ 5°C. At the start of each sampling day, the SPME fibre was conditioned according to manufacturers instructions (1 h at 250°C), followed by a fibre blank run. Prior to sampling, the inoculated Reacsyn^{TM} vessels were flushed to clear them of any accumulated volatiles or plasticiser by removal of the foam bung within a sterile laminar flow system for 10 min (following Hynes et al., 2007). The vessel was then removed from the laminar flow, the SPME fibre was inserted through the bung and exposed to the headspace of the inner chamber. After 1 h the fibre was withdrawn and immediately injected into the GC/MS.

4.2.5 GC/MS analysis of volatiles

Volatile profiles were resolved using a gas chromatograph containing a 30 m VF23ms polar column (0.25 mm internal diameter, 0.25 μm film thickness; Varian, Palo Alto, CA, USA), with helium at 55 kPa acting as a carrier gas. The column was coupled to a quadrupole mass spectrometer in Electron Impact (EI) mode working from m/z 35 to 400 with a source temperature of 200°C and interface temperature of 280°C. Compounds were desorbed for 2 min at 220°C in a split/splitless injection port, working in splitless mode. Analysis was performed using the temperature programme: start 45°C, increasing by 3°C min^{-1} until 200°C, then 5 min at 200°C to ensure flushing through of all compounds.

As no internal standard could be used, a terpene mixture of known concentration was

used as an external standard to assess machine performance. A terpene mixture was appropriate because the profile of volatile compounds emitted from interacting fungi was expected to contain sesquiterpenes. The mixture consisted of (1R)-fenchone, (1S)-verbenone and α-huumulene, each at a concentration of 0.5 mg ml^{-1} in hexane solvent. The terpene standard (1 μl) was injected by an autosampler into the injection port at 220°C with the machine working in split mode. The temperature programme used to resolve the mixture was: 40°C for 5 min, ramping to 250°C at 4.5°C min^{-1}. The mass spectrometer scan was widened to m/z 28 – 400, with a 2 min solvent delay at the start of each run to avoid damage by copious amounts of hexane.

Peaks of interest were integrated using Masslab v1.4 (Thermofinnigan, Manchester, UK). The terpene run for each sampling day was used to calibrate machine performance by comparison of the total area of all peaks present in each sample. The ratio of the three peaks (corresponding to the three terpenes contained in the mixture) was consistent in every standard run, so the total integrated area was comparable. As the same quantity of terpene standard was injected on each day, any differences in the total area of integrated peaks were attributable to changes in machine performance, and experimental peak areas were adjusted accordingly, and designated terpene adjusted units (TAU). However, for the treatments involving *B. adusta* and *H. fasciculare*, terpene standards were not used so an internal calibration was performed by standardising chromatograms to a malt control peak at RT (retention time) 12.1 min, with peak areas designated malt adjusted units (MAU). These have been included as rough values only to show qualitative rather than quantitative changes.

Identifications of the peaks of interest were made by comparison of mass spectra with library entries (National Institute of Standards and Technology v.12 mass spectral database) and retention times. However, these are putative identifications only, as absolute identification would require preparation of individual reference compounds which is beyond the scope of this work.

4.2.6 Statistical analysis

Radial extension rate was determined as the slope of a linear regression of extension versus time. Comparisons of daily radial extension rates were performed using one-way ANOVA with Tukey-Kramer a posteriori analysis (Minitab v.15). The evolution of each volatile produced by *S. gausapatum* or the TvSg interaction (as peak area, in TAU, over time) was compared by one-way ANOVA (Minitab v.15) of the area under the curve as determined by KaleidaGraph software (Synergy Software, Reading, PA, USA). Evolution of each volatile produced during interactions involving *B. adusta* and *H. fasciculare* (as peak area, in MAU) was compared using one-way ANOVA or two-way t-tests (Minitab v.15).

4.3 Results

4.3.1 Effect of VOCs produced during interactions on extension rates of target fungi

There was no significant difference ($P \leq 0.05$) between the extension rates for *T. versicolor*, *S. gausapatum* and *H. fasciculare*, when grown above self-paired controls compared to growth over agar (Table 4.1). However, the extension rate of *B. adusta* was significantly ($P \leq 0.05$) greater when grown above its own self-pairing. *T. versicolor* extended significantly ($P \leq 0.05$) faster when grown above the self-pairings of *S. gausapatum*, *B. adusta* and *H. fasciculare*, and interacting mycelia of *T. versicolor* and *B. adusta*. Growth of *S. gausapatum* and *H. fasciculare* were generally not affected by the interaction plates, although in two instances there were significant ($P \leq 0.05$) differences in extension rate when compared with growth above their self-pairings, but not when compared with agar alone. *S. gausapatum* extension rate decreased over *B.*

adusta paired with *H. fasciculare*, while that of *H. fasciculare* was greater when grown above *T. versicolor* paired with *S. gausapatum*. Over half of the interactions tested were associated with a significant ($P \leq 0.05$) decrease in extension rate of *B. adusta* when compared to its self-pairing but not compared to agar alone.

Table 4.1: Effects of VOCs produced by interactions on the growth of four 'target' species

Interaction plate	Percentage change in growth of 'target' species			
	T. versicolor	*S. gausapatum*	*B. adusta*	*H. fasciculare*
Agar	6.1 mm d^{-1}	5.2 mm d^{-1}	9.4 mm d^{-1}	2.5 mm d^{-1}
T. versicolor vs T. versicolor	100	99	104	100
S. gausapatum vs S. gausapatum	108[a]	102	109	102
B. adusta vs B. adusta	107[a]	100	111[a]	102
H. fasciculare vs H. fasciculare	107[a]	98	98[b]	100
T. versicolor vs S. gausapatum	99	99	105	105[b]
T. versicolor vs B. adusta	107[a]	102	103[b]	103
T. versicolor vs H. fasciculare	99	100	98[b]	103
S. gausapatum vs B. adusta	99	103	98[b]	103
S. gausapatum vs H. fasciculare	101	100	97[b]	103
B. adusta vs H. fasciculare	102	96[b]	98[b]	97

Changes in extension rate are expressed as percentages relative to the agar control. [a], significantly different ($P \leq 0.05$) to the agar control; [b], significantly different to the respective self-pairing.

4.3.2 General observations on VOC production during inter-specific interactions

VOC profiles were generally qualitatively consistent between replicates. VOC peaks have been designated numbers based on their order of elution, including compounds present in the malt broth (Figure 4.1; Tables 4.2 and 4.3), and these have putative identifications (Table 4.4).

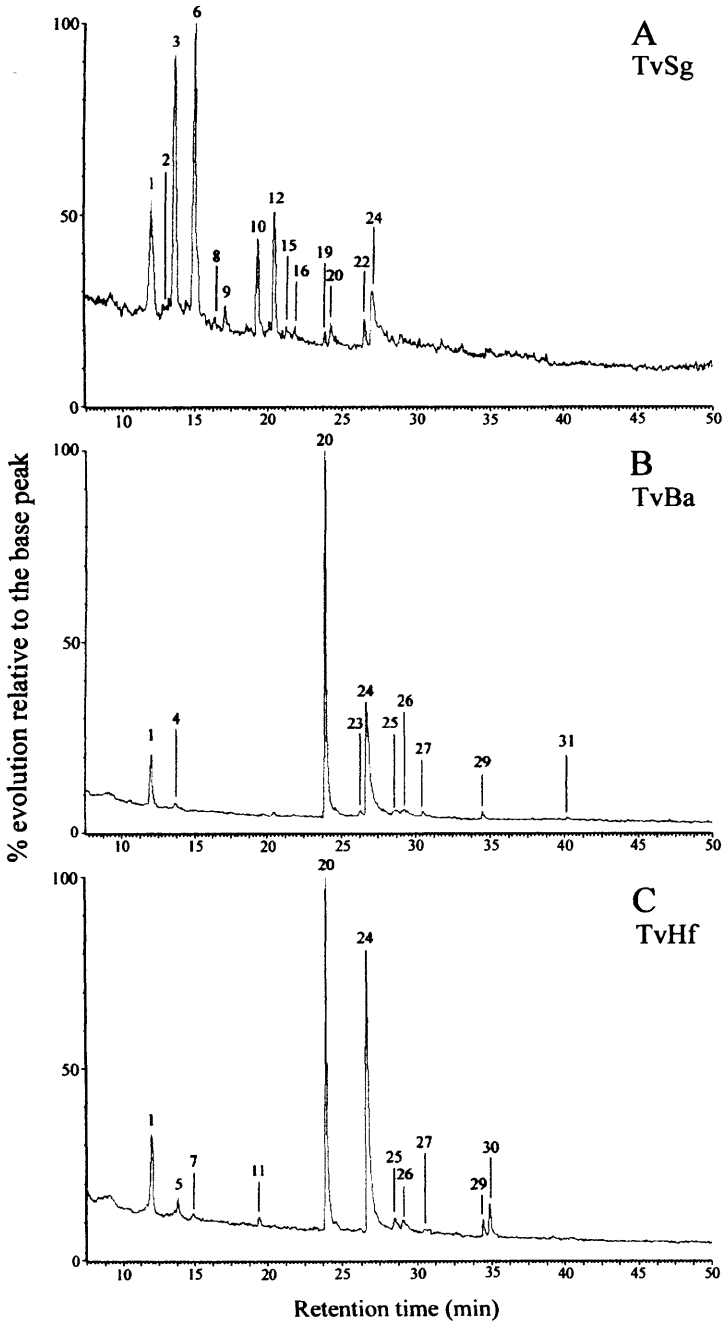

Figure 4.1: VOC profiles after mycelia of *T. versicolor* met with: A, *S. gausapatum*; B, *B. adusta*; C, *H. fasciculare*. Mycelia had met for 6 d in A, and 1 d in B and C. Peaks are numbered according to order of elution, as detailed in Tables 4.2 and 4.3.

4.3.3 VOCs in malt control

Four VOCs were detected in the malt broth control, with average retention times of 12.1, 24.2, 26.9 and 34.7 min (Peaks 1, 20, 24 and 29; Figure 4.1; Tables 4.2 and 4.3). Preliminary identifications suggest they are plasticisers emitted by the Reacsyn^{TM} vessels: dibutylbenzene (molecular weight [M_w] 190), dimethylbenzaldehyde (M_w 135), a long-chain hydrocarbon of M_w 168 (possibly dodecene) and bis(1,1-dimethylethyl)-phenol (M_w 206; Table 4.4).

4.3.4 VOCs from *T. versicolor* growing alone (Tv) and in self-pairing (TvTv)

No VOCs specific to *T. versicolor* were detected during sampling, with the exception of one VOC (Peak 26) produced by Tv after 19 d. This peak was not present in TvTv (Table 4.2). Otherwise, both Tv and TvTv profiles were identical to those from the malt control.

4.3.5 *S. gausapatum* growing alone (Sg), in self-pairing (SgSg) and interacting with *T. versicolor* (TvSg)

There were no quantitative or qualitative differences in the VOC profile of Sg compared with SgSg, hence single inocula were omitted in subsequent experiments (Table 4.2). Five VOCs specific to *S. gausapatum* were detected (Table 2). Peak 8 (RT 16.6 min), putatively identified as methylbenzoate or anisaldehyde (M_w 136), and Peak 9 (RT 17.13 min), a benzaldehyde-containing compound, were present sporadically in all treatments containing *S. gausapatum*. Peak 22 (RT 26.5 min), methyl ester-dimethylbenzoic acid, was the largest *S. gausapatum*-specific peak present after 3 d.

Table 4.2: Comparison of the evolution of fungal volatiles over time for the interactions between *T. versicolor* (Tv) and *S. gausapatum* (Sg) and their single-species controls

Peak	RT		Tv					TvTv					Sg					SgSg					TvSg				
no.	(min)	Day	1	3	6	11	19	1	3	6	11	19	1	3	6	11	19	1	3	6	11	19	1	3	6	11	19
1[a]	12.1		54	25	31	35	23	35	21	26	25	14	43	27	31	27	41	39	36	38	31	61	34	27	79	136	23
			(19)	(4)	(5)	(2)	(2)	(7)	(2)	(5)	(7)	(1)	(14)	(5)	(3)	(6)	(24)	(3)	(10)	(6)	(8)	(32)	(13)	(6)	(20)	(46)	(5)
2	13.1		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	19	7	*
																									(1)	(2)	
3	13.6		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	146	175	*
																									(45)	(94)	
6	15		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	38	478	*
																									(27)	(60)	
8	16.6		*	*	*	*	*	*	*	*	*	*	*	*	14	8	*	*	*	14	*	*	*	*	*	11	*
															(2)	(2)				(5)						(1)	
9	17.1		*	*	*	*	*	*	*	*	*	*	9	*	*	*	*	*	*	*	*	*	*	*	*	18	*
													(2)													(8)	
10	19.3		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	41	59	*
																									(12)	(32)	
12	20.4		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	30	232	*
																									(7)	(44)	
15	21.3		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	9	*
																										(3)	
16	21.8		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	14	*	*
																									(5)		
19	23.8		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	8	*
																										(1)	
20[a]	24.2		133	145	52	49	28	38	128	24	35	20	106	88	92	18	9	70	92	61	15	14	109	121	45	17	7
			(42)	(20)	(12)	(2)	(4)	(20)	(12)	(6)	(5)	(3)	(0)	(17)	(24)	(6)	(0)	(31)	(16)	(15)	(2)	(1)	(30)	(24)	(6)	(7)	(1)
22	26.5		*	*	*	*	*	*	*	*	*	*	*	11	140	39	41	*	22	156	76	42	*	7	27	16	*
														(1)	(18)	(11)	(3)		(1)	(50)	(11)	(13)		(3)	(7)	(7)	
24[a]	26.9		189	152	96	90	75	137	116	70	59	52	165	135	146	95	59	243	173	141	88	72	82	133	101	99	58
			(90)	(31)	(17)	(19)	(14)	(55)	(21)	(8)	(31)	(8)	(0)	(22)	(23)	(29)	(4)	(205)	(53)	(51)	(5)	(15)	(62)	(26)	(23)	(13)	(3)
25	28.8		*	*	*	*	*	*	*	*	*	*	4	*	*	*	*	*	*	*	*	*	*	*	5	*	*
													(1)												(3)		
26	29.3		*	*	*	*	9	*	*	*	*	*	5	*	*	*	*	*	*	*	*	*	*	*	*	*	*
							(2)						(3)														
28	31.1		*	*	*	*	*	*	*	*	*	*	*	*	9	24	26	*	*	13	30	27	*	*	8	*	*
															(2)	(5)	(3)			(4)	(6)	(7)			(1)		
29[a]	34.7		15	6	*	*	*	5	6	*	*	*	*	7	4	13	*	*	8	11	*	*	9	5	5	*	*
			(7)	(1)				(2)	(2)					(1)	(1)	(0)			(3)	(10)			(3)	(1)	(3)		

Peak areas for each volatile given in Terpene Adjusted Units (TAU), standard error of the means are given in parentheses. Identifications of peaks are given in Table 4. *, not detected; [a], present in malt control treatments.

Table 4.3: Comparison of the evolution of fungal volatiles over time for the interactions between *T. versicolor* (Tv) and *B.adusta* (Ba) or *H. fasciculare* (Hf), and single-species controls

Peak	RT	BaBa		TvBa			HfHf		TvHf			Malt blank				
no.	(min)	Day 3	6	3	6	11	3	6	3	6	11	1	3	6	11	19
1[a]	12.1	48 (4)	48 (4)	51 (9)	51 (9)	51	67 (26)	67 (26)	67 (15)	67 (15)	67	39	17	16	38	31
4	13.7	12 (0.4)	6 (1)	13 (5)	11 (5)	2	*	*	*	*	*	*	*	*	*	*
5	14	*	*	*	*	*	4 (2)	17 (6)	4 (1)	16 (5)	9	*	*	*	*	*
7	15.1	*	*	*	*	*	*	6 (2)	*	6 (2)	*	*	*	*	*	*
11	19.6	*	*	*	*	*	0.5 (0.3)	9 (5)	0.4 (0)	8	34	*	*	*	*	*
13	20.6	*	*	*	*	*	*	0.8 (0)	*	0.6 (0.6)	*	*	*	*	*	*
14	20.6	2 (0.4)	1 (0.3)	1 (0.7)	*	5	*	*	*	*	*	*	*	*	*	*
17	22	3 (0.2)	0.2 (0)	2 (0.2)	0.7 (0.6)	*	*	*	*	*	*	*	*	*	*	*
18	23.1	3 (0.1) (0.1)	1 (0.3) (0.3)	3 (0.5) (0.5)	1 (0.7) (0.7)	*	*	*	*	*	*	*	*	*	*	*
20[a]	24.2	157 (6)	309 (30)	258 (65)	252 (56)	100	193 (68)	193 (53)	276 (68)	159 (29)	94	51	53	31	87	63
21	26.2	2 (0.2)	*	2 (0.4)	*	*	*	*	*	*	*	*	*	*	*	*
23	26.5	*	*	*	4 (2)	0.71	*	*	*	*	*	*	*	*	*	*
24[a]	26.9	236 (21)	175 (30)	261 (38)	174 (37)	169	256 (99)	206 (61)	336 (84)	207 (53)	172	69	134	132	272	135
25	28.8	19 (10)	*	18 (4)	*	*	7 (3)	12 (3)	22 (16)	9 (1)	*	*	*	*	*	*
26	29.3	6 (6)	*	24 (4)	*	*	11 (3)	15 (5)	5 (3)	10 (4)	*	*	*	*	*	*
27	30.7	*	2 (1)	*	2 (1)	*	2 (0.3)	4 (2)	1 (0.5)	7 (0.6)	*	*	*	*	*	*
29[a]	34.7	28 (3)	5 (0.8)	22 (2)	5 (1)	5	17 (6)	17 (9)	24 (7)	8 (1)	8	*	11	10	42	6
30	35.1	*	*	*	*	*	4 (2)	*	1 (0)	8 (0)		*	*	*	*	*
31	39.7	*	16 (7)	*	2 (1)	*	*	*	*	*	*	*	*	*	*	*
32	47.3	39 (38)	*	0.2 (0.1)	0.7 (0.6)	*	*	*	*	*	*	*	*	*	*	*

Peak areas for each volatile given in Malt Adjusted Units (MAU), with the exception of the malt control where Terpene Adjusted Units (TAU) are used. Standard error of the means are given in parentheses, except for day 11 of BaBa, TvBa, HfHf and TvHf where only one replicate was performed. Identifications of peaks are given in Table 4. *, not detected; [a], present in malt control treatments.

Table 4.4: Putative identifications of fungal volatiles detected in experimental treatments

Peak no.	Putative identification			Treatments present in
	Name	Formula	M_w	
1[a]	dibutylbenzene	$C_{14}H_{22}$	190	Malt control
2	isomer of (1)	$C_{14}H_{22}$	190	TvSg
3	5-methyl,1,3-cyclohexadiene	C_7H_{10}	94	TvSg
4	PDMS contamination	-	-	BaBa, TvBa
5	methylbenzoate OR anisaldehyde	$C_8H_8O_2$	136	HfHf, TvHf
6	aromatic	?	204?	TvSg
7	alkane, at least C_7	C_7+	99+	HfHf, TvHf
8	methylbenzoate OR anisaldehyde isomer of (5)	$C_8H_8O_2$	136	Sg, SgSg, TvSg
9	benzaldehyde structure	?	?	Sg, TvSg
10	α-myrcene	$C_{10}H_{16}$	136	TvSg
11	monoterpene or derivative	$C_{10}H_{16}$	136	HfHf, TvHf
12	isomer of (6)	?	204?	TvSg
13	peaks too small	-	-	HfHf, TvHf
14	alkene	C_nH_{2n}	128+	BaBa, TvBa
15	peaks too small	-	-	TvSg
16	peaks too small	-	-	TvSg
17	alkenol	$C_nH_{2n}O$	140+	BaBa, TvBa
18	peaks too small	-	-	BaBa, TvBa
19	peaks too small	-	-	TvSg
20[a]	dimethylbenzaldehyde	$C_9H_{10}O$	135	Malt control
21	peaks too small	-	-	BaBa, TvBa
22	dimethylbenzoic acid, methyl ester-	$C_{10}H_{12}O_2$	164	Sg, SgSg, TvSg
23	peaks too small	-	-	TvBa
24[a]	alkene, at least C_{12}	C_{12}+	168+	Malt control
25	alkenol	$C_nH_{2n}O$	168+	Sg, TvSg, BaBa, TvBa, HfHf, TvHf
26	alkenol	$C_nH_{2n}O$	168+	Tv, Sg, BaBa, TvBa, HfHf, TvHf
27	monoterpene(possibly carene)	$C_{10}H_{16}$	136	HfHf, TvHf, BaBa, TvBa
28	methoxybenzoic acid, methyl ester-	?	166	Sg, SgSg, TvSg
29[a]	phenol, 2,4-bis(1,1-dimethyl)	$C_{14}H_{22}O$	206	Malt control
30	quinolinium-type compound	?	?	HfHf, TvHf
31	peaks too small	-	-	BaBa, TvBa
32	bis(2-ethylhexyl)phthalate (plasticiser)	$C_{24}H_{38}O_4$	390	BaBa, TvBa

[a], present in malt control treatments

The related compound methylester-methoxybenzoic acid (M_w 166, Peak 28, RT 31.1 min) was present from 6 d onwards in Sg and SgSg, but only at the 6 d sampling in TvSg. Peaks 25 and 26, both putatively long-chain alkenols, were detected in Sg after 1 d, but were present in larger quantities in other interactions.

Eight VOCs specific to TvSg were detected (Table 4.2) following mycelial contact and pigment production. Six of these were detected after 6 d: Peak 2, another isomer of dibutylbenzene (different to Peak 1; M_w 190); Peak 3, 5-methyl,1,3-cyclohexadiene (M_w 94); Peak 6, identified generally as having an aromatic structure, of which Peak 12 is an isomer; Peak 10, bearing strong similarity to α-myrcene (M_w 136); and Peak 16, which was too small for identification. A further two VOCs were detected after 11 d: Peaks 15 and 19, which were both too small to allow confident identification. Peaks 8 and 22, detected in Sg and SgSg, were also detected in TvSg but at significantly lower concentrations. Peak 9 was unusual as its total production in Sg was significantly ($P \leq 0.05$) lower than in TvSg (Figure 4.2). Peak 25, a putative alkenol, was also present in the VOC profile of Sg, HfHf, TvHf, BaBa and TvBa, and appears in TvSg after 6 d only.

4.3.6 *B. adusta* self-pairing (BaBa) and interacting with *T. versicolor* (TvBa)

Five VOCs were specific to *B. adusta* (Table 4.3): Peak 4, an alkene; Peak 17, an alkenol; Peaks 18, 21 and 31 were present at too low a concentration to be confidently identified. Peak 32 was possibly a plasticiser contaminant (bis(2-ethylhexyl)phthalate). Peaks 25 and 26, both alkenols, and Peak 27, a monoterpene (putatively carene), were also present but not specific to *B. adusta*, also being found in interactions involving *H. fasciculare*. One VOC specific to the TvBa interaction was detected following mycelial contact: Peak 23 (RT 26.5 min, but too small for identification) in two replicates at 6

d declining considerably by 11 d (Table 4.3).

4.3.7 *H. fasciculare* self-pairings (HfHf) and interacting with *T. versicolor* (TvHf)

Five *H. fasciculare*-specific VOCs were detected (Table 4.3): Peak 5, either methylbenzoate or an isomer of the anisaldehyde occurring in TvSg Peak 8; Peak 7, an alkane with at least a C_7 chain length; Peak 11, a monoterpene or derivative; Peak 30, a quinolinium-type compound with a distinctive spectral pattern at m/z 302; Peak 13, too small for identification. Peaks 25, 26 and 27 were also present in *H. fasciculare* profiles though not specifically, also being present in the profiles of *B. adusta* and TvSg. No VOCs were specific to TvHf.

4.3.8 Changes in VOC profiles over time

VOC concentrations usually increased and then declined as interactions proceeded (Figure 4.2, Tables 4.2 and 4.3). Of the interaction-specific VOCs produced in TvSg, maximal production was at 11 d (Peaks 2, 3, 6, 10, 12, 15, 19 and 25) with none detectable by 19 d (with the exception of Peak 2 and Peak 25 which peak at 6 d; Figure 4.2). The *S. gausapatum*-specific VOCs (Peaks 8, 22 and 28) declined but did not disappear by 19 d in Sg and SgSg (Table 4.2). There was no significant difference ($P > 0.05$) in total VOC evolution of *S. gausapatum*-specific compounds Peaks 8, 22 and 28 between Sg and SgSg (Figure 4.3). However, for Peaks 22 and 28 there was a significant decrease in total evolution by SgSg (Figure 4.3). In BaBa and TvBa, Peaks 16 and 18 declined dramatically between 3 d and 6 d (Table 4.3). For HfHf Peaks 5 and 11 occurred at a significantly ($P \leq 0.05$) higher concentration at the later sampling day, while for TvHf Peaks 5, 11 and 27 increased significantly ($P \leq 0.05$) between 3 d

and 6 d (Table 4.3). Only three *B. adusta*-specific VOCs were still detectable at 11 d in TvBa (Peaks 4, 14 and 23), and only two (Peaks 5 and 11) out of the six (Peaks 5, 7, 11, 13, 27 and 30) *H. fasciculare*-specific VOCs were detectable at 11 d (Table 4.3).

The malt extract Peaks 20 and 24 declined in the controls and all treatments over the course of the experiment (Figure 4.3). The evolution of malt Peak 29 also decreased over sampling days in BaBa, TvBa and TvHf (Table 4.3). In contrast, there was no change in malt Peak 1 in the presence of fungi, except in TvSg where there was a significant ($P \leq 0.05$) increase between 6 d and 11 d (Table 4.2).

4.4 Discussion

4.4.1 Species specificity

These results highlight the species-specific nature of both volatile production and susceptibility to non-self VOCs. For example, there was no detectable change in VOC profile during interactions between *T. versicolor* and *H. fasciculare*, and only one interaction-specific VOC (too small to be identified) was produced during the interaction between *T. versicolor* and *B. adusta*. Nor did the quantity of species-specific VOCs produced differ between intra- and inter-specific pairings of these species. In contrast, following mycelial contact between *T. versicolor* and *S. gausapatum*, eight interaction-specific VOCs were identified. No VOCs specific to *T. versicolor* were detected in any of the interactions, whereas the other species examined produced at least five species-specific VOCs both during self-pairings and during interactions. It is unlikely that *T. versicolor* is responsible for the production of any of the 11 VOCs produced during interaction with *S. gausapatum* as none of these VOCs recur in pairings between *T. versicolor* and other species. Any volatile-mediated antagonism produced

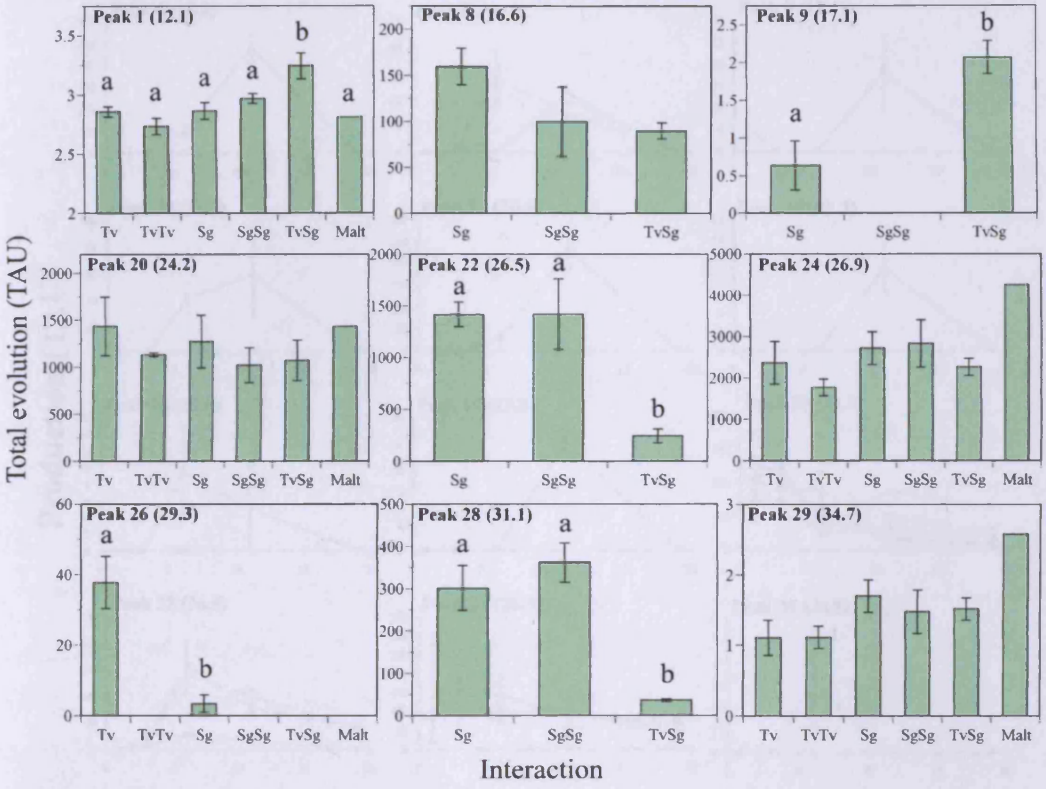

Figure 4.2: Total evolution of VOC peaks over 19 d for interactions involving *T. versicolor* (Tv) and *S. gausapatum* (Sg). Values are means of 3 replicates, with standard error of the mean, of total under the curve produced over 19 d. For each peak, bars with different letters are significantly ($P \leq 0.05$) different from each other, no letters on a graph indicates no significant differences ($P > 0.05$) between treatments. Log transformation was required to normalise data for peaks 1, 9 and 29 for statistical tests.

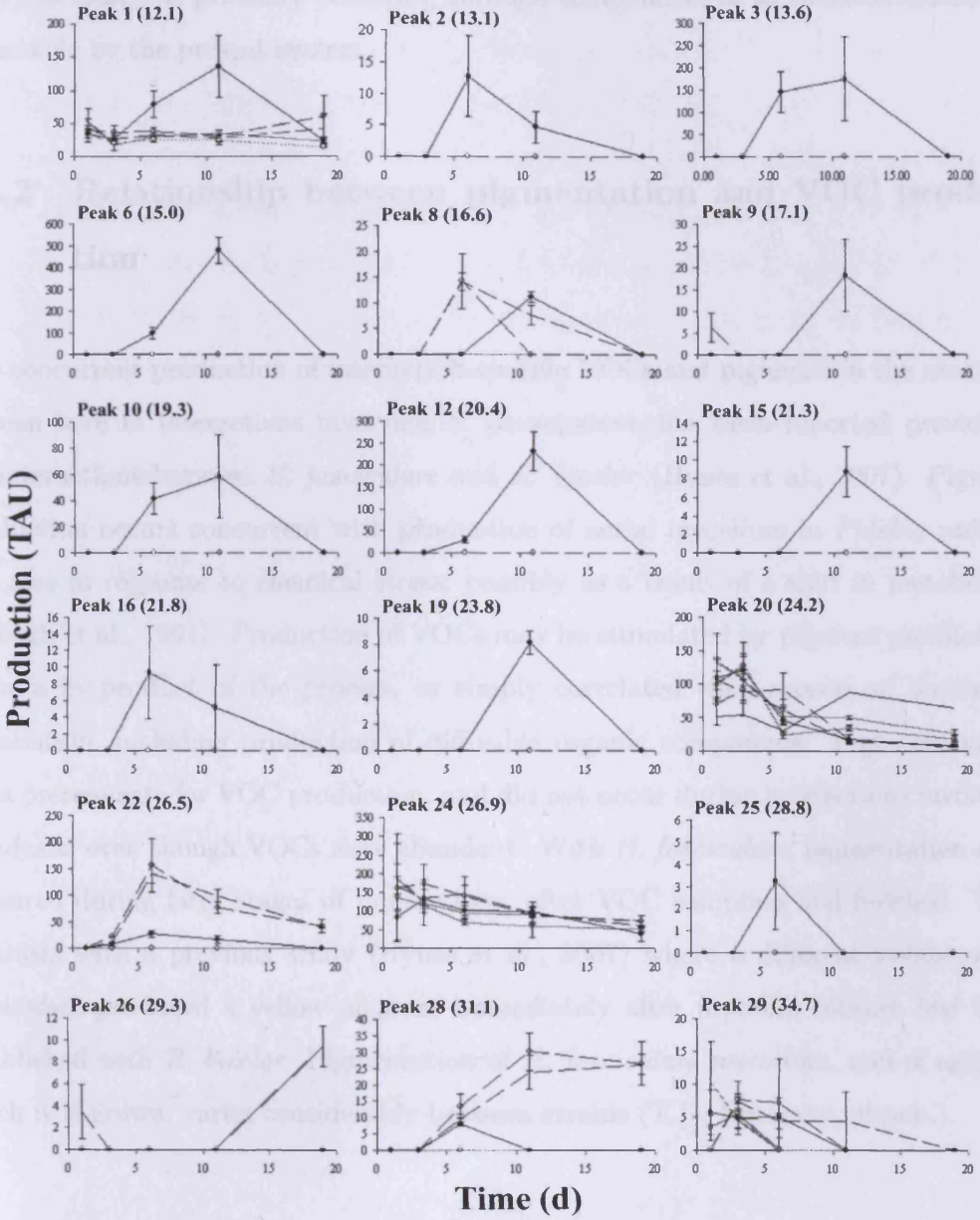

Figure 4.3: Production of VOCs specific to interactions among and between *T. versicolor* (Tv) and *S. gausapatum* (Sg), over time. Values are means of 3 replicates with standard error of the mean. Dotted lines, Tv; Dotted lines with open circles, TvTv; dashed lines, Sg; dashed lines with open circles, SgSg; solid lines with solid diamonds, TvSg; malt control, solid line. Peaks are numbered according to order of elution (with RT in parentheses), as detailed in Table 4.2.

by *T. versicolor* is probably occurring through compounds, or at concentrations, undetectable by the present system.

4.4.2 Relationship between pigmentation and VOC production

The concurrent production of interaction-specific VOCs and pigments in the medium, as seen here in interactions involving *S. gausapatum*, has been reported previously for interactions between *H. fasciculare* and *R. bicolor* (Hynes et al., 2007). Pigment production occurs concurrent with production of aerial mycelium in *Phlebia radiata*, and also in response to chemical stress; possibly as a result of a shift in metabolism (Griffith et al., 1994). Production of VOCs may be stimulated by pigment production, or be a by-product of the process, or simply correlated with aspects of secondary metabolism, including production of diffusible organic compounds. Pigmentation is not a prerequisite for VOC production, and did not occur during interactions involving *B. adusta*, even though VOCs were abundant. With *H. fasciculare*, pigmentation only appeared during later stages of interactions, after VOC sampling had finished. This contrasts with a previous study (Hynes et al., 2007) where a different isolate of *H. fasciculare* produced a yellow pigment immediately after mycelial contact had been established with *R. bicolor*. Pigmentation of *H. fasciculare* mycelium, and of agar on which it is grown, varies considerably between strains (T.D. Rotheray, unpub.).

4.4.3 Antagonism at a distance and temporal changes

While there is clearly long-distance signalling, as evidenced by changes in extension rates in the double plate experiment, no gross morphological changes were evident nor interaction-specific volatiles produced prior to mycelial contact. The latter concurs

with the previous study on VOCs produced during interactions between *R. bicolor* and *H. fasciculare* (Hynes et al., 2007). However, in view of the numerous reports of antagonism at a distance (Boddy, 2000; Woodward and Boddy, 2008), it seems extremely unlikely that this is always the case. Indeed, other VOCs may be present in the interactions used in the current study, and possibly produced prior to mycelial contact. SPME fibre-coatings differ in their detection limits for specific compounds – the PDMS fibre-coating used here was chosen as it is especially good at absorbing terpenes, known to be involved in fungal interactions (Hynes et al., 2007). A much wider range of VOCs might be revealed using additional sampling devices and analytical approaches (Ewen et al., 2004).

The general pattern of VOC production suggests an increase with mycelial volume, with a decrease by later samplings. The decrease may be due to limited nutrients or space, but may equally reflect the stage of the interaction. Thus, cessation in production of both *S. gausapatum*- and interaction-specific VOCs by 19 d in TvSg correlates with complete replacement of the former by *T. versicolor*. The general trend of decrease in malt extract peaks in all combinations implies degradation of these compounds by the fungi. White rot fungi are known to be able to degrade a wide range of chemicals including plastics (Sutherland et al., 1997). The single exception to this trend – the increase in dibutylbenzene (Peak 1) in TvSg relative to the malt control – may result from fungal production, as a different isomer of dibutylbenzene (Peak 2) is also produced during the interaction.

4.4.4 Function of VOCs

Although the majority of VOCs produced during the interaction between *H. fasciculare* and *R. bicolor* in a previous study were sesquiterpenes (Hynes et al., 2007), in the present study only three monoterpenes were identified: α-myrcene (Peak 10)

specific to the TvSg interaction; putative carene (Peak 27) common to both *B. adusta* and *H. fasciculare*; and a putative unidentified monoterpene (Peak 11) specific to *H. fasciculare*. Terpenes are ecologically significant, in some cases possessing antifungal activity which could constitute an antagonistic mechanism during fungal interactions (Viiri et al., 2001). The effect of monoterpenes on fungal growth varies. Carene and myrcene in the volatile phase are known to inhibit *Heterobasidion* and *Leptographium* species, and the terpene-rich volatiles from tomato leaves inhibit the phytopathogens *Alternaria alternata* and *Botrytis cinerea* (Hamilton-Kemp et al., 1992; Zamponi et al., 2006). However, terpene mixtures characteristic of conifer wood stimulate the growth of conifer-pathogenic fungi (Gao et al., 2005). Monoterpenes could also function to repel predators, as has been reported for *Collembola* insects grazing bacteria and fungi in soil (Ladygina et al., 2005).

The biological functions of several of the other VOCs identified are known. Many are scent compounds, including benzoic acid derivatives (Peaks 22 and 28) – components of the volatile oil of sage, and of the *Agaricus blazei* volatile fraction (Stijve et al., 2002). Benzoic acid methyl ester (Peak 28), another VOC identified from *S. gausapatum*, is part of the volatile profile of *Tricholoma matsutake* (pine mushroom; Cho et al., 2007). Several VOCs were identified as alkenols of different chain lengths (Peaks 17, 25 and 26), and two long-chain hydrocarbons were putatively identified (Peaks 7 and 14). Alkenols are common fungal VOCs. 1-Octen-3-ol is responsible for the characteristic odour of mushrooms (Ziegenbein et al., 2006), while undecanal and 2-decen-1-ol are components of the VOC profile of chicory, which has antifungal activity (Bais et al., 2003). The production of the same alkenol peaks in cultures of different species indicates these are general fungal VOCs. They could thus play a role in alerting fungi to the presence of foreign mycelia.

Whilst the nature of Peaks 5 and 8 could not be elucidated further than either methylbenzoate or isomers of anisaldehyde, both have interesting implications. Methylben-

zoate is also found in the VOC profile of the brown-rot basidiomycete *Coniophora puteana*, and its derivatives are known to possess antifungal activity (Woodward et al., 1993; Wolf, 1951). Anisaldehyde was one of the most abundant VOCs produced by cultures of six species of the basidiomycete *Pleurotus* spp., and was produced by cultures of *Ischnoderma benzoinum, Clitocybe odora, Lentinellus cochleatus* and *Agaricus essettei* (Berger et al., 1987; Gutierrez et al., 1994; Rapior et al., 2002). Thought to act as a redox-cycling agent for H_2O_2 production, anisaldehyde could play several roles in the highly metabolically active interaction zone, or in long-distance signalling of the presence of another mycelium (Guillen and Evans, 1994).

The quinolinium-like compound identified from *H. fasciculare* (Peak 30) was also produced during interactions between *H. fasciculare* (although a different isolate to the one used here) and *R. bicolor* (Hynes et al., 2007). Quinolinium compounds, and the related quinolines, have high antifungal activity, e.g. the quinoline alkaloid viridicatin produced by *Penicillium* sp. has been used to drive development of commercial fungicides (Bringmann et al., 1997). Moreover, fungi can also degrade a variety of quinoline-type compounds (Kaiser et al., 1996).

Clearly, a variety of fungal metabolites, with equally diverse ecological functions, are produced both constitutively and during interactions by wood-decay fungi. These compounds have potential roles as antifungal volatile chemical weaponry or as 'infochemcials' (Wheatley et al., 1997).

