Effects of Volatiles and Stress on Growth, Enzyme Activity and Gene Expression of *Trametes versicolor*

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Abbreviations

5'-RACE	Rapid amplification of 5' complementary DNA ends
A	Adenine
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Ba	*Bjerkandra adusta* isolate BK1
BLAST	Basic local alignment search tool
C	Cytosine
cDNA	Complementary DNA
DDHF3	*Hypholoma fasciculare* isolate DDHF3
DDHF4	*Hypholoma fasciculare* isolate DDHF4
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOCs	Diffusible organic compounds
ds cDNA	Double stranded cDNA
DTT	Dithio-1,4-threitol
EDTA	Ethylenediamine tetraacetic acid
Es	*Eutypa spinosa*
EST	Expressed sequenced tags
EtBr	Ethidium bromide
Ff	*Fomes fomentarius*
G	Guanine
GC	Gas chromatograph
GC-MS	Gas chromatrography-mass spectrometry
Hf3	*Hypholoma fasciculare* Hf DD3
Hf4	*Hypholoma fasciculare* Hf DD4
IPTG	Isopropyl-1-thio-ß-D-galactopyranoside
Lacc	Laccase gene
LiP	Lignin peroxidase
MA	Malt extract agar
MGED	Microarray gene expression data

MnP	Manganese peroxidase
mRNA	Messenger RNA
MS	Mass spectrometer
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
Nox	NADPH gene
NVBI	National Centre for Biotechnology Information
NERC	Natural Environment Research Council
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PEI	Polyethylenimine
polyA	polyadenylated
Rb	*Resinicium bicolor*
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT	Retention time
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
SDS	Sodium docecyl sulphate
SE	Standard error
SEM	Standard error of mean
Sg	*Stereum gausapatum*
Sg/Sg	*S. gausapatum* vs *S. gausapatum*
SH	Subtractive hybridisation
Sh	*Stereum hirsutum*
Ss cDNA	Single stranded cDNA
SSH	Suppression subtractive hybridisation
T	Thymine
TAE	Tri-acetate-EDTA
TrEMBL	Translated sequences from the EMBL database
Tv	*Trametes versicolor*
TvD1	*Trametes versicolor* isolate D1
Tv/Bk	*T. versicolor* vs *B. adusta* interaction
Tv/Hf	*T. versicolor* vs *H. fasciculare* interaction
Tv/Sg	*T. versicolor* vs S. gausapatum interaction

Tv/Tv	*T. versicolor* vs *T. versicolor* self pairing
U	Uracil
Vc	*Vuilleminia comedens*
VOCs	Volatile organic compounds

Abstract

Lignin degrading (white-rot) basidiomycete fungi are major agents of carbon cycling and play a key role in maintaining forest ecosystems. *Trametes versicolor* is a white-rot fungus of both industrial and ecological interest. The aims of this study were to investigate the effects of biotic and abiotic stress factors on growth, ligninolytic enzyme (laccase) production and gene expression in white-rot fungi, using *T. versicolor* as a model for most of the work. Mycelial interactions are important in defining community structure in wood-rotting fungi, and during these interactions volatile organic compounds (VOCs) are produced which can affect the growth of other fungi, at a distance. The effects of different combinations of interacting fungi on *T. versicolor* growth and ligninolytic enzyme production showed that effects were variable. Effects of VOCs from interaction on wood blocks were more significant than those on agar.

Abiotic stress factors affect fungal metabolism and thereby regulate their biological activity. This study investigated the effect of abiotic stress factors on *T. versicolor* using different temperatures (low and high), osmotic pressure (KCl) and nutrients (no and low nitrogen). Growth rate, laccase production and expression of three genes (FRA19, Nox, and Lacc) were mainly reduced when the abiotic stress was imposed, although there was some variability in gene expression. Laccase is encoded by a gene family and differential expression of gene family members under stress treatments was investigated. Some evidence for an over-representation of ? group sequences following stress treatments was found. Future directions are discussed to further investigate the roles of biotic and abiotic stress in regulating the growth and underlying biological processes of white rot fungi.