Chapter 5

Combative ability and enzyme production by homokaryons and heterokaryons of *T. versicolor*

5.1 Introduction

The life cycle of a typical wood decay basidiomycete involves two mycelial stages. Spore germination produces a homokaryotic mycelium, which, following hyphal fusion and nuclear exchange with a mating-type compatible conspecific may form a heterokaryotic mycelium. The two haploid gametic types of nuclei are maintained indefinitely during vegetative growth, fusing only for karyogamy and meiosis which yields homokaryotic spores. The heterokaryotic state can also break down through the formation of asexual homokaryotic conidia (James et al., 2008; Stenlid, 2008). The heterokaryotic state is similar to diploidy in that two haploid genomes reside in each cell with full opportunity for genetic complementation, but is crucially different because the two haploid genomes

remain separated in different nuclei (Clark and Anderson, 2004).

The duration of the homokaryotic phase for a mycelium inhabiting woody organic resources is determined by a variety of factors, most important of which are the local abundance of the species and its mating type structure. The homokaryotic phase persists until a compatible conspecific is encountered, and there is evidence of an inverse relationship between the number of colonies of a species in the field and the number that are homokaryons (Stenlid, 1994). Homokaryons are often considered short-lived with the heterokaryotic phase dominating the life cycle, but this is less likely to be the case in areas where mating potential is restricted as the homokaryotic phase would necessarily persist for much longer. For common species, e.g. *T. versicolor*, homokaryons might be expected to be short-lived. This has been observed in areas of high spore fall with a large area of resource open to colonisation (Williams et al., 1981). However there is also evidence of homokaryons, including those of *T. versicolor*, persisting in woody resources in the field for several years (Coates and Rayner, 1985; Garbelotto et al., 1997; Redfern et al., 2001; Stenlid, 2008).

Homokaryons and heterokaryons are subject to largely similar stresses during colonisation of, and proliferation within, woody organic resources, such as obtaining carbon and nutrients from recalcitrant lignified tissues, and antagonistic interspecific interactions. Whilst competition is regarded as a major factor influencing the distribution and abundance of wood decay fungi, only a few ecological studies have considered the competitive ability of homokaryons (Crockatt et al., 2008). It has been hypothesised that the temporary nature of homokaryons requires less combative ability than heterokaryons (e.g., Gardes et al., 1990), but the reverse was found for several species including the rare *Hericium coralloides* (Crockatt et al., 2008). However, these results are species-specific as no differences in combative ability were found between homo- and heterokaryotic mycelia of *Aleurodiscus (Acanthophysellum) lividocoeruleus* (Fryar et al., 2002). It has also been suggested that the greater genetic adaptive potential of

heterokaryons relative to homokaryons may increase the capacity to tolerate different stresses and thus improve combative ability (Clark and Anderson, 2004).

Differences in growth and decay rate between homokaryons and heterokaryons have also been reported, although again no clear pattern emerges. Heterokaryons of *Lenzites (Gloeophyllum) trabea, Phellinus weirii* and *Pleurotus ostreatus* generally grew more rapidly than homokaryons (Bezemer, 1973; Hansen, 1979), but there were no differences between different karyotic states of *A. lividocoeruleus* or *H. coralloides* (Fryar et al., 2002; Crockatt et al., 2008). Overall, no differences in decay rate were found between different karyotic states of *Gloeophyllum trabeum* (Costa and Kerruish, 1965; Amburgey, 1970). Heterokaryons of *P. weirii* decayed wood more rapidly than homokaryons (Hansen, 1979), but the reverse was true for *Poria (Antrodia) vaillantii* and *Elfvingia (Ganoderma) applanata* (Aoshima, 1954; Costa and Kerruish, 1965). Decay rate by *Serpula lacrymans* was less in 57% (of 138) of heterokaryons compared to their component homokaryons, intermediate in 33% and greater in 10% (Elliott et al., 1979). Also, homokaryons and heterokaryons sometimes have different morphologies, e.g. *Schizophyllum commune* and *S. lacrymans* (Clark and Anderson, 2004; Kauserud et al., 2006).

In a limited study the ability of homokaryons and heterokaryons of *T. versicolor* to break down lignin was compared (Addleman and Archibald, 1993). There were no differences in ligninolytic enzyme production or delignification between homokaryons and heterokaryons, though homokaryons were better able to bleach kraft pulps, but this was based on a very small sample size of isolates especially selected for high enzyme production. However, in *S. commune*, laccase activity was detected in heterokaryotic cultures only, implying different regulation in the different phases (de Vries et al., 1986). The greater genetic potential of heterokaryons may enable production of a more diverse range of isozymes of a particular enzyme, increasing substrate range, stress mediation, and adaptability to different resources. For example, two *Coriolus (Trametes) hirsutus*

homokaryons produced different laccase isozymes, both of which were expressed by the heterokaryotic product of their mating (Kojima et al., 1990).

In the limited number of previous studies performed so far there is no evidence of a trend for either karyotic state being more combative, growing faster or decaying wood more rapidly than the other. However, these studies have usually compared only one or two characters, rather than a wide suite. The aim of the study reported here was to compare a range of characters of *T. versicolor* homokaryons and heterokaryons, including extension rate, wood decay rate, ligninolytic enzyme (laccase and peroxidases) production, and combative ability. As a secondary coloniser (Boddy, 2001), when *T. versicolor* basidiospores arrive at a woody resource it is already colonised, so homokaryons would be expected to have good combative ability. Likewise heterokaryons must also be combative to avoid replacement. Thus no differences in combative ability are hypothesised. Neither is there any reason for expecting extension rates of homo- and heterokaryons to differ. However, since the main role of homokaryons is initial establishment, presumably usually of a small focus, whereas that of heterokaryons is persistence and utilisation of the woody resource, it is hypothesised that heterokaryons will produce larger amounts of ligninolytic enzymes.

5.2 Materials and Methods

5.2.1 Fungal isolates

Primary and secondary mycelia of *Trametes versicolor*, and heterokaryons of other wood decay Ascomycota and Basisdiomycota (Table 5.1) were maintained on 2% (w/v) malt agar (MA; 15 g l^{-1} Lab M Agar no. 2 (Lab M, Bury, Lancashire, UK), 20 g l^{-1} Munton and Fison Spray Malt Light (Munton Plc, Stowmarket, Suffolk, UK)).

Table 5.1: Details of *Trametes versicolor* isolates, and isolates against which they were paired in agar culture.

Ecological role	Species	Strain	Source	Isolated by	Date isolated/ created
Early secondary colonisers on standing and fallen wood	*Trametes versicolor*	*Natural heterokaryons*			
		TvD4	*Quercus robur* Ccc	L. Boddy	
		TvD2	*Q. robur* Ccc	L. Boddy	
		TvJHC		J. Heilmann-Clausen	
		TvA	*Fagus sylvatica* Ccc	J. Hiscox	Apr 08
		Natural homokaryons		J. Hiscox	Apr 08
		A1			
		A2			
		A3			
		A4			
		A5			
		A6			
		A7			
		A8			
		Artificial heterokaryons		C. Hibbert	Oct 08
		A1xA2			
		A3xA7			
		A4xA6			
		A5xA8			
		De-dikaryotised homokaryons		J. Hunt	Apr 08
		D2*1			
		D2*2			
		D2*3			
		D2*4			
	Bjerkandera adusta	MA313	WGP	M. Ainsworth	
Primary colonisers, latently present in standing trunks and attached branches	*Stereum gausapatum*	Sg1	*Q. robur* Ccc	L. Boddy	
	Vuilleminia comedens	Vc1	*Q. robur* Ccc	L. Boddy	
	Stereum hirsutum	Sh1			
	Daldinia concentrica[a]	Dc290594			
	Eutypa spinosa[a]	Es1	*F. sylvatica* Ccc	S. J. Hendry	Sep 05
Heart rotters	*Fomes fomentarius*	JHC001-201	*F. sylvatica* Denmark	J. Heilmann-Clausen	
Later secondary cord-forming colonisers	*Hypholoma fasciculare*	GTWV2	South Wales	G. Tordoff	2003
		DD2		D. Donnelly	
	Phanerochaete velutina	Pv29			
Tooth fungus[b]	*Hericium coralloides*	HcMA129-9	*F. sylvatica* Ccc	M. Crockatt	Nov 05

Ccc: Cardiff University Culture Collection; a: *Ascomycota*, all others are *Basidiomycota*; b: ecological role not entirely clear, found both in central heart regions and outer sapwood (Boddy and Wald, 2002); WGP: Windsor Great Park, UK.

'Natural' homokaryotic mycelia were isolated following germination of single spores from the fruit body of isolate TvA. Small pieces of mature hymenium of fruit body isolate TvA were stuck to the lid of a sterile, empty Petri dish with petroleum jelly and left to deposit spores overnight. Spores were suspended in sterile distilled water and diluted so that when spread on 9 cm non-vented Petri dishes (Greiner Bio-one, Austria) containing high clarity 2% MA (20 g l^{-1} Lab M Agar no.1; 15 g l^{-1} Lab M malt extract) there was approximately one spore per field of view at $\times 10$ magnification. Plates were incubated upside down, at 20°C in darkness. The position of germinating spores, that were well-separated from other hyphae, were marked using a dummy objective. The dummy objective consists of a thin metal tube mounted onto an objective lens which directly corresponds to the field of view through the $\times 10$ objective. This tube can be lowered into the agar to cut a plug of agar around a chosen spore. The plug of agar containing the germinating spore was transferred to a fresh agar plate with a sharpened tungsten wire. Cultures were checked for clamp connections.

'Artificial' homokaryotic mycelia were generated through de-dikaryotisation of isolate TvD2 by Dr Julie Hunt (Cardiff lab). As the two nuclei in a heterokaryon remain distinct, it is possible to recover the two haploid components by protoplast formation and regeneration. TvD2 heterokaryotic mycelia was grown in liquid culture (agar plugs inoculated into malt broth; 20 g l^{-1} Lab M malt extract in distilled water) at 20°C in the dark. Protoplasts were generated following the methods outlined in Sato et al. (1998). Briefly, week-old mycelium was extracted by filtration, blended and incubated in a buffer solution containing cellulase and chitinase to permeabilise cell walls. These protoplasts were subjected to restriction enzyme-mediated integration (REMI). The principle of REMI involves restriction enzymes penetrating the cells and nuclear membranes and cleaving chromosomal DNA in vivo at specific restriction sites. The free chromosomal DNA ends generated can then be ligated by host cells to plasmid DNA which has been linearised by the restriction enzymes (Sato et al., 1998). The vector inserted contained a gene conferring hygromycin resistance which allowed

for selection of successful transformants. These transformants were checked for the presence of clamp connections to ensure they were de-dikaryotised. The mating types (and hence which of the D2 nuclei they contain) of these homokaryotic mycelia were identified by pairing them against each other and monitoring whether the colonies merged or formed clamp connections. No clamp connections were observed in any of the pairings so we can assume they all contain the same nucleus and the mycelia have just merged as self-pairings.

'Natural' heterokaryotic isolates were obtained by fruit body tissue isolations: small pieces of tissue (avoiding the hymenium) were surface-sterilised by flaming and placed onto 2% MA. 'Artificial' heterokaryons were generated by randomly pairing homokary ons on agar plates to produce stable heterokaryotic mycelia (Table 5.1).

5.2.2 Extension rates

Plugs (6 mm diam.), cut from the actively growing margin of a colony using a No. 3 cork borer, were inoculated centrally onto 9 cm non-vented Petri dishes (Greiner Bio-one, Austria) of 2% MA. Colony diameter in two dimensions perpendicular to each other was measured daily, to 0.1 mm using Vernier callipers (Swiss Precision Instruments Inc, CA, USA).

5.2.3 Wood decay rates

Beech (*Fagus sylvatica*) blocks (2 × 2 × 1 cm) were cut from a freshly felled trunk (Wentwood, Newport, UK) and stored at −18°C until required. Blocks were defrosted by soaking overnight in distilled water and sterilised by autoclaving three times. They were then incubated on 14 cm Petri dishes (Greiner, Bio-one, Austria) of 2% MA, fully

colonised by *T. versicolor*, at 20°C in the dark. Density (g cm^{-3}) of wood blocks was determined destructively at the start and after 10 months incubation (10 replicates) and percentage weight loss estimated. Unfortunately this was not performed for 4 of the isolates due to contamination.

5.2.4 Interspecific interactions

Interactions were set up between all isolates of *T. versicolor* and 10 other species (Table 5.1), isolated from wood or fruit bodies, on 2% MA in 9 cm non-vented Petri dishes. Fungi were inoculated as 6 mm diameter plugs 30 mm apart, and plates were incubated in darkness at 20°C. Timing of inoculation depended on extension rate and where necessary was staggered to ensure that mycelia met in the centre of the dish. Three replicates were performed for each combination. Once colonies met, interactions were observed approximately weekly, with final outcomes recorded after 8 to 10 weeks. Outcomes were recorded as either deadlock (where neither mycelium captured territory from the other), replacement (where one fungus grew over and through the other so that it was no longer recoverable by re-isolation) or partial replacement, mutual partial replacement (where both mycelia gain some of the territory of the other), or overgrowth (where the one fungus grew over the other but did not replace it). Outcomes were confirmed by re-isolation from the base of the agar, which was essential for distinguishing between any replacement and overgrowth.

The outcome of each pairing was given a score, as an aid to comparison of combative ability (following Crockatt et al., 2008): replacement of the antagonist by *T. versicolor* was assigned +2; partial replacement of the antagonist, +1; deadlock, 0; partial replacement of *T. versicolor*, -1; complete replacement of *T. versicolor*, -2. Mutual partial replacement was scored as -1 and +1, so a net score of 0. Where either antagonist had killed but not (yet) replaced the other, the outcome was scored as for replacement,

and where overgrowth but not replacement had occurred the outcome was scored as deadlock. Cumulative scores were determined for each isolate by addition of scores from all replicate interactions.

5.2.5 Enzyme assays

Isolates of *T. versicolor* were grown on 16 ml 2% MA in 9 cm non-vented Petri dishes in darkness at 20°C. A plug of agar with mycelium (12 mm diameter; mycelial coverage 112 mm^2; made using a no. 8 cork borer) was removed from just behind the growing margin of a 7 d-old colony. Each plug was cut into c. 12 pieces and transferred to a 1.5 ml Eppendorf tube, to which 1 ml of deionised water was added. Tubes were shaken overnight at 4°C, then 0.8 ml of extract was removed and centrifuged for 10 min at 8,000 *g* at 4°C to pellet any debris. Extracts were also made from 6-month-old colonised wood blocks; these were roughly chopped (pieces less than 2 × 2 × 2 mm, and c. 100 mg wet weight) and extracts prepared in 1.5 ml Eppendorf tubes as for agar cultures. Extracts were kept at 4°C and used within 12 h. The wet weight of the agar plus mycelium, and the dry weight of the wood shavings plus mycelium, were used to normalise the data from agar and wood block cultures respectively. Three replicates were performed for each isolate.

Enzyme assays were performed according to the methods described in Section 2.2.4.

5.2.6 Statistical analysis

Colony extension rates during the log phase of extension were estimated by linear regression and compared using one-way ANOVA, or Kruskal-Wallis when non-normal, in Minitab (v.15). Percentage density loss between isolates was compared in the same

way, and so were differences in enzyme activity. Correlation between extension rate, enzyme activity, competitive score and decay rate was assessed using Spearmans' rank correlation in Minitab.

5.3 Results

5.3.1 Extension rates

Radial extension rate ranged from 4.1 to 6.4 mm d^{-1}. Overall, natural heterokaryon extension rate was significantly ($P \leq 0.05$) greater than that of homokaryons (Figure 5.1A), however there was no significant difference ($P > 0.05$) between the mean extension rate of the natural homokaryons and 'artificial' heterokaryons (the mating products of the homokaryons; Figure 5.1A), nor between the natural homokaryons, the 'artificial' heterokaryons and their 'parent' natural heterokaryon TvA (not shown in figure; $P > 0.05$). The extension rate of the artificial heterokaryons were similar to at least one of the component homokaryons (Figure 5.1B). The heterokaryon tended to be more similar to the faster of the two homokaryons, for example in combinations A3xA7 and A5xA8 where extension rate of both the faster homokaryon and the heterokaryon were significantly ($P \leq 0.05$) higher than the slower homokaryon. Dedikaryotised homokaryons grew significantly ($P \leq 0.05$) slower than the other groups (Figure 5.1A) but only D2*1 had a significantly lower extension rate compared to its parent heterokaryon TvD2 (Figure 5.1C).

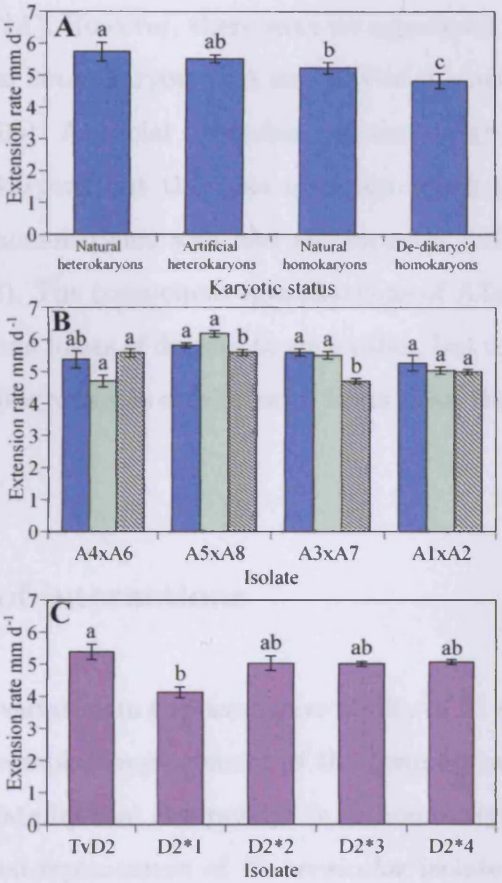

Figure 5.1: **A**: The mean extension rates of *T. versicolor* natural heterokaryons, artificial heterokaryons, natural homokaryons and de-dikaryotised homokaryons. **B**: Comparing the mean extension rates of *T. versicolor* natural homokaryons and the heterokaryon products of their matings. Blue bars, heterokaryons; green bars, 1st component homokaryon (e.g. for A3xA7 this would be A3); striped bars, 2nd component homokaryon. **C**: Comparison of the extension rates of the natural heterokaryon TvD2 and de-dikaryotised mutants D2*1 to *4. Different letters indicate a significant ($P \leq 0.05$) difference in extension rate.

5.3.2 Decay rates

Overall, heterokaryons effected a significantly greater decay of beech wood blocks than homokaryons (Figure 5.2A). However, there were no significant differences between the density loss from natural heterokaryon TvA and any of the natural homokaryons (not shown in figure; $P > 0.05$). Artificial heterokaryons caused greater weight losses than their component homokaryons but this was only significant ($P \leq 0.05$) for A1xA2, where the component homokaryons also had significantly different decay abilities to each other (Figure 5.2B). The component homokaryons of A3xA7 and A5xA8 did not cause significantly different losses of density to each other, but in both combinations one of the component homokaryons was significantly lower than the artificial heterokaryon (Figure 5.2B).

5.3.3 Outcome of interactions

There was considerable variation in the combative ability of *T. versicolor* isolates. Outcomes ranged between complete replacement of the competitor to partial replacement of the *T. versicolor* isolate by that competitor (e.g. homokaryon A8 vs. *F. fomentarius*), and between partial replacement of *T. versicolor* isolate to partial replacement of the competitor (e.g. heterokaryon TvD4 vs. *H. coralloides*; Table 5.2). Against this set of antagonists, *T. versicolor* was a successful combatant, completely replacing its opponent in over half of all interactions, and with 18 out of the 20 isolates having positive cumulative scores. The mean score for all isolates was 24.2 (Table 5.2).

Overall, there were no significant differences ($P > 0.05$) between the proportion of successful interaction outcomes (i.e. where *T. versicolor* replaced or partially replaced the competitor) attained by homokaryons and heterokaryons (Figure 5.3). In some cases the artificial heterokaryons outperformed both component homokaryons: for ex-

Table 5.2: Outcomes of interactions on agar between *Trametes versicolor* homokaryons and heterokaryons and 11 antagonist wood decay species.

Isolate	*Stereum gausapatum*	*Stereum hirsutum*	*Daldinia concentrica*	*Hericium coralloides*	*Hypholoma fasciculare* GTWV2	*Hypholoma fasciculare* DD2	*Bjerkandera adusta*	*Vuilleminia comedens*	*Eutypa spinosa*	*Fomes fomentarius*	*Phanerochaete velutina*	Cumulative score	Rank
Natural heterokaryons													
TvD4	R	R	R(2)PR	PR(2)pr	pr*	pr	pr	R	R	PR	PR(2)D	26	11
TvD2	R	R	R(2)PR	pr(2)D	r	r(2)pr	pr(2)r	R	R	D	PR(2)D	15	17
TvJHC	R	R	R	OG	r*	r	pr(2)r	R	R	D	R	20	15
TvA	R	R	R	MPR(2)PR	pr*(2)R	PR(2)R	PR(2)R	R(2)PR	R	R(2)D	R(2)PR	47	2
Artificial heterokaryons													
A4xA6	R	R	R	OG	MPR	R(2)PR	pr(2)D	PR(2)R	R	D(2)PR	R(2)MPR	37	3
A5xA8	R	R	D	MOG	r	R	r	R	R	PR(2)D	R	26	11
A3xA7	R	R	R	PR+og	r*(2)pr	PR(2)MPR	r(2)pr	R	R	R(2)D	R	35	4
A1xA2	R	R	R	pog	pr	R	r(2)pr	R(2)PR	R	D	PR(2)R	31	6
Natural homokaryons													
A1	R	R	R	MPOG	pr*	pr(2)r	pr(2)O/G	PR(2)R	R	PR	R	28	7
A2	R	R	R	OG	r*	r	r(2)pr	R	R	D	R	19	16
A3	R	R	R	PR	r*	r(2)MPR	PR	R(2)PR	R	R	R	57	1
A4	PR	R	PR(2)OG	pr+OG(2)pr	r	r	r	R(2)PR	PR(2)R	pr(2)D	o/g	-3	19
A5	R	R	PR(2)R	MPOG	r(2)pr*	R	r(2)pr	R	R	PR(2)R	R	34	5
A6	R(2)PR	R	R	OG	r	pr(2)R	r(2)pr	R	R	R	R(2)MPR	28	7
A7	R(2)PR	R	R	r	MPR(2)r*	PR	r	R	R	D(2)PR	R	24	13
A8	R	R	R	D(2)MPR	r	r(2)MPR	r	R(2)PR	R	pr(2)R	PR(2)r	14	18
De-dikaryotised homokaryons													
D2*1	PR(2)D	POG	OG(2)R	r(2)D	r	r	r	r	D	D(2)pr	r	-31	20
D2*2	R	R	R	MOG	r*	r(2)pr	r(2)pr	R	R	PR	R	23	14
D2*3	R(2)PR	R	R	pr+OG	r	r	PR+pog	R	R	R	R	27	9
D2*4	R(2)PR	R	R	D	r	r(2)pr	pog(2)r	R	R	R	R(2)PR	27	9
Cumulative scores	109	114	102	-10	-92	-29	-69	119	112	46	82		

R: replacement of competitor by *T. versicolor*; PR: partial replacement of competitor by *T. versicolor*; D: deadlock; MPR: mutual partial replacement; OG overgrowth by *T. versicolor*; og: overgrowth of *T. versicolor* by competitor; pr: partial replacement of *T. versicolor* by competitor; r: replacement of *T. versicolor* by competitor. Hf: *Hypholoma fasciculare*. * indicates *H. fasciculare* overgrowth where *T. versicolor* has been killed but not yet replaced. The numbers in brackets are the number of replicates with that particular outcome (three replicates were performed for each combination).

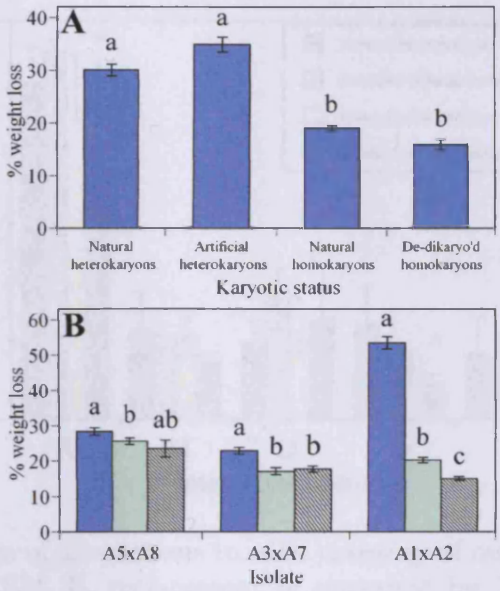

Figure 5.2: **A**: The mean % weight loss of beech wood blocks after 10 months colonisation by *T. versicolor* natural heterokaryons, artificial heterokaryons, natural homokaryons and de-dikaryotised homokaryons. **B**: Comparing the mean weight loss of beech wood blocks after 10 months colonisation by *T. versicolor* natural homokaryons and the heterokaryon products of their matings. Blue bars, heterokaryons; green bars, 1st component homokaryon (e.g. for A3xA7 this would be A3); striped bars, 2nd component homokaryon. Different letters indicate significant ($P \leq 0.05$) differences in density loss.

ample, A4 and A6 were both replaced by *H. fasciculare* GTWV2, but the outcome with their mating product A4xA6 was mutual partial replacement or partial overgrowth by *H. fasciculare* (Figure 5.4 A-C). Another example is the replacement of A2 by *H. fasciculare* DD2, the mutual partial replacement with A1, compared to the complete replacement of *H. fasciculare* DD2 by A1xA2 (Figure 5.4 D-F). Generally, the combative ability of the artificial heterokaryon was similar to that of their 'strongest' component homokaryon, for example A8 is replaced by *H. fasciculare* DD2 whilst A5 and A5xA8 both replace *H. fasciculare* DD2 (Figure 5.4 G-I).

TvD2 (the isolate used in other chapters) was not very successful in interactions rel-

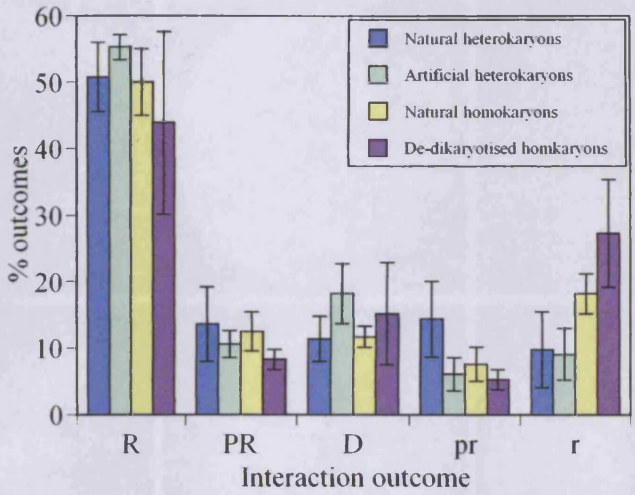

Figure 5.3: Percentage of interactions in each category of outcomes. Bars are mean % for each group ± SEM. R, replacement of opponent by *T. versicolor*; PR, partial replacement of opponent by *T. versicolor*; D/MPR, deadlock or mutual partial replacement; pr, partial replacement of *T. versicolor* by opponent; r, replacement of *T. versicolor* by opponent. There were no significant differences in the mean % total outcomes in each category for the different karyotic states.

ative to the other *T. versicolor* isolates, ranking 17th out of 20 (Table 5.2). TvD2 was outperformed by the de-dikaryotised mutants D2*2, D2*3 and D2*4, which had similar competitive rankings; however TvD2 was much more successful than D2*1. For example, the outcome of interactions with *Phanerochaete velutina* Pv29: partial replacement of *P. velutina* by heterokaryon TvD2, complete replacement by D2*2, *3 and *4, but complete replacement of D2*1 by the competitor (Figure 5.4 J-L).

No differences were observed in homokaryon or heterokaryon morphology during interactions. There were, however, notable variations in interaction morphology of A5 and A5xA8: fluid-filled balls of mycelium, c. 0.5 cm in diameter, were produced behind the interaction zones in older interactions.

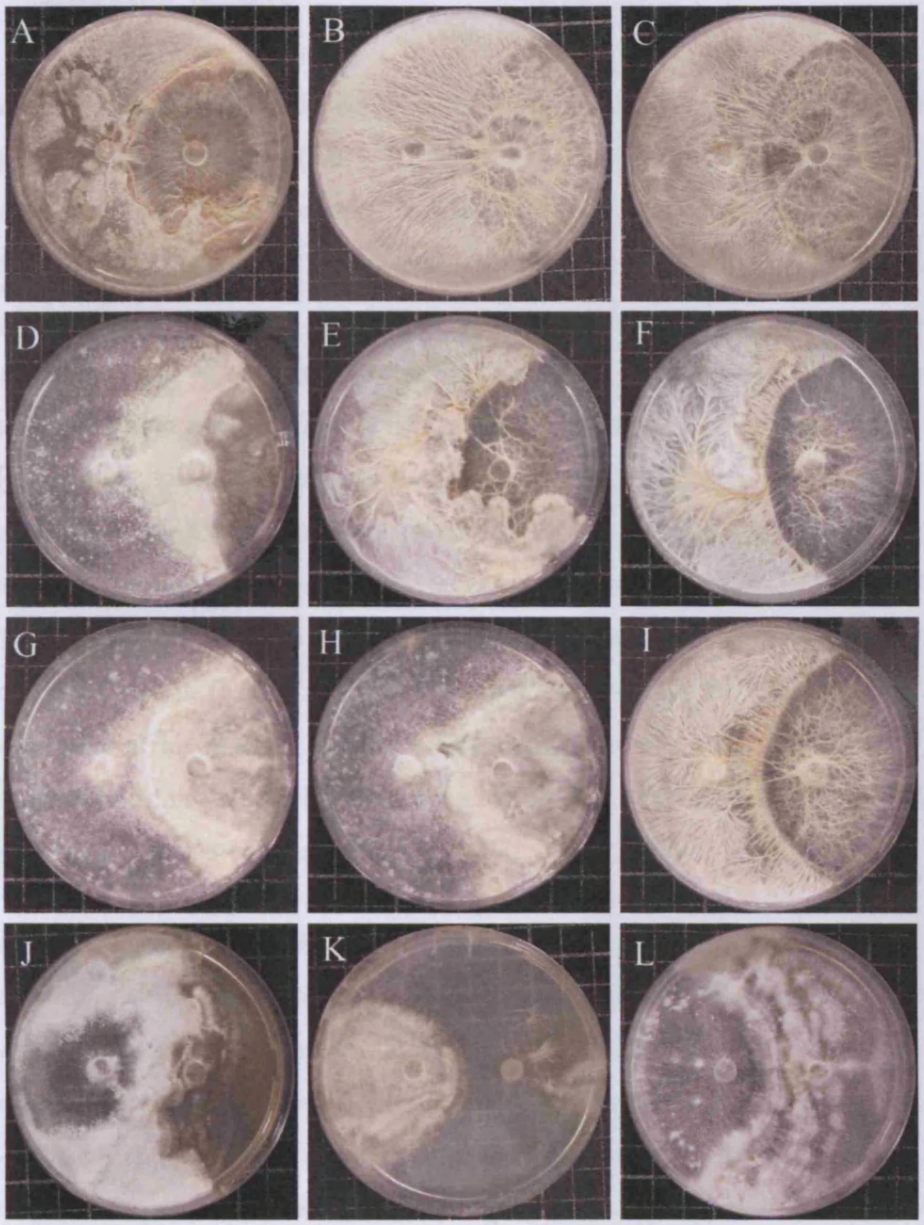

Figure 5.4: Differences in interaction outcomes at 2 months between *T. versicolor* heterokaryons and their component homokaryons. *T. versicolor* is always on the left hand side. A-C: heterokaryon A4xA6 (A) and homokaryons A4 (B) and A6 (C) vs. *H. fasciculare* GTWV2; D-E: heterokaryon A1xA2 (D) and homokaryons A1 (E) and A2 (F) vs. *H. fasciculare* DD2; G-I: heterokaryon A5xA8 (G) and homokaryons A5 (H) and A8 (I) vs. *H. fasciculare* DD2; J-L: natural heterokaryon TvD2 (J) and de-dikaryotised homokaryons D2*1 (K) and D2*3 (L) vs. *P. velutina* Pv29.

5.3.4 Enzyme assays

All enzymes assayed for were detected in agar cultures, except manganese peroxidase (MnP) and lignin peroxidase (LiP). The production of enzymes in agar culture varied widely between isolates (Table 5.3). There were no significant differences between natural homokaryons and their mated products – the artificial heterokaryons – in activity of any of the enzymes. However, when considered individually, artificial heterokaryons often had significantly lower enzyme activity than one of their component homokaryons in agar culture. The most dramatic was A4xA6, which had significantly lower ($P \leq 0.05$) activity of β-glucosidase, chitinase and acid phosphatase than its component homokaryon A4, but similar activity to its other component homokaryon A6 (Figure 5.5). The opposite occurred for laccase activity, with no significant difference ($P > 0.05$) in activity between A4xA6 and A4, but both had significantly ($P \leq 0.05$) lower activity than A6 (Figure 5.5). De-dikaryotised homokaryon D2*1 had the lowest laccase activity of all isolates in agar culture, but for all other enzymes D2*1 agar cultures displayed higher activity than that of the other de-dikaryotised homokaryons and heterokaryon TvD2 (Table 5.3).

There was much greater variability in enzyme activity between replicates of wood block cultures compared to agar cultures (Table 5.4). MnP activity was detected in 13 of the 20 isolates in wood block culture, but again no LiP activity was detected. Whereas agar cultures of homokaryon A4 showed significantly higher chitinase, acid phosphatase and β-glucosidase activity than all other natural homokaryons, no significant differences in activity of A4 enzymes were found in wood block culture extracts (Figure 5.5). Where there were significant differences in activity of a particular enzyme between artificial heterokaryons and a component homokaryon in agar culture, these were not consistent with activities in wood block culture (Figure 5.5). For example, artificial heterokaryon A4xA6 had significantly higher laccase activity than either component homokaryon in wood block culture, but was significantly lower than A6 in agar culture (Figure 5.5).

Table 5.3: Extracellular enzyme activities of *Trametes versicolor* homokaryon and heterokaryon agar extracts.

Isolate	Activity mU g^{-1} wet weight					
	Laccase	MnP	Peroxidase	Glucosidase	Chitinase	Acid Phosphatase
Natural heterokaryons						
TvD4	181.8 ± 22.6	0.0 ± 0.0	8.0 ± 0.3	10.8 ± 2.1	1.8 ± 0.3	2.8 ± 0.3
TvD2	194.6 ± 37.2	0.0 ± 0.0	6.6 ± 0.5	5.9 ± 1.3	1.5 ± 0.2	2.0 ± 0.1
TvJHC	204.8 ± 30.3	0.0 ± 0.0	4.9 ± 0.1	11.4 ± 1.5	2.4 ± 0.4	4.9 ± 0.9
TvA	406.2 ± 49.4	0.0 ± 0.0	5.2 ± 0.2	31.9 ± 2.7	1.9 ± 0.2	2.8 ± 0.4
Average	*226.3^{a} ± 27.7*	*0.0 ± 0.0*	*5.7^{a} ± 0.5*	*15.0^{a} ± 3.1*	*1.9^{a} ± 0.1*	*3.1^{a} ± 0.4*
Artificial heterokaryons						
A4xA6	348.9 ± 0.5	0.0 ± 0.0	7.1 ± 0.7	20.5 ± 1.7	1.6 ± 0.2	3.1 ± 0.2
A5xA8	267.7 ± 19.4	0.0 ± 0.0	6.2 ± 1.0	26.8 ± 2.1	1.1 ± 0.1	3.6 ± 0.2
A3xA7	343.7 ± 11.4	0.0 ± 0.0	7.8 ± 0.9	24.3 ± 1.2	1.5 ± 0.2	2.9 ± 0.3
A1xA2	279.0 ± 60.5	0.0 ± 0.0	5.1 ± 0.4	27.6 ± 0.9	1.3 ± 0.3	2.9 ± 0.3
Average	*303.1^{ab} ± 17.6*	*0.0 ± 0.0*	*6.6^{a} ± 0.5*	*24.8^{b} ± 1.1*	*1.4^{b} ± 0.1*	*3.1^{a} ± 0.1*
Natural homokaryons						
A1	224.2 ± 38.0	0.0 ± 0.0	4.9 ± 0.4	13.8 ± 1.1	0.8 ± 0.1	2.8 ± 0.2
A2	305.1 ± 26.8	0.0 ± 0.0	6.2 ± 0.1	7.9 ± 0.7	0.9 ± 0.0	2.4 ± 0.2
A3	327.5 ± 30.1	0.0 ± 0.0	5.6 ± 0.2	7.0 ± 0.6	1.2 ± 0.0	2.6 ± 0.2
A4	299.1 ± 9.0	0.0 ± 0.0	7.9 ± 0.2	57.2 ± 2.3	17.8 ± 2.3	15.2 ± 0.4
A5	294.7 ± 6.6	0.0 ± 0.0	5.3 ± 0.2	24.7 ± 3.3	1.3 ± 0.2	1.5 ± 1.0
A6	537.0 ± 46.4	0.0 ± 0.0	8.5 ± 0.6	11.6 ± 1.2	1.2 ± 0.1	3.0 ± 0.3
A7	264.8 ± 4.9	0.0 ± 0.0	6.8 ± 1.1	13.2 ± 0.9	1.4 ± 0.1	2.9 ± 0.3
A8	446.2 ± 19.6	0.0 ± 0.0	7.2 ± 0.3	7.9 ± 0.6	1.1 ± 0.2	5.0 ± 0.2
Average	*328.6^{b} ± 19.4*	*0.0 ± 0.0*	*6.5^{a} ± 0.3*	*17.9^{ab} ± 3.3*	*3.2^{ab} ± 1.2*	*4.4^{a} ± 0.9*
De-dikaryotised homokaryons						
D2*1	171.0 ± 30.2	0.0 ± 0.0	20.1 ± 1.8	15.0 ± 1.8	4.0 ± 1.1	14.8 ± 3.6
D2*2	411.3 ± 60.9	0.0 ± 0.0	8.2 ± 0.6	3.4 ± 0.3	1.2 ± 0.2	2.7 ± 0.3
D2*3	365.9 ± 17.6	0.0 ± 0.0	5.4 ± 0.0	3.2 ± 0.2	1.3 ± 0.2	2.5 ± 0.1
D2*4	446.5 ± 44.7	0.0 ± 0.0	9.0 ± 0.6	3.4 ± 0.2	1.3 ± 0.3	2.5 ± 0.1
Average	*375.3^{b} ± 47.4*	*0.0 ± 0.0*	*10.7^{b} ± 1.7*	*6.2^{c} ± 1.6*	*2.0^{ab} ± 0.4*	*5.6^{a} ± 1.8*
Agar	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0

Values given are mean activity of 3 replicate cultures, ± standard error of the mean (SEM). Different letters imply significant differences in group average activity. 'Peroxidase' refers to Mn-independent peroxidases.

Table 5.4: Extracellular enzyme activities of *Trametes versicolor* homokaryon and heterokaryon colonised beech wood block extracts.

Isolate	Activity mU g^{-1} dry weight					
	Laccase	MnP	Peroxidase	Glucosidase	Chitinase	Acid Phosphatase
Natural heterokaryons						
TvD4	474.8 ± 250.4	51.2 ± 11.2	64.1 ± 32.5	71.6 ± 16.9	18.3 ± 3.0	22.1 ± 8.2
TvD2	4.3 ± 3.0	0.0 ± 0.0	12.3 ± 7.1	50.4 ± 18.6	18.0 ± 4.5	49.8 ± 1.8
TvJHC	207.1 ± 168.9	0.0 ± 0.0	57.7 ± 10.8	43.6 ± 20.5	11.6 ± 3.3	34.2 ± 7.8
TvA	293.7 ± 111.6	17.1 ± 13.3	81.5 ± 17.4	48.9 ± 19.3	5.1 ± 1.1	10.1 ± 1.7
Average	*245.0^{ab} ± 85.5*	*17.1^{ab} ± 7.3*	*53.9^{a} ± 11.3*	*53.6^{a} ± 8.7*	*13.3^{a} ± 2.1*	*29.0^{ab} ± 5.1*
Artificial heterokaryons						
A4xA6	278.1 ± 19.7	50.1 ± 12.3	90.3 ± 2.9	23.8 ± 2.7	2.6 ± 0.7	11.2 ± 4.0
A5xA8	452.1 ± 199.3	19.9 ± 11.9	63.1 ± 7.2	68.7 ± 22.7	12.8 ± 2.3	20.9 ± 5.4
A3xA7	95.2 ± 28.4	28.7 ± 16.8	79.5 ± 12.0	20.7 ± 2.4	1.8 ± 0.2	13.8 ± 0.1
A1xA2	86.9 ± 57.9	10.7 ± 3.1	73.3 ± 11.7	26.6 ± 6.7	4.6 ± 0.9	20.2 ± 1.9
Average	*290.3^{ab} ± 92.1*	*23.2^{b} ± 6.4*	*76.6^{a} ± 4.9*	*45.6^{a} ± 12.3*	*6.0^{b} ± 1.4*	*16.5^{a} ± 1.9*
Natural homokaryons						
A1	97.1 ± 50.1	0.0 ± 0.0	47.7 ± 19.6	92.2 ± 34.1	14.5 ± 3.9	9.9 ± 2.9
A2	212.5 ± 83.2	0.5 ± 0.5	54.6 ± 22.5	21.9 ± 4.5	2.7 ± 0.4	10.4 ± 4.1
A3	843.5 ± 102.7	5.9 ± 3.1	89.3 ± 7.7	32.1 ± 13.1	5.7 ± 2.3	16.2 ± 4.0
A4	79.8 ± 36.7	0.0 ± 0.0	131.3 ± 11.1	20.6 ± 13.0	1.6 ± 0.2	9.7 ± 2.3
A5	718.9 ± 104.4	20.7 ± 11.9	46.1 ± 7.0	61.3 ± 13.0	5.2 ± 1.6	18.4 ± 3.2
A6	70.4 ± 18.9	6.6 ± 6.6	48.1 ± 12.9	17.2 ± 6.9	4.9 ± 0.6	11.8 ± 3.1
A7	372.8 ± 47.3	0.0 ± 0.0	22.4 ± 5.1	30.8 ± 3.5	3.8 ± 0.2	22.8 ± 2.9
A8	428.9 ± 28.9	0.0 ± 0.0	173.9 ± 40.7	45.5 ± 4.1	21.5 ± 3.6	21.4 ± 1.6
Average	*353.0^{a} ± 61.3*	*4.2^{a} ± 2.0*	*76.7^{a} ± 11.6*	*45.9^{a} ± 7.9*	*7.5^{b} ± 1.5*	*15.1^{a} ± 1.4*
De-dikaryotised homokaryons						
D2*1	305.7 ± 30.4	0.0 ± 0.0	29.4 ± 4.0	66.4 ± 54.9	15.2 ± 11.1	56.4 ± 32.9
D2*2	29.2 ± 4.5	0.5 ± 0.5	18.6 ± 7.2	7.7 ± 3.5	3.8 ± 1.7	43.0 ± 16.4
D2*3	473.5 ± 258.3	11.0 ± 0.6	45.5 ± 11.4	40.5 ± 7.9	6.4 ± 0.6	21.0 ± 0.8
D2*4	24.3 ± 7.0	1.2 ± 1.2	3.8 ± 0.7	32.6 ± 7.7	11.4 ± 1.7	197.6 ± 35.8
Average	*208.2^{b} ± 79.9*	*3.2^{a} ± 1.4*	*24.3^{b} ± 5.5*	*36.8^{a} ± 13.5*	*9.2^{ab} ± 2.8*	*79.5^{b} ± 23.6*
Wood	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.4	0.7 ± 0.2	1.0 ± 0.0	4.8 ± 2.5

Values given are mean activity of 3 replicate cultures, ± standard error of the mean (SEM). Different letters imply significant differences between the group averages. 'Peroxidase' refers to Mn-independent peroxidases.

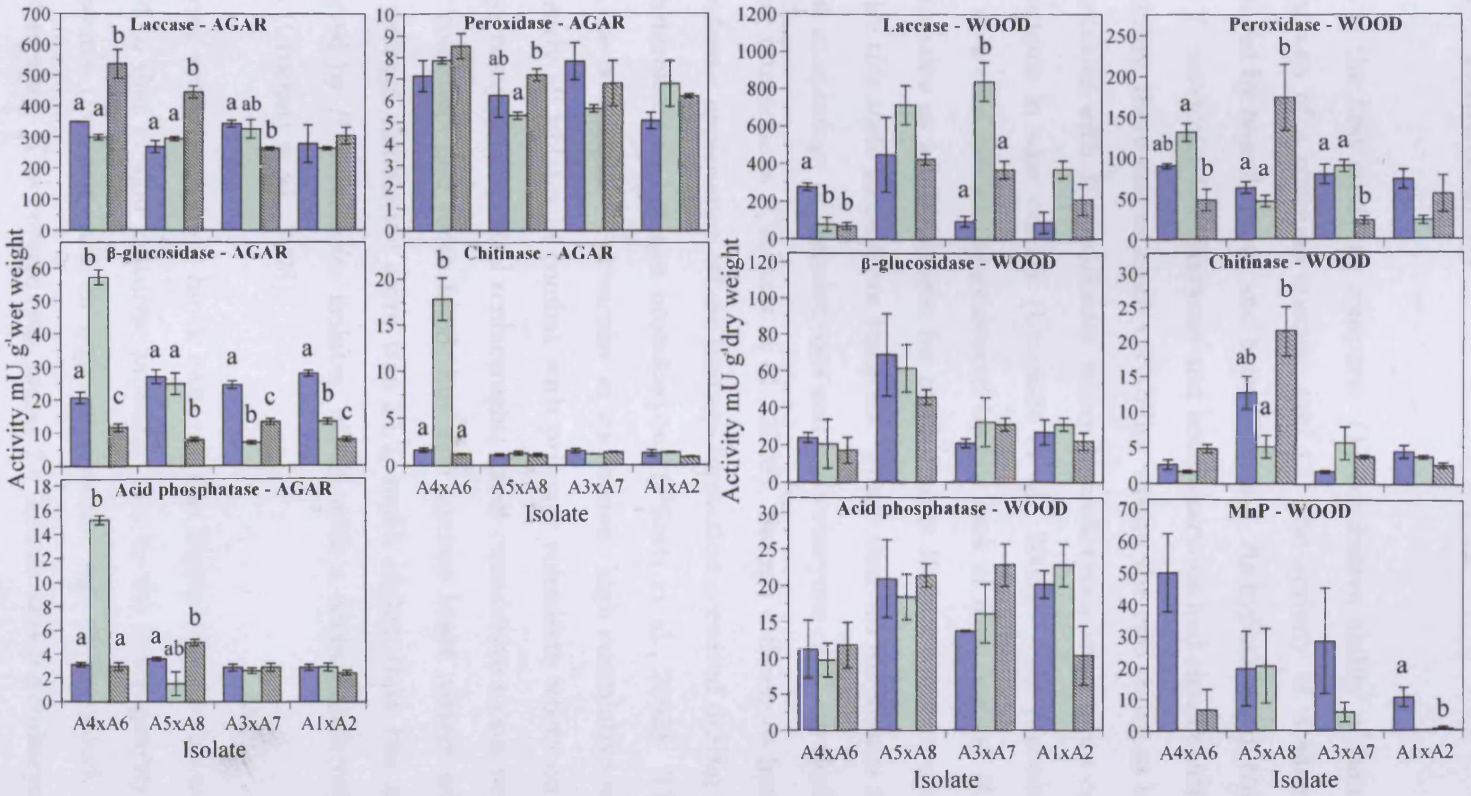

Figure 5.5: Extracellular enzyme activity in agar and wood block cultures of artificial heterokaryons and their component homokaryons. Blue bars, artificial heterokaryon; green bars, 1st component homokaryons; striped bars, 2nd component homokaryons. Bars are mean activity of three replicates ± SEM. MnP activity was detected in wood block cultures only. Different letters indicate a significant ($P \leq 0.05$) difference in activity between artificial heterokaryons and their component homokaryons. No letters indicates no significant differences ($P > 0.05$).

5.4 Discussion

This is the first study to compare: (1) combative ability of homokaryons and heterokaryons of a common species, and (2) the activity of a wide range of enzymes produced by homokaryons and heterokaryons. As hypothesised, there was no evidence that *T. versicolor* homokaryons and heterokaryons had significantly different combative ability, there was as much variability within the two states as between them. This is contrasts with *H. coralloides* where homokaryons were more combative than heterokaryons in agar culture (Crockatt et al., 2008). This probably reflects the fact that as a rare species (as evidenced by the lack of fruit bodies) *H. coralloides* probably remains as a homokaryon for much longer than a common species and thus may require this state to be more vigorous. There were no differences between the macroscopic morphology of homokaryons and heterokaryons of *T. versicolor* or *H. coralloides* during interactions (Crockatt et al., 2008), though differences have been reported in *Ganoderma applanatum* where barrage formation occurred during interactions involving heterokaryons but not homokaryons (Pilotti et al., 2002). The results correlate with the position of *T. versicolor* in succession: high cumulative scores were achieved by nearly all isolates in combat with primary colonisers where outcomes were nearly always complete or partial replacement; lower cumulative scores were achieved against later colonisers and tooth fungi, but also against heart rotters which is unexpected. The average cumulative score was 24.2, much higher than the average score of 2.6 achieved by *H. coralloides* isolates, albeit with a slightly different profile of antagonists (Crockatt et al., 2008).

Enzyme activity of wood block cultures was highly variable between replicates compared to that of agar cultures, probably due to the heterogeneity of wood blocks as a resource (i.e. variation in nutrient content, age of the wood, orientation of vessel elements). The reverse was found for homo- and heterokaryons of *P. ostreatus*, where ligninolytic enzyme activity was less variable between isolates when grown on

wheat straw compared to growth on agar (Eichlerová et al., 2000). The was as much variability in enzyme activity between homokaryons of *T. versicolor* as there was between heterokaryons. This contrasts with *Lentinus tigrinus* and *P. ostreatus*, where protoplast- and basidiospore-generated homokaryons had higher variability in enzyme activity compared to heterokaryotic isolates (Homolka et al., 1995; Eichlerová and Homolka, 1999).

The pattern of enzyme activity produced by an isolate relative to other isolates was also different during growth on agar compared to growth on wood blocks, which might be expected as the strategy of enzyme production would differ on a nutrient-rich resource such as malt agar compared to nutrient-limiting beech wood. During growth on agar, natural homokaryon A4 generated high levels of chitinase, acid phosphatase and β-glucosidase activity; these enzymes function to release bound nutrients from the substrate. So the increased production of these enzymes by A4 on a nutrient-rich resource may imply problems with nutrient uptake and utilisation, which is supported because A4 was the slowest growing isolate and second weakest combatant of all the isolates. The lack of MnP activity in agar culture is surprising, but might be explained by a low manganese content of the substrate. MnP activity was present in younger colonies of *T. versicolor* on agar but not in older colonies (Section 2.3.3), perhaps as available Mn^{2+} was depleted.

Overall, there were no differences in radial extension rates of homo- and heterokaryons, as hypothesised. Similarly, extension rates in *H. coralloides* did not significantly differ between homokaryons and heterokaryons (Crockatt et al., 2008). This contrasts with *Gloeophyllum trabeum, Phellinus weirii* and *P. ostreatus* where heterokaryon extension rates were greater than those of homokaryons (Bezemer, 1973; Hansen, 1979; Eichlerová and Homolka, 1999). Differences in extension rates between homokaryons and heterokaryons implies a difference in control of gene expression in each ploidy phase (Clark and Anderson, 2004), which we can infer is not the case for *T. versicolor*.

Decay rates follow a similar pattern: overall, *T. versicolor* heterokaryons had greater decay ability than homokaryons, but there were no significant differences in decay rate of the natural homokaryons and the 'parent' heterokaryon TvA, and no significant differences between the artificial heterokaryons and one or both component homokaryons. Nor was there a significant difference between extension rates of the de-dikaryotised homokaryons and the 'parent' heterokaryon TvD2, with the exception of D2*1. The lack of significant correlations between decay rate and enzyme activity in wood block cultures implies other factors are responsible for the differences in decay rate, or that there is a more complex relationship between lignin decomposition and weight loss.

Comparison of characteristics of artificial heterokaryons with their component homo karyons indicated that where the homokaryons significantly differ in a particular character, the artificial heterokaryon is similar to one of the homokaryons in this character rather than being intermediate. This occurred for agar enzyme activity, extension rate, decay rate and combative ability, but not for wood block enzyme activity where differences may be masked by substrate heterogeneity. The heterokaryon was not consistently similar to either homokaryon, for example laccase activity of heterokaryon A4xA6 was very similar to that of A4, whereas chitinase and acid phosphatase activities were very similar to A6. Also, heterokaryon enzyme activity was rarely significantly higher than activity of both component homokaryons. The trend for mated heterokaryons to resemble one component homokaryon rather than be intermediate also occurs in *H. coralloides* extension rates, although the extension rate of the heterokaryons was occasionally faster than either component homokaryon (Crockatt et al., 2008). This would appear to suggest regulation of different genes in a dominant-recessive system, where heterokaryons are functionally equivalent to diploids. This further confirms balanced nuclear ratios within *T. versicolor* (1:1) because if there were over-representation of one constituent nucleus, the heterokaryon would resemble the corresponding homokaryon across all traits (James et al., 2008).

Chapter 6

Synthesis and future work

6.1 Synthesis

Interspecific basidiomycete interactions are inevitable and are of key importance to decomposition processes in woodland ecosystems. They determine the pattern of fungal colonisation of wood and affect decay rate, mineralisation and nutrient translocation (Holmer and Stenlid, 1997; Boddy, 2001; Wells, 2002). However, the processes underlying mycelial interactions are not fully understood. The aims of this research were to relate differences in interaction processes – the production of ligninolytic enzymes, and volatile and diffusible organic compounds – with the outcomes of interactions. The response of a single species, *T. versicolor*, was compared during interactions where it was successful (replacement of *S. gausapatum* or deadlock with *B. adusta*) with interactions where it was not successful (replacement by *H. fasciculare*). This was addressed through staining and assaying ligninolytic enzyme production (Chapter 2), analysing expression of key genes (Chapter 3), analysing production and effects of interaction volatile organic compound (VOC) profiles (Chapter 4), and how combative

ability varies within a species (Chapter 5).

6.1.1 Activity of laccase and MnP/MRP were not related to interaction outcome

There were no clear patterns of laccase and MnP/MRP activity in interactions with different outcomes. Large increases in laccase activity occurred during interactions (Section 2.3.4), which agrees with previous findings for *T. versicolor* (Freitag and Morrell, 1992; Baldrian, 2004). Laccase activity was consistently highest at the interface between the competitors (the interaction zone), with the extent of the increase varying depending on the competitor (Section 2.3.4), which has also been reported for other fungi including *R. bicolor, P. ostreatus* and *S. hirsutum* (Iakovlev and Stenlid, 2000; Chi et al., 2007; Peiris, 2009). Away from the interaction zone, there were few changes in laccase activity in other regions of interacting *T. versicolor* mycelia compared to *T. versicolor* self-pairings (Section 2.4.3). This implies a specific role for laccase at interaction zones.

The detected MnP activity was not supported by MnP gene expression; instead it is likely that this activity was attributable to MRP (Mn-repressed peroxidase; Section 3.4). MRP could act like MnP or LiP, and although its transcription is repressed by Mn^{2+} this does not exclude it from acting like MnP in the assay conditions (Johansson et al., 2002; Kim et al., 2005). However further tests would be necessary to confirm this (see Section 6.2), so the term 'MnP/MRP activity' will be used when referring to assay results. The increases in MnP/MRP activity that were detected during interactions (Section 2.3.4) have not previously been reported for *T. versicolor*, although there are reports of MnP increases in interactions involving other species (Baldrian, 2004; Ferreira-Gregorio et al., 2006; Chi et al., 2007). In interactions 8 d after mycelia had met, MnP/MRP was detected in all regions of interacting *T. versicolor* mycelia, but

not in *T. versicolor* self-pairings (Section 2.3.4). This implies that MnP/MRP has interaction-specific functions in regions not in immediate contact with the competitor, although activity was consistently highest at the interaction zone.

6.1.2 Levels of laccase production were not related to combative ability

Laccase production varied in different species. It has been suggested that saprotrophic fungi from mid to late successional stages (i.e. more combative species) have higher laccase activity than those from earlier stages (Iakovlev and Stenlid, 2000). This did not occur here as *S. gausapatum*, a primary coloniser, had roughly similar levels of laccase production during growth alone to those of *H. fasciculare*, a late-stage cord-forming species (Section 2.3.4). *T. versicolor* had far higher levels of laccase production than *H. fasciculare*, despite being a late primary to early secondary coloniser (Section 2.3.4; Boddy, 2001). This could suggest that despite its up-regulation at interaction zones, laccase is not central to combative and/or defensive abilities. However, it must be considered that different species may differ in their ability to increase production of laccase during interactions, which may provide a competitive advantage.

6.1.3 Changes in expression of ligninolytic genes were not dramatic

Few previous studies have attempted to link the activity of ligninolytic enzymes to their gene expression during interactions. Here, changes in gene expression relative to *T. versicolor* self-pairings were small (Section 3.3.3), which agrees with the findings of Eyre (2007). Overall, it seems likely that any changes in gene expression were transient, and missed by sampling 2 d after mycelia had met. The increased levels of laccase and

MnP/MRP activity in 2 d interactions suggest that any upregulation of gene expression occurred before this point (Section 2.3.4), although rapid increases in activity could also be generated by release of laccase and peroxidase from wall-bound or intracellular storage (including Dittmer et al., 1997; Valášková and Baldrian, 2006). Increased gene expression may also occur before mycelia make physical contact, through detection of the competitor by its VOCs or DOCs (diffusible organic compounds; Wheatley et al., 1997; Boddy, 2000; Woodward and Boddy, 2008). Serial sampling points should be performed in future to pinpoint any increases in expression: before mycelia make contact, just after the first contact between competitor hyphae, and at regular intervals from then on.

Serial sampling of laccase expression has been performed for liquid culture interactions involving *S. hirsutum* (Peiris, 2009). Unlike the results of the present study, a lag between mycelial contact and increased expression was detected; however, mixing of liquid cultures involves gross damage to the mycelia of both competitors which probably affects their interaction responses (initially, at least). The greatest increase in laccase expression detected during *S. hirsutum* interactions was only two-fold that of the expression in self-pairings (Peiris, 2009), which corresponds to the small increases in expression reported in Chapter 3 (Section 3.3.3). Similarly, up-regulation of laccase and peroxidase genes has not been reported in microarray expression analyses of interactions, perhaps because any up-regulation fell under the cut-off point for significance (Iakovlev et al., 2004; Adomas et al., 2006; Eyre, 2007). This suggests that large increases in laccase and peroxidase gene expression do not occur; perhaps because they are not necessary. Laccase and peroxidases are highly stable in the extracellular environment (Baldrian, 2006), which can be seen in Chapter 2 (Section 2.3.6), where diffusibles from liquid cultures were added to blank agar plates. The uninoculated control plates still had laccase activity one week after the diffusibles had been filter-sterilised and added. This enzymic stability may mean that small increases in production can lead to accumulation of large amounts of enzymes in the extracellular

environment.

6.1.4 Different isozymes may be expressed during interactions

Another explanation for the discrepancy between levels of expression and levels of activity is the production of different isozymes of laccase and peroxidases during interactions. Different isozymes may be specific for different substrates, and perhaps confer adaptability to different environmental conditions (Martinez, 2002; Necochea et al., 2005; Baldrian, 2006; Kersten and Cullen, 2007). Thus the overall level of gene expression may not increase, but the profile of isozymes expressed may change. Expression of different laccase, LiP and MRP isozymes by *T. versicolor* during interaction with *S. gausapatum* were analysed by cloning PCR products (Section 3.3.4). Expression of LiP and MRP isozymes did not differ compared to expression in the *T. versicolor* self-pairing. Several different laccase isozymes were expressed in both the interaction and the self-pairing, but the sample sizes were too small to be confident of any differences or similarities between the expression profiles (Sections 3.3.4 and 3.4). This is an important direction for future work (see Section 6.2). Finally, increased production of laccase and peroxidase mediators (e.g. veratryl alcohol), or secretion of different types of mediators, may increase enzyme efficiency without necessitating increased production of the enzymes themselves (Eggert et al., 1996; Tadesse et al., 2008).

6.1.5 Comparing results from assays, staining and gene expression

The enzyme assays, staining and gene expression experiments performed tell similar, though not always identical, stories (Chapters 2 and 3). Each method measured a different fraction of the total laccase or peroxidase production by *T. versicolor* dur-

ing interactions. Laccase and peroxidases may be secreted as extracellular enzymes, or wall-associated, or stored intracellularly (Garcia et al., 1987; Dittmer et al., 1997; Schlosser et al., 1997; Velázquez-Cedeño et al., 2004; Valášková and Baldrian, 2006). RT-PCR measured production of all ligninolytic enzymes by *T. versicolor* (Section 3.3.3); enzyme assays measured the extracellular fraction (Section 2.3.4); staining measured both the extracellular and wall-associated fractions (Section 2.3.2). Also, the staining results for laccase, MnP and LiP show the accumulation of enzyme activity over time (dyes were part of the growth media), whereas the enzyme assays and RT-PCR were more of a snapshot of activity/expression at the time of sampling. This may explain any discrepancies between the results. Generally, though, all three methods agree with the key findings: increases in activity of laccase and peroxidases occurred at the interaction zone, with few increases in activity elsewhere in interacting *T. versicolor* mycelia; and changes in activity were not related to interaction outcome.

6.1.6 Peroxidase activity in culture extracts of homo- and heterokaryotic isolates was perhaps attributable to LiP activity

Activity of manganese-independent peroxidases was detected in single cultures of *T. versicolor* at 7 d (timed from inoculation) on agar, but not in *T. versicolor* during self-pairings or interactions (Sections 2.3.4 and 5.3.4). This Mn-independent peroxidase activity is unlikely to be attributable to MRP, because, as mentioned previously, MRP is likely to behave like MnP under the assay conditions. However, no MnP activity was detected in the *T. versicolor* single cultures (Section 4.3.4). The peroxidases of *T. versicolor* have been well-studied, with reports of LiP, MnP and MRP production (including Johansson and Nyman, 1993; Johansson et al., 1993; Jönsson and Nyman, 1994; Jönsson et al., 1994; Collins et al., 1999). The peroxidase activity detected

may result from production of a new type of *T. versicolor* peroxidase, but this seems unlikely. It may also have been caused by activity of LiP, or through problems with the assay. The absence of this type of peroxidase activity from 2 and 8 d cultures of *T. versicolor* may be due to slight differences in substrate composition which have previously been shown to regulate peroxidase production in a range of species (Gold and Alic, 1993; Kaal et al., 1995; Schlosser et al., 1997; Galhaup et al., 2002; Mikiashvili et al., 2005).

In wood block cultures, high levels of MnP activity and Mn-independent peroxidase activity occurred within the same sample (Section 5.3.4). Again, it is impossible to tell whether the 'MnP' activity is the result of MnP or MRP. The Mn-independent peroxidase activity levels were so high they are unlikely to have been caused by assay problems (Section 5.3.4). If the activity was attributable to LiP, then this should have been picked up by the LiP assay. However, preliminary assays of cultures grown on oak sawdust were unsuccessful due to the high tannin content of extracts, which persisted even after filtering (results not shown). Although the beech wood cultures used in the present study did not contain visible levels of tannins, any that were present might have interfered with the UV absorption necessary for the LiP assay.

6.1.7 Patterns of enzyme activity differ between interactions grown on different substrates

Production of laccase and peroxidases by *T. versicolor* differed during growth on malt agar and during growth in beech wood blocks (Sections 2.3.4-5 and 5.3.4). Malt agar is very rich in nutrients, whereas in wood nutrients are limiting and lignin decomposition is necessary for fungi to gain access to utilisable carbon and nitrogen sources (Rayner and Boddy, 1988). Carbon and nitrogen availability, and the presence of phenolic compounds and lignin derivatives, affect ligninolytic enzyme production (Schlosser et al.,

1997; Galhaup et al., 2002; Mikiashvili et al., 2005). It stands to reason that different sets of enzymes would be produced during growth in such different conditions. Production of ligninolytic enzymes during interactions involving *T. versicolor* occurring in wood should ideally be investigated, to see whether there is a link between interaction outcome and enzyme production. Interaction outcomes on agar are not always consistent with those that occur in natural substrates (Rayner and Todd, 1979; Holmer and Stenlid, 1993; Owens et al., 1994; Wells, 2002), and perhaps the stimulation of different enzymes on different substrates is partly responsible for this.

Production of different enzymes may affect the combative ability of a mycelium. Enriching malt agar with Mn^{2+} changed the production of laccase and peroxidases during interactions, with production of MnP assumed as MRP would be repressed by the high levels of Mn^{2+} present (Section 2.3.5). Growth on Mn^{2+}-enriched agar altered the outcome of interactions with *H. fasciculare*, from replacement of *T. versicolor* by *H. fasciculare* to deadlock. The production of MnP could have increased *T. versicolor* combative ability. Although the high Mn^{2+} content used in this experiment (5 to 10 times higher than in wood and leaf litter; Baldrian et al., 2005) means the result is not ecologically relevant, it shows the importance of the abiotic environment on enzyme production.

Conditions within woody tissues vary, and therefore so does ligninolytic enzyme production by fungal colonisers. Mn^{2+} levels in wood tissues vary depending on the type of wood, removal by previous colonisers, or the state of decay. As decay of wood progresses, the available Mn^{2+} decreases as it is oxidised to MnO_2 (Collins et al., 1999). Levels of available carbon and nitrogen within wood also change as decay progresses, as easily utilisable resources are used up or accumulated within mycelia (Rayner and Boddy, 1988; Griffith and Boddy, 1991). As combative ability may be determined (at least in part) by enzyme production, this again highlights the dynamic equilibrium existing between environmental variables and success in interactions (Rayner and Boddy,

1988; Boddy, 2000).

6.1.8 Stimulation and inhibition by VOCs and DOCs

Fungal volatile and diffusible organic compounds (VOCs and DOCs) may play a variety of roles during mycelial interactions (including Holmer and Stenlid, 1997; Wheatley et al., 1997; Wheatley, 2002; Heilmann-Clausen and Christensen, 2003). No VOCs specific to *T. versicolor* were detected in any of the interactions, whereas the other species examined produced at least five species-specific VOCs (Section 4.3). It seems unlikely that *T. versicolor* would not produce a specific VOC profile (Fäldt et al., 1999; Wu et al., 2005), and future work should adjust methodology in order to detect it (see Section 6.2). Production of VOCs does not seem to be related to combative ability, as *T. versicolor* is more combative than the VOC-producing *S. gausapatum*, but less combative than the VOC-producing *H. fasciculare* (Sections 4.3.4-6). Interaction-specific VOCs were detected in the interaction between *T. versicolor* and *S. gausapatum* only, and there were no large increases in constitutive VOCs during interactions either. There does not appear to be a link between VOC production and interaction outcome, rather VOCs vary depending on the combination of species interacting (Section 4.3). Although several of the identified VOCs had potential antifungal properties (e.g. terpenes and quinolines; Bringmann et al., 1997; Viiri et al., 2001), the effect on remote mycelia was often a stimulation of extension rate (Section 4.3.1). The VOCs may thus be acting as 'infochemicals' signalling the presence of an opponent (Wheatley et al., 1997). Increased extension rate when exposed to competitor VOCs may have evolved to maximise the territory possessed by a mycelium prior to contact with the opponent. Increased extension rate when exposed to 'self' VOCs could function as a competitive response to conspecifics, a signal of a potential mate, or a larger 'self' colony (Wheatley et al., 1997).

In contrast, both inhibition and stimulation of extension rate occurred in response to diffusible organic compounds (DOCs) from other species and interactions (Section 2.3.6). This may be linked to successional stage, with later stage colonisers adapted to DOCs from their predecessors (Holmer and Stenlid, 1997; Heilmann-Clausen and Christensen, 2003; Heilmann-Clausen and Boddy, 2005). *T. versicolor* extension rate was stimulated by DOCs from the *S. gausapatum* self-pairing (replacement of *S. gausapatum*), but inhibited by DOCs from the interaction between *T. versicolor* and *H. fasciculare* (replacement of *T. versicolor*; Section 2.3.6). *H. fasciculare*, a late-stage coloniser, was not significantly stimulated or inhibited by the DOCs from any of the other species (Section 2.3.6). Interestingly, all of the test fungi were inhibited by DOCs from *F. fomentarius* (Section 2.3.6), a heart rotter with the ecological strategy of inhabiting heartwood for long periods of time (Rayner and Boddy, 1988). It has intermediate combative ability, and perhaps maintains its territory by excluding other species through production of antagonistic DOCs. Analysis of the chemical composition of these DOCs could increase understanding of the processes occurring at the interaction zone, or reveal new metabolites that could have industrial potential.

6.1.9 Mycelial contact is necessary to stimulate production of laccase, but not MnP/MRP

Interaction responses may occur before and/or after the competing mycelia establish contact (Boddy, 2000). Prior to mycelial contact, no interaction-specific VOCs were detected, which concurs with the previous study on VOCs produced during interactions between *H. fasciculare* and *R. bicolor* (Section 4.3; Hynes et al., 2007). Mycelial contact is necessary to stimulate laccase production, as there were no increases in laccase activity in *T. versicolor* cultures exposed to DOCs from other fungi (although in several cases there was inhibition of laccase activity relative to controls; Section 2.3.6).

Also, there was no upregulation of laccase activity during the interaction between *T. versicolor* and *F. fomentarius*, where hyphal contact is not established for at least a month due to non-contact inhibition (Section 2.3.4). However, increased MnP/MRP activity was detected at the interaction interface between *T. versicolor* and *F. fomentarius* (Section 2.3.4), and also in response to DOCs from two interactions (Section 2.3.6). This all implies that physical contact between mycelia is necessary to stimulate increased production of laccase and interaction-specific VOCs by *T. versicolor*, but that DOCs or other fungal-derived compounds (e.g. chitin fragments) are able to stimulate production of MnP/MRP. Further work should test whether VOCs are able to stimulate increases in enzyme activity in remote mycelia.

6.1.10 Functions of ligninolytic enzymes during interactions – some suggestions

Defining the functions of ligninolytic enzymes during interactions was not an aim of this work. However, certain roles or functions can be excluded based on some of the results. Firstly, laccase and peroxidases do not appear to function to increase nutrient acquisition, in order to satisfy the increased metabolic demand of combat. There were no increases in activity of nutritionally-related enzymes (glucosidase, chitinase and acid phosphatase) during interactions compared to self-pairings (Section 2.3.4), which would be expected if increased nutrient acquisition was occurring. Also, the increases in laccase and MnP/MRP activities were limited to the interaction zone (Section 2.3.4), whereas we would expect mycelium-wide increases to maximise nutrient uptake. Laccase does not appear to function to break down antagonistic compounds in *T. versicolor*, as no increases in activity occurred in response to competitor diffusibles (Section 2.3.6). However, MnP/MRP was upregulated in response to DOCs from certain fungi, so this could be functioning in detoxification (Section 2.3.6).

Laccases and peroxidases could function to generate protective extracellular melanins during interactions (Bell and Wheeler, 1986; Henson et al., 1999). Melanins are usually pigmented dark brown to black, but the heterogeneous melanins produced by saprotrophic basidiomycetes are derived from phenols in their environment which may lead to different coloured pigments (Bell and Wheeler, 1986). Orange pigmentation occurs at the interaction zone during interactions with *S. gausapatum* (Section 2.3.1), which also has the highest levels of laccase and MnP/MRP activity (Section 2.3.4). However, there is also increased laccase and MnP/MRP activity at the interaction zone with *B. adusta* (Section 2.3.4), but no pigmentation appears over the duration of this interaction (Section 2.3.1). If laccases and peroxidases are involved in melanin production during interactions it is a species-specific response.

Increased production of laccase and peroxidases could function to mediate oxidative stress at the interaction zone. ROS have previously been associated with interactions zones (Tornberg and Olsson, 2002; Silar, 2005); here, staining showed accumulations of superoxide at interaction zones and around invasive hyphal cords, whereas there was very little accumulation during growth alone (Section 2.3.2). NADPH oxidases, important in the generation of ROS, were upregulated during interactions between *T. versicolor* and all three competitors (Eyre, 2007), indicating that this response occurs irrespective of interaction outcome. Ligninolytic enzyme activity increased in cultures where oxidative stress was artificially induced (Jaszek et al., 2006a,b; Zhao et al., 2009) and could mediate this stress by removing H_2O_2, or through formation of melanins (Henson et al., 1999). However, ligninolytic enzymes are also capable of generating oxidative stress; ROS are generated during lignin decomposition, and as such are central to the fungal lifestyle (Hammel et al., 2002). Clearly, a balance must exist between the different roles of ligninolytic enzymes at interaction zones.

Finally, the increases in activity could occur concomitant with morphological changes (de Vries et al., 1986; Rayner et al., 1994). Increases in activity of laccase and

MnP/MRP were highest at the interaction zones (Section 2.3.4), which were the sites of major morphological change. Staining showed that general peroxidase activity and superoxide accumulation were localised to barrages, hyphal cords and aerial mycelium (Section 2.3.1). Laccase and peroxidase activity may be necessary to form bonds between hyphal walls, conferring structural strength to the hyphal assemblages formed during interactions (de Vries et al., 1986; Bell and Wheeler, 1986).

6.1.11 Production of VOCs may support laccase and peroxidase activity

Aromatic VOCs produced by fungi may also play a role in lignin degradation. VA (veratryl alcohol) is an aromatic compound with a well-known role in LiP activity, and has been reported in several species, including *T. versicolor* (Okamoto et al., 2002). Much less is known about other aromatic compounds produced by white rot fungi, although production of benzyl alcohol, benzaldhyde and anisyl (4-methoxybenzyl) compounds have been reported (Okamoto et al., 2002). Of the VOCs identified in Chapter 5, two were putatively identified as benzoic acid compounds, one with a benzaldehyde structure, and two putative anisaldehyde (or methylbenzoate) compounds (Table 5.4). It has been suggested that VA and anisaldehyde act as redox cycling agents, involved in the production of H_2O_2, along with other intermediates of their oxidation such as benzaldehyde and benzoic acids (Guillen and Evans, 1994). In this process, VA and anisaldehyde are oxidised by aryl-alcohol oxidase (AAO) which generates H_2O_2, then are reduced (returned to 'native' state) by the action of aryl-alcohol dehydrogenase (AAD; Gutierrez et al., 1994). AAO activity was shown to increase in parallel to anisaldehyde production in cultures of *P. ostreatus*, which immediately preceded increases in MnP activity (Okamoto et al., 2002). Production of anisaldehyde may thus support peroxidase activity. The putative anisaldehyde compound was produced

constitutively by *S. gausapatum* and *H. fasciculare*, and levels of production did not increase during interactions relative to self-pairings (Sections 5.3.5 and 5.3.7). However, during interactions there is half the mycelial volume available for VOC production compared to during self-pairings, which could suggest upregulation of anisaldehyde production during interactions, although it is of course impossible to rule out production by *T. versicolor*.

Monoterpenes have been reported to increase laccase activity in *H. annosum*, especially when the fungus was exposed to β-pinene and carene (Flodin, 1979). α-pinene and D-limonene have also been demonstrated to improve the catalytic efficiency of laccase from *T. versicolor, T. hirsuta* and *Botrytis cinerea* (Tzialla et al., 2009). Two monoterpenes, putatively identified as carene and α-myrcene, were identified in fungal VOC profiles (Table 5.4). Production of α-myrcene was specific to the interaction with *S. gausapatum*, and carene was produced constitutively by *B. adusta* and *H. fasciculare*, and increased during interaction with *H. fasciculare* relative to self-pairings (Section 5.3). Increased laccase production in response to terpenes may function to detoxify the terpene itself, or, as wood is often rich in terpenoid compounds, to stimulate lignin decomposition. The production of interaction-specific myrcene could explain why laccase activity is so high during interactions with *S. gausapatum* (Section 2.3.4).

6.1.12 Homokaryons have similar combative and enzyme producing abilities to heterokaryons

No differences were apparent between homokaryon and heterokaryon combative ability, enzyme production, decay ability or extension rate (Chapter 4). There was as much variation within the karyotic states as between them. As *T. versicolor* is a common species, the heterokaryon is the state that would predominate in woody tissues; however, as no differences were found between homokaryons and heterokaryons, it would be

as ecologically relevant to use *T. versicolor* homokaryons in future experiments. This would have many advantages for transformations or genetic studies as there is a single copy of the genome present. Comparing mated heterokaryons with their component homokaryons revealed that the heterokaryon behaves as a functional diploid (Section 4.4; James et al., 2008). The mycelium can thus be thought of as an 'individualistic mycelium' (Rayner, 1991), analogous to a genetic individual rather than a situation where there is conflict between the constituent haploid nuclei (James et al., 2008).

6.2 Future work

Although using agar culture provides many advantages, it is ecologically unrealistic, and it would be better to study interactions within woody tissues. Sawdust or milled wood could be used, or ideally wood blocks as the structure of wood affects patterns of fungal colonisation. Wood block cultures are significantly more complicated in terms setting up interactions and interpreting outcomes, but this is the best way of establishing patterns of mycelial enzyme and VOC production, as both are regulated by substrate (e.g. Wheatley et al., 1997; Bruce et al., 2000; Mikiashvili et al., 2005). Enzyme extraction from wood blocks and sawdust cultures works well (e.g., Chapter 4; Mikiashvili et al., 2005; Baldrian et al., 2005) with production of enzymes a lot more variable than in agar culture (Chapter 4). VOC profiles have recently been shown to be more complex than those from agar cultures, probably due to the presence of specific inducers (N. Elariebi, pers. comm.).

The production of laccase is one of the most interesting aspects to pursue, as the reason for such large upregulation during interactions is unclear. Generating strains of fungi where laccase genes are overexpressed or knocked out might help towards understanding the function of laccase upregulation and what affect it has on combative ability. Knocking out laccase genes would prove considerably more difficult than

overexpressing them, due to the multiplicity of genes (Conesa et al., 2002; Martinez, 2002), however using RNAi could (in theory) downregulate a whole family of closely related genes (Nakayashiki, 2005; Echeverri and Perrimon, 2006). There has, however, been limited success in transforming basidiomycetes, which are considered 'less genetically tractable' compared to other fungi (e.g. the Ascomycetes; Rodgers et al., 2009). However, successful transformation of basidiomycetes with peroxidase genes have been reported (including Kim et al., 2002, 2005), and *T. versicolor* has recently been successfully transformed by J. Hunt (HJR lab), which opens the way for these experiments. Chemical inhibition of laccase by sodium azide could potentially be used (Zollner, 1993; Johannes and Majcherzczyk, 2000), but restricting the effects of the inhibitor to a single competitor would be difficult and the chemical may have other unwanted effects on growth.

Inhibition and overexpression of peroxidase genes to clarify their role during interactions should also be investigated, but first the production of different types of peroxidase during interactions and growth alone needs to be clarified. This could be achieved by assays combined with gene expression analysis, but ideally peroxidases should be extracted from culture media and separated by column chromatography and/or native PAGE (polyacrylamide gel electrophoresis; as in Jordaan et al., 2004), following which in-gel assays could be performed to confirm oxidation of different substrates. Different peroxidases could thus be identified by specific assays.

Profiling the laccase isozymes produced during interactions compared to growth alone is the ideal next step. The present study showed that a range of laccase isozymes are expressed during growth alone, and during interactions (Section 3.3.4), but a much larger sample size would be needed to see whether there are any patterns of isozyme production. This could be achieved using the same method as in Chapter 3, cloning and sequencing PCR products. However, RFLP (restriction fragment length polymorphism) analysis of PCR products would be an efficient (and cost-effective) way of

distinguishing between large numbers of transcripts. PCR-RFLP has previously been used to characterise expression of multigene families (e.g. Lurin and Jouanin, 2003), and also to identify a new laccase gene in a species of *Trametes* (González et al., 2003). Upregulation of laccases is believed to be a general response to stress (Baldrian, 2006), so profiling laccase isozyme production in temperature- or oxidatively-stressed mycelia would show whether different isozymes are produced in response to different stresses.

Mycelia were shown to produce constitutive and interaction-specific VOCs (Chapter 4), although many of the compounds detected could not be identified. Standards could be used to provide a more definite identification of the compounds, or individual compounds could be analysed by NMR (nuclear magnetic resonance). No VOCs were detected from *T. versicolor*, which could be attributable to the isolate or methodology used. SPME (the technique used) can detect compounds at very low concentrations, but has the major disadvantage that not all of the volatiles are adsorbed (Jelen, 2003; Ewen et al., 2004). Other techniques, such as dynamic headspace sampling which consists of the constant removal of headspace VOCs in a carrier gas and adsorption onto Tenax mesh or activated carbon, may give a fuller profile of VOCs (Ewen et al., 2004), as a wide range of polar and non-polar volatiles can be collected.