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Chapter 1: Introduction

1.1 Fungal communities in nature

Without fungi, terrestrial ecosystems on Planet Earth would not function. Fungi play key roles as mycorrhizal symbionts, lichens, endophytes, pathogens and saprotrophs. Mycorrhizal symbiots play a major role in the uptake of nutrients by plants, and in protection against drought stress and against pathogens. Lichens - a mutualistic relationship between a fungus and a photobiont - are significant primary producers in extreme environments. Endophytes also often confer advantages to plants in stressful environments, against pathogens, and against invertebrate and vertebrate grazers. Pathogens, in contrast, harm plants and introduce plant materials to the decomposition process sooner than normal. Saprotrophs decompose dead organic matter and release nutrients for use by other organisms, including plants. This thesis concentrates on saprotrophs that are able to decompose wood (Chapela and Boddy, 1988; Boddy, 1993; Boddy, 1999; Boddy, 2000; Bretherton *et al*., 2006; Rotheray *et al*., 2008).

Both soil and woodland environments have high fungal diversity. The woodland ecosystem comprises a complex interaction between the abiotic and biotic environment resulting in dynamic communities. Fungi play a main role in woodland ecosystems, together with bacteria and invertebrates. Wood litter is the most abundant carbon source on the planet, and saprotrophic basidiomycetes are the major agents of

wood decomposition. Their mycelia are universal in forest soils where they perform a range of key ecological roles to colonize wood and interact with each other, and with animals forming decay communities (Boddy and Rayner, 1983; Boddy 1993, 1999; Boddy & Watkinson 1995, Cromack & Caldwell, 1992; Cairney, 2005). They are the main agents of nutrient recycling, though they are considered to be highly conservative of obtained nutrients, representing an extensive nutrient reservoir in woodland ecosystems (Wells & Boddy, 2002).

Interactions between fungi can be affected by a number of mechanisms. Chemical signalling can play a major role in recognition systems between these fungi, and fungal interactions can be affected by the environmental conditions (Boddy, 2000). Environmental conditions and substrate or host specificity set the fundamental role of all fungi, but, competition appears to play a key role in determining the final role for many fungal species (Peay *et al.,* 2008), as in many other organisms. Microclimatic factors such as temperature, water potential, pH, moisture content and gaseous establishment can also influence fungal extension rate, production of enzymes, germination of spores, morphology, the decay rate and the outcome of fungal interactions (Rayner & Boddy, 1988).

Climate change may alter community structures as varying temperatures and other abiotic factors have differential effects on different species and on outcomes of interactions between species. Fungi have adapted and evolved to a changeable environment, with various morphologies, physiologies and ecological strategies.

These differences maintain and shape community structure, and structural heterogeneities and differences in spatiotemporal distribution of nutrients influence fungi at all levels, from hyphae to communities (Boswell *et al.*, 2007).

Besides, interactions occur between fungi and other organisms such as between invertebrates and bacteria as well as between themselves. Invertebrate feeding can alter the foraging strategies of some fungal species (Tordoff *et al.*, 2006), and affect the outcome of their interactions with other fungi (Bretherton *et al.*, 2006; Tordorff *et al.*, 2007; Rotheray *et a*l., 2010; Crowther *et al.*, 2011) Bacteria may benefit from fungal company but fungi may themselves be repressed by their presence and *vice versa* (Romaní *et al.*, 2006; De Boer *et al.*, 2007) which may influence their interactions with other species.

1.2 The role of basidiomycetes and ascomycetes in wood decay

Decomposition is a vital process that results in nutrient recycling by making resources in animal and plant material available for utilisation by a wide range of organisms. Most simple sugars are easily metabolised, but more complex compounds such as hemicellulose, cellulose, lignin and chitin are less easily decomposed and can only be utilised by specific species. Saprotrophic basidiomycetes are the main agents of wood decomposition in terrestrial ecosystems, though ascomycetes also take this role. They break down the more recalcitrant compounds into simple forms, which are then available to other organisms (Boddy, 2001). They are essential in carbon and nitrogen cycling within ecosystems, and have a role in humus formation (Jasalavich *et al.*, 2000).