Diffusibles produced during growth alone and during interactions caused stimulation and inhibition of extension rates in remote mycelia (Section 2.3.6). Interaction-specific DOCs have been detected using HPLC (J. Hynes, unpublished; Rayner et al., 1994), but not identified. Extracellular DOCs could be extracted from liquid-grown cultures, or by extracting from agar (if necessary, concentrating the sample e.g. by freeze-drying and methanol extraction). The extracts could then be separated by chromatographic techniques such as HPLC (high performance liquid chromatography) or GC, and compounds identified by mass spectrometry or NMR. Separated compounds could be collected and tested for activity against mycelia of different species. Metabolite profiles of interacting mycelia have previously been analysed by GC/MS (Peiris et al., 2008),

which could be repeated for interactions involving *T. versicolor*. Metabolite profiling gives an instantaneous snapshot of the entire physiology of the tissue, but there is a low rate of compound identification, especially as fungi often produce unusual compounds: although *S. hirsutum* metabolite profiles were information-rich (120+ metabolite peaks detected per sample), the majority of these peaks were unidentified (Peiris et al., 2008).

Obvious gross morphological changes can be seen during interactions (e.g. barrages, hyphal cords), but little is known about the processes occurring at the level of individual hyphae. It is difficult to use microscopic methods to distinguish between hyphae of different species, so transformation of isolates with GFP or RFP is being trialled by J. Hunt (HJR lab). Detection of any regions of cell death (for example at the interaction zone) could be achieved by using a stain that differentiates between living and dead cells, such as Sytox green, in combination with a stain that stains all tissues such as Calcofluor white (Green et al., 2000).

Further work comparing homokaryon and heterokaryon characteristics should use a wider range of isolates than used in Chapter 4, and, if possible, homokaryons isolated directly from the field. Although there were no differences in combative ability between homo- and heterokaryons, this does not mean they are equally capable of dealing with other stresses such as temperature or osmotic stress. The greater genetic potential of heterokaryons may make them more adaptable to environmental conditions (Clark and Anderson, 2004), which would be a good direction for future work. Perhaps the most interesting result of the homokaryon work was that the main *T. versicolor* isolate used throughout was one of the least 'fit' in terms of combativeness and enzyme production. Isolate TvD2 is fairly old, and years of maintenance in the lab may have resulted in accumulation of mutations etc. For any future experiments, use of a younger, more recently acquired, isolate would be a good idea.

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Appendix A

Further detail on laccase and peroxidase

A.1 Laccase

Laccase catalyses the 4 electron reduction of O_2 to H_2O, coupled with four single-electron substrate oxidation steps, which has led to the analogy of a four-electron 'biofuel' cell, where laccase operates like a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen (Thurston, 1994). Bound oxygen intermediates appear to be involved in this process (Baldrian, 2006). The presence of four copper ions is an absolute requirement for optimal activity, and there have been suggestions that laccase substrate specificity is determined by the redox potential of these copper ions (Piontek et al., 2002). The high reduction potential of fungal laccases, and the large range in reduction potentials between isozymes, are not fully understood, but are thought to be controlled by structural determinants (Jönsson et al., 1995).

A.1.1 Crystal structure of laccase

Typical fungal laccase is approximately 60-70 kDa with an acidic isoelectric point around pH 4.0 (Baldrian, 2006). Most fungal laccases are monomeric proteins, although several exhibit a homodimeric structure, the enzyme being composed of two identical subunits with a molecular weight typical of monomeric laccases (Baldrian, 2006). Like most extracellular fungal enzymes, laccases are glycoproteins with the extent of glycosylation ranging from 10-20%. In addition to its structural role, glycosylation may protect the enzyme from proteolytic degradation (Baldrian, 2006). Modeling of the crystal structure of *T. versicolor* laccase (TvL) showed a total of seven N-acetyl glucsoamine moeities at 5 distinct N-glycosylation sites (Piontek et al., 2002). Glycosylation is one of the biggest problems for the heterologous production of the enzyme, and is extremely difficult to overcome.

Laccases are ancient enzymes with a highly conserved catalytic site, despite which the rest of the protein shows wide variability (Mayer and Staples, 2002). The crystal structures of several fungal laccases, including *Coprinus cinereus*, *T. versicolor*, *Melanocarpus albomyces* and *Rigidoporus lignosus* have recently been solved by X-ray diffraction (Bukh et al., 2006). These laccases share similar topology: a monomer organised in three sequentially arranged domains, each of which is of a similar β-barrel type architecture with only small amounts of α-helix (Piontek et al., 2002). Both N-terminal and C-terminal amino acids benefit from hydrogen bonding networks to the rest of the protein, providing rigidity (Piontek et al., 2002). Two disulfide bridges are present, one of which forms a bridge between the first and second domains (Jönsson et al., 1995). The presence of four cupric ions, each coordinated to a single polypeptide chain, is an absolute requirement for optimal activity (Baldrian, 2006), and the regions that coordinate the Cu^{2+} ions are perfectly conserved (Asiegbu et al., 2004). Laccase contains at least one T1 (type 1) copper, embedded in domain 3, which is the primary oxidation site. There are also one T2 and two T3 coppers arranged in a trinuclear

cluster situated between domains 1 and 3. The T3 site is where reduction of molecular oxygen occurs (Piontek et al., 2002). One electron is abstracted from the substrate at the T1 site, progressively leading to the reduction of all four Cu(II) ions to Cu(I). Re-oxidation of the cuprous ions to Cu(II) occurs via electron donation to an O_2 molecule complexed at the T2/T3 cluster, leading to the formation of water without the release of toxic peroxide intermediates (Tadesse et al., 2008). The three copper types can be differentiated by their spectroscopic behaviour. Site T1 is a blue copper ion with strong absorption around 600 nm. T2 exhibits features typical of normal copper, with only weak absorption in the visible region but is EPR-active (electron paramagnetic resonance spectrum). The T3 coppers are characterised by an absorption band around 330 nm and are EPR silent due to their strong antiferromagnetic coupling bridged by a hydroxide (Solomon et al., 1996; Piontek et al., 2002).

The function of the T1 site in the enzyme is long range intramolecular electron transfer, shuttling electrons from the substrate to the trinuclear cluster along a His-Cys-His tripeptide pathway which is highly conserved among blue multicopper oxidases (Piontek et al., 2002). Primary amino acid sequence homology shows that the conserved ligands for the T1 site are two histidine nitrogens and a cysteine thiolate sulphur (Solomon et al., 1996). The peptide ligands of the trinuclear cluster are eight histidines which occur in a highly conserved pattern of four HXH motifs (Mayer and Staples, 2002). The electrostatic surface potential distribution of TvL revealed a dominance of negative charges, which is in accordance with the acidic pI of c. 3.5. The crystal structure shows that the substrate binds in a small, negatively charged cavity near the copper T1 site. The oxygen-reducing site at the T2/T3 cluster has access to the solvent through two channels, which are well suited to allow fast access of dioxygen molecules to the trinuclear cluster and subsequent easy release of water (Piontek et al., 2002).

The reason for the very high reduction potentials, as well as the large range in reduction

potentials among fungal laccases remains to be determined, but it is thought to be controlled by structural determinants. A mechanism has been suggested where the redox potential of the T1 cation is altered by stretching of the hydrogen bond between the metal and the ligating amino acid (Piontek et al., 2002). Other possible factors influencing reduction potentials of fungal laccase T1 sites are electrostatic interactions between the metal site and the protein and the water environment (Solomon et al., 1996).

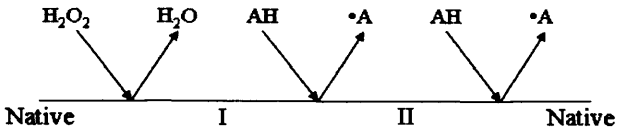
A.2 Peroxidase: mechanism of action

A single-substrate reversible reaction can be formulated as:

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES} \rightleftharpoons \mathrm{E} + \mathrm{P}$$

where E is the enzyme, S is the substrate and P is the product. However, peroxidases catalyse a 2-substrate, 2-product reaction, for which enzyme-substrate complexes have not been detected, and the only detectable intermediates are covalent compounds. Two-substrate, two-product reactions occurs when: (1) one substrate binds, followed by a first-product release, resulting in a modified enzyme to which (2) a second substrate binds, followed by second-product release. This is also known as the 'Ping-pong mechanism' (Dunford, 1999). Ping-pong reactions are usually reversible, with the consequence that there is a finite upper limit in rate. Peroxidase kinetics have similarities to Ping-pong kinetics, with the difference that the reactions are usually irreversible. This is often misunderstood; the observed irreversibility does not mean the back reaction has zero rate, but that its rate is not measurable. The rate of reaction appears to have no upper limits, the larger the concentration of reactants, the faster the reaction (Dunford, 1999). No equilibrium between reactants and products is formed because the

reaction is irreversible. The peroxidase reaction can be diagrammatically represented, as shown below.



The peroxidase enzyme is oxidised from its native state to Compound I, after one reduction step Compound II is formed, then another reduction step returns the enzyme to its native state. AH is the reduced form of the substrate, ·A is the oxidised form of the substrate. The products are shown departing as fast as the substrate comes into contact with the enzyme because complexes between substrates and enzymes are very short-lived and difficult or impossible to detect. There are several mechanisms for a peroxidase reaction, the most common of which is:

$$(Fe^{3+})Pox + H_2O_2 \rightarrow \underbrace{(Fe(IV) = O)Pox^{\star}}_{Compound\ I} + H_2O$$

$$(Fe(IV) = O)Pox^{\star} + AH \rightarrow \underbrace{(Fe(IV) = O)Pox}_{Compound\ II} + \cdot A$$

$$(Fe(IV) = O)Pox + AH \rightarrow (Fe^{3+})Pox + \cdot A$$

where the resting enzyme [(Fe^{3+})Pox] reacts with H_2O_2 in a two-electron transfer reaction that results in Compound I formation. Compound I has one reducing equivalent at the oxyl-ferric iron [Fe(IV)=O] and the other forms a cation radical [Pox*]. Compound I is then reduced by the substrate AH in two sequential one-electron steps through Compound II (Conesa et al., 2002). The sum of the 3-step peroxidase reaction is:

$$H_2O_2 + 2AH_2 \longrightarrow 2H_2O + 2 \cdot A$$

A.3 Crystal structure of peroxidases

Ligninolytic peroxidases are globular proteins formed by 11-12 predominantly α-helices in two domains delimiting a central cavity harboring the heme group. The protein structure is stabilised by 8 cysteine residues forming 4 disulfide bridges, with a fifth bridge in the C-terminal region of MnP, and two Ca^{2+} binding sites (Dunford, 1999; Martinez, 2002). 53 residues are conserved in all fungal peroxidases. Some of these are probably required to attain correct protein folding, and there are four clusters of conserved residues are located around the 2 structural Ca^{2+}, and at the distal and proximal sides of the heme cavity (Martinez, 2002). Differences in glycosylation are in some cases responsible for the appearance of isoforms (Conesa et al., 2002), but the glycan groups are not essential for activity. LiPs have molecular masses of approximately 40 kDa, whilst MnPs are comparatively larger, with molecular masses of 47-60 kDa.

The heme group is sandwiched between an N-terminal and a C-terminal helix and rests at the bottom of the space formed by the surfaces of both structures (Conesa et al., 2002). The iron coordination and the residues involved are conserved amongst most peroxidases, with nearly all plant and fungal peroxidases have the prosthetic group ferriprotoporphyrin IX, a structure of 4 pyrrole rings joined by methene bridges with iron(III) at the centre and (due to displacement of 2 hydrogen atoms) a net charge of +1. The native resting enzyme has a 5-coordinate iron(III), where positions 1 to 4 are occupied by nitrogen atoms from the pyrrole rings, and the fifth, on the proximal side of the heme, is the site of attachment to the apoprotein via the imidazole side chain of a histidine residue in a relationship best described as covalent (Dunford, 1999). Another, distal histidine, assisted by an asparagine residue, participates in the transfer of oxidising equivalents from H_2O_2 to the heme (Conesa et al., 2002). The

vacant sixth coordinate position of iron(III) is located on the distal side of the heme, in a cavity that is the site of peroxidase reactions. Common ligands such as cyanide and azide can occupy this position and thus act as effective peroxidase inhibitors (Dunford, 1999). The low pH dependency of the redox potential of peroxidases could indicate participation of a carboxylic acid side chain in the catalytic reaction, and several residues have been proposed to play this role in LiP and in MnP (Sundaramoorthy et al., 1994).

In most peroxidases direct access to the heme is restricted, but the heme edge is available for substrate interactions (Kishi et al., 1996; Doyle et al., 1998). An exposed tryptophan (Trp) related to VA oxidation is a conserved residue around the heme pocket in LiP (Doyle et al., 1998). Trp171 (numbering from *P. chrysosporium* LiP) is present in all LiPs, is redox active, and is an alternative binding site for VA and other aromatic substrates; replacement of this residue by site-directed mutagenesis results in loss of VA-oxidising ability and inclusion of a Trp into MnP resulted in an enzyme which could oxidise VA (Doyle et al., 1998; Timofeevski et al., 1999). In MnP there are three conserved acidic residues around the heme pocket – two glutamic acids and one aspartic acid (Glu35, Glu39 and Asp179 in *P. chrysosporium* MnP) – which are involved in Mn^{2+} oxidation and Compound II reduction, but not in the reaction with phenolic substrates or with H_2O_2 (Kishi et al., 1996). VP contains both sets of residues for VA and Mn^{2+} binding, which has been confirmed in *P. eryngii*. Peroxidases from *P. ostreatus, B. adusta* and *T. versicolor* show putative Mn^{2+} and VA oxidation sites in their sequences, with the so-called Mn-repressed peroxidase (MRP) of *T. versicolor* also potentially able to show VP-type properties (Ruiz-Dueñas et al., 2009).

Appendix B

Ligninolytic enzyme activity staining

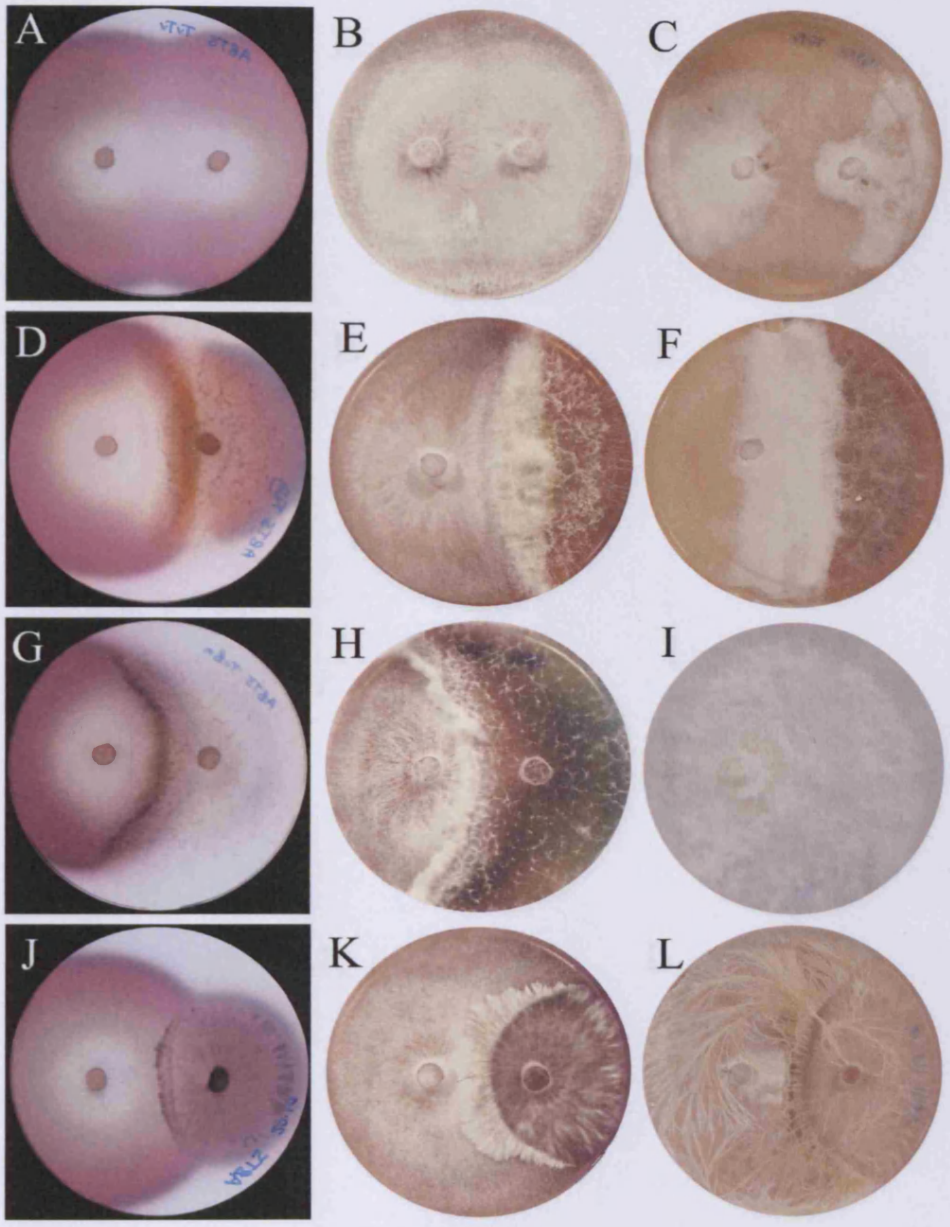


Figure B.1: Localisation of laccase activity by growth on 2% MA containing ABTS. *T. versicolor* is on the left in all pairings. A-C, *T. versicolor* self-pairings; D-F, vs. *S. gausapatum*; G-I, vs. *B. adusta*; J-L, vs. *H. fasciculare* GTWV2. Laccase activity causes formation of a green, then violet colouration in the agar. Photos taken after 2 d, (using a lightbox; A, D, G and J), after 8 d (B, E, H and K), and after 2 months (C, F, I and L).

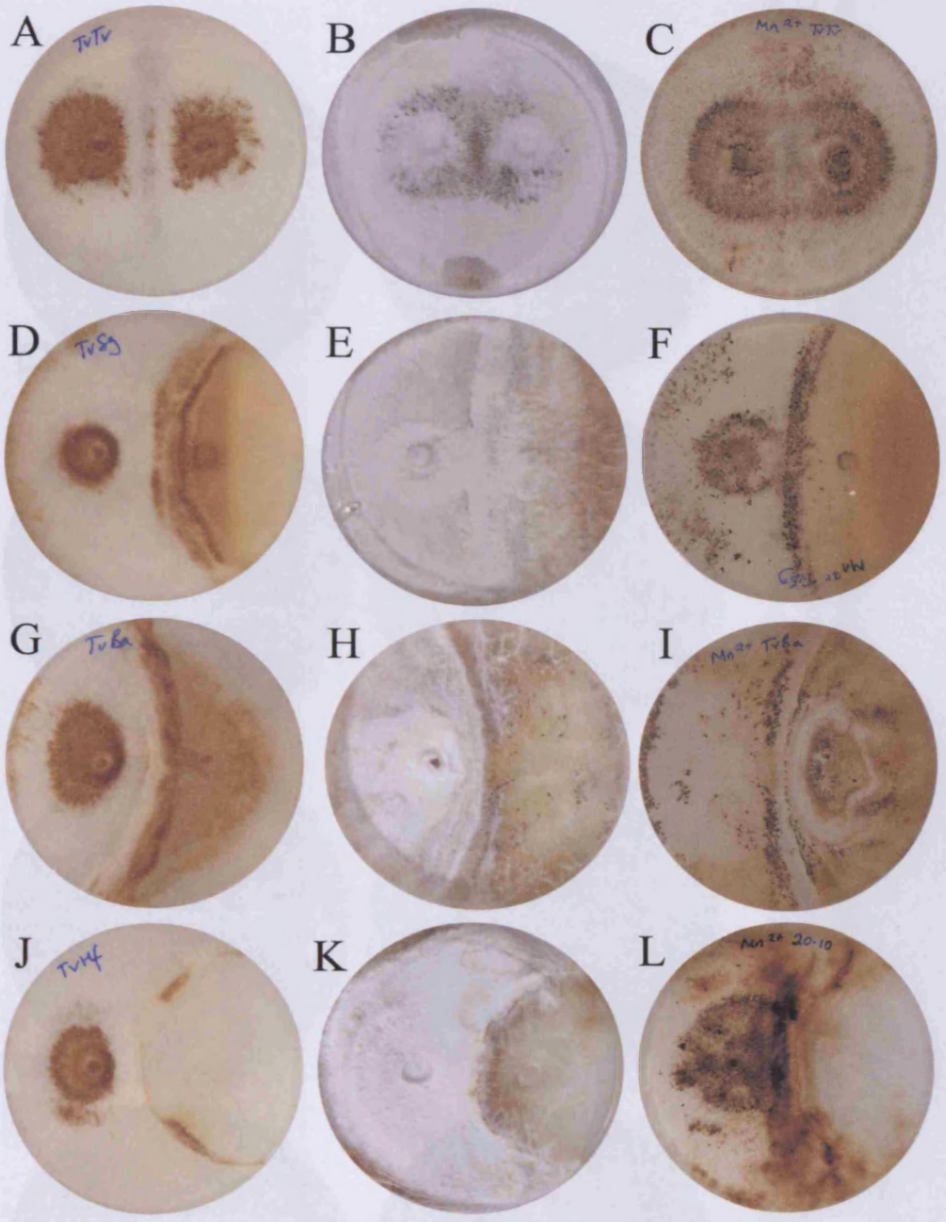


Figure B.2: Localisation of MnP activity by growth on 2% MA containing $MnCl_2$. *T. versicolor* is on the left in all pairings. A-C, *T. versicolor* self-pairings; D-F, vs. *S. gausapatum*; G-I, vs. *B. adusta*; J-L, vs. *H. fasciculare* GTWV2. MnP activity causes formation of a brown precipitate in the agar. Photos taken after 8 d from the bottom (agar view; A, D, G and J), and from the top (mycelium view; B, E, H and K), and after 2 months (agar view; C, F, I and L).

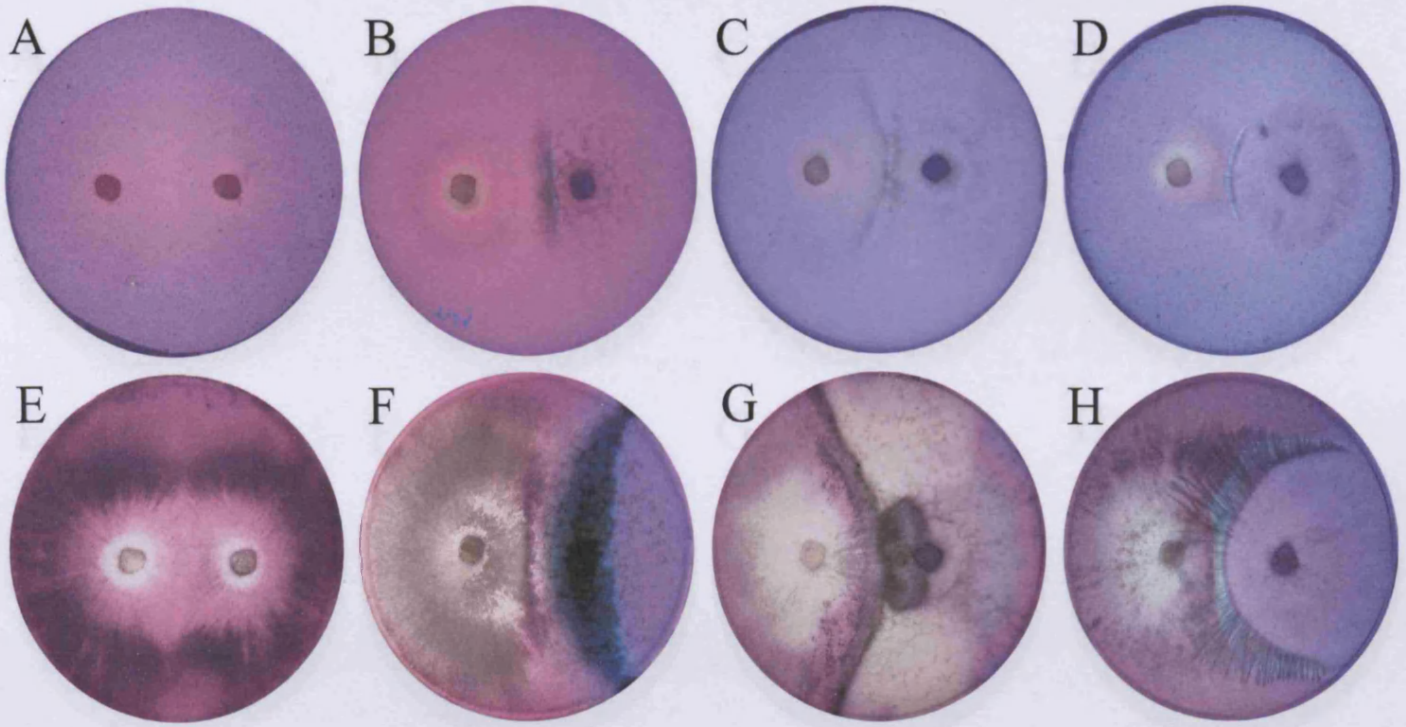


Figure B.3: Localisation of LiP activity by growth on 2% MA containing the dye Azure B. *T. versicolor* is on the left in all pairings; all photos taken using a lightbox. A and E, *T. versicolor* self-pairings; B and F, vs. *S. gausapatum*; C and G, vs. *B. adusta*; D and H, vs. *H. fasciculare* GTWV2. LiP activity causes decolourisation of the blue dye. Photos taken after 2 d (A-D), and after 8 d (E-H)

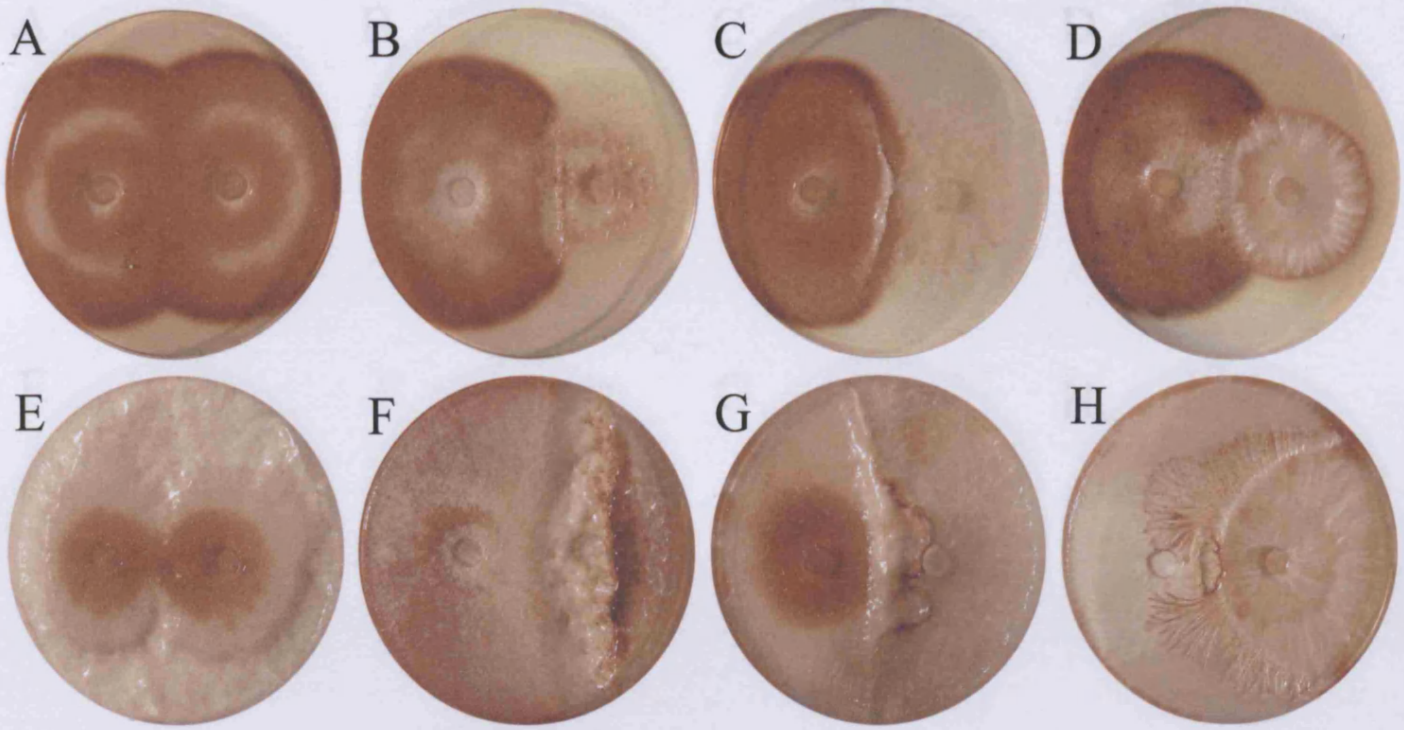


Figure B.4: Localisation of peroxidase activity by staining with DAB. *T. versicolor* is on the left in all pairings. A and E, *T. versicolor* self-pairings; B and F, vs. *S. gausapatum*; C and G, vs. *B. adusta*; D and H, vs. *H. fasciculare* GTWV2. Oxidase activity leads to deposition of a red-brown precipitate. Photos taken after 2 d (A-D), and after 8 d (E-H)

Figure B.5: Localisation of superoxide by staining with NBT. *T. versicolor* is on the left in all pairings. A and E, *T. versicolor* self-pairings; B and F, vs. *S. gausapatum*; C and G, vs. *B. adusta*; D and H, vs. *H. fasciculare* GTWV2. The reaction of NBT with superoxide leads to deposition of a purple-blue precipitate. Photos taken after 2 d (A-D), and after 8 d (E-H)

Appendix C

Laccase and peroxidase sequence information and gene expression data

Table C.1: Laccase sequences used for primer design

Genbank accession code	Type	Other name?	DNA sequence			cDNA sequence			Reference
			no. bases	Primers predicted to bind	Length of amp'd seq	no. bases	Primers predicted to bind	Length of amp'd seq	
AJ746240	Lacc	klc1	1298	Lacc-F: 90%	-	1018	Lacc-F: 90%	-	Cheong et al. (2006)
AM422387	Lacc	klc2	-	-	-	1784	Lacc-F: 90% Lacc-R: 100%	377	Yeo et al. (2008)
X84683	Lacc	lcc1	2800	Lacc-F: 90% Lacc-R: 100%	483	1563	Lacc-F: 90% Lacc-R: 100%	377	Jönsson et al. (1995)
AB212733	Lacc	lac3	-	-	-	1563	Lacc-F: 90% Lacc-R: 100%	374	Fujihiro et al. dir. sub. 2005
D84235	Lacc	-	3099	Lacc-F: 66% Lacc-R: 100%	521	1581	Lacc-F: 90% Lacc-R: 100%	388	Mikuni and Morohoshi (1997)
U44431	Lacc	lccIV	-	-	-	2561	Lacc-F: 85% Lacc-R: 95%	388	Ong et al. dir. sub. 1996
AB212734	Lacc	lac4	-	-	-	1584	Lacc-F:95% Lacc-R: 100%	388	Fujihiro et al. dir. sub. 2005
AY243857	Lacc	lcc6	194	-	-	144	-	-	Pointing et al. (2005)
AB212732	Lacc	lac2	-	-	-	1560	Lacc-F: 100% Lacc-R: 100%	379	Fujihiro et al. dir. sub. 2005
AY693776	Lacc	lcc1	-	-	-	1560	Lacc-F: 100% Lacc-R: 100%	379	Necochea et al. (2005)
AJ627252	Lacc	lcc	-	-	-	1560	Lacc-F: 100% Lacc-R: 100%	379	Kapoor et al. dir. sub. 2004
AY049725	Lacc	lac1	-	-	-	2408	Lacc-F: 100% Lacc-R: 100%	379	O'Callaghan et al. (2002)
U44851	Lacc	lcc1	2127	Lacc-F: 85% Lacc-R: 100%	441	1560	Lacc-F: 100% Lacc-R: 100%	379	Ong et al. dir. sub. 1996
U44430	Lacc	lcc1	-	-	-	1932	Lacc-F: 100% Lacc-R: 100%	379	Ong et al. (1997)
AY204503	Lacc	-	198	-	-	143	-	-	Garcia-Mena et al. (2005)
AF414109	Lacc	lac1	-	-	-	1563	Lacc-F: 100% Lacc-R: 100%	379	Jolivalt et al. (2005)
AY081188	Lacc	laccIII	3963	Lacc-F: 95% Lacc-R: 100%	379	1563	Lacc-F: 95% Lacc-R: 100%	379	Schuren et al. dir. sub. 2002
Y18012	Lacc	-	-	-	-	1563	Lacc-F: 100% Lacc-R: 100%	379	Jonsson and Nyman dir. sub. 1998
AB212731	Lacc	lac1	-	-	-	1563	Lacc-F: 100% Lacc-R: 100%	379	Fujihiro et al. dir. sub. 2005
D13372	Lacc	cvl3	2684	Lacc-F: 100% Lacc-R: 95%	379	1563	Lacc-F: 100% Lacc-R: 95%	379	Mikuni et al. dir. sub. 1996

Dir. sub., direct submission to GenBank; amp'd, amplified. Where primers are predicted to bind to accessioned sequences, the % homology between the primer sequence and the accessioned sequences are given.

Table C.2: Lignin peroxidase and Mn-repressed peroxidase sequences used for primer design

Genbank accession code	Type	Other name?	DNA sequence			cDNA sequence			Reference
			no. bases	Primers predicted to bind	Length of amp'd seq	no. bases	Primers predicted to bind	Length of amp'd seq	
Z30666	LiP	LPGIII	1889	LiP-F: 95% LiP-R: 100%	350	1104	LiP-F: 95% LiP-R: 100%	353	Johansson and Nyman (1996)
Z30667	LiP	LiP7	1971	LiP-F: 85% LiP-R: 78%	601	1107	LiP-F: 85% LiP-R: 78%	543	Johansson and Nyman (1993)
AB158478	LiP	cvlip	-	-	-	1104	LiP-F: 100% LiP-R: 100%	353	Iimura dir. sub. 2000
M91818	LiP	TMTVLG2A	2042	LiP-F: 100% LiP-R: 100%	353	1104	LiP-F: 100% LiP-R: 100%	353	Black and Reddy dir. sub. 1991
X75655	LiP	LPGI + LPGII	5799						Jönsson and Nyman (1994)
		LPGII	-	LiP-F: 95% LiP-R: 100%	353	1104	LiP-F: 95% LiP-R: 100%	353	
		LPGI	-	LiP-R: 95%	-	1119	LiP-R: 95%	-	
M64993	LiP	TMTLIGPERO	2140	LiP-R: 95%	-	1119	LiP-R: 95%	-	Jönsson and Nyman (1992)
Z31011	LiP	LPGIV (LP12)	2581		348	1119	LiP-F: 85% LiP-R: 95%	348	Johansson and Nyman (1993)
M55294	LiP	VLG1	2092	-	-	-	-	-	Black and Reddy (1991)
AY243871	LiP		429	-	-	-	-	-	Pointing et al. (2005)
AY243872	LiP		429	-	-	-	-	-	Pointing et al. (2005)
AJ745080	MRP	-	-	-	-	1395	MRP-F: 100% MRP-R: 100%	134	Kim et al. (2005)
AF008585	MRP	-	-	-	-	1362	MRP-F: 100% MRP-R: 100%	135	Collins et al. (1999)

Dir. sub., direct submission to GenBank; amp'd, amplified. Where primers are predicted to bind to accessioned sequences, the % homology between the primer sequence and the accessioned sequences are given.

Table C.3: MnP sequences used for primer design

Genbank accession code	Type	Other name?	DNA sequence			cDNA sequence			Reference
			no. bases	Primers predicted to bind	Length of amp'd seq	no. bases	Primers predicted to bind	Length of amp'd seq	
AJ745879	MnP	-	-	-	-	1313	MnP-R: 85% MnP2-R: 85%	-	Kim et al. dir. sub. 2004
Z30668	MnP	MPGI	2125	MnP-F: 100% MnP-R: 95% MnP2-R: 90%	MnP: 294	1098	MnP-F: 100% MnP-R: 95% MnP2-R: 90%	MnP: 211	Johansson and Nyman (1993)
D86493	MnP	CVMNP	2739	MnP-F: 100% MnP-R: 100%	MnP: 292	1098	MnP-F: 100% MnP-R: 100% MnP2-R: 90%	MnP: 211	Iimura dir. sub. 2000
AY677128	MnP	cmp1	-	-	-	1361	MnP-R: 65% MnP2-R: 95%	-	Jun and Kim dir. sub. 2004
AY677129	MnP	cmp2	-	-	-	1312	MnP-F: 100% MnP-R: 100% MnP2-R: 95%	MnP: 211	Jun and Kim dir. sub. 2004
AY677130	MnP	cmp3	-	-	-	1275	MnP2-F: 95% MnP2-R: 95% MnP-R: 85%	MnP2: 186	Jun and Kim dir. sub. 2004
AY677131	MnP	cmp5	-	-	-	1253	MnP-R: 95% MnP2-R: 90%	-	Jun and Kim dir. sub. 2004
AF102515	MnP	MNP2	-	-	-	1133	MnP2-F: 100% MnP2-R:100% MnP-R: 85%	MnP2: 186	Cullen et al. dir. sub. 1998
Z54279	MnP	PGVII	2235	MnP2-F: 100% MnP2-R: 100% MnP-R: 85%	MnP2: 202	1095	MnP2-F: 100% MnP2-R: 100% MnP-R: 85%	MnP2: 186	Johansson and Nyman dir. sub. 1995

Dir. sub., direct submission to GenBank; amp'd, amplified. Where primers are predicted to bind to accessioned sequences, the % homology between the primer sequence and the accessioned sequences are given.

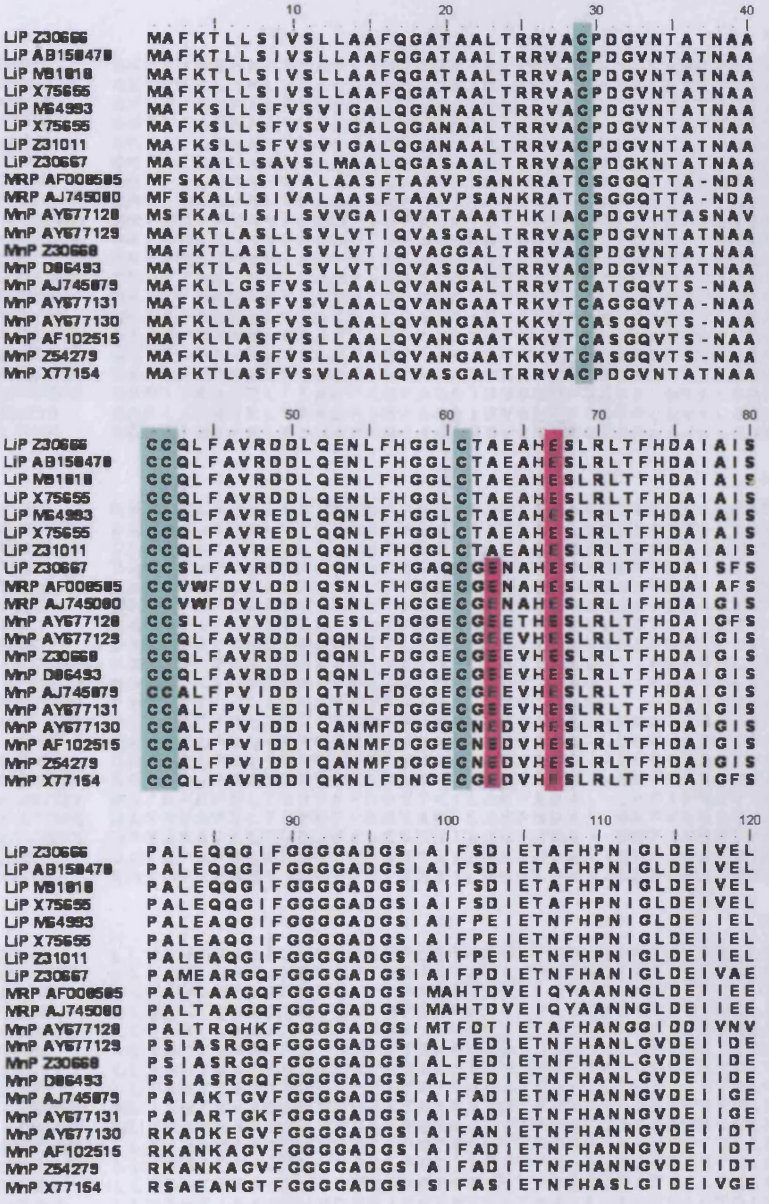


Figure C.1: Mulitple alignment of *T. versicolor* peroxidase amino acid sequences (360 of 376 amino acids shown). The sequences are labelled according to whether they are lignin peroxidase (LiP), manganese peroxidase (MnP) or Mn-repressed peroxidase (MRP), along with their accession numbers. More detail on individual sequecnes can be found in Tables C.1, C.2 and C.3. Pink highlighting, the three acidic residues involved in Mn^{2+} binding; yellow highlighting, the conserved tryptophan residue involved in the interaction with veratryl alcohol; green highlighting, the eight cysteine residues conserved in all heme peroxidases.

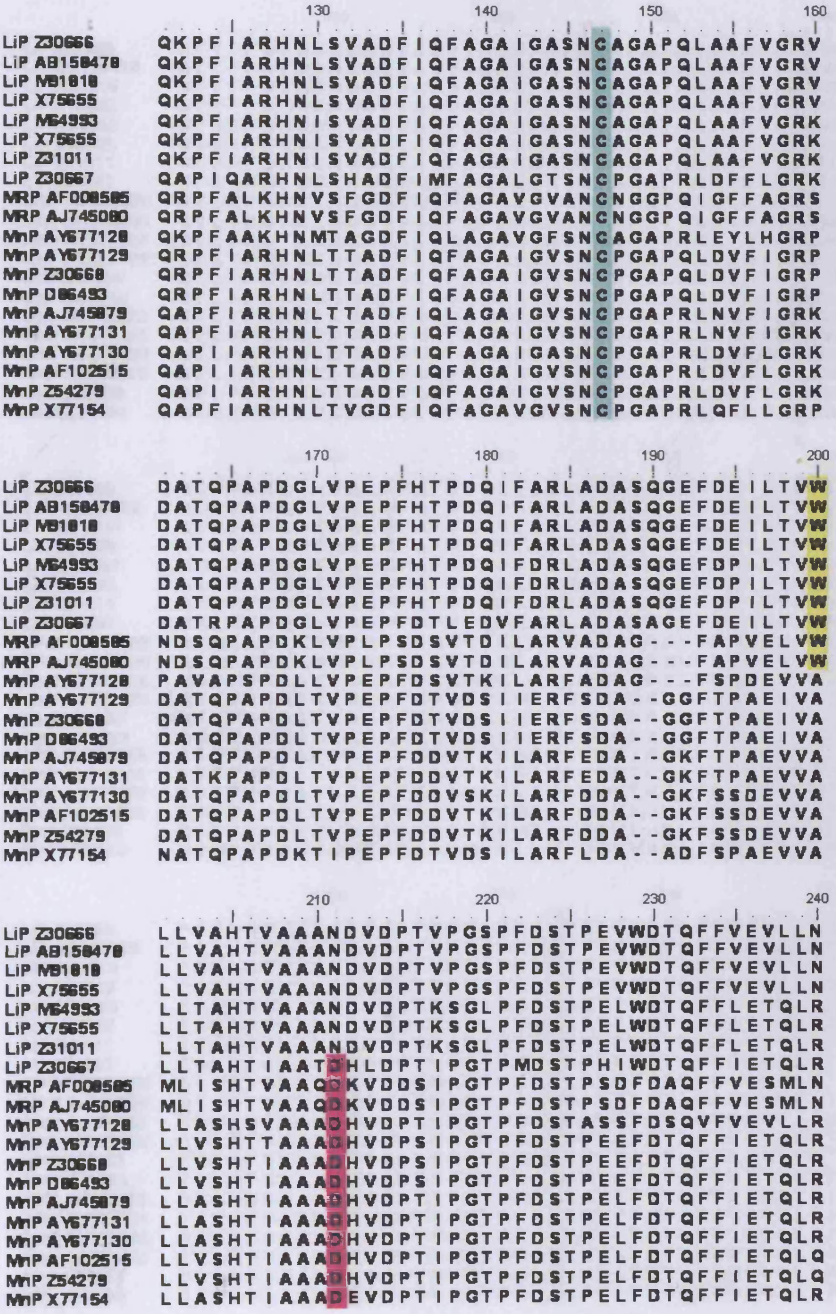


Figure C.1 cont'd

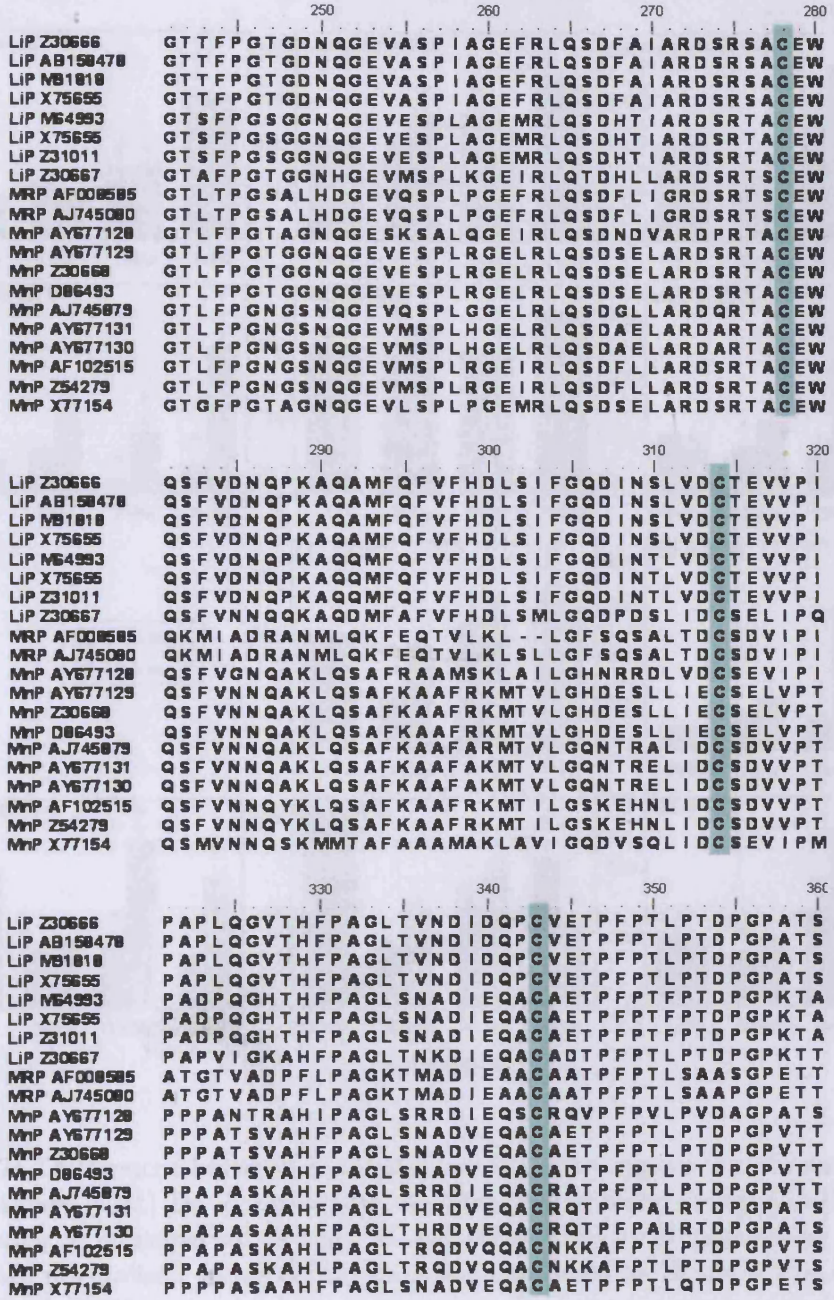


Figure C.1 cont'd

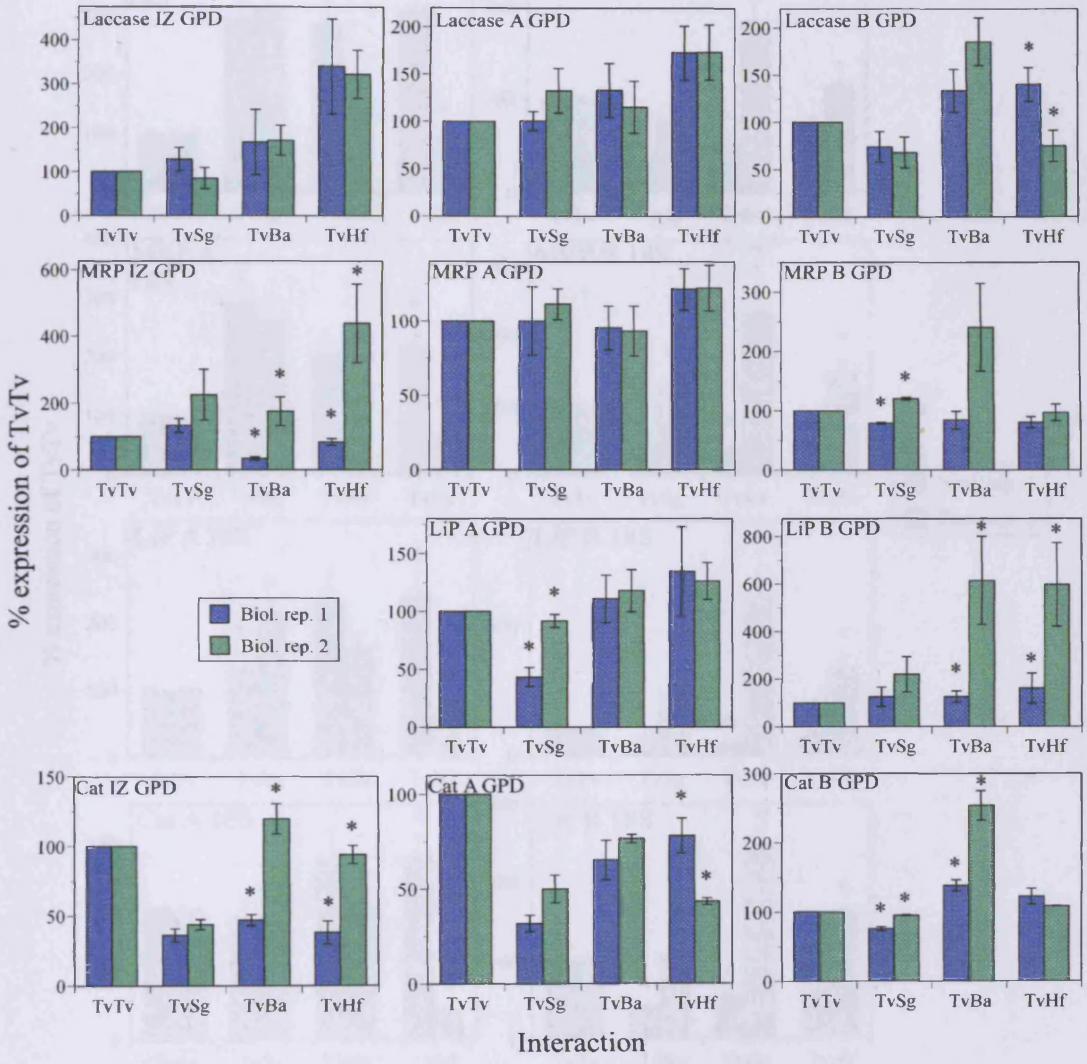


Figure C.2: Differences between biological replicates in expression of laccase, MRP, LiP and catalase (Cat) by *T. versicolor* mycelia during self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *B. adusta* (TvBa) or *H. fasciculare* (TvHf) at 2 d. Data are normalised using GPD. Data are expressed as % transcript abundance relative to TvTv cDNA samples from each region. Bars are the mean of the three technical replicates ± SEM. * indicates a significant ($P \leq 0.05$) difference in expression between biological replicates.

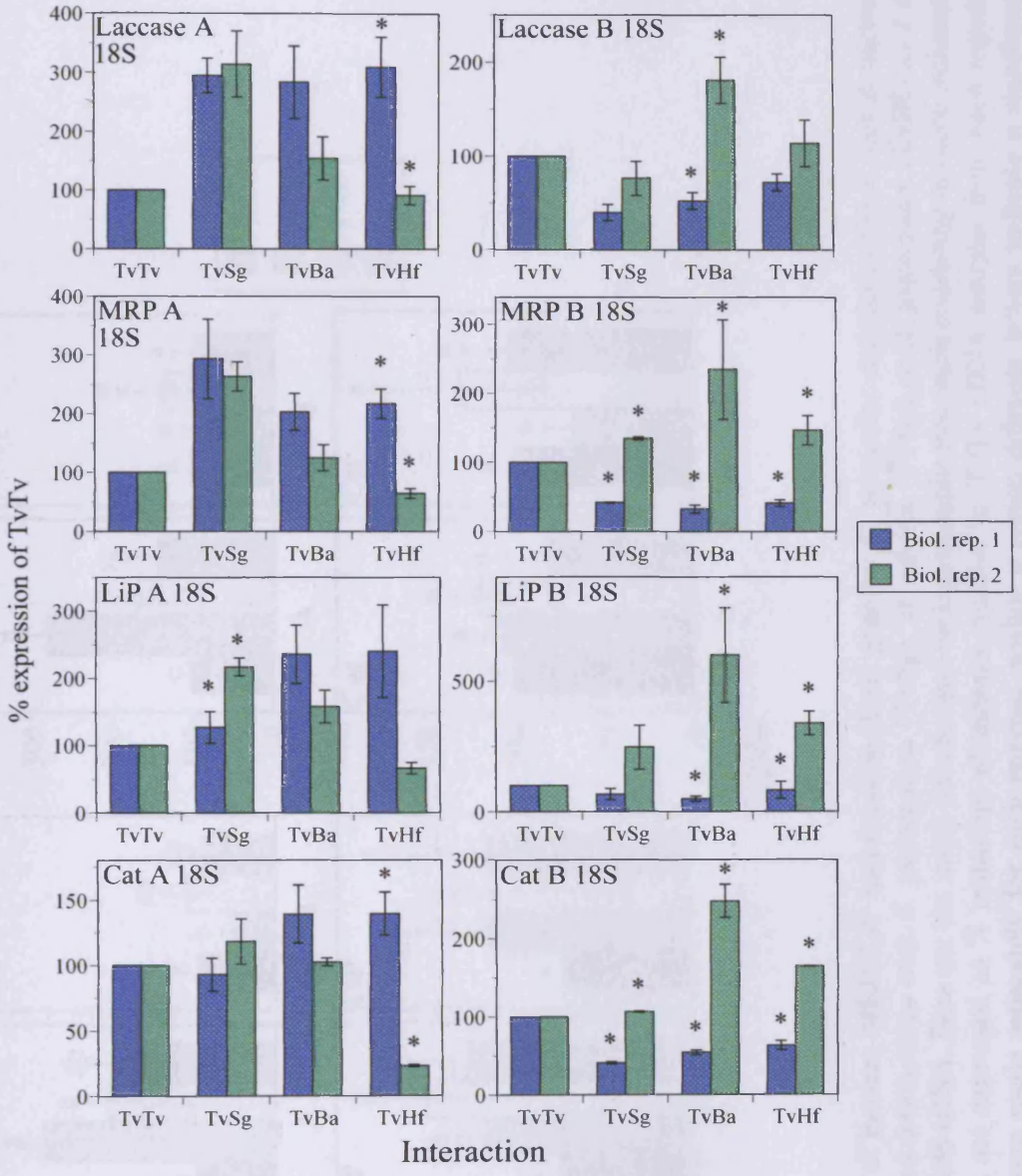


Figure C.3: Differences between biological replicates in expression of laccase, MRP, LiP and catalase (Cat) by *T. versicolor* mycelia during self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *B. adusta* (TvBa) or *H. fasciculare* (TvHf) at 2 d. Data are normalised using 18S rRNA. Data are expressed as % transcript abundance relative to TvTv cDNA samples from each region. Bars are the mean of the three technical replicates ± SEM. * indicates a significant ($P \leq 0.05$) difference in expression between biological replicates.

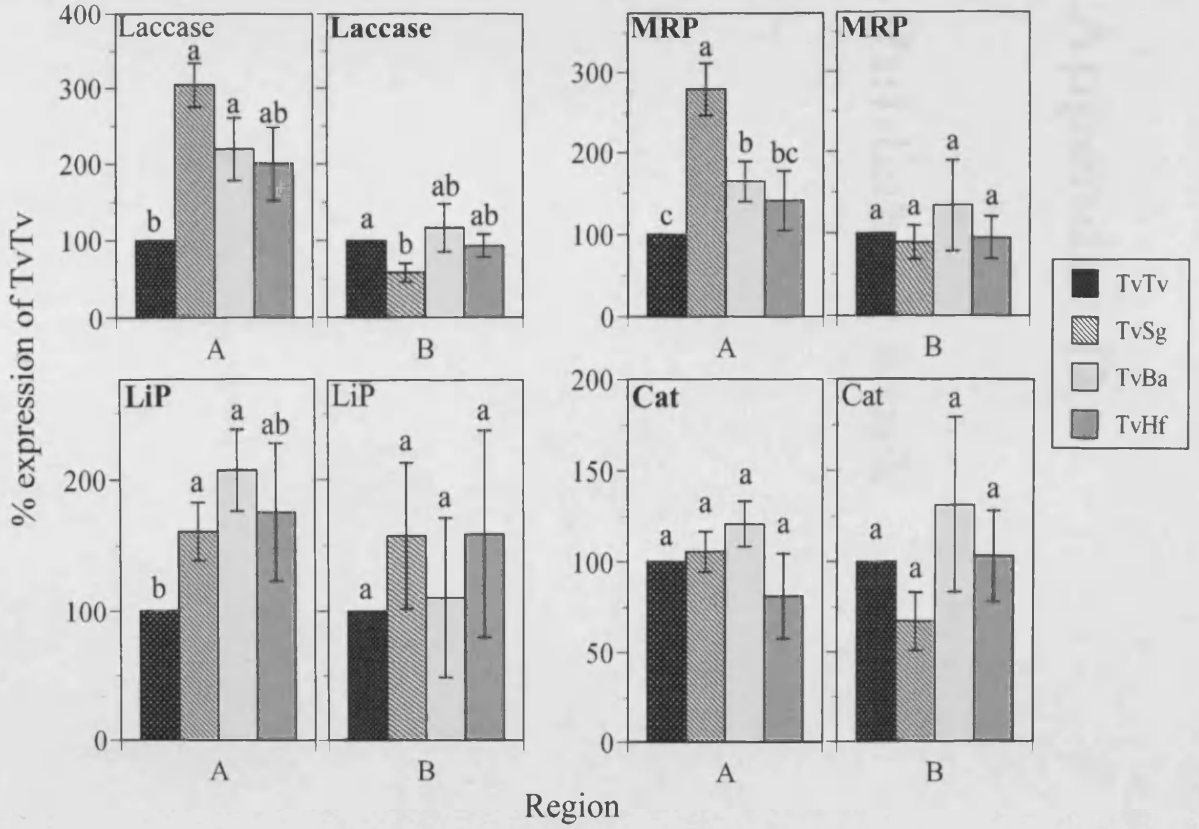


Figure C.4: Expression of laccase, MRP, LiP and catalase (Cat) gene by *T. versicolor* mycelia in the A and B regions of self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *B. adusta* (TvBa) or *H. fasciculare* (TvHf) at 2 d. Data were normalised to 18S rRNA. Bars are the mean data for two biological replicates, each consisting of three technical replicates, ± SEM. Data are expressed as % transcript abundance relative to TvTv cDNA samples from each region. Statistical comparisons were made separately for each enzyme, within a region different letters indicate a significant difference ($P \leq 0.05$).

Appendix D

Published work

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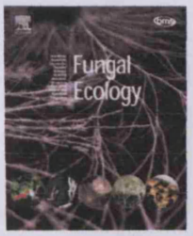

Changes in volatile production during interspecific interactions between four wood rotting fungi growing in artificial media

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ABSTRACT

Effects of volatile organic compounds (VOCs) produced during interspecific mycelial interactions were examined by measuring extension rate of 'target' fungi growing in agar plates taped above two interacting mycelia – *Bjerkandera adusta*, *Hypholoma fasciculare*, *Stereum gausapatum* and *Trametes versicolor* in all combinations. Extension rates of *T. versicolor*, *S. gausapatum* and *H. fasciculare* above self-pairings were not significantly different ($P > 0.05$) to growth above agar controls, but the extension rate of *B. adusta* was significantly ($P \leq 0.05$) greater. Extension rates of *T. versicolor* and *B. adusta* were often significantly greater above inter-specific interactions and above other species compared with growth above self or agar controls. VOC production was quantified and qualified, by gas chromatography–mass spectrometry (GC/MS), over the course of interactions with *T. versicolor* replacing *S. gausapatum*, deadlocking with *B. adusta* and replaced by *H. fasciculare*. VOC production was species- and interaction-specific. It varied over the time course of interactions, and changes in production were correlated with production of pigments in interactions involving *S. gausapatum*. VOCs included 3 monoterpenes, benzoic acid, alkenols of different chain lengths, two long-chain hydrocarbons and a quinolinium-like compound. Their possible roles are discussed.

Introduction

Inter- and intra-specific interactions between wood-decay basidiomycetes are inevitable where their niches overlap (Rayner & Boddy 1988; Boddy 2000; Woodward & Boddy 2008). The outcome determines the size of territory held by a mycelium, and hence access to nutrients. It is a major driver of community change, and affects decay rate (Owens *et al.* 1994; Boddy 2000). Recognition as 'non-self' (where there is no mating compatibility) by basidiomycete mycelia triggers antagonistic responses including changes in colony morphology at the interaction zone (the area of physical contact between competing mycelia) and elsewhere, growth rate, and production and release of extracellular enzymes and secondary metabolites (Rayner & Boddy 1988; White & Boddy 1992; Griffith *et al.* 1994; Score *et al.* 1997). Effects commonly occur following mycelial contact, but sometimes also prior to contact (Schoeman *et al.* 1996; Wheatley *et al.* 1997; Savoie *et al.* 2001; Humphris *et al.* 2002). The drivers of this 'distance antagonism' are volatile and diffusible secondary chemicals, which may continue to function following mycelial contact. Whereas aqueous diffusion of secondary metabolites through the substratum could mediate local antagonism, production of volatile organic compounds (VOCs) could act over much greater distances (Rayner *et al.* 1994; Wheatley *et al.* 1997; Heilmann-Clausen & Boddy 2005).

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All basidiomycetes probably constitutively produce profiles of VOCs from mycelium and fruit bodies (Fäldt *et al.* 1999; Wu *et al.* 2005), and these have been used chemotaxonomically and in the detection of fungi within food and building timber (Nilsson *et al.* 1996). VOC profiles are reproducible but dependent on several factors including substratum composition (Wheatley *et al.* 1997; Bruce *et al.* 2000; Wheatley 2002; Ewen *et al.* 2004), temperature (Tronsmo and Dennis 1978; Jelen 2002), pH (Chen *et al.* 1984) and growth stage (Nilsson *et al.* 1996; Fäldt *et al.* 1999; Jelen 2002; Wu *et al.* 2005). The VOC profile of a fungus may also alter qualitatively and/or quantitatively during interactions, depending on the combination of species involved (Griffith *et al.* 1994; de Jong and Field 1997; Hynes *et al.* 2007). However, it is not known how the profile alters during interactions having different outcomes. Basidiomycete VOC profiles comprise a diverse range of chemical classes, including alcohols, aldehydes, ketones, terpenes and aromatic compounds (Maga 1981; Sunesson *et al.* 1995; Nilsson *et al.* 1996; Breheret *et al.* 1997; Wheatley *et al.* 1997; Rosecke *et al.* 2000; Humphris *et al.* 2001; Humphris *et al.* 2002; Ewen *et al.* 2004). Each of these chemical classes contains compounds with known antifungal activity, perhaps most notably the sesquiterpenes of which many have antifungal properties and are involved in the defence responses of both plants and fungi (Stadler & Sterner 1998; Fäldt *et al.* 1999; Abraham 2001; Cheng *et al.* 2005). The antagonistic potential of the VOC profile of a fungus depends on the constituent chemicals, their relative amounts, and the susceptibility of the combatant. VOC-mediated effects during interactions are subtle, and are often manifested as changes in extension rate or enzyme activity (Strobel *et al.* 2001), and effects can be stimulatory as well as inhibitory (Dick & Hutchinson 1966; Schoeman *et al.* 1996; Mackie & Wheatley 1999).

VOC profiles of individual fungi can change over time, as can those produced during the time course of interactions (Griffith *et al.* 1994; Wu *et al.* 2005; Hynes *et al.* 2007). Following mycelial contact between the basidiomycetes *Hypholoma fasciculare* and *Resinicium bicolor*, 10 VOCs were identified that were not detected in single-species controls (Hynes *et al.* 2007). The majority of the VOCs identified were sesquiterpenoids, perhaps further indicating a defensive role of VOCs, since many sesquiterpenes are known to have antifungal activity or roles in plant defence (Rostelien *et al.* 2000; Viiri *et al.* 2001; Hynes *et al.* 2007).

This study aims to determine whether the VOC profile of a species changes when faced with different antagonists, during interactions with different outcomes. Effects of VOCs were examined by measuring extension rate of 'test' fungi in agar plates taped above two interacting mycelia – *Bjerkandera adusta*, *H. fasciculare*, *Stereum gausapatum* and *Trametes versicolor* in all combinations. These species would be likely to encounter each other in the natural environment, and during interaction with *T. versicolor* result in different outcomes. VOC production was quantified and qualified, by solid-phase microextraction followed by analysis using gas chromatography–mass spectrometry (GC/MS), over the course of interactions with *T. versicolor* replacing *S. gausapatum*, deadlocking with *B. adusta* and replaced by *H. fasciculare*. Determining which of the interacting pair produces which volatiles is, of course, not possible.

Materials and methods

Fungal cultures

Strains of the wood-decay basidiomycetes *T. versicolor* (CvD2), *S. gausapatum* (Sg1), *H. fasciculare* (HfGTWV2) and *B. adusta* (Bk1), from Cardiff University culture collection, were maintained on 0.5 % (w/v) malt agar (MA; 5 g Munton & Fison spray malt, 15 g Lab M agar no. 2 per litre distilled water) in 9 cm plastic non-vented Petri dishes, incubated upside down at 20 °C in the dark.

Effect of VOCs produced during inter-specific interactions on mycelial extension rate

Paired interactions of *T. versicolor* (Tv), *S. gausapatum* (Sg), *B. adusta* (Ba) and *H. fasciculare* (Hf) were set up on 0.5 % MA in all combinations. Agar plugs (6 mm diam.), cut from the actively growing mycelial margin, were inoculated face down on the agar 30 mm away from a competitor. Fungi were inoculated at the same time except for *H. fasciculare* that was added 2 d before its competitor, to ensure that mycelia were of similar size when they met. Five days after the mycelia had met, the Petri dish lid was discarded and the plate attached to a lidless dish containing 0.5 % MA, onto which a single plug of a target species had been freshly inoculated. This method was used successfully in the past to examine effects of VOCs produced by single species (Dick and Hutchinson 1966; Schoeman *et al.* 1996; Wheatley *et al.* 1997; Mumpuni *et al.* 1998; Mackie and Wheatley 1999; Humphris *et al.* 2002; Stinson *et al.* 2003).

Dishes were attached using masking tape and incubated in the dark at 20 °C. The dish bearing the interaction was always placed on the bottom (i.e. below the plate bearing the target species) to eliminate the possibility of diffusibles dripping down onto the target species. All species involved in the interactions were also used as target species. Extension of the target species was measured daily, until growth was 10 mm from the edge of the plate, by taking two measurements of colony diameter at 90° to each other. Controls consisted of (1) the target species inoculated onto a sterile agar plate; and (2) self-pairings of the target species. Five replicates were made.

Interspecific interaction systems for VOC analysis

The method followed Hynes *et al.* (2007). In brief, Reacsyn™ fermentation vessels (BioDiversity, Enfield, UK) – compartmentalised polypropylene bottles that allow sampling of both the headspace and the growth medium – were used. Mycelia were grown on a Fluorpore™ membrane (1 µm pore size; Millipore, Watford, Kent, UK) on top of a porous polypropylene/polyester support, which rested on the surface of 35 ml 0.5 % malt extract broth (Lab M MC23 malt extract). The growth chamber was stoppered with a gas permeable polyurethane foam bung which was removed to aerate the chamber, and through which sampling apparatus was inserted.

T. versicolor was paired in triplicate with either *S. gausapatum*, *B. adusta* or *H. fasciculare* by inoculating 6 mm diam.

agar plugs 20 mm apart on the membrane. Self-pairings were also made. *T. versicolor* and *S. gausapatum* were also inoculated as single plugs, in three replicates. A vessel containing only malt broth was included in the analysis to identify any non-fungal volatiles present. Vessels were incubated at 20 °C in the dark, held in racking trays.

Sampling VOCs

Sampling times were guided by Hynes *et al.* (2007) and a preliminary experiment. For the *T. versicolor* and *S. gausapatum* interaction (and all related control treatments), volatiles were sampled after 1, 3, 6, 11 and 19 d. Mycelial contact was made after 5 d. Based on the results of this experiment, sampling after 1 and 19 d was omitted for the treatments involving *B. adusta* and *H. fasciculare*.

Headspace VOCs were collected by solid-phase microextraction (SPME), a method previously used successfully with fungi (Nilsson *et al.* 1996; Fäldt *et al.* 1999; Jelen 2003; Demyttenaere *et al.* 2004; Ewen *et al.* 2004; Hynes *et al.* 2007). A 100 μm PDMS fibre (Supelco, Poole, Dorset, UK) was used at 20 ± 5 °C. At the start of each sampling day the SPME fibre was conditioned according to manufacturers' instructions (1 h at 250 °C), followed by a fibre blank run. Prior to sampling, the inoculated Reacsyn™ vessels were flushed to clear them of any accumulated volatiles or plasticiser by removal of the foam bung within a sterile laminar flow system for 10 min (following Hynes *et al.* 2007). The vessel was then removed from the laminar flow, the SPME fibre was inserted through the bung and exposed to the headspace of the inner chamber. After 1 h the fibre was withdrawn and immediately injected into the GC/MS.

GC/MS analysis of volatiles

Volatile profiles were resolved using a gas chromatograph containing a 30 m VF23ms polar column (0.25 mm ID, 0.25 μm film thickness; Varian, Palo Alto, CA, USA), with helium at 55 kPa acting as a carrier gas. The column was coupled to a quadrupole mass spectrometer in Electron Impact (EI) mode working from *m/z* 35 to 400 with a source temperature of 200 °C and interface temperature of 280 °C. Compounds were desorbed for 2 min at 220 °C in a split/splitless injection port, working in splitless mode. Analysis was performed using the temperature programme: start 45 °C, increasing by 3 °C min^{-1} until 200 °C, then 5 min at 200 °C.

As no internal standard could be used, a terpene mixture of known concentration was used as an external standard to assess machine performance. A terpene mixture was appropriate because the profile of volatile compounds emitted from interacting fungi was expected to contain sesquiterpenes. The mixture consisted of (1R)-fenchone, (1S)-verbenone and α-humulene, each at a concentration of 0.5 mg ml^{-3} in hexane solvent. The terpene standard (1 μl) was injected by an autosampler into the injection port at 220 °C with the machine working in split mode. The temperature programme used to resolve the mixture was: 40 °C for 5 min, ramping to 250 °C at 4.5 °C min^{-1}. The mass spectrometer scan was widened to *m/z* 28–400, with a 2 min solvent delay at the start of each run to avoid damage by copious amounts of hexane.

Peaks of interest were integrated using Masslab v1.4 (Thermofinnigan, Manchester, UK). The terpene run for each sampling day was used to calibrate machine performance by comparison of the total area of all peaks present in each sample. The ratio of the three peaks (corresponding to the three terpenes contained in the mixture) was consistent in every standard run, so the total integrated area was comparable. As the same quantity of terpene standard was injected on each day, any differences in the total area of integrated peaks were attributable to changes in machine performance, and experimental peak areas were adjusted accordingly, and designated 'terpene adjusted units' (TAU). However, for the treatments involving *B. adusta* and *H. fasciculare*, terpene standards were not used so an internal calibration was performed by standarising chromatograms to a malt control peak at RT 12.1 min, with peak areas designated 'malt adjusted units' (MAU). These have been included as rough values only to show qualitative rather than quantitative changes.

Identifications of the peaks of interest were made by comparison of mass spectra with library entries (National Institute of Standards and Technology v2.1 mass spectral database) and retention times. However, these are putative identifications only as absolute identification would require preparation of individual reference compounds which is beyond the scope of this work.

Statistical analysis

Radial extension rate was determined as the slope of a linear regression of extension *versus* time. Comparisons of daily radial extension rates were performed using one-way ANOVA with Tukey-Kramer *a posteriori* analysis (Minitab v14). The evolution of each volatile produced by *S. gausapatum* or the TvSg interaction (as peak area, in TAU, over time) was compared by one-way ANOVA (Minitab v14) of the area under the curve as determined by KaleidaGraph software (Synergy Software, Reading, PA, USA). Evolution of each volatile produced during interactions involving *B. adusta* and *H. fasciculare* (as peak area, in MAU) was compared using one-way ANOVA or two-way t-tests (Minitab v14).

Results

Effect of VOCs produced during interactions on extension rates of target fungi

There was no significant difference ($P > 0.05$) between the extension rates for *T. versicolor*, *S. gausapatum* and *H. fasciculare*, respectively, when grown above self-paired controls compared to growth over agar (Table 1). However, the extension rate of *B. adusta* was significantly ($P \leq 0.05$) greater when growing above its own self-pairing. *T. versicolor* extended significantly ($P \leq 0.05$) faster when grown above the self-pairings of *S. gausapatum*, *B. adusta* and *H. fasciculare*, and interacting mycelia of *T. versicolor* and *B. adusta*. Growth of *S. gausapatum* and *H. fasciculare* was generally not affected by the interaction plates, although in two instances there were significant ($P \leq 0.05$) differences in extension rate when compared with

Table 1 – Effects of VOCs produced by interactions on the growth of four 'target' species

Interaction plate	Percentage change in growth of 'target' species			
	T. versicolor	*S. gausapatum*	*B. adusta*	*H. fasciculare*
Agar	6.1 mm d^{-1}	5.2 mm d^{-1}	9.4 mm d^{-1}	2.5 mm d^{-1}
T. versicolor vs T. versicolor	100	99	104	100
S. gausapatum vs S. gausapatum	108[a]	102	109	102
B. adusta vs B. adusta	107[a]	100	111[a]	102
H. fasciculare vs H. fasciculare	107[a]	98	98[b]	100
T. versicolor vs S. gausapatum	99	99	105	105[b]
T. versicolor vs B. adusta	107[a]	102	103[b]	103
T. versicolor vs H. fasciculare	99	100	98[b]	103
S. gausapatum vs B. adusta	99	103	98[b]	103
S. gausapatum vs H. fasciculare	101	100	97[b]	103
B. adusta vs H. fasciculare	102	96[b]	98[b]	97

Changes in extension rate are expressed as percentages relative to the agar control.
a Significantly different ($P \leq 0.05$) to the agar control.
b Significantly different ($P \leq 0.05$) to the respective self-pairing.

growth above their self-pairings, but not when compared with agar alone. *S. gausapatum* extension rate decreased over *B. adusta* paired with *H. fasciculare*, while that of *H. fasciculare* was greater when grown above *T. versicolor* paired with *S. gausapatum*. Over half of the interactions tested were associated with a significant ($P \leq 0.05$) decrease in extension rate of *B. adusta* when compared to its self-pairing but not compared to agar alone.

General observations on morphology and VOC production during interspecific interactions

In all three interspecific interactions, mycelial morphology changed following contact. Mycelia met after 3 d in agar culture, or 5 d after inoculation into Reacsyn™ vessels. Mycelial barrages formed at the interaction zone in *T. versicolor vs S. gausapatum* (TvSg) and *T. versicolor vs B. adusta* (TvBa) 1 d after mycelia had met, but later in *T. versicolor vs H. fasciculare* (TvHf). A bright yellow-orange pigment was present in the agar in the interaction zone in TvSg just after contact, but no pigmentation developed in TvBa, and did not occur until later stages in TvHf, after VOC sampling had finished (Fig 1). Morphology and VOC profiles were generally qualitatively consistent between replicates. VOC peaks have been designated numbers based on their order of elution, including compounds present in the malt broth (Tables 2 and 3), and these have putative identifications (Table 4).

VOCs in malt control

Four VOCs were detected in the malt broth control, with average retention times of 12.1, 24.2, 26.9 and 34.7 min (Peaks 1, 20, 24 and 29; Tables 2 and 3). Preliminary identifications suggest they are plasticisers emitted by the Reacsyn™ vessels: dibutylbenzene (molecular weight [M_w] 190), dimethylbenzaldehyde (M_w 135), a long-chain hydrocarbon of M_w 168 (possibly dodecene) and bis(1,1-dimethylethyl)-phenol (M_w 206; Table 4).

VOCs from T. versicolor growing alone (Tv) and self-pairing (TvTv)

No VOCs specific to *T. versicolor* were detected during sampling, with the exception of one VOC (Peak 26) produced by Tv after 19 d. This peak was not present in TvTv (Table 2). Otherwise, both Tv and TvTv profiles were identical to those from the malt control.

S. gausapatum growing alone (Sg), in self-pairing (SgSg) and interacting with T. versicolor (TvSg)

There were no qualitative or quantitative differences in the VOC profile of Sg compared with SgSg, hence single inocula were omitted in subsequent experiments (Table 2). Five VOCs specific to *S. gausapatum* were detected (Table 2). Peak 8 (RT 16.58 min), putatively identified as methylbenzoate or anisaldehyde (M_w 136), and Peak 9 (RT 17.13 min), a benzaldehyde-containing compound, were present sporadically in all treatments containing *S. gausapatum*. Peak 22 (RT 26.47 min), dimethylbenzoic acid, methyl ester-, was the largest *S. gausapatum*-specific peak present after 3 d. The related compound methoxybenzoic acid, methyl ester- (M_w 166; Peak 28; RT 31.1 min) was present from 6 d onwards in Sg and SgSg, but only at the 6 d sampling in TvSg. Peaks 25 and 26, both putative long-chain alkenols, were detected in Sg after 1 d, but were present in larger quantities in other interactions (see below).

Eight VOCs specific to TvSg were detected (Table 2) following mycelial contact and pigment production. Six of these were detected after 6 d: Peak 2, another isomer of dibutylbenzene (different to Peak 1; M_w 190); Peak 3, 5-methyl,1,3-cyclohexadiene (M_w 94); Peak 6, identified generally as having an aromatic structure, of which Peak 12 is an isomer; Peak 10, bearing a strong similarity to α-myrcene (M_w 136); and Peak 16, which was too small for identification. A further two VOCs were detected after 11 d: Peaks 15 and 19, which were both too small to allow confident identification. Peaks 8, 9 and 22, detected in Sg and SgSg, were also detected in

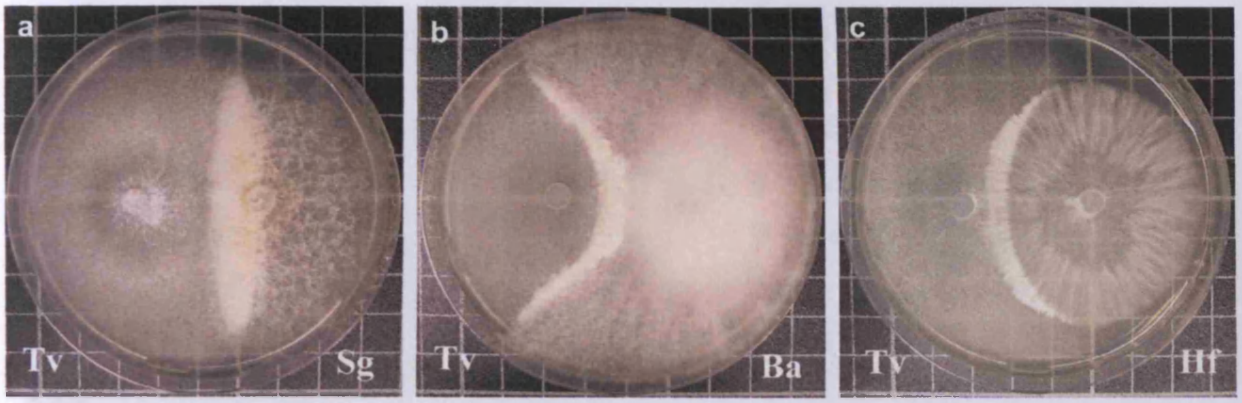

Fig 1 – Interactions involving *Trametes versicolor* (left) against: a, *S. gausapatum*; b, *Bjerkandera adusta*; and c, *Hypholoma fasciculare* 4 d after mycelia had met.

TvSg but at significantly lower concentrations. Peak 9 was unusual as its total production in Sg was significantly ($P \leq 0.05$) lower than in TvSg. Peak 25, a putative alkenol, was also present in the VOC profile of Sg, HfHf, TvHf, BaBa and TvBa, and appears in TvSg after 6 d only.

B. adusta self-pairing (BaBa) and interacting with T. versicolor (TvBa)

Five VOCs were specific to *B. adusta* (Table 3): Peak 4, an alkene; Peak 17, an alkenol; Peaks 18, 21 and 31 were present at too low a concentration to be confidently identified. Peak 32 was possibly a plasticiser contaminant (bis(2-ethylhexyl) phthalate). Peaks 25 and 26, both alkenols, and Peak 27 – a monoterpene, putatively carene, were also present but not specific to *B. adusta*, also being found particularly in interactions involving *H. fasciculare*. One VOC specific to the TvBa interaction was detected following mycelial contact: Peak 23 (RT 26.52 min, but too small for identification) in two replicates at 6 d declining considerably by 11 d (Table 3).

H. fasciculare self-pairings (HfHf) and interacting with T. versicolor (TvHf)

Five *H. fasciculare*-specific VOCs were detected (Table 3): Peak 5, either methylbenzoate or an isomer of the anisaldehyde occuring in TvSg Peak 8; Peak 7, an alkane with at least a C_7 chain length; Peak 11, a monoterpene or derivative; Peak 30, a quinolinium-type compound with a distinctive spectral pattern at *m/z* 302; Peak 13 – too small for identification. Peaks 25, 26 and 27 were present in *H. fasciculare* profiles though not specifically, also being present in the profiles of *B. adusta* and TvSg. No VOCs were specific to TvHf.

Changes in VOC profiles over time

VOC concentrations usually increased and then declined as interactions proceeded (Table 2 and 3). Of the interaction-specific VOCs produced in TvSg, maximal production was at 11 d (Peaks 2, 3, 6, 10, 12, 15, 19 and 25) with none detectable by 19 d (with the exception of Peak 2 and Peak 25 which peak at 6 d). The *S. gausapatum*-specific VOCs (Peaks 8, 22, and 28) declined but did not disappear by 19 d in Sg and SgSg (Table 2). There was no significant difference ($P > 0.05$) in total VOC evolution of *S. gausapatum*-specific compounds Peaks 8, 22 and 28 between Sg and SgSg. However, for Peaks 22 and 28 there was a significant decrease in the total evolution by TvSg. In BaBa and TvBa, Peaks 16 and 18 declined dramatically between 3 d and 6 d (Table 3). For HfHf Peaks 5 and 11 occurred at a significantly ($P \leq 0.05$) different (higher) concentration at the later sampling day, while for TvHf Peaks 5, 11 and 27 increased significantly ($P \leq 0.05$) between 3 d and 6 d. Only three *B. adusta*-specific VOCs were still detectable at 11 d in TvBa (Peaks 4, 14 and 23), and only two (Peaks 5 and 11) out of the six (Peaks 5, 7, 11, 13, 27 and 30) *H. fasciculare*-specific VOCs were detectable at 11 d (Fig 2).

The malt extract Peaks 20 and 24 declined in the controls and all treatments over the course of the experiment (Table 3). The total evolution of malt Peak 29 also significantly ($P \leq 0.05$) decreased relative to the controls in TvSg (Table 2), and also decreased over sampling days in BaBa, TvBa, and TvHf. In contrast, there was no change in malt Peak 1 in the presence of fungi, except in TvSg where there was a significant ($P \leq 0.05$) increase between 6 d and 11 d.

Discussion

Species specificity

The study highlights the species-specific nature of both volatile production and susceptibility to non-self VOCs. For example, there was no detectable change in VOC profile during interactions between *T. versicolor* and *H. fasciculare*, and only one interaction-specific VOC (too small to be identified) was produced during the interaction between *T. versicolor* and *B. adusta*. Nor did the quantity of species-specific VOCs produced differ between intra- and inter-specific pairings of these species. In contrast, following mycelial contact between *T. versicolor* and *S. gausapatum*, eight interaction-specific VOCs were identified. No VOCs specific to *T. versicolor* were detected in any of the interactions, whereas the other species

Table 2 – Comparison of the evolution of fungal volatiles over time for the interaction between *Trametes versicolor* (Tv) and *Stereum gausapatum* (Sg) and their single-species controls

Peak no.	RT (min)	Tv					TvTv					Sg					SgSg					TvSg				
		Day 1	Day 3	Day 6	Day 11	Day 19	Day 1	Day 3	Day 6	Day 11	Day 19	Day 1	Day 3	Day 6	Day 11	Day 19	Day 1	Day 3	Day 6	Day 11	Day 19	Day 1	Day 3	Day 6	Day 11	Day 19
1[a]	12.1	54 (19)	25 (4)	31 (5)	35 (2)	23 (2)	35 (7)	21 (2)	26 (5)	25 (7)	14 (1)	43 (14)	27 (5)	31 (3)	27 (6)	41 (24)	39 (3)	36 (10)	38 (6)	31 (8)	61 (32)	34 (13)	27 (6)	79 (20)	136 (46)	23 (5)
2	13.1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	19 (1)	7 (2)	*
3	13.6	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	146 (45)	175 (94)	*
6	15.0	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	98 (27)	478 (60)	*
8	16.6	*	*	*	*	*	*	*	*	*	*	*	*	14 (2)	8 (2)	*	*	*	14 (5)	*	*	*	*	*	11 (1)	*
9	17.1	*	*	*	*	*	*	*	*	*	*	9 (2)	*	*	*	*	*	*	*	*	*	*	*	*	18 (8)	*
10	19.3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	41 (12)	59 (32)	*
12	20.4	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	30 (7)	232 (44)	*
15	21.3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	9 (3)	*
16	21.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	14 (5)	*	*
19	23.8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	8 (1)	*
20[a]	24.2	133 (42)	145 (20)	52 (12)	49 (2)	28 (4)	98 (20)	128 (12)	24 (6)	35 (5)	20 (3)	106 (0)	88 (17)	92 (24)	18 (6)	9 (0)	70 (31)	92 (16)	61 (15)	15 (2)	14 (1)	109 (30)	121 (24)	45 (6)	17 (7)	7 (1)
22	26.5	*	*	*	*	*	*	*	*	*	*	*	11 (1)	140 (18)	99 (11)	41 (3)	*	22 (1)	156 (50)	76 (11)	42 (13)	*	7 (3)	27 (7)	16 (7)	*
24[a]	26.9	189 (90)	152 (31)	96 (17)	90 (19)	75 (14)	137 (55)	116 (21)	70 (8)	59 (31)	52 (8)	165 (0)	135 (22)	146 (23)	95 (29)	59 (4)	243 (205)	173 (53)	141 (51)	88 (5)	72 (15)	82 (62)	133 (26)	101 (23)	99 (13)	58 (3)
25	28.8	*	*	*	*	*	*	*	*	*	*	4 (1)	*	*	*	*	*	*	*	*	*	*	*	5 (3)	*	*
26	29.3	*	*	*	*	9 (2)	*	*	*	*	*	5 (3)	*	*	*	*	*	*	*	*	*	*	*	*	*	*
28	31.1	*	*	*	*	*	*	*	*	*	*	*	*	9 (2)	24 (5)	26 (3)	*	*	13 (4)	30 (6)	27 (7)	*	*	8 (1)	*	*
29[a]	34.7	15 (7)	6 (1)	*	*	*	5 (2)	6 (2)	*	*	*	*	7 (1)	4 (1)	13 (0)	*	*	8 (3)	11 (10)	*	*	9 (3)	5 (1)	5 (3)	*	*

* = Not detected.

Peak areas for each volatile given in Terpene Adjusted Units (TAU), standard error of the means are given in parentheses. Identifications of peaks are given in Table 4.

a Present in malt control treatments.

Table 3 – Comparison of the evolution of fungal volatiles over time for the interactions between *T. versicolor* (Tv) and *Bjerkandera adusta* (Ba) or *H. fasciculare* (Hf), and single-species controls

Peak no.	RT (min)	BaBa		TvBa			HfHf		TvHf			Malt blank				
		Day 3	Day 6	Day 3	Day 6	Day 11	Day 3	Day 6	Day 3	Day 6	Day 11	Day 1	Day 3	Day 6	Day 11	Day 19
1[a]	12.1	48 (4)	48 (4)	51 (9)	51 (9)	51	67 (26)	67 (26)	67 (15)	67 (15)	67	39	17	16	38	31
4	13.7	12 (0.4)	6 (1)	13 (5)	11 (5)	2	•	•	•	•	•	•	•	•	•	•
5	14.0	•	•	•	•	•	4 (2)	17 (6)	4 (1)	16 (5)	9	•	•	•	•	•
7	15.1	•	•	•	•	•	•	6 (2)	•	6 (2)	•	•	•	•	•	•
11	19.6	•	•	•	•	•	0.5 (0.3)	9 (5)	0.4 (0)	8 (1)	34	•	•	•	•	•
13	20.6	•	•	•	•	•	•	0.8 (0)	•	0.6 (0.6)	•	•	•	•	•	•
14	20.6	2 (0.4)	1 (0.3)	1 (0.7)	•	5	•	•	•	•	•	•	•	•	•	•
17	22.0	3 (0.2)	0.2 (0)	2 (0.2)	0.7 (0.6)	•	•	•	•	•	•	•	•	•	•	•
18	23.1	3 (0.1)	1 (0.3)	3 (0.5)	1 (0.7)	•	•	•	•	•	•	•	•	•	•	•
20[a]	24.2	157 (6)	309 (30)	258 (65)	252 (56)	99	193 (68)	193 (53)	276 (68)	159 (29)	94	51	53	31	87	63
21	26.2	2 (0.2)	•	2 (0.4)	•	•	•	•	•	•	•	•	•	•	•	•
23	26.5	•	•	•	4 (2)	1	•	•	•	•	•	•	•	•	•	•
24[a]	26.9	236 (21)	175 (30)	261 (38)	174 (37)	169	256 (99)	206 (61)	336 (84)	207 (53)	172	69	134	132	272	135
25	28.8	19 (10)	•	18 (4)	•	•	7 (3)	12 (3)	22 (16)	9 (1)	•	•	•	•	•	•
26	29.3	6 (6)	•	24 (4)	•	•	11 (3)	15 (5)	5 (3)	10 (4)	•	•	•	•	•	•
27	30.7	•	2 (1)	•	2 (1)	•	2 (0.3)	4 (2)	1 (0.5)	7 (0.6)	•	•	•	•	•	•
29[a]	34.7	28 (3)	5 (0.8)	22 (2)	5 (1)	5	17 (6)	17 (9)	24 (7)	8 (1)	8	•	11	10	42	6
30	35.1	•	•	•	•	•	4 (2)	•	1 (0)	8 (0)		•	•	•	•	•
31	39.7	•	16 (7)	•	2 (1)	•	•	•	•	•	•	•	•	•	•	•
32	47.3	39 (38)	•	0.2 (0.1)	0.7 (0.6)	•	•	•	•	•	•	•	•	•	•	•

• = Not detected.

Peak areas for each volatile are given in Malt Adjusted Units (MAU), with the exception of the malt control where Terpene Adjusted Units (TAU) are used. Standard error of the means are given in parentheses, except for day 11 of BaBa, TvBa, HfHf and TvHf where only one replicate was performed. Identifications of peaks are given in Table 4.

a Present in malt control treatments.

Table 4 – Putative identifications of fungal volatiles detected in experimental treatments

Peak no.	Putative identification			Treatments present in
	Name	Formula	M_w	
1[a]	Dibutylbenzene	$C_{14}H_{22}$	190	Malt control
2	Isomer of (1)	$C_{14}H_{22}$	190	TvSg
3	5-Methyl,1,3-cyclohexadiene	C_7H_{10}	94	TvSg
4	PDMS contamination	–	–	BaBa, TvBa
5	Methylbenzoate OR anisaldehyde	$C_8H_8O_2$	136	HfHf, TvHf
6	Aromatic	?	204?	TvSg
7	Alkane, at least C7	C_7+	99+	HfHf, TvHf
8	Methylbenzoate OR anisaldehyde isomer of (5)	$C_8H_8O_2$	136	Sg, SgSg, TvSg
9	Benzaldehyde structure	?	?	Sg, TvSg
10	α-Myrcene	$C_{10}H_{16}$	136	TvSg
11	Monoterpene or derivative	$C_{10}H_{16}$	136	HfHf, TvHf
12	Isomer of (6)	?	204?	TvSg
13	Peaks too small	–	–	HfHf, TvHf
14	Alkene	C_nH_{2n}	128+	BaBa, TvBa
15	Peaks too small	–	–	TvSg
16	Peaks too small	–	–	TvSg
17	Alkenol	$C_nH_{2n}O$	140+	BaBa, TvBa
18	Peaks too small	–	–	BaBa, TvBa
19	Peaks too small	–	–	TvSg
20[a]	Dimethylbenzaldehyde	$C_9H_{10}O$	135	Malt control
21	Peaks too small	–	–	BaBa, TvBa
22	Dimethylbenzoic acid, methyl ester-	$C_{10}H_{12}O_2$	164	Sg, SgSg, TvSg
23	Peaks too small	–	–	TvBa
24[a]	Alkene, at least C12	C_{12}+	168+	Malt control
25	Alkenol	$C_nH_{2n}O$	168+	Sg, TvSg, BaBa, TvBa, HfHf, TvHf
26	Alkenol	$C_nH_{2n}O$	168+	Tv, Sg, BaBa, TvBa, HfHf, TvHf
27	Monoterpene (possibly carene)	$C_{10}H_{16}$	136	HfHf, TvHf, BaBa, TvBa
28	Methoxybenzoic acid, methyl ester-	?	166	Sg, SgSg, TvSg
29[a]	Phenol, 2,4-bis(1,1-dimethyl)	$C_{14}H_{22}O$	206	Malt control
30	Quinolinium-type compound	?	?	HfHf, TvHf
31	Peaks too small	–	–	BaBa, TvBa
32	Bis(2-ethylhexyl)phthalate (plasticiser)	$C_{24}H_{38}O_4$	390	BaBa, TvBa

a Present in malt control treatments.

examined produced at least five species-specific VOCs both during self-pairings and during interactions. It is unlikely that *T. versicolor* is responsible for the production of any of the 11 VOCs produced during interaction with *S. gausapatum* as none of these VOCs recur in pairings between *T. versicolor* and other species. We conclude that any volatile-mediated antagonism produced by *T. versicolor* is occurring through compounds, or at concentrations, undetectable by the present system. Lack of production of VOCs does not appear to be related to combative ability, as *T. versicolor* is more combative than the VOC-producing *S. gausapatum*, but less combative than the VOC-producing *H. fasciculare*.

Relationship between pigmentation and VOC production

The concurrent production of interaction-specific VOCs and pigments in the medium, as seen here in interactions involving *S. gausapatum*, has been reported previously for interactions between *H. fasciculare* and *R. bicolor* (Hynes *et al.* 2007). Pigment production occurs concurrent with production of aerial mycelium in *P. radiata*, and also in response to chemical stress; possibly as a result of a shift in metabolism (Griffith *et al.* 1994). Production of VOCs may be stimulated by pigment production, or be a by-product of the process, or simply correlated with aspects of secondary metabolism, including production of diffusible organic compounds. Pigmentation is not a prerequisite for VOC production, and did not occur during interactions involving *B. adusta*, even though VOCs were abundant. With *H. fasciculare*, pigmentation only appeared during later stages of interactions, after VOC sampling had finished. This contrasts with a previous study (Hynes *et al.* 2007) where a different isolate of *H. fasciculare* produced a yellow pigment immediately after mycelial contact had been established with *R. bicolor*. Pigmentation of *H. fasciculare* mycelium, and of agar on which it is growing, varies considerably between strains (T.D. Rotheray, unpublished data).

Antagonism at a distance and temporal changes

While there is clearly long-distance signalling, as evidenced by changes in extension rates in the double plate experiment, no gross morphological changes were evident nor interaction-specific volatiles produced prior to mycelial contact. The latter concurs with the previous study on VOCs produced during

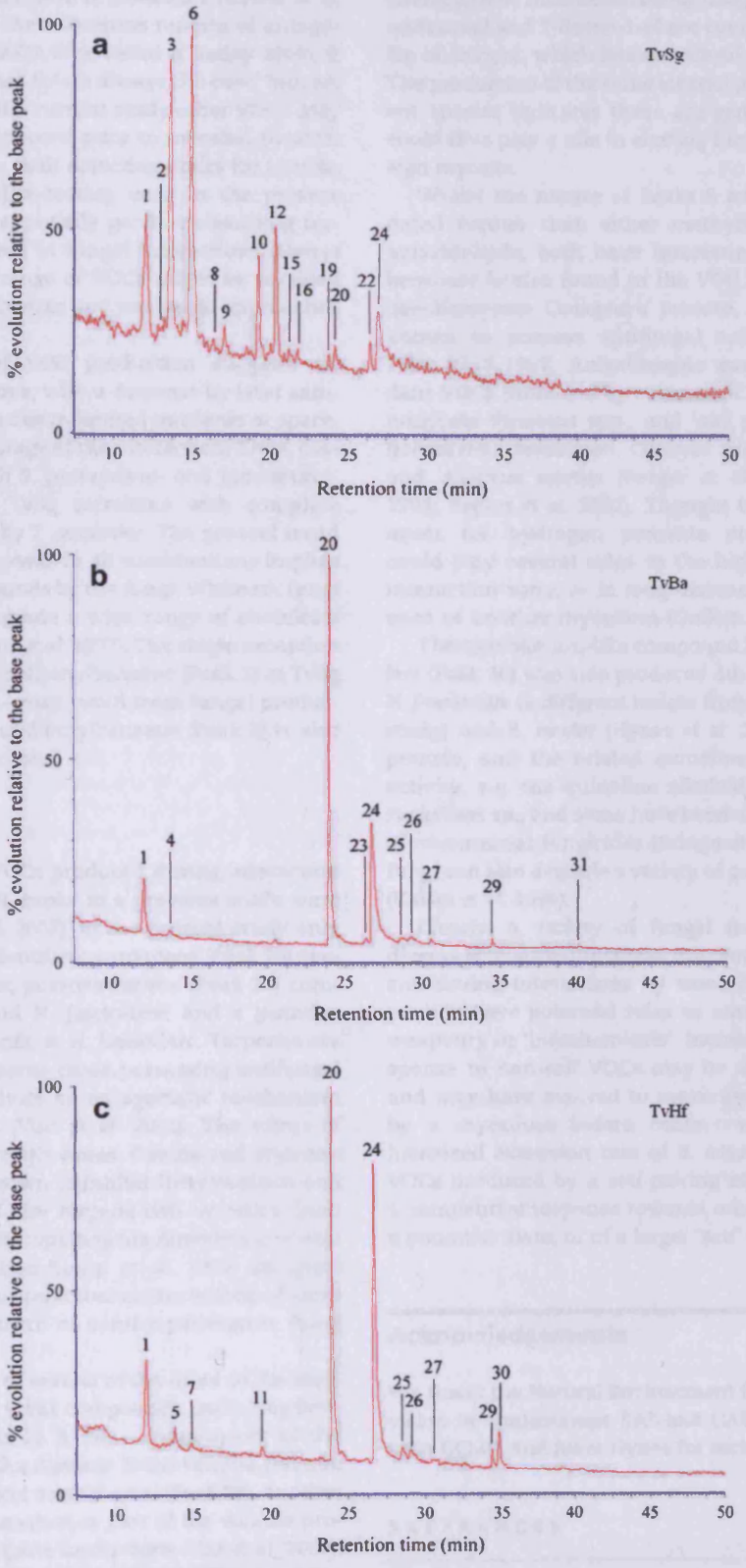

Fig 2 – VOC profiles after mycelia of *Trametes versicolor* met with: a, *Stereum gausapatum*; b, *Bjerkandera adusta*; and c, *Hypholoma fasciculare*. Mycelia had met for 6 d in a, 1 d in b and c. Peaks are numbered according to order of elution, as detailed in Table 2.

interactions between R. *bicolor* and H. *fasciculare* (Hynes *et al.* 2007). However, in view of the numerous reports of antagonism at a distance (Boddy 2000; Woodward & Boddy 2008), it seems extremely unlikely that this is always the case. Indeed, even in the interactions in the current study other VOCs may be present, and possibly produced prior to mycelial contact. SPME fibre-coatings differ in their detection limits for specific compounds – the PDMS fibre-coating used in the present study was chosen as it is especially good at absorbing terpenes, known to be involved in fungal interactions (Hynes *et al.* 2007). A much wider range of VOCs might be revealed using additional sampling devices and analytical approaches (Ewen *et al.* 2004).

The general pattern of VOC production suggests an increase with mycelial volume, with a decrease by later samplings. The decrease may be due to limited nutrients or space, but equally may reflect the stage of the interaction. Thus, cessation in production of both S. *gausapatum*- and interaction-specific VOCs by 19 d in TvSg correlates with complete replacement of the former by T. *versicolor*. The general trend of decrease in malt extract peaks in all combinations implies degradation of these compounds by the fungi. White rot fungi are known to be able to degrade a wide range of chemicals including plastics (Sutherland *et al.* 1997). The single exception to this trend – the increase in dibutylbenzene (Peak 1) in TvSg relative to the malt control – may result from fungal production, as a different isomer of dibutylbenzene (Peak 2) is also produced during the interaction.

Function of VOCs

Although the majority of VOCs produced during interaction between H. *fasciculare* and R. *bicolor* in a previous study were sesquiterpenes (Hynes *et al.* 2007), in the present study only three monoterpenes were identified: α-myrcene (Peak 10) specific to the TvSg interaction; putative carene (Peak 27) common to both B. adusta and H. *fasciculare*; and a putative monoterpene (Peak 11) specific to H. *fasciculare*. Terpenes are ecologically significant, in some cases possessing antifungal activity which could constitute an antagonistic mechanism during fungal interactions (Viiri *et al.* 2001). The effect of monoterpenes on fungal growth varies. Carene and myrcene in the volatile phase are known to inhibit *Heterobasidion* and *Leptographium* species, and the terpene-rich volatiles from tomato leaves inhibit the phytopathogens *Alternaria alternata* and *Botrytis cinerea* (Hamilton-Kemp *et al.* 1992; Zamponi *et al.* 2006). However, terpene mixtures characteristic of conifer wood stimulate the growth of conifer-pathogenic fungi (Gao *et al.* 2005).

The biological functions of several of the other VOCs identified are known. Many are scent compounds, including benzoic acid derivatives (Peaks 22 & 28 – components of the volatile oil of sage, and of the *Agaricus blazei* volatile fraction (Stijve *et al.* 2002). Benzoic acid methyl ester (Peak 28), another VOC identified from S. *gausapatum*, is part of the volatile profile of *Tricholoma matsutake* (pine mushroom; Cho *et al.* 2007). Several VOCs were identified as alkenols of different chain lengths (Peaks 17, 25 and 26), and two long-chain hydrocarbons were putatively identified (Peaks 7 and 14). Alkenols are common fungal VOCs. 1-Octen-3-ol is responsible for the characteristic mushroom odour (Ziegenbein *et al.* 2006), while undecanal and 2-decen-1-ol are components of the VOC profile of chicory, which has antifungal activity (Bais *et al.* 2003). The production of the same alkenol peaks in cultures of different species indicates these are general fungal VOCs. They could thus play a role in alerting fungi to the presence of foreign mycelia.

Whilst the nature of Peaks 5 and 8 could not be elucidated further than either methylbenzoate or isomers of anisaldehyde, both have interesting implications. Methylbenzoate is also found in the VOC profile of the brown-rot basidiomycete *Coniophora puteana*, and its derivatives are known to possess antifungal activity (Woodward *et al.* 1993; Wolf 1951). Anisaldehyde was one of the most abundant VOCs produced by cultures of six species of the basidiomycete *Pleurotus* spp., and was produced by cultures of *Ischnoderma benzoinum*, *Clitocybe odora*, *Lentinellus cochleatus* and *Agaricus essettei* (Berger *et al.* 1987; Gutierrez *et al.* 1994; Rapior *et al.* 2002). Thought to act as a redox-cycling agent for hydrogen peroxide production, anisaldehyde could play several roles in the highly metabolically active interaction zone, or in long-distance signalling of the presence of another mycelium (Guillen & Evans 1994).

The quinolinium-like compound identified from H. *fasciculare* (Peak 30) was also produced during interactions between H. *fasciculare* (a different isolate from that used in the present study) and R. *bicolor* (Hynes *et al.* 2007). Quinolinium compounds, and the related quinolines, have high antifungal activity, e.g. the quinoline alkaloid viridicatin produced by *Penicillium* sp., and some have been used to drive development of commercial fungicides (Bringmann *et al.* 1997). Moreover, fungi can also degrade a variety of quinoline-type compounds (Kaiser *et al.* 1996).

Clearly, a variety of fungal metabolites, with equally diverse ecological functions, are produced both constitutively and during interactions by wood-decay fungi. These compounds have potential roles as antifungal volatile chemical weaponry or 'infochemicals'. Increased extension rate in response to non-self VOCs may be an example of the latter, and may have evolved to maximise the territory possessed by a mycelium before contact with an opponent. The increased extension rate of B. *adusta* when exposed to the VOCs produced by a self-pairing of itself could function as a competitive response towards other mycelia, or a signal of a potential mate, or of a larger 'self' colony.

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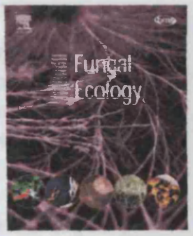

Monokaryons and dikaryons of *Trametes versicolor* have similar combative, enzyme and decay ability

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ABSTRACT

Heterokaryons (mated mycelia) are commonly used to study basidiomycete ecology, with little known about the relative abilities of homokaryotic (unmated) mycelia. *Trametes versicolor* is a common wood decay basidiomycete, which fruits prolifically, producing a high proportion of viable basidiospores that germinate readily. The 'fitness' of monokaryotic *T. versicolor* mycelia was compared to that of dikaryons in terms of extension rate, decay rate, combative ability, and production of ligninolytic enzymes in agar culture and during growth on beech wood blocks. Eight monokaryons, four natural dikaryons and four artificially synthesised dikaryons (created by pairing monokaryotic cultures) were compared, and paired against 11 wood decay species to assess combative ability. There were no significant differences between monokaryons and dikaryons of *T. versicolor* in any of the characters examined, with as much variation within the karyotic groups as there was between them. When artificial dikaryons and their component monokaryons were considered individually, the dikaryon resembled one of the component monokaryons rather than being intermediate. This implies that dikaryons behave as functional diploids.

Introduction

The life cycle of a typical wood decay basidiomycete involves two mycelial stages. Spore germination produces a primary, unmated mycelium (monokaryon/homokaryon), which, following hyphal fusion and nuclear exchange with a mating-type compatible conspecific may form a secondary mycelium (dikaryon/heterokaryon). The two haploid gametic types of nuclei are maintained indefinitely during vegetative growth, fusing only for karyogamy and meiosis which yields homokaryotic spores. The heterokaryotic state can also break down through the formation of asexual homokaryotic conidia (Hui *et al.* 1999 and references within). The heterokaryotic state is similar to diploidy in that two haploid genomes reside in each cell with full opportunity for genetic complementation, but is crucially different because the two haploid genomes remain separated in different nuclei (Clark & Anderson 2004).

The duration of the homokaryotic phase for a mycelium inhabiting woody organic resources is determined by a variety of factors, most important of which are probably the local abundance of the species and its mating-type structure. The homokaryotic phase persists until a compatible conspecific is encountered and there is evidence of an inverse relationship between the number of colonies of a species in the field and the number that are homokaryons (Stenlid 1994). Homokaryons are often considered short-lived with the heterokaryotic phase dominating the life cycle, but this is less likely to be the case in areas where mating potential is restricted as the homokaryons would necessarily persist for much longer. For common species, e.g. *Trametes versicolor*, homokaryons (in this species known to be monokaryons) might be expected to

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be short-lived. This has been observed in areas of high spore fall with a large area of resource open to colonisation (Williams *et al.* 1981). However, there is also evidence of homokaryons, including *T. versicolor* monokaryons, persisting in woody resources in the field for several years (Coates & Rayner 1985; Garbelotto *et al.* 1997; Redfern *et al.* 2001; Stenlid 2008).

Homokaryons and heterokaryons are subject to largely similar stresses during colonisation of, and proliferation within, woody organic resources, such as obtaining carbon and nutrients from recalcitrant lignified tissues, and antagonistic interspecific interactions. Antagonistic interactions between wood decay basidiomycetes occur between individuals competing for territory and the resources within (Boddy 2000). Whilst competition is regarded as a major factor influencing the distribution and abundance of wood decay fungi, only a few studies have considered the competitive ability of homokaryons (Crockatt *et al.* 2008). It has been hypothesised that the temporary nature of homokaryons requires less combative ability than heterokaryons (e.g. Gardes *et al.* 1990), but the reverse was found for several species including the rare *Hericium coralloides* (Crockatt *et al.* 2008). However, these results are species-specific as no differences in combative ability were found between homo- and heterokaryotic mycelia of *Acanthophysellum lividocoeruleum* (Fryar *et al.* 2002). It has also been suggested that the greater adaptive genetic potential of heterokaryons relative to homokaryons may increase the capacity to tolerate different stresses and thus improve combative ability (Clark & Anderson 2004).

Differences in growth and decay rate between homokaryons and heterokaryons have also been reported, although again no clear pattern emerges. Heterokaryons of *Gloeophyllum trabeum*, *Phellinus weirii* and *Pleurotus ostreatus* generally grew more rapidly than homokaryons (Bezemer 1973; Hansen 1979; Eichlerová & Homolka 1999), but there were no differences between different karyotic states of *A. lividocoeruleum* or *H. coralloides* (Fryar *et al.* 2002; Crockatt *et al.* 2008). Overall, no differences in decay rate were found between different karyotic states of *G. trabeum* (da Costa & Kerruish 1965; Amburgey 1970). Heterokaryons of *P. weirii* decayed wood more rapidly than homokaryons (Hansen 1979), but the reverse was true for *Antrodia vaillantii* and *Ganoderma applanatum* (Aoshima 1954; da Costa & Kerruish 1965). Decay rate by *Serpula lacrymans* was less in 57 % (of 138) of heterokaryons compared to their component homokaryons, intermediate in 33 % and greater in 10 % (Elliott *et al.* 1979). Homokaryons and heterokaryons sometimes have different morphologies, e.g. *Schizophyllum commune* and *S. lacrymans* (Clark & Anderson 2004; Kauserud *et al.* 2006).

T. versicolor produces high levels of ligninolytic enzymes including laccase and peroxidases which are crucial to the decomposition of woody tissues. These non-specific, highly oxidative enzymes attack the lignin polymer, allowing access to bound cellulose and hemicellulose nutrient sources. They also play a variety of roles in fruiting, morphological changes and stress mediation, and are known to increase in activity during interspecific interactions (Baldrian 2004; Ferreira-Gregorio *et al.* 2006; Chi *et al.* 2007). In a limited study the ability of monokaryons and dikaryons of *T. versicolor* to break down lignin was compared (Addleman & Archibald 1993). There were no differences in enzyme production or delignification between monokaryons and dikaryons though monokaryons were better able to bleach kraft pulp, but this was based on a very small sample size of isolates especially selected for high enzyme production. However, in *S. commune*, laccase activity was detected in heterokaryotic cultures only, implying different regulation in the different phases (de Vries *et al.* 1986). The greater genetic potential of heterokaryons may enable production of a more diverse range of isozymes of a particular enzyme, increasing substrate range, stress mediation, and adaptability to different resources. For example, two *Trametes hirsuta* homokaryons produced different laccase isozymes, both of which were expressed by the heterokaryotic product of their mating (Kojima *et al.* 1990).

In the limited number of previous studies performed so far there is no evidence of a trend for either karyotic state being more combative, growing faster or decaying wood more rapidly than the other. However, these studies have usually compared only one or two characters, rather than a wide suite. The aim of the study reported here was to compare a range of characters of *T. versicolor* monokaryons and dikaryons, including extension rate, wood decay rate, ligninolytic enzyme (laccase and peroxidases) production, and combative ability. As a secondary coloniser (Rayner & Boddy 1988), when *T. versicolor* basidiospores arrive at a woody resource it is already colonised, so monokaryons would be expected to have good combative ability. Likewise dikaryons must also be combative to avoid replacement. Thus no differences in combative ability are hypothesised. Neither is there any reason for expecting extension rates of mono- and dikaryons to differ. However, since the main role of monokaryons is initial establishment, presumably usually of a small focus, whereas that of dikaryons is persistance and utilisation of the woody resource, it is hypothesised that dikaryons will produce larger amounts of ligninolytic enzymes.

Methods

Isolates

Monokaryons and dikaryons of *T. versicolor*, and heterokaryons of other wood decay Ascomycota and Basidiomycota (Table 1) were maintained on 2 % (w/v) malt agar (MA: 15 $g\,l^{-1}$ Lab M Agar no. 2 (Lab M, Bury, Lancs., UK), 20 $g\,l^{-1}$ Munton and Fison Spray Malt Light (Munton Plc, Stowmarket, Suffolk, UK)). Monokaryotic isolates were obtained by collecting spores from the fruit body of natural dikaryon TvA on a glass slide. Spores were suspended in sterile distilled water and diluted so that when 25 µl was spread onto a 2 % MA plate there was approximately 1 spore per field of view at ×100 magnification. The plates were incubated at 20 °C in darkness for 1–2 d, and single, well-spaced germinating spores were transferred to fresh 2 % MA plates, by removing plugs with a sharpened tungsten wire. Cultures were checked for lack of clamp connections. 'Natural' dikaryotic isolates were obtained by fruit body tissue isolations. 'Artificial' dikaryons were

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Table 1 – Details of *Trametes versicolor* isolates, and isolates against which they were paired in agar culture

Ecological role[a]	Species	Strain	Source	Isolated by	Date isolated/ created
Early secondary colonisers on standing and fallen wood	*Trametes versicolor*	**Natural heterokaryons**			
		TvD4	*Quercus robur* Ccc	L. Boddy	
		TvD2	*Q. robur* Ccc	L. Boddy	
		TvJHC		J. Heilmann-Clausen	
		TvA	*Fagus sylvatica* Ccc	J. Hiscox	Apr-08
		Natural homokaryons		J. Hiscox	Apr-08
		A1			
		A2			
		A3			
		A4			
		A5			
		A6			
		A7			
		A8			
		Artificial heterokaryons		C. Hibbert	Oct-08
		A4 × A6			
		A5 × A8			
		A3 × A7			
		A1 × A2			
	Bjerkandera adusta	MA313	WGP	M. Ainsworth	
Primary colonisers, latently present in standing trunks and attached branches	*Stereum gausapatum*	Sg1	*Q. robur* Ccc	L. Boddy	
	Vuilleminia comedens	Vc1	*Q. robur* Ccc	L. Boddy	
	Stereum hirsutum	Sh1			
	Daldinia concentrica[b]	Dc290594			
	Eutypa spinosa[b]	Es1	*F. sylvatica* Ccc	S. J. Hendry	Sep-05
Heart rotters	*Fomes fomentarius*	JHC001-201	*F. sylvatica*, Denmark	J. Heilmann-Clausen	
Later secondary cord-forming colonisers	*Hypholoma fasciculare*	GTWV2	Wenvoe, South Wales	G. Tordoff	2003
		DD2		D. Donnelly	
	Phanerochaete velutina	Pv29			
Tooth fungus[c]	*Hericium coralloides*	HcMA129-9	*F. sylvatica*, WGP	M. Crockatt	Nov-05

Ccc: Cardiff University Culture Collection; WGP: Windsor Great Park, UK.
a Rayner & Boddy (1988).
b Ascomycota, all others are Basidiomycota.
c Ecological role not entirely clear, found both in central heart regions and outer sapwood (Boddy & Wald 2002).

generated by randomly pairing monokaryons on agar plates to produce stable dikaryotic mycelia (Table 2).

Extension rates

Plugs (6 mm diam.), cut from the actively growing margin of a colony using a no. 3 cork borer, were inoculated centrally onto 9 cm non-vented Petri dishes (Greiner Bio-one, Austria) of 2 % MA. Colony diameter in two dimensions perpendicular to each other was measured daily, to 0.1 mm using vernier callipers (Swiss Precision Instruments Inc., CA, USA) for all isolates.

Wood decay rates

Beech (*Fagus sylvatica*) blocks (2 × 2 × 1 cm) were cut from a freshly felled trunk (Wentwood, Newport, UK) and stored at −18 °C until required. Blocks were defrosted by soaking overnight in distilled water and sterilised by autoclaving three times. They were then incubated on 14 cm Petri dishes (Greiner Bio-one, Austria) of 2 % MA, fully colonised by *T. versicolor*, at 20 °C in the dark. Density (g cm^{-3}) of wood blocks was determined destructively at the start and after 10 months incubation (10 replicates), and percentage weight loss estimated.

Interspecific interactions

Interactions were set up between all isolates of *T. versicolor* and 10 other species (Table 1), isolated from wood or fruit bodies (identified by colony or fruit body morphology), on 2 % MA in 9 cm non-vented Petri dishes. Fungi were inoculated as 6 mm diameter plugs 30 mm apart, and plates were incubated in darkness at 20 °C. Timing of inoculation depended on extension rate, and where necessary was staggered to ensure that mycelia met in the centre of the dish. Three replicates were performed for each combination. Once colonies met, interactions were observed approximately weekly, with final outcomes recorded after 8–10 weeks. Outcomes were recorded as either deadlock (where neither mycelium captured territory from the other), replacement (where one fungus grew over and through the other so that it was no longer recoverable by re-isolation) or partial replacement, mutual partial replacement (where both fungi gained some of the territory of the other), or overgrowth (where one fungus grew over the other

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Table 2 – Outcomes of interactions on agar between *Trametes versicolor* homokaryons and heterokaryons and 11 antagonist wood decay species

Isolate	*Stereum gausapatum*	*Stereum hirsutum*	*Daldinia concentrica*	*Hericium coralloides*	*Hypholoma fasciculare*		*Bjerkandera adusta*	*Vuilleminia comedens*	*Eutypa spinosa*	*Fomes fomentarius*	*Phanerochaete velutina*	Cumulative scores	Rank
					GTWV2	DD2							
Natural heterokaryons													
TvD4	R	R	R(2)PR	PR(2)pr	pr*	pr	pr	R	R	PR	PR(2)D	26	9
TvD2	R	R	R(2)PR	pr(2)D	r	r(2)pr	pr(2)r	R	R	D	PR(2)D	15	14
TvJHC	R	R	R	OG	r*	r	pr(2)r	R	R	D	R	20	13
TvA	R	R	R	MPR(2)PR	pr*(2)R	PR(2)R	PR(2)R	R(2)PR	R	R(2)D	R(2)PR	47	2
Artificial heterokaryons													
A4 × A6	R	R	R	OG	MPR	R(2)PR	pr(2)D	PR(2)R	R	D(2)PR	R(2)MPR	37	3
A5 × A8	R	R	D	MOG	r	R	r	R	R	PR(2)D	R	26	9
A3 × A7	R	R	R	PR+og	r*(2)pr	PR(2)MPR	r(2)pr	R	R	R(2)D	R	35	4
A1 × A2	R	R	R	pog	pr	R	r(2)pr	R(2)PR	R	D	PR(2)R	31	6
Natural homokaryons													
A1	R	R	R	MPOG	pr*	pr(2)r	pr(2)OG	PR(2)R	R	PR	R	28	7
A2	R	R	R	OG	r*	r	r(2)pr	R	R	D	R	19	12
A3	R	R	R	PR	r*	r(2)MPR	PR	R(2)PR	R	R	R	57	1
A4	PR	R	PR(2)OG	pr+OG(2)pr	r	r	r	R(2)PR	PR(2)R	pr(2)D	og	−3	16
A5	R	R	PR(2)R	MPOG	r(2)pr*	R	r(2)pr	R	R	PR(2)R	R	34	5
A6	R(2)PR	R	R	OG	r	pr(2)R	r(2)pr	R	R	R	R(2)MPR	28	7
A7	R(2)PR	R	R	r	MPR(2)r*	PR	r	R	R	D(2)PR	R	24	11
A8	R	R	R	D(2)MPR	r	r(2)MPR	r	R(2)PR	R	pr(2)R	PR(2)r	14	15
Cumulative scores	91	96	82	−5	−68	−7	−58	107	94	32	71		

R: replacement of competitor by *T. versicolor*; PR: partial replacement of competitor by *T. versicolor*; D: deadlock; MPR: mutual partial replacement; OG overgrowth by *T. versicolor*; og: overgrowth of *T. versicolor* by competitor; pr: partial replacement of *T. versicolor* by competitor; r: replacement of *T. versicolor* by competitor. * Indicates *H. fasciculare* overgrowth where *T. versicolor* has been killed but not yet replaced. Numbers in brackets indicate how many replicates had this outcome.

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but did not replace it). Outcomes were confirmed by re-isolation from the base of the agar, which was essential for distinguishing between replacement and overgrowth.

The outcome of each pairing was given a score, as an aid to comparison of combative ability (following Crockatt *et al.* 2008): replacement of the antagonist by *T. versicolor* was assigned +2; partial replacement of the antagonist, +1; deadlock, 0; partial replacement of *T. versicolor*, −1; complete replacment of *T. versicolor*, −2. Mutual partial replacement was scored as −1 and +1, so a net score of 0. Where either antagonist had killed but not (yet) replaced the other, the outcome was scored as for replacement, and where overgrowth but not replacement had occurred the outcome was scored as deadlock. Cumulative scores were determined for each isolate by addition of scores from all replicate interactions.

Enzyme assays

Isolates of *T. versicolor* were grown on 16 ml 2 % MA in 9 cm non-vented Petri dishes in darkness at 20 °C. A plug of agar with mycelium (12 mm diameter; mycelial coverage 112 mm^2; made using a no. 8 cork borer) was removed from just behind the growing margin of a 7 d-old colony. Each plug was cut into c. 12 pieces and transferred to a 1.5 ml Eppendorf tube, to which 1 ml of deionised water was added. Tubes were shaken overnight at 4 °C, then 0.8 ml of extract was removed and centrifuged for 10 min at 8,000 g at 4 °C to pellet any debris. Extracts were also made from 6-month-old colonised beech wood blocks; these were roughly chopped (pieces < 2 × 2 × 2 mm, and c. 100 mg wet weight) and extracts prepared in 1.5 ml Eppendorfs as for agar cultures. Extracts were kept at 4 °C and used on the day of harvest. The wet weight of the agar plus mycelium, and the dry weight of the wood shavings plus mycelium, were used to normalise the data from agar and wood block cultures respectively. Three replicates were performed for each isolate.

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) in a citrate-phosphate buffer (100 mM citrate, 200 mM phosphate, pH 5.0), following Niku-Paavola *et al.* (1990). The formation of green colouration was followed spectrophotometrically at 420 nm. Activity of manganese peroxidase (MnP; EC 1.11.1.13) was assayed according to Ngo & Lenhoff (1980) in succinate-lactate buffer (100 mM, pH 4.5). MBTH (3-methyl-2-benzothiazoline-hydrazone hydrochloride) and DMAB (3-(dimethyl amino)-benzoic acid) were oxidatively coupled by MnP action, and formation of purple colouration followed spectrophotometrically at 590 nm. The results were corrected by activities of samples: (1) without manganese, where manganese sulphate was replaced by EDTA (ethylene diamine tetraacetate) to chelate Mn present in the extract (Mn-independent peroxidase activity); and (2) in the absence of hydrogen peroxide to allow detection of activity of oxidases but not peroxidases. Lignin peroxidase (EC 1.11.1.14) activity was measured by monitoring the oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde in a 250 mM tartaric acid buffer (pH 2.5) containing 5 mM H_2O_2 (Collins *et al.* 1996). The change in absorbance at 310 nm was followed using a Helios UV-vis spectrophotometer (Tecan, USA). One unit of enzyme activity was defined as the amount of enzyme releasing 1 mM of product per min.

The activity of 1,4-β-glucosidase (EC 3.2.1.21) was assayed in microplates using *p*-nitrophenyl-β-D-glucoside (pNPG, Megazyme Ltd, Ireland). The reaction mixture comprised 160 μl of 1.2 M pNPG in 50 mM sodium acetate buffer (pH 5.0) and a 40 μl sample. Reaction mixtures were incubated at 40 °C for 120 min. The reaction was stopped by addition of 0.5 M sodium carbonate, and absorbance read at 400 nm. Enzyme activity was calculated using the molar extinction coefficient of *p*-nitrophenol (11,600 M cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute. Activity of phosphomonoesterase (acid phosphatase; EC 3.1.3.2) and 1,4-N-acetylglucosaminidase (chitinase; EC 3.2.1.50) were assayed using *p*-nitrophenyl phosphate (pNPP; Megazyme International Ltd, Ireland) and *p*-nitrophenyl N-acetyl-β-D-glucosaminide (pNPN; Megazyme International Ltd, Ireland), respectively, using the same method. Spectrophotometric measurements for MnP and laccase were made in a Bioscreen II plate reader, those for LiP made in a UV-vis spectrophotometer (Helios, Tecan, USA), and all others in a Dynex microplate reader (Dynex Technologies Ltd., Sussex, UK).

Statistical analysis

Colony extension rates during the log phase of extension were estimated by linear regression and compared using one-way ANOVA, or Kruskal–Wallis when non-normal, in Minitab (v.15). Percentage density loss between isolates was compared in the same way, and so were differences in enzyme activity. Correlation between extension rate, enzyme activity, competitive score and decay rate was assessed using Spearmans rank correlation in Minitab.

Results

Extension rates

There was high variability in radial extension rate, ranging from 4.1 to 6.4 mm d^{-1}. Overall, natural dikaryon extension rate was significantly ($P \leq 0.05$) greater than that of monokaryons (5.7 ± 0.3 mm d^{-1} and 5.2 ± 0.2 mm d^{-1} respectively; mean ± SEM). However, there was no significant difference ($P > 0.05$) between the mean extension rate of the natural monokaryons and 'artificial' dikaryons (the mating products of the monokaryons; 5.5 ± 0.1 mm d^{-1}), nor between the natural monokaryons and their 'parent' dikaryon TvA (5.1 ± 0.2 mm d^{-1}). The extension rate of the artificial dikaryons was similar to at least one of the component monokaryons (Fig 1A). The dikaryon tended to be more similar to the faster of the two monokaryons, for example in combinations A3 × A7 and A5 × A8 where extension rate of both the faster monokaryon and the dikaryon were significantly different ($P \leq 0.05$) from the slower monokaryon (Fig 1A).

Decay rates

Overall, dikaryons effected significantly ($P \leq 0.05$) greater decay of beech wood blocks than monokaryons (natural heterokaryons, 30.1 ± 1.1 % weight loss; artificial dikaryons, 35 ± 1.4 %; natural monokaryons, 19 ± 0.5 %; mean ± SEM).

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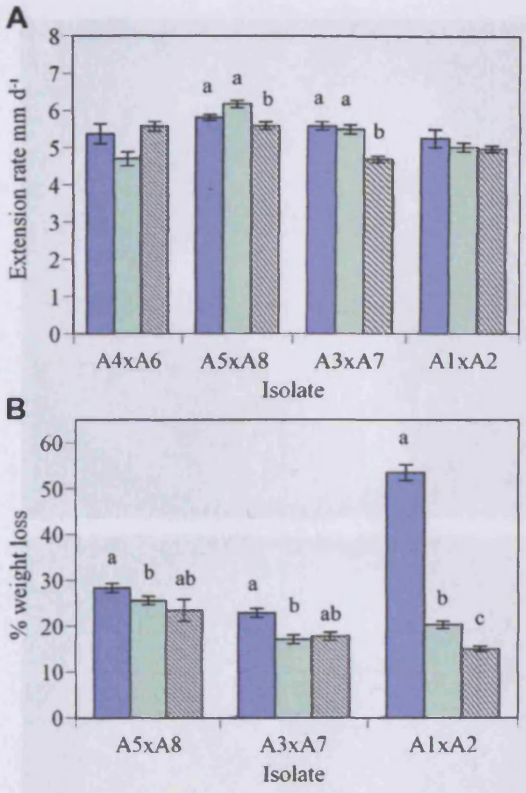

Fig 1 – A: Mean extension rates of *T. versicolor* natural homokaryons and the heterokaryon products of their matings. B: Mean % weight loss of beech wood blocks after 10 months colonisation by *T. versicolor* natural homokaryons and the heterokaryon products of their matings. Dark grey bars, heterokaryons; light grey bars, 1st component homokaryon (e.g. for A3 × A7 this would be A3); striped bars, 2nd component homokaryon. Different letters indicate significant ($P \leq 0.05$) differences in extension rate/% weight loss between component homokaryons and the related heterokaryon.

However, there were no significant differences ($P > 0.05$) between the weight loss from natural dikaryon TvA (18.3 ± 1.2 % weight loss) and any of the natural monokaryons. Artificial dikaryons caused greater weight losses than their component monokaryons but this was only significant ($P \leq 0.05$) for A1 × A2, where the component monokaryons also had significantly different decay abilities to each other (Fig 1B). The component monokaryons of A3 × A7 and A5 × A8 did not cause significantly different weight losses to each other, but in both combinations decay by one of the component monokaryons was significantly lower ($P \leq 0.05$) than the artificial dikaryon (Fig 1B).

Outcome of interactions

There was considerable variation in the combative ability of *T. versicolor* isolates. Outcomes ranged between complete replacement of the competitor to partial replacement of the *T. versicolor* isolate by that competitor (e.g. monokaryon A8 *vs.* *Fomes fomentarius*), and between partial replacement of the *T. versicolor* isolate to partial replacement of the competitor (e.g. dikaryon TvD4 *vs.* *H. coralloides*; Table 2). Against this set of antagonists, *T. versicolor* was a successful combatant, completely replacing its opponent in over half of all interactions, and with 15 out of the 16 isolates having positive cumulative scores. The mean score for all isolates was 27.3 (out of a maximum of 66 if *T. versicolor* had replaced all competitors in all replicates, and a minimum of −66 if *T. versicolor* had been replaced by all competitors in all replicates; Table 2).

Overall, there were no significant differences ($P > 0.05$) between the proportion of successful interaction outcomes (i.e. where *T. versicolor* replaced or partially replaced the competitor) attained by monokaryons and dikaryons, both natural and artificial (Supplementary Fig 1). In some cases the artificial dikaryons outperformed both component dikaryons: for example, the complete replacement of A2 by *Hypholoma fasciculare* DD2, the mutual partial replacement with A1, compared to the complete replacement of *H. fasciculare* DD2 by A1 × A2 (Fig 2; Table 2). In another example, A4 and A6 were both replaced by *H. fasciculare* GTWV2, but the outcome with their mating product A4 × A6 was mutual partial replacement or partial overgrowth by *H. fasciculare* (Table 2). Generally, the combative ability of the artificial dikaryon was similar to that of their "strongest" component monokaryon, for example A8 was replaced by *H. fasciculare* DD2 whilst A5 and A5 × A8 both replaced *H. fasciculare* DD2 (Table 2).

Enzyme assays

All enzymes assayed for were detected in agar cultures, except manganese peroxidase (MnP) and lignin peroxidase (LiP). The production of enzymes in agar culture varied widely between isolates (Table 3). There were no significant differences ($P > 0.05$) between natural monokaryons and their mated products – the artificial dikaryons – in activity of any of the enzymes. However, when considered individually, artificial dikaryons often had significantly lower enzyme activity than one of their component monokaryons in agar culture. The most dramatic was A4 × A6, which had significantly lower ($P \leq 0.05$) activity of 1,4-β-glucosidase, chitinase and acid phosphatase than its component monokaryon A4, but similar activity to its other component monokaryon A6 (Table 3; Supplementary Fig 2). The opposite occurred for laccase activity, with no significant difference ($P > 0.05$) in activity between A4 × A6 and A4, but both had significantly ($P \leq 0.05$) lower activity than A6 (Table 3; Supplementary Fig 2).

There was much greater variability in enzyme activity between replicates of wood block cultures than in agar cultures (Table 4). MnP activity was detected in 10 of the 16 isolates in wood block culture, but again no LiP activity was detected. Whereas agar cultures of monokaryon A4 had significantly ($P \leq 0.05$) higher chitinase, acid phosphatase and 1,4-β-glucosidase activity than all other natural monokaryons, no significant differences ($P > 0.05$) in activity of A4 enzymes were found in wood block culture extracts (Table 4; Supplementary Fig 2). Where there were significant differences in activity of a particular enzyme between artificial dikaryons and a component monokaryon in agar culture, these were not consistent with activities in wood block culture

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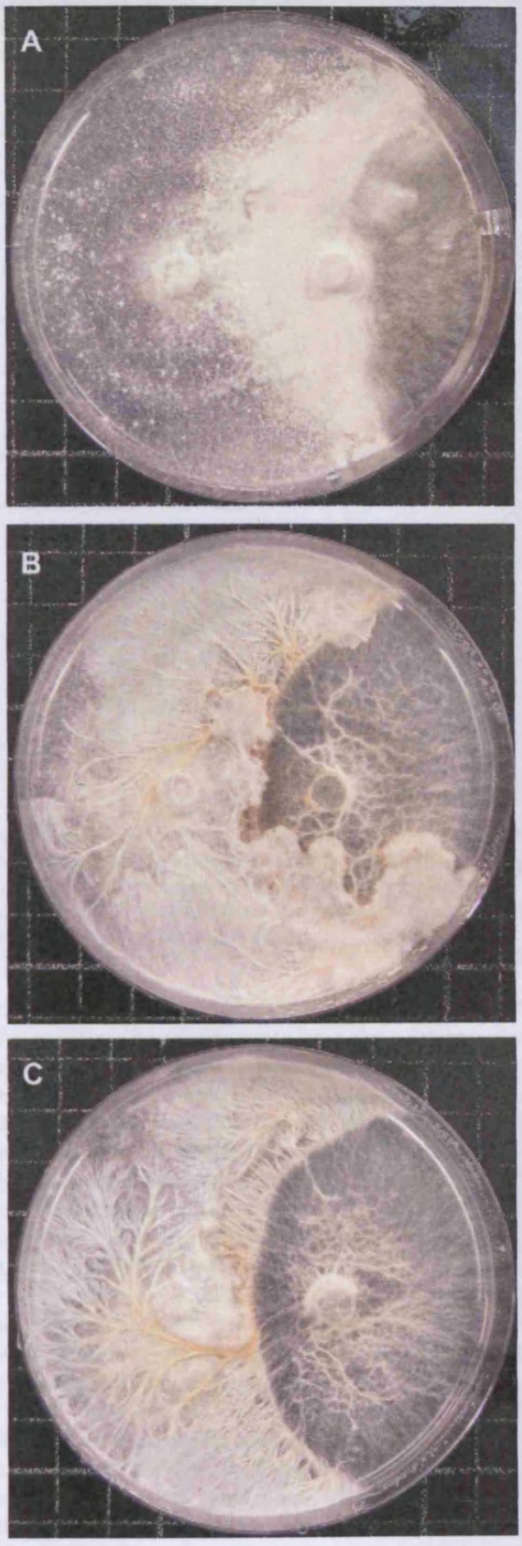

Fig 2 – Differences in interaction outcomes at 2 months between *T. versicolor* heterokaryons and their component homokaryons. *T. versicolor* is always on the left hand side. Heterokaryon A1 × A2 (A) and homokaryons A1 (B) and A2 (C) vs. *H. fasciculare* DD2.

(Table 4; Supplementary Fig 2). For example, artificial dikaryon A4 × A6 had significantly ($P \leq 0.05$) higher laccase activity than either component monokaryon in wood block culture, but was significantly ($P \leq 0.05$) lower than A6 in agar culture.

Discussion

This is the first study to compare: (1) combative ability of monokaryons and dikaryons of a common species, and (2) the activity of a wide range of enzymes produced by monokaryons and dikaryons. As hypothesised, there was no evidence that *T. versicolor* monokaryons and dikaryons had significantly different combative ability; there was as much variability within the two states as between them. This contrasts with *H. coralloides* where homokaryons were more combative than heterokaryons in agar culture (Crockatt *et al.* 2008). This probably reflects the fact that as a rare species (as evidenced by the lack of fruit bodies) *H. coralloides* probably remains as a homokaryon for much longer than a common species and thus may require this state to be more vigorous. Macroscopic morphological changes during interactions did not differ between karyotic states in *T. versicolor* as they did with *H. coralloides* (Crockatt *et al.* 2008), though in *Ganoderma orbiforme* barrage formation occurred only in heterokaryon interactions and homokaryon morphology was linked to mating type (Pilotti *et al.* 2002). The results correlate with the position of *T. versicolor* in succession: high cumulative scores were achieved by nearly all isolates in combat with primary colonisers where outcomes were nearly always complete or partial replacement; lower cumulative scores were achieved against later colonisers and tooth fungi, but also against heart rotters. The latter was unexpected as heart rotters are primary colonisers and *T. versicolor* might be expected to replace them (Rayner & Boddy 1988); evidently the heart rotters were defensively combative. The average cumulative score of 27.3 was much higher than the mean score of 2.6 achieved by *H. coralloides* isolates, albeit with a slightly different profile of antagonists (Crockatt *et al.* 2008).

Enzyme activity of wood block cultures was highly variable between isolates, and between biological replicates, compared to that of agar cultures, probably due to the heterogeneity of wood blocks as a resource (e.g. variation in nutrient content, decay state of the wood, orientation of vessel elements). The reverse was found for homo- and heterokaryons of *P. ostreatus*, where ligninolytic enzyme activity was less variable between isolates when grown on wheat straw compared to growth on agar (Eichlerová *et al.* 2000). There was as much variability between monokaryons of *T. versicolor* as there was between dikaryons. This contrasts with results from *Lentinus tigrinus* and *P. ostreatus*, where protoplast- and basidiospore-generated homokaryons had higher variability in enzyme activity compared to heterokaryotic isolates (Homolka *et al.* 1995; Eichlerová & Homolka 1999).

The pattern of enzyme activity produced by an isolate relative to other isolates was also different during growth on agar compared to during growth on wood blocks, which might be expected as the strategy of enzyme production would differ on a nutrient-rich resource such as malt agar compared to nutrient-limiting beech wood. During growth on agar, natural monokaryon A4 generated high levels of chitinase, acid

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Table 3 – Extracellular enzyme activities of *Trametes versicolor* homokaryon and heterokaryon agar extracts

Isolate	Activity mU g^{-1} wet weight				
	Laccase	Peroxidase*	β-glucosidase	Chitinase	Acid phosphatase
Natural heterokaryons					
TvD4	181.8 ± 22.6	8.0 ± 0.3	10.8 ± 2.1	1.8 ± 0.3	2.8 ± 0.3
TvD2	194.6 ± 37.2	6.6 ± 0.5	5.9 ± 1.3	1.5 ± 0.2	2.0 ± 0.1
TvJHC	204.8 ± 30.3	4.9 ± 0.1	11.4 ± 1.5	2.4 ± 0.4	4.9 ± 0.9
TvA	406.2 ± 49.4	5.2 ± 0.2	31.9 ± 2.7	1.9 ± 0.2	2.8 ± 0.4
Average	226.3^{a} ± 27.7	5.7^{a} ± 0.5	15.0^{a} ± 3.1	1.9^{a} ± 0.1	3.1^{a} ± 0.4
Artificial heterokaryons					
A4 × A6	348.9 ± 0.5	7.1 ± 0.7	20.5 ± 1.7	1.6 ± 0.2	3.1 ± 0.2
A5 × A8	267.7 ± 19.4	6.2 ± 1.0	26.8 ± 2.1	1.1 ± 0.1	3.6 ± 0.2
A3 × A7	343.7 ± 11.4	7.8 ± 0.9	24.3 ± 1.2	1.5 ± 0.2	2.9 ± 0.3
A1 × A2	279.0 ± 60.5	5.1 ± 0.4	27.6 ± 0.9	1.3 ± 0.3	2.9 ± 0.3
Average	303.1^{ab} ± 17.6	6.6^{a} ± 0.5	24.8^{b} ± 1.1	1.4^{b} ± 0.1	3.1^{a} ± 0.1
Natural homokaryons					
A1	224.2 ± 38.0	4.9 ± 0.4	13.8 ± 1.1	0.8 ± 0.1	2.8 ± 0.2
A2	305.1 ± 26.8	6.2 ± 0.1	7.9 ± 0.7	0.9 ± 0.0	2.4 ± 0.2
A3	327.5 ± 30.1	5.6 ± 0.2	7.0 ± 0.6	1.2 ± 0.0	2.6 ± 0.2
A4	299.1 ± 9.0	7.9 ± 0.2	57.2 ± 2.3	17.8 ± 2.3	15.2 ± 0.4
A5	294.7 ± 6.6	5.3 ± 0.2	24.7 ± 3.3	1.3 ± 0.2	1.5 ± 1.0
A6	537.0 ± 46.4	8.5 ± 0.6	11.6 ± 1.2	1.2 ± 0.1	3.0 ± 0.3
A7	264.8 ± 4.9	6.8 ± 1.1	13.2 ± 0.9	1.4 ± 0.1	2.9 ± 0.3
A8	446.2 ± 19.6	7.2 ± 0.3	7.9 ± 0.6	1.1 ± 0.2	5.0 ± 0.2
Average	328.6^{b} ± 19.4	6.5^{a} ± 0.3	17.9^{ab} ± 3.3	3.2^{ab} ± 1.2	4.4^{a} ± 0.9
Agar	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0

Values given are mean activity of 3 replicate cultures, ±standard error of the mean (SEM). *, Manganese-independent peroxidase. MnP activity was not detected. Different letters imply significant differences in group average activity. Scores for each isolate are cumulative for all replicates against all antagonists. Scores for each replicate were −2 when T. versicolor was replaced, −1 when it was partially replaced, 0 for outcomes of deadlock, 1 when T. versicolor partially replaced its opponent and 2 when it completely replaced its opponent. Mutual replacement was scored as both −1 and +1, i.e. net score of 0.

phosphatase and 1,4-β-glucosidase activity; these enzymes function to release bound nutrients from the substrate. So the increased production on a nutrient-rich resource may imply problems with nutrient uptake and utilisation, which is supported because A4 was the slowest growing isolate and weakest combatant of all the isolates. The low scores of A4 may have distorted the results for monokaryons. The lack of MnP activity in agar cultures is surprising, but might be explained by a low manganese content of the substrate. MnP activity was present in younger colonies of T. versicolor on agar, but production ceased by 7 d (results not shown), perhaps as available Mn^{2+} was depleted.

Overall, there were no differences in radial extension rates of mono- and dikaryons, as hypothesised. Similarly, extension rates in *H. coralloides* did not significantly differ between homokaryons and heterokaryons (Crockatt *et al.* 2008). This contrasts with *G. trabeum*, *P. weirii* and *P. ostreatus* where heterokaryon extension rates were greater than those of homokaryons (Bezemer 1973; Hansen 1979; Eichlerová & Homolka 1999). Differences in extension rates between monokaryons and dikaryons imply a difference in control of gene expression in each karyotic phase (Clark & Anderson 2004), which we can infer is not the case for *T. versicolor*. Decay rates follow a similar pattern: overall, *T. versicolor* dikaryons had greater decay ability than monokaryons, but there were no significant differences in decay rate by the natural monokaryons and the 'parent' dikaryon TvA, and no significant differences between the artificial dikaryons and one or both component monokaryons. The lack of significant correlations between decay rate and enzyme activity in wood block cultures implies other factors are responsible for the differences in decay rate, or that there is a more complex relationship between lignin decomposition and weight loss.

Comparison of characteristics of artificial dikaryons with their component monokaryons indicated that where the monokaryons significantly differ in a particular character, the artificial dikaryon is similar to one of the monokaryons in this character rather than being intermediate. This occurred for agar enzyme activity, extension rate, decay rate and combative ability, but not for wood block enzyme activity where differences may be masked by substrate heterogeneity. The dikaryon was not consistently similar to either monokaryon, for example laccase activity of dikaryon A4 × A6 was very similar to that of monokaryon A4, whereas chitinase and acid phosphatase activities were very similar to A6. Also, dikaryon enzyme activity was rarely significantly higher than activity of both component monokaryons. The trend for mated heterokaryons to resemble one component homokaryon rather than be intermediate also occurred in *H. coralloides* extension rates, although the extension rate of the heterokaryons was

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Table 4 – Extracellular enzyme activities of *Trametes versicolor* homokaryon and heterokaryon colonised beech wood block extracts

Isolate	Activity mU g^{-1} dry weight					
	Laccase	MnP	Peroxidase*	1,4-β-glucosidase	Chitinase	Acid phosphatase
Natural heterokaryons						
TvD4	474.8 ± 250.4	51.2 ± 11.2	64.1 ± 32.5	71.6 ± 16.9	18.3 ± 3.0	22.1 ± 8.2
TvD2	4.3 ± 3.0	0.0 ± 0.0	12.3 ± 7.1	50.4 ± 18.6	18.0 ± 4.5	49.8 ± 1.8
TvJHC	207.1 ± 168.9	0.0 ± 0.0	57.7 ± 10.8	43.6 ± 20.5	11.6 ± 3.3	34.2 ± 7.8
TvA	293.7 ± 111.6	17.1 ± 13.3	81.5 ± 17.4	48.9 ± 19.3	5.1 ± 1.1	10.1 ± 1.7
Average	245.0^{a} ± 85.5	17.1^{ab} ± 7.3	53.9^{a} ± 11.3	53.6^{a} ± 8.7	13.3^{a} ± 2.1	29.0^{a} ± 5.1
Artificial heterokaryons						
A4 × A6	278.1 ± 19.7	50.1 ± 12.3	90.3 ± 2.9	23.8 ± 2.7	2.6 ± 0.7	11.2 ± 4.0
A5 × A8	452.1 ± 199.3	19.9 ± 11.9	63.1 ± 7.2	68.7 ± 22.7	12.8 ± 2.3	20.9 ± 5.4
A3 × A7	95.2 ± 28.4	28.7 ± 16.8	79.5 ± 12.0	20.7 ± 2.4	1.8 ± 0.2	13.8 ± 0.1
A1 × A2	86.9 ± 57.9	10.7 ± 3.1	73.3 ± 11.7	26.6 ± 6.7	4.6 ± 0.9	20.2 ± 1.9
Average	290.3^{a} ± 92.1	23.2^{b} ± 6.4	76.6^{a} ± 4.9	45.6^{a} ± 12.3	6.0^{b} ± 1.4	16.5^{a} ± 1.9
Natural homokaryons						
A1	97.1 ± 50.1	0.0 ± 0.0	47.7 ± 19.6	92.2 ± 34.1	14.5 ± 3.9	9.9 ± 2.9
A2	212.5 ± 83.2	0.5 ± 0.5	54.6 ± 22.5	21.9 ± 4.5	2.7 ± 0.4	10.4 ± 4.1
A3	843.5 ± 102.7	5.9 ± 3.1	89.3 ± 7.7	32.1 ± 13.1	5.7 ± 2.3	16.2 ± 4.0
A4	79.8 ± 36.7	0.0 ± 0.0	131.3 ± 11.1	20.6 ± 13.0	1.6 ± 0.2	9.7 ± 2.3
A5	718.9 ± 104.4	20.7 ± 11.9	46.1 ± 7.0	61.3 ± 13.0	5.2 ± 1.6	18.4 ± 3.2
A6	70.4 ± 18.9	6.6 ± 6.6	48.1 ± 12.9	17.2 ± 6.9	4.9 ± 0.6	11.8 ± 3.1
A7	372.8 ± 47.3	0.0 ± 0.0	22.4 ± 5.1	30.8 ± 3.5	3.8 ± 0.2	22.8 ± 2.9
A8	428.9 ± 28.9	0.0 ± 0.0	173.9 ± 40.7	45.5 ± 4.1	21.5 ± 3.6	21.4 ± 1.6
Average	353.0^{a} ± 61.3	4.2^{a} ± 2.0	76.7^{a} ± 11.6	45.9^{a} ± 7.9	7.5^{b} ± 1.5	15.1^{a} ± 1.4
Wood	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.4	0.7 ± 0.2	1.0 ± 0.0	4.8 ± 2.5

Values given are mean activity of 3 replicate cultures, ±standard error of the mean (SEM). *, Manganese-independent peroxidase. Different letters imply significant differences between the group averages.

occasionally faster than either component homokaryon (Crockatt *et al.* 2008). This would appear to suggest regulation of different genes in a dominant–recessive system, where heterokaryons are functionally equivalent to diploids. This further implies that nuclear ratios within *T. versicolor* are balanced (1:1) because if there were over-representation of one constituent nucleus, the dikaryon would resemble the corresponding monokaryon across all traits (James *et al.* 2008). The mycelium can thus be thought of as an 'individualistic mycelium' (Rayner 1991), analogous to a genetic individual rather than a situation where there is genomic conflict between the constituent 'haploid' nuclei (James *et al.* 2008).

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Supplementary material

Supplementary material associated with this article can be found in online version at doi:10.1016/j.funeco.2010.02.003.

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Changes in oxidative enzyme activity during interspecific mycelial interactions involving the white-rot fungus *Trametes versicolor*

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ABSTRACT

Interspecific fungal antagonism leads to biochemical changes in competing mycelia, including up-regulation of oxidative enzymes. Laccase, manganese peroxidase (MnP), manganese-repressed peroxidase (MRP) and lignin peroxidase (LiP) gene expression and enzyme activity were compared during agar interactions between *Trametes versicolor* and five other wood decay fungi resulting in a range of interaction outcomes from deadlock to replacement of one fungus by another. Increased laccase and Mn-oxidising activities were detected at all interaction zones, but there were few changes in activity in regions away from the interaction zone in *T. versicolor* mycelia compared to self-pairings. Whilst no LiP activity was detected in any pairing, low level LiP gene expression was detected. MnP activity was detected but not expression of MnP genes; instead, MRP could explain the observed activity. No relationship was found between extent of enzyme activity increase and interaction outcome. Similarities between patterns of gene expression and enzyme activity are discussed.

1. Introduction

Fungal competition for territory and the resources within is brought about by antagonistic mycelial interactions and is inevitable where there is overlap between the niches of different species or strains (Rayner and Boddy, 1988; Boddy, 2000; Woodward and Boddy, 2008). Interactions are crucial determinants of fungal community development, and affect decay rate and carbon turnover in woodland ecosystems (Owens et al., 1994; Boddy, 2000). There are two main interaction outcomes: replacement occurs when one fungus gains the territory of another; deadlock occurs when neither fungus gains headway. A spectrum of outcomes can occur between replacement and deadlock, for example, partial replacement of one competitor followed by deadlock, or mutual partial replacement by both competitors.

Antagonism leads to mycelial morphological changes, metabolic changes, and the production and release of secondary metabolites and extracellular enzymes (Rayner and Boddy, 1988; Griffith et al., 1994; Score et al., 1997; Iakovlev et al., 2004). There is considerable evidence for up-regulation of oxidative enzymes during antagonistic interactions between wood-decay species (Freitag and Morrell, 1992; White and Boddy, 1992; Iakovlev and Stenlid, 2000; Savoie and Mata, 1999; Baldrian, 2004; Chi et al., 2007; Ferreira-Gregorio et al., 2007). The extent of the increase in activity differs, depending on the innate enzyme producing ability of a species, and the combination of species interacting (Iakovlev and Stenlid, 2000; Chi et al., 2007). Within interacting mycelia, spatial patterns of activity are inconsistent between interaction combinations, although some general trends have been observed. For example up-regulation of mycelial laccase in response to interaction lessened with distance from the interaction zone (Iakovlev and Stenlid, 2000). As yet, no study has fully investigated the links between oxidative enzyme activity during interactions and interaction outcome.

The main oxidative enzymes secreted by white rot basidiomycetes are laccase (phenoloxidase), manganese peroxidase (MnP) and lignin peroxidase (LiP). Laccase acts to oxidise a variety of phenolic compounds and aromatic diamines (Leonowicz et al., 2001; Mayer and Staples, 2002), whilst peroxidases utilise hydrogen peroxide (H_2O_2) to catalyse the oxidation of a range of organic and inorganic compounds. MnP and LiP differ in their reducing substrates: LiP catalyses the oxidation of a wide range of aromatic non-phenolic compounds; MnP catalyses the Mn-mediated oxidation of phenolic lignin compounds (Hofrichter, 2002; Martinez, 2002; Martinez et al., 2005). Other enzymes such as versatile peroxidase (VP), which combines features of MnP and LiP, may also play lesser roles. While the production of LiP and VP appears to be limited to a small group of white rot fungi, MnP is produced by most white rot and litter-colonising basidiomycetes (Morgenstern et al., 2008) and its genes have been recently detected in mycorrhizal fungi as well (Bodeker et al., 2009). Though their primary role is in lignin decomposition, the

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highly oxidative and non-specific nature of laccase and peroxidases enables a variety of other potential roles. Laccase activity increased dramatically during interactions (White and Boddy, 1992; Iakovlev and Stenlid, 2000; Baldrian, 2004; Chi et al., 2007; Ferreira-Gregorio et al., 2007), and also in fungal cultures exposed to abiotic stress such as elevated temperature (Fink-Boots et al., 1999), heavy metals (Baldrian et al., 2005), and oxidative stress (Jaszek et al., 2006). MnP activity increased in certain interactions, although not to the same extent as laccase activity (Baldrian, 2004), and also in response to oxidative stress (Jaszek et al. 2006). General peroxidase activity increased in response to heat shock (Fink-Boots et al., 1999), but there have been no reports of stress-induced increases in LiP activity.

Production of laccase and MnP in response to stress implies a defensive function during interactions. A typical result of antagonistic interactions is oxidative stress, and accumulation of reactive oxygen species (ROS) and H_2O_2 is associated with interaction zones (Tornberg and Olsson, 2002; Silar, 2005). Increased laccase and MnP production occurred in response to chemically-induced oxidative stress in cultures of *Trametes versicolor*, *Fomes* and *Tyromyces* spp. (Jaszek et al., 2006; Jaszek et al., 2006a), and activity increases occurred along with superoxide accumulation in heat-shocked cultures of *T. versicolor*, *Cerrena unicolor* and *Abortiporus biennis* (Fink-Boots et al., 1999). Laccase and peroxidases could mediate oxidative stress by removing H_2O_2 or generating melanins which protect hyphae from ROS (Henson et al., 1999). Melanins may also participate in the absorption of toxic compounds, which could also be detoxified by laccase; laccase is known to detoxify fungicides in yeast (Baldrian, 2006). Laccase and peroxidases also have roles in morphogenesis (de Vries et al., 1986; Thurston, 1994) and may be involved in the formation of defensive barrages or invasive hyphal cords (Griffith et al., 1994).

Aside from a defensive function, increased laccase and peroxidase activity during interactions may imply increased acquisition of nutrients, and there is evidence of increased production of other enzymes involved in nutrition. Activity of β-glucosidase, which primarily functions to hydrolyse the β1–4 bonds between glucose monomers (such as the bonds found in cellulose), increased during interactions between *T. versicolor* and *Trichoderma harzianum* (Freitag and Morrell, 1992). Acid phosphatase occurs in ascomycetes and basidiomycetes, and functions to release P_i from organophosphates. Acid phosphatase activity could function during interactions to release bound organophosphates from captured opponent mycelium (Reyes et al., 1990). Chitinases function to hydrolyse the β1–4 bonds between N-acetylglucosamine monomers in chitin chains. Increased chitinase activity occurred during interactions involving *Hypholoma fasciculare*, *Resinicium bicolor* and *Coniophora arida* (Lindahl and Finlay, 2006). The chitinous cell walls of previous colonisers would be an important nitrogen source for later colonisers (Patil et al., 2000). Other roles of chitinases during interactions include morphogenetic changes, cell wall modifications, or permeabilisation of competitor cell walls to allow entry of toxic or antifungal compounds (Lindahl and Finlay, 2006).

Agar cultures have been used qualitatively to stain for laccase activity (White and Boddy, 1992; Iakovlev and Stenlid, 2000), and to quantify laccase activity during the interaction between *Heterobasidion annosum* and *R. bicolor* (Iakovlev and Stenlid, 2000). Although agar plates are not an ideal substrate in ecological terms, they allow sampling of different regions within the mycelia of interacting fungi.

The aim of the present study was to test whether laccase and peroxidases act as important determinants of the outcome of competition by direct action, or whether they are only an indirect consequence of stress or higher nutrient demand. To address this question, the spatial distribution of enzyme activities, their gene transcription, and the activities of other enzymes linked to nutrition were investigated during interactions involving *T. versicolor* that result in a range of outcomes. The role of oxidative enzymes as a defence mechanism against biotic and abiotic stress leads to the hypothesis that oxidative enzyme production is highest in interactions where *T. versicolor* is replaced by the competitor, compared to where it is successful (replaces or deadlocks with the competitor), and that enzyme activity declines as distance from the interaction zone increases. The effect of enhancing MnP production on laccase activity and interaction outcomes was also tested.

2. Methods

2.1. Isolates and culture conditions

Strains of *T. versicolor* (TvD2) and five other wood decay fungi were obtained from the Cardiff University Fungal Ecology Group culture collection (Table 1). Isolates were chosen to display a range of interaction outcomes during interactions with *T. versicolor* (Table 1). Cultures were maintained on 2% (w/v) malt agar (MA; 20 g Munton and Fison spray malt; 20 g Lab M agar No. 2; distilled water) in 9 cm plastic non-vented Petri dishes (Greiner Bio-one, Austria), incubated upside down at 20 °C in the dark. For each interspecific pairing 6 mm diameter plugs were removed from the margins of actively growing colonies using a No. 3 cork borer and inoculated 30 mm apart on 2% MA in 9 cm non-vented Petri dishes. Slower-growing species were inoculated earlier to ensure that competing mycelia met in the centre of the plate. Self-pairings of all isolates were used as controls.

2.2. Localisation of enzyme activity by staining

Interspecific interactions involving *T. versicolor* vs. *S. gausapatum*, *H. fasciculare* or *B. adusta*, and self-pairings, were set up in triplicate. Localisation of laccase activity was determined in 9 cm Petri dishes of 2% MA plus 250 mg l^{-1} 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), which is oxidised to form a green/violet product. MnP activity was localised by growth on 2% MA plus 0.5 mM manganese chloride ($MnCl_2 \cdot 4H_2O$), which is oxidised to form a brown precipitate (Steffen et al., 2000). Photographs of plates were taken, with a Nikon Coolpix camera, at 2 d, 8 d, 1 month and 2 months after establishment of mycelial contact, and interaction outcomes assessed after 2 months. For ABTS plates photos were taken on a lightbox in a dark room. Interaction outcomes were assessed visually after 2 months.

General peroxidase activity was visualized at three times, following methods adapted from Silar (2005). Briefly, 2, 5 and 8 d-old interactions on 2% MA in 9 cm Petri dishes were flooded with 2.5 mM diaminobenzidine (DAB) in phosphate buffer (0.1 M, pH 6.9), and incubated with gentle rotation for 30 min. The stain

Table 1
Competitor isolates and the outcome of their interaction with *T. versicolor* D2.

Ecological role	Species	Strain	Outcome of interaction with *T. versicolor*
Primary coloniser	*Stereum gausapatum*[b]	Sg1	Replacement by *T. versicolor*
	Daldinia concentrica[a]	290495	Replacement by *T. versicolor*
Early secondary coloniser	*Bjerkandera adusta*[b]	MA313	Deadlock/partial replacement by *B. adusta*
Heart rotter	*Fomes fomentarius*[b]	Ff1	Deadlock
Late secondary coloniser	*Hypholoma fasciculare*[b]	GTWV2	Replacement by *H. fasciculare*

[a] Ascomycota.
[b] Basidiomycota.

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was drained off and the location of a red precipitate was recorded after 4 h. Control plates were supplemented with buffer only.

2.3. Enzyme assays

Interspecific interactions between *T. versicolor* and the five combatant species (Table 1), plus all relevant self-pairing controls, were set up as previously described on 16 ml 2% MA in 9 cm non-vented Petri dishes. Material was removed for extraction at 2 d and 8 d after establishment of mycelial contact for combinations involving *S. gausapatum*, *B. adusta* and *H. fasciculare* – 8 d interactions showed the greatest differences in activity between treatments so material was removed at 8 d only for other interactions. Six plugs (5 mm diam.) were removed with a No. 2 cork borer from each of four regions of the interacting mycelia: the interaction zone itself (IZ); just behind the interaction zone in *T. versicolor* (A); 30–40 mm from the interaction zone within the *T. versicolor* mycelium (B); and just behind the interaction zone in the competitors' mycelium (C; Fig. 1). Total area of mycelium extracted was 118 mm^2 per region (approx. 200 mg wet weight). For self-pairings of combatant species, plugs were removed from the interaction zone only. Interactions were also set up on 16 ml 2% MA enriched with 0.5 mM $MnCl_2 \cdot 4H_2O$, and plugs removed from 8 d-old interactions.

Plugs were cut in half and transferred to a 1.5 ml Eppendorf tube, to which 1 ml deionised water was added. Tubes were shaken gently at 4 °C overnight, then 0.8 ml of extract removed and centrifuged (8000g) for 10 min at 4 °C to pellet any debris. Extracts were kept at 4 °C and assays performed immediately. Three replicate plates for each region, isolate and time-point were used. Data were normalised to the wet weight of agar plus mycelium extracted for each sample.

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of ABTS in citrate–phosphate buffer (100 mM citrate, 200 mM phosphate, pH 5.0), following Bourbonnais and Paice (1990). Formation of green coloration was followed spectrophotometrically at 420 nm. Manganese peroxidase (MnP; EC 1.11.1.13) activity was assayed according to Ngo and Lenhoff (1980) in succinate-lactate buffer (100 mM, pH 4.5), where MBTH (3-methyl-2-benzothiazoline-hydrazone hydrochloride) and DMAB (3-(dimethyl amino)-benzoic acid) were oxidatively coupled by MnP action, and formation of purple coloration followed spectrophotometrically at 590 nm. The results were corrected by activities of samples: (1) without manganese, where manganese sulphate was replaced by EDTA (ethylene diamine tetraacetate) to chelate Mn^{2+} present in the extract allowing detection of Mn^{2+}-independent peroxidases; and (2) in the absence of hydrogen peroxide (H_2O_2) to allow detection of activity of oxidases but not peroxidases. Lignin peroxidase (LiP; EC 1.11.1.14) activity was measured according to Collins et al. (1996) by monitoring the oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde in 250 mM tartaric acid buffer (pH 2.5) containing 5 mM H_2O_2, by the change in absorbance at 310 nm. One unit of enzyme activity (U) was defined as the amount of enzyme releasing 1 mM of product per min.

The activity of 1,4-β-glucosidase (EC 3.2.1.21) was assayed in microplates using *p*-nitrophenyl-β-D-glucoside (pNPG, Glycosynth, UK) as described previously (Valášková et al., 2007). The reaction mixture comprised 160 µl of 1.2 M pNPG in sodium acetate buffer (50 mM, pH 5.0) and a 40 µl sample. Reaction mixtures were incubated for 120 min at 40°C. The reaction was stopped by addition of 0.5 M sodium carbonate, and absorbance read at 400 nm. Enzyme activity was calculated using the molar extinction coefficient of *p*-nitrophenol (11,600 $M\ cm^{-1}$). One unit of enzyme activity (U) was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per minute. Activity of 1,4-N-acetylglucosaminidase (NAG) and phosphomonoesterase (acid phosphatase) were assayed using *p*-nitrophenyl N-acetyl-β-D-glucosaminide (pNPN; Glycosynth, UK) and *p*-nitrophenyl phosphate (pNPP; Glycosynth, UK), respectively, using the same method. Spectrophotometric measurements were made in a Bioscreen C II plate reader (Oy Growth Curves Ab Ltd., Finland) for laccase and MnP, a UV–vis spectrophotometer (Helios, Tecan, USA) for LiP, and all others in a Dynex Revelation microplate reader (Dynex Technologies Ltd., Sussex, UK).

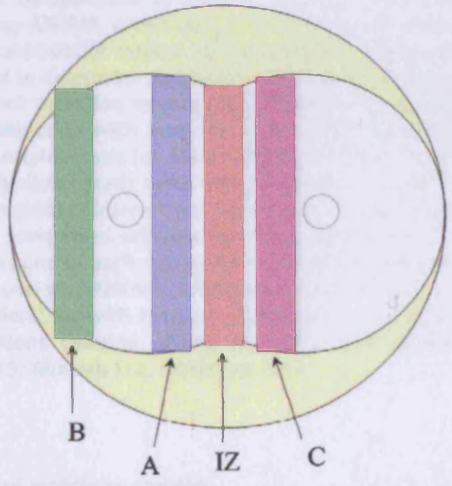

Fig. 1. Sampling regions in interacting mycelia for enzyme extraction. *T. versicolor* is on the left. Six agar plugs were removed from each of four different regions. Region 'IZ' corresponds to the interaction zone; 'A' is just behind the interaction zone in the *T. versicolor* mycelium; 'B' is further back in the *T. versicolor* mycelium; 'C' is just behind the interaction zone in the mycelium of the competitor.

2.4. RNA extraction

Mycelia of *T. versicolor* were allowed to interact with *S. gausapatum*, *B. adusta* and *H. fasciculare* for 2 and 8 d. Mycelium was skimmed from the surface of the agar from regions IZ, A and B (Fig. 1), with a sterile spatula. Each RNA extraction "biological" replicate used material derived from 30–50 plates; two biological replicates were performed for each region of each interaction at each time point. Mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle, and RNA was extracted using 2 ml tri-reagent (Sigma–Aldrich, Dorset, UK) according to manufacturer's instructions. After DNase treatment with RQ1 DNase (Promega, Southampton, UK), RNA was reverse-transcribed using M-MLV RNase H(-) Reverse Transcriptase (Promega) and oligo(dT) (Promega). cDNA presence was confirmed by PCR using the 18S rRNA primers EF4, GGA AGG GRT GTA TIT ATT AG, and fung5, GTA AAA GTC CTG GTT CCC C (Smit et al., 1999). The success of DNase treatment was confirmed by PCR using the treated RNA, where a lack of PCR product indicated successful DNase treatment.

2.5. Primer design

Laccase and peroxidase sequences were downloaded from GenBank (The National Centre for Biotechnology Information, NCBI, Bethesda, MD, USA: http://www.ncbi.nlm.nih.gov) and aligned using BioEdit. Primers were designed to subgroup level rather than to individual sequences, using the programs Primer 3 (http://www.frodo.wi.mit.edu) and Oligoanalyzer (http://www.idtdna.com). Primer sequences were (5′–3′): *lacc*, Lacc-F CTT CAA CGG CAC CAA CTT CTT, Lacc-R GAA GTC GAT GTG GCA GTG GAG; *MnP*, MnP-F CAT CTC TCC TTC CAT CGC CTC, MnP-R GGG GCA GTT GCT GAC ACC; *MRP*, MRP-F CAG GAC AAG GTT GAC GAC T, MRP-R GGA CTG AAG ACG GAA CTC; *LiP*, LiP-F ACG AAA TCT TGA CCG TGT G,

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LiP-R GAG CGA GTT GAT GTC CTG. Specificity to *T. versicolor* sequences was confirmed by PCR with competitor cDNA (Supplementary Fig. 1). The amplified regions ranged between approx. 170–350 bp. Primer specificity was confirmed by cloning and sequencing of PCR products (results not shown).

2.6. Semi-quantitative RT-PCR

Reactions were perfomed in a Techne Flexigene thermocycler (Bibby Scientific, Staffordshire, UK) using Hotstar Taq polymerase (Qiagen, Crawley, UK), and the program: 94 °C for 15 min, [94 °C for 1 min, T_m for 1 min, 72 °C for 1 min] × 30–35 cycles, 72 °C for 6 min. T_m (annealing temperature) used was 50 °C for *MRP* and *MnP* primers, 55 °C for *lacc*, *LiP* and glyceraldehyde 3-phosphate dehydrogenase (*GPD*) primers. At least three replicates were performed for each primer set with each cDNA sample to eliminate equipment variability. PCR products were separated by agarose gel electrophoresis and quantified using the Gene Genius bioimaging system and GeneSnap software (Syngene, Synoptics Ltd., Cambridge, UK).

Normalisation was performed to *GPD* trancript levels using the general fungal primers: GPD-F, GTT CAA GTA CGA CTC CGT CCA; GPD-R, ACT TTT CGG TGG TGG TGA AG (designed by the authors from published sequences). Normalisation of *T. versicolor* cDNA from the interaction zone, where there was also the presence of competitor cDNA, was performed to *T. versicolor*-specific *GPD* sequences obtained with the primers TvGPD-F, ACC GCA TAC ATC CTA ATC TCG; and GPD-R (as above; designed by the authors from published sequences). Reactions were cycled as above, and product quantitation from the *GPD* target used to normalise results for the other primer sets. Cycle number was optimised and limited for each primer set and cDNA batch combination, by running dilution series of the cDNA and checking for linear responses. This ensured that reactions were in the exponential phase at a particular cycle number, allowing product quantitation to be considered semi-quantitative with respect to transcript abundance. This methodology has previously been used successfully for a range of experimental systems (including Parfitt et al., 2005; Wagstaff et al., 2005; Orchard et al., 2005; Price et al., 2008).

2.7. Statistical analyses

Statistical comparisons of enzyme activity were performed using one-way ANOVA, where data met the required assumptions, with significant results further tested using the Tukey–Kramer *a posteriori* test to determine significant differences between means. If data did not meet the assumptions of ANOVA, Kruskal–Wallis tests in combination with *post hoc* Mann–Whitney *U*-tests were used. Two-sample *t*-tests (or Mann–Whitney *U*-tests if data were non-normally distributed), were used to compare enzyme activity in different regions of interactions with the equivalent regions in *T. versicolor* or competitor self-pairings. Gene expression relative to other interactions in each region for each primer set were compared using one-way ANOVA (following arcsine transformation of percentage data), or with Kruskal–Wallis based on data meeting the assumptions detailed above. All tests were performed in Minitab (v. 15; Minitab Ltd., Coventry, UK).

3. Results

3.1. Location of activity by staining

Strong enzyme activity was associated with interaction zones in all three pairings irrespective of outcome (Fig. 2). However, for laccase activity, there was clearing at the interaction zone in TvSg (interaction with *S. gausapatum*, resulting in replacement by *T. versicolor*) and TvBa (interaction with *B. adusta* resulting in deadlock), but not in TvHf (interaction with *H. fasciculare*, resulting in replacement of *T. versicolor*; Fig. 2A, D and G). Clearing indicated greater laccase production in these interactions (with further oxidation of ABTS to a colourless derivative). There was distinct association of MnP activity along the length of the interaction zones in TvSg and TvBa (Fig. 2B and E), but it was patchy along the TvHf interaction zone (replacement by *H. fasciculare*) with activity in regions where *H. fasciculare* invasive hyphal cords began to develop (Fig. 2 H). The presence of Mn^{2+} affected the interaction outcome of TvHf, which resulted in deadlock, rather than the replacement of *T. versicolor* by *H. fasciculare* that occurred in the absence of Mn^{2+}. General peroxidase activity was associated with interaction zones again irrespective of interaction outcome (Fig. 2C, F and I).

3.2. Enzyme activity of T. versicolor self-pairings on 2% MA

Activity of laccase, MnP, β-glucosidase, NAG and acid phosphatase were detected in culture extracts of *T. versicolor*; production by competitors varied (Table 2). LiP and Mn-independent peroxidase activity were not detected either 2 or 8 d after contact in *T. versicolor* self-pairings or during interactions (Table 2). There were no significant differences ($P > 0.05$) in laccase activity in any region of *T. versicolor* self-pairings at 2 or 8 d after contact (Table 2), but for the other enzymes detected there were significant differences ($P \leqslant 0.05$) in activity levels in different regions of the *T. versicolor* self-pairing (Table 2).

3.3. Enzyme activity

Differences in enzyme activity between regions were evident, with highly localised production at the interaction zone (Table 2). Differences in enzyme activity between interactions, or between regions within an interaction, were more pronounced at 8 d than 2 d (Tables 2 and 3; Suppl Fig. 2). At 8 d, laccase and MnP activities were highest in the IZ region in all interactions except TvFf (interaction with *F. fomentarius*; Table 2, Suppl Fig. 3). There were no significant differences ($P > 0.05$) in laccase or MnP activity between the A and B regions of any interaction, with the single exception of 8 d TvSg where laccase activity in the A region was significantly ($P \leqslant 0.01$) higher than in the B region (Table 2). Thus in general there was a sharp drop from the IZ to regions behind the IZ. The activity in the C region, i.e. the competitor mycelium, varied depending on the species. In all interactions bar one (TvDc) the extracted C regions were colonised by competitor only. However, in TvDc, MnP was detected in the C region which implies overgrowth of *D. concentrica* by *T. versicolor*; as an ascomycete, *D. concentrica* would not produce peroxidases capable of oxidising Mn^{2+}. This was not visually discernible when extractions were performed.

For all enzymes detected, there was significantly ($P \leqslant 0.05$) higher IZ enzyme activity in interactions compared to self-pairings of at least one of the competitors (Table 2). However, in TvFf non-contact inhibition occurred and the activity of all enzymes were distinctly lower in the IZ, A and C regions compared to other interactions (Table 2). There were few differences in activity in the A and B regions of interactions compared to the equivalent regions in *T. versicolor* self-pairings, with the notable exception of TvSg B region which had significantly ($P \leqslant 0.01$) lower activity of all enzymes except MnP compared to *T. versicolor* self-pairing B region (Table 2). For β-glucosidase, NAG and acid phosphatase, differences in activity in different regions of interacting *T. versicolor* mycelia were small and varied depending on the competitor (Table 2).

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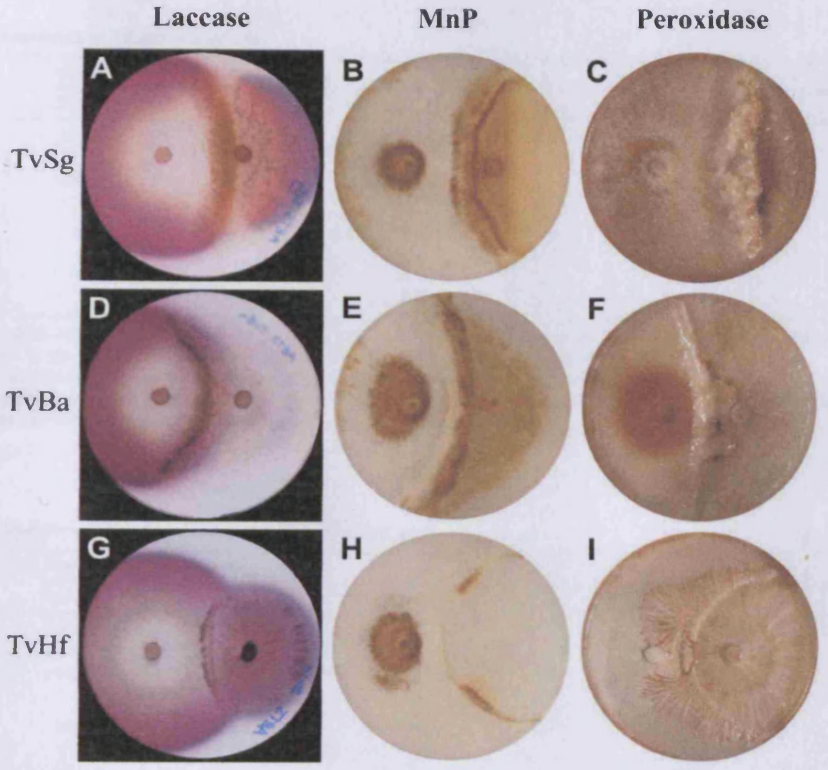

Fig. 2. Localisation of laccase, MnP and peroxidase revealed by staining interactions involving *T. versicolor* on 2% MA. *T. versicolor* is on the left in all pairings. (A–C), vs. *S. gausapatum*; (D–F), vs. *B. adusta*; (G–I), vs. *H. fasciculare*. A, D and G, laccase activity generates a purple stain, 2 d-old interactions; B, E and H, MnP activity results in deposition of a brown precipitate, 8 d-old interactions; C, F and I, peroxidase reacts with DAB to form a red-brown precipitate, 8 d-old interactions.

Table 2
Enzyme activity of *T. versicolor* and competitors in 8 d-old interactions.

Enzyme	Region	Activity mU g^{-1} wet weight										
		TvTv	SgSg	TvSg	DcDc	TvDc[a]	BaBa	TvBa	FfFf	TvFf	HfHf	TvHf
Laccase	IZ	216.0	27.3	3218.9*,†a	0.0	877.8*,†a	1.0	871.6*,†a	0.7	141.8†a	31.4	417.1*,†a
	A	238.3	–	449.8*b	–	263.4 b	–	376.6*ab	–	149.1 a	–	253.6 b
	B	243.7	–	74.5*c	–	189.7 b	–	222.2 b	–	215.5 a	–	202.7 bc
	C	–	–	0.2 †d	–	221.7†b	–	5.9 c	–	3.3 †b	–	112.6†c
MnP	IZ	0.0	2.7	33.6*,†a	0.0	10.4*,†a	3.9	11.0*,†a	0.0	5.9*,†a	8.9	8.7*
	A	0.0	–	7.7*ab	–	0.9 b	–	6.8*ab	–	0.0 b	–	6.1*
	B	0.0	–	2.6*b	–	0.0 b	–	4.7*b	–	0.0 b	–	6.3*
	C	–	–	6.0 b	–	3.8†a	–	6.0 ab	–	0.2 b	–	6.6
β-glucosidase	IZ	5.6 a	12.9	11.7*a	15.7	4.9†	4.6	37.7*,†a	2.8	2.0*a	6.2	3.4†a
	A	7.8 a	–	4.5*b	–	6.4*	–	6.0 b	–	4.4*b	–	10.5 b
	B	11.1 b	–	1.3*b	–	6.0	–	8.9 b	–	7.4*c	–	8.3 c
	C	–	–	3.9 †b	–	7.3†	–	2.4 c	–	4.1 b	–	7.3 c
NAG	IZ	0.4 a	13.3	3.7 a	49.1	1.0†a	3.8	10.0*a	9.4	1.1 a	0.6	1.5*,†a
	A	0.5 a	–	0.8 b	–	0.9 a	–	0.8 b	–	0.4 b	–	1.7*a
	B	1.0 b	–	0.3*b	–	0.8 a	–	0.8 b	–	0.7 ab	–	1.2 b
	C	–	–	4.7*a	–	19.5†b	–	1.0†b	–	10.4 c	–	0.1 b
Acid phosphatase	IZ	2.1 a	9.4	19.5*,†a	53.2	8.4*,†a	0.4	10.0*,†a	3.2	4.4*	3.9	2.9†a
	A	2.8 ab	–	2.0 b	–	3.7 b	–	1.8 b	–	3.2	–	2.9 a
	B	4.3 b	–	0.9*c	–	3.0 b	–	1.5*b	–	4.6	–	2.1*a
	C	–	–	10.5 d	–	44.7 c	–	0.4 c	–	3.8	–	6.0†b

Values are means of 3 replicates. –, samples not taken from this region.
* Significant difference ($P \leq 0.05$) in activity compared with the corresponding region in the *T. versicolor* self-pairing (TvTv).
[a] Competitor probably overgrown by *T. versicolor*; IZ, interaction zone; A, the region adjacent to the interaction zone within *T. versicolor* mycelium; B, the region further back within *T. versicolor*; C, the region adjacent to the interaction zone within competitor mycelium (see Fig. 1); NAG, 1,4-N-acetylglucosaminidase.
† Significant difference ($P \leq 0.05$) in activity compared with the competitor self-pairing; different letters indicate significant differences ($P < 0.05$) in enzyme activity between different regions within an interaction (no letters indicates no significant differences, $P > 0.05$).

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Table 3
Enzyme activity of *T. versicolor* and competitors in 2 d-old interactions.

Enzyme	Region	Activity mU g^{-1} wet weight						
		TvTv	SgSg	TvSg	BaBa	TvBa	HfHf	TvHf
Laccase	IZ	318.9	6.2	907.2*,†a	0.5	487.7†a	41.0	615.8*,†a
	A	261.6	–	330.7 b	–	349.4*b	–	333.0 b
	B	303.4	–	329.2 b	–	361.5 b	–	368.0 b
	C	–	–	12.2 †c	–	10.1 c	–	78.3†c
MnP	IZ	6.6 a	0.0	37.3*,†a	3.1	13.3†	25.9	15.6†a
	A	1.0 b	–	0.0 b	–	7.5*	–	1.8 b
	B	0.5 b	–	0.9 c	–	9.1*	–	4.0 b
	C	–	–	1.0†c	–	9.6†	–	5.4†b

Values are means of 3 replicates. –, samples not taken from this region; IZ, interaction zone; A, the region adjacent to the interaction zone within *T. versicolor* mycelium; B, the region further back within *T. versicolor*; C, the region adjacent to the interaction zone within competitor mycelium (see Fig. 1).
* Significant difference ($P \leqslant 0.05$) in activity compared with the corresponding region in the *T. versicolor* self-pairing (TvTv);
† Significant difference ($P \leqslant 0.05$) in activity compared with the competitor self-pairing; different letters indicate significant differences ($P < 0.05$) in enzyme activity between different regions within an interaction (no letters indicates no significant differences, $P > 0.05$).

Table 4
Enzyme activity of *T. versicolor* and competitors in 8 d-old interactions on Mn-enriched agar.

Enzyme	Region	Activity mU g^{-1} wet weight						
		TvTv	SgSg	TvSg	BaBa	TvBa	HfHf	TvHf
Laccase	IZ	109.6	30.5	3060.5*,†a	0.1	340.5*,†a	9.1	1273.2*,†a
	A	102.3	–	170.7 b	–	125.7 b	–	201.8*b
	B	116.7	–	31.3*c	–	53.2*c	–	60.8*c
	C	–	–	12.6 c	–	0.1 d	–	8.4 d
MnP	IZ	29.1 ab	1.5	193.8*,†a	70.7	57.4*	53.3	110.6*,†a
	A	35.3 a	–	61.9*b	–	49.2*	–	38.4 b
	B	19.9 b	–	49.9*b	–	49.0*	–	32.8 bc
	C	–	–	5.8†c	–	53.7	–	20.8†c

Values are means of 3 replicates. –, samples not taken from this region; IZ, interaction zone; A, the region adjacent to the interaction zone within *T. versicolor* mycelium; B, the region further back within *T. versicolor*; C, the region adjacent to the interaction zone within competitor mycelium (see Fig. 1).
* Significant difference ($P \leqslant 0.05$) in activity compared with the corresponding region in the *T. versicolor* self-pairing (TvTv).
† Significant difference ($P \leqslant 0.05$) in activity compared with the competitor self-pairing; different letters indicate significant differences ($P < 0.05$) in enzyme activity between different regions within an interaction (no letters indicates no significant differences, $P > 0.05$).

3.4. Enzyme activity during interactions on Mn^{2+}-enriched agar

Growth on Mn^{2+}-enriched agar stimulated laccase production in *T. versicolor* mycelia during interaction with *H. fasciculare* (Tables 2 and 4); laccase activities in the IZ and A regions of TvHf were significantly ($P \leqslant 0.05$) higher than the equivalent regions in TvTv on Mn^{2+}-enriched agar, which did not occur during growth on 2% MA. *H. fasciculare* laccase activity appeared to be repressed on the Mn^{2+}-enriched agar since there was down-regulation in the TvHf C region relative to growth on 2% MA (Tables 2 and 4). Growth on Mn^{2+}-enriched agar stimulated MnP activity in *T. versicolor* self-pairings, and during interactions the pattern of MnP activity at the IZ was similar to the pattern of laccase activity: all interactions had significantly ($P \leqslant 0.01$) higher activity than TvTv, with TvSg > TvHf > TvBa (Table 4). Similar patterns of activity in laccase and MnP did not occur in the A and B regions (Table 4).

3.5. Expression of laccase, MRP and LiP genes during interactions

lacc gene expression was detected in all regions of all the interactions and the TvTv self-pairing. There was significantly ($P \leqslant 0.01$) higher *lacc* expression in the IZ region of TvHf compared to TvTv IZ, and this higher expression was maintained in the A region but not in the B region (Fig. 3). In the B region, expression in TvBa was significantly higher, and expression in TvSg B significantly lower ($P \leqslant 0.05$) than in TvTv B (Fig. 3). No significant differences in *MRP* expression were detected in any region, although transcript levels were higher in the IZ regions of TvSg and TvHf compared to TvTv IZ (Fig. 3). *LiP* transcripts were detected in regions A and B though not at the IZ. However, a higher cycle number was required to measure *LiP* RT-PCRs than with for *lacc* and *MRP* primers, which may suggest a lower transcript abundance. In the A region, expression of *LiP* was similar in TvTv and TvBa, significantly ($P \leqslant 0.05$) higher in TvHf and significantly ($P \leqslant 0.01$) lower in TvSg compared to TvTv (Fig. 3). Expression of *LiP* in the B region was similar in TvTv and TvSg, with higher expression in TvBa B and TvHf B, but these differences were not significant ($P > 0.05$; Fig. 3).

No *MnP* transcripts were detected at 2 d using MnP-F/R primers. When tested on *T. versicolor* genomic DNA and cDNA, MnP-F/R amplified sequences from genomic DNA but not cDNA (Suppl Fig. 1B). Two other sets of primers specific for *MnP*, from Johansson et al. (2002), also did not detect expression in *T. versicolor* cDNA (results not shown) indicating that indeed expression was absent or at undetectably low levels.

4. Discussion

4.1. Oxidative enzyme activity increased during interactions but was not linked to interaction outcome

Of the enzymes assayed, laccase showed the largest increase in activity during interactions: 14-fold that of self-pairings in the TvSg interaction zone. This large laccase response agrees with previous findings for *T. versicolor* during interactions (White and Boddy, 1992; Baldrian, 2004; Baldrian, 2006). However, the increased MnP activity during interactions had not been previously reported in *T. versicolor*, although it has been reported in other species

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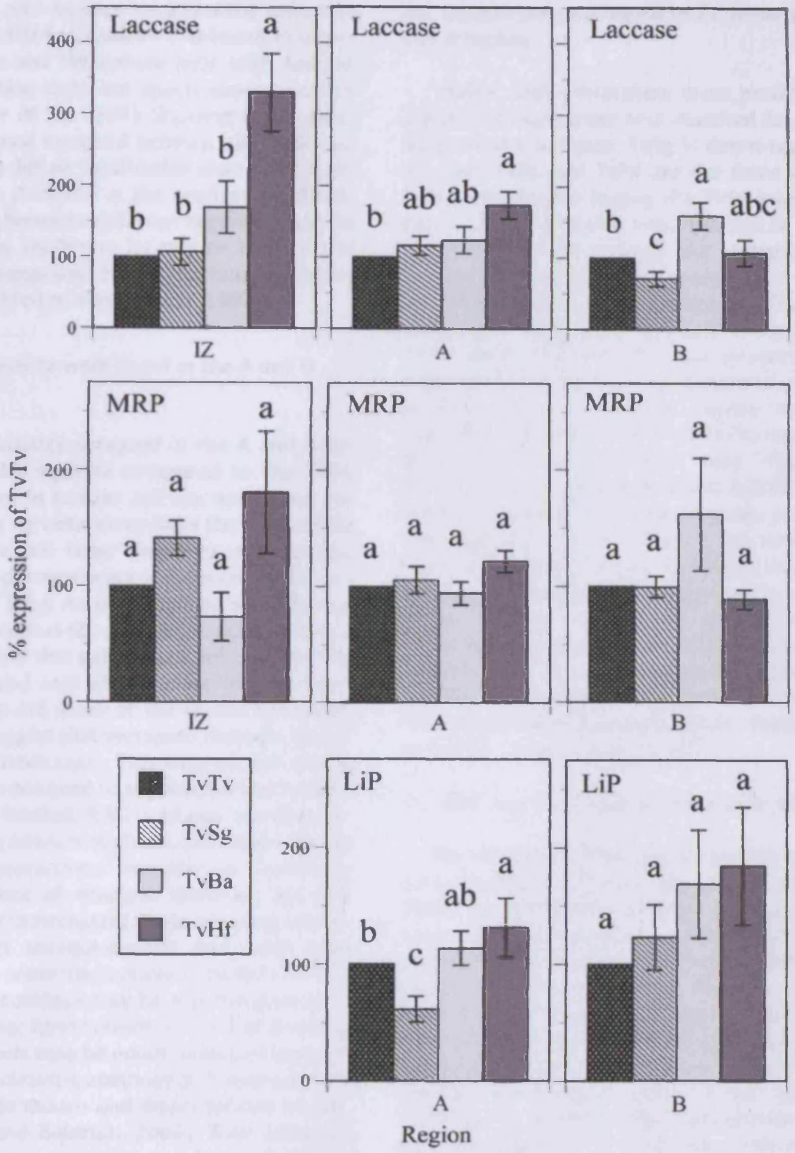

Fig. 3. Expression of laccase, MRP and LiP genes by *T. versicolor* mycelia during self-pairings (TvTv) and interactions with *S. gausapatum* (TvSg), *B. adusta* (TvBa) or *H. fasciculare* (TvHf) at 2 d. Bars are the mean data for two biological replicates, each consisting of three technical replicates, ±SEM. Data are expressed as% transcript abundance of TvTv cDNA samples from each region. Different letters indicate significant differences ($P \leq 0.05$).

(Baldrian, 2004; Chi et al., 2007). Enzyme activity was higher at the interaction zone than in other regions of the interaction, as has been previously reported (Iakovlev and Stenlid, 2000). However, the extent of this increase varied because of production by the competitor and/or modulation of the *T. versicolor* response based on the identity of the competitor. Different levels of enzymatic response to different competitors has been reported for a variety of interactions, including those involving *Pleurotus ostreatus* (Iakovlev and Stenlid, 2000; Chi et al., 2007).

There were no clear patterns of laccase and MnP activity in interactions with different outcomes; similar patterns of activity occurred in the two interactions where *T. versicolor* replaced the competitor (TvSg and TvDc), one of the two interactions which resulted in deadlock (TvBa), and in the interaction that resulted in replacement of *T. versicolor* (TvHf). Enzyme activity in the interaction TvFf, which resulted in deadlock, was strikingly different to other interactions due to non-contact inhibition, with a clear zone occurring between the competing mycelia. These results differ from liquid culture interactions involving *S. hirsutum* (demonstrated to be the likely source of laccase), where oxidative enzyme activity was much higher in interactions where *S. hirsutum* was replaced than where it was successful (Peiris, 2009). It has been suggested that saprotrophic fungi from later successional stages (i.e. more combative species) have higher laccase activity than those from earlier stages (Iakovlev and Stenlid, 2000), but this did not occur here as *S. gausapatum*, a primary coloniser, had roughly similar levels of laccase production during growth alone to those of *H. fasciculare*, a late-stage cord-forming species. *T. versicolor* had far higher levels of laccase production than *H. fasciculare*, despite being a late primary to early secondary coloniser.

4.2. Enzyme activity was highly localised to different regions within interactions

The increases in oxidative enzyme activity at the IZ were highly localised, which could result from very specific production of

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enzymes and their co-factors, and implies very limited diffusion from the point of secretion. Similar localisation was found in interactions involving *H. fasciculare* and *Peniophora lycii*, with laccase activity highest in the interaction zone but much lower in other areas of the mycelium (Rayner et al., 1994). Staining for laccase, MnP and peroxidase also showed localised activity, although the laccase staining indicated more diffuse localisation than did the assays most probably due to the diffusion of the reaction products. The stark contrasts in activity between different regions could be caused by secretion of enzyme inhibitors by one or both of the antagonists, which has been suggested for interactions between *T. versicolor* and *T. harzianum* (Freitag and Morrell, 1992).

4.3. Minor changes in enzyme activity were found in the A and B regions of interactions

Minor changes in enzyme activity occurred in the A and B regions of interacting *T. versicolor* mycelia compared to the TvTv self-pairing. Qualitative changes in laccase activity have been reported in regions of interacting mycelia away from the interaction zone, but these changes were not large (Iakovlev and Stenlid, 2000). Here, the only notable changes were the decreases in laccase activity in the B region of TvSg. An overall pattern for laccase and MnP activity can be expressed as IZ > A ⩾ B (i.e. activity in the IZ was greater than in A or B), but this pattern did not consistently occur for β-glucosidase, NAG and acid phosphatase. Whilst some significant increases in activity did occur at the IZ, the decreases in activity in A and B regions suggest that increased nutrient acquisition does not occur during interactions. This may be due to the rich substrate: other regions do not need to support the interaction zone because nutrients are not limited. NAG is an exo-cleaving enzyme, involved in the final degradation of chitin, and might be expected to increase during interactions in order to hydrolyse competitor cell walls as a source of nitrogen. However, the rich growth medium may make this unnecessary. Endo-cleaving chitinases, however, could function antagonistically and have been shown to increase in activity in some interactions (Lindahl and Finlay, 2006). Production of β-glucosidase may be repressed on malt agar, where there is no cellulose; furthermore, activity of β-glucosidase and NAG in culture extracts may be underestimated because significant amounts of the exo-cleaving enzymes of *T. versicolor* are mycelium-associated to provide mono- and disaccharides for uptake into hyphae (Valášková and Baldrian, 2006). Thus although no increases in secreted enzyme activity were detected during interactions, increased production of enzymes involved in nutrient acquisition cannot be excluded.

4.4. Overall, changes in gene expression were not dramatic

Despite the large changes detected in enzyme activity between interacting *T. versicolor* mycelia and self-pairings, changes in gene expression were not dramatic. This agrees with the microarray data for the *T. versicolor* A region during interactions, where few targets were up- or down-regulated by more than twofold during interactions (C.A. Eyre et al., unpub'd data). Transcriptional changes are thought to either be relatively minor compared to growth alone, or transient (C.A. Eyre et al., unpub'd data) reflecting the dynamic nature of mycelial responses. However, laccase and peroxidase enzymes are stable in the extracellular environment; high activity can thus be a result of a relatively short period of increased expression, and, with hyphal contact having been established 2 d prior to sampling, this window may have been missed. Also, expression of different isozymes with higher activity may have occurred. Changes in gene expression may also have been missed due to the methodology used.

4.5. Laccase gene expression at 2 d is similar to activity at 8 d in the A and B regions

Whilst RNA extractions were performed on 2 d cultures, the pattern of expression best matched laccase activity from 8 d cultures. In the B region, TvSg is down-regulated compared to TvTv, whereas TvBa and TvHf are the same or higher. In the A region, activity of laccase during the TvSg interaction at 8 d was greater than in TvTv, and this was reflected in slightly higher expression of the transcripts (though not statistically significant). Likewise the enzyme and transcript patterns for TvBa match, though again not fully supported by the statistics. The major difference was between activity in the A region of TvHf at 8 d, which was the same as TvTv, while the expression was elevated. The most striking difference was at the IZ; here the pattern of expression did not resemble activity at 2 or 8 d. Laccase activity increased relative to TvTv in TvSg and TvBa but not TvHf, whereas transcript levels of TvSg and TvBa were not different from TvTv but were higher in TvHf. However, at the IZ interpretation is difficult due to potential contribution from both interacting species, whereas the transcript levels were specific to *T. versicolor*. A lag between gene expression and enzyme activity is normal and has been reported in interacting cultures of *P. ostreatus* (Velázquez-Cedeño et al., 2007). Other discrepancies between expression and activity of laccase could be explained by rapid release of laccase from intracellular stores, thus not involving *de novo* transcription. Dramatic increases in laccase activity, attributable to activation of latent forms, have previously been reported in *Lentinula edodes* during interaction with *Trichoderma* sp. (Savoie et al., 1998).

4.6. MRP, not MnP, may be responsible for the detected Mn-oxidation

The increase in MnP activity during interaction differs from previous findings for *T. versicolor* (Freitag and Morrell, 1992; Baldrian, 2004), but it has been found for several other white rot species (Chi et al., 2007; Ferreira-Gregorio et al., 2007). MnP was detected in all regions of 8 d-old interacting mycelia but not in self-pairings, with activity consistently highest at the IZ, implying a specific role for MnP during interactions. However, there was an apparent lack of MnP gene expression, which was supported by detection of MRP expression, indicating low levels of manganese in the agar which would repress transcription of MnP genes (Collins et al., 1999; Johansson et al., 2002). MRP can function like MnP and LiP, with active sites capable of oxidising veratryl alcohol (LiP), and Mn^{2+} (MnP), similar to the versatile peroxidase (VP) that occurs in a range of species (Ruiz-Dueñas et al., 2009). Whilst MRP has previously been isolated under conditions of Mn-repression, this transcriptional control does not exclude the ability of the enzyme to act like MnP when Mn^{2+} levels are rapidly increased such as in the assay substrate solution (Collins et al., 1999). MRP could thus be responsible for the MnP activity detected in the assays. The LiP-like isozyme LiP7 can also function like both LiP and MnP (Johansson et al., 1993), so could also contribute to the detected MnP activity, and would not have been detected by the LiP primers used.

MRP transcript levels resembled more closely patterns of Mn-oxidation at 2 d rather than 8 d. In the IZ, MnP activity was elevated in TvSg and TvHf interactions and an increase was also seen in transcripts of MRP, although not statistically significant. The enzyme activity in TvBa at the IZ was less well correlated with the transcripts, which appeared repressed. In the A and B regions, TvSg and TvHf enzyme activities did not differ from TvTv and the same is true for transcript levels. The only significant change in enzyme activity compared to TvTv in the A and B regions was in the TvBa interaction, where activity was higher; transcripts were a little elevated in the B region though again not significantly. This might

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indicate a more rapid production of active secreted enzyme from transcripts for MRP compared to laccase.

4.7. Expression of LiP is not reflected in enzyme activity

LiP enzyme activity was not detected during growth alone or during interactions, but LiP gene expression was at measurable levels. Higher expression of LiP compared to TvTv occurred in TvHf in both A and B regions, and in TvBa in the B region (though not fully supported statistically). This might indicate a role for LiP during interactions. The lack of detectable activity was either attributable to low production (as suggested by the high cycle number necessary for RT-PCR), or caused by lower sensitivity of the assay. LiP has not previously been identified in the response to biotic stresses such as interactions (Baldrian, 2004). There is some evidence that, despite its extracellular role, LiP is stored intracellularly, which may explain why expression was detected from whole mycelia whereas there was no detectable secreted activity (Garcia et al., 1987).

4.8. Addition of Mn^{2+} to the medium increased oxidative enzyme production

Mn^{2+} is known to regulate MnP and MRP in *T. versicolor*, and it also alters the production of laccase (Mikiashvili et al., 2005). On Mn-enriched agar, there was a similar pattern of Mn-oxidation and laccase activity at the interaction zone, implying similar regulation of these enzymes in this region when there is ample Mn^{2+}. MnP (rather than MRP) is likely to be the cause of the observed activity due to the abundance of Mn^{2+} in the substrate, but this has not been confirmed by gene expression analysis. At the TvSg and TvHf interaction zones, laccase activity relative to TvTv was much higher during growth on Mn-enriched agar compared to growth on 2% MA, but activity levels were similar in TvBa relative to TvTv on both substrates. This may be caused by different susceptibility of the competitor to Mn^{2+}, or stimulation of laccase production by increased levels of quinone products of MnP activity, to function in their detoxification similar to the suggested role of laccase during lignin decomposition (Solomon et al., 1996; Thurston, 1994). The differences in laccase and MnP activity in the A and B regions of interactions during growth on Mn-enriched agar suggests that whilst the roles of MnP and laccase at the interaction zone may be similar, roles in other areas of the mycelium are not. Abiotic conditions have previously been shown to alter interaction outcomes (Rayner and Boddy, 1988; Boddy, 2000). Here, Mn^{2+} levels changed the outcome of interactions with *H. fasciculare* from replacement of *T. versicolor* to deadlock. This may have been caused by increased MnP production by *T. versicolor*, or the effects of Mn^{2+} on other factors. The Mn^{2+} levels used here were 5 to 10 times those in wood or leaf litter (Baldrian et al., 2005), but although not ecologically relevant, this manipulation of enzyme production shows the importance of abiotic conditions on oxidative enzyme production and suggests a possible link between enzyme production and interaction outcome.

5. Conclusions

There were large increases in the activity of laccase and MnP at interaction zones, but, as hypothesised, there were no large changes in activity in other regions of interacting *T. versicolor* mycelia. However, the hypothesis that laccase production is highest in interactions where *T. versicolor* is unsuccessful has to be rejected, since interaction outcome did not appear to affect the extent of the increase in laccase or MnP activity at the interaction zone, nor did it appear to affect the pattern of activity in other regions of the *T. versicolor* mycelia. Overall the small changes in laccase transcript levels compared to large changes in enzyme activity suggest transient changes in gene expression and the accumulation of stable enzymes over time. The lack of MnP gene expression but detection of MnP activity – peroxidase-mediated Mn-oxidation – is likely attributable to MRP, which has similar patterns of expression to the detected activity.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.03.007.

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