Three major types of wood decay can be recognized - white, brown and soft rot. The first two types are caused by a large number of basidiomycetes and ascomycetes (Rayner & Boddy, 1988, Boddy, 1999, 2000). In white-rot the wood gains a bleached appearance as lignin as well as cellulose and hemicellulose components are broken down. The hyphae sit on the wood wall in the cell lumen and diffusion of enzymes from these leads to formation of erosion grooves or troughs. In brown-rot wood is stained brown, cracks cubically, becomes friable and eventually powdery. Hemicellulose and cellulose are removed, but the lignin is only modified slightly (Rayner & Boddy 1988). Soft rots are caused largely by certain ascomycetes which usually attack wood with high or fluctuating moisture content. They cause decay more slowly than do basidiomycetes, generally higher in hardwoods than in soft. Again lignin decomposition is either slow or absent and left as a residue or in modified form (Ohkuma *et al.*, 2001).

Saprotrophic basidiomycetes are abundant in forests degrading cellulose, lignin and lignocellulose (Sharma, 2007). These compounds can be degraded by the production of enzymes that can be produced by wood decay fungi which are able to break them down to be utilised by other organisms, these fungi are considered as the most important decomposers in terresterial ecosystems (Boddy, 2001). These enzymes can be produced also during mycelial interactions of fungi.

1.3 Fungal community development

In nature, wood decomposition is rarely effected by a single species, but by communities of different species (Boddy, 1999). These communities are not static but change with time. All organisms in their natural environment encounter a range of other organisms. Some are involved in primary resource capture, which is the process where the pioneer species gain access for the first time to uncolonised resources, and others in secondary resource capture, which involves replacement of fungi already present in the wood, and hence alters the community structure (Rayner & Boddy, 1988, Boddy, 2001; Boddy & Heilmann-Clausen, 2008). The development of communities can be influenced by four main factors: stress aggregation, stress alleviation, disturbance and combat - interspecific competition for space and nutrients (Rayner & Boddy, 1988; Boddy & Heilmann Clausen, 2008).

Living trees are protected from fungal colonization by chemical and physical factors such as the host resistance of living cells, and the high content of water in sapwood. Fungi can penetrate living trees via wounds, insect vectors or after breakage of branches (Rayner & Boddy, 1988; Boddy, 2001; Niemela *et al.*, 2002). Wood is usually already colonised and decay has begun by the time it reaches the forest floor. After falling to the forest floor the fungal communities face a swift change in microclimate conditions which will affect the community present, and other fungi will start to colonize (Boddy, 2008). The interacting fungi are able to retain and protect territory in resources through either aggressive combative mechanisms, or non-selective replacement, which is a secondary capture that shows no clear association between preceding species and succeeding species (Cooke & Rayner, 1984). These interactions and replacements play a crucial role in changes in communities with time

(Rayner & Boddy, 1988; Dowson *et al.*, 1988a; Holmer & Stenlid, 1993; Boddy, 2000; Boddy, 2001; Donnelly & Boddy, 2001).

Inter-specific interactions between saprotrophic basidiomycetes are a major factor in their ecology, affecting substratum colonisation and access to nutrients (Boddy 2000; Woodward & Boddy 2008), as well as wood decay rate and the succession of species (Boddy & Heilmann-Clausen 2008). Arrival at the resource is the initial stage of colonisation for a wood decay fungus, followed by establishment within it. Arrival can be as spores, or as mycelium, gaining access to woody tissues by means of bark discontinuities such as branch stubs or wounds, or from the development of latent propagules (Rayner & Boddy, 1988; Boddy, 2001). After arrival, the fungi would start to colonise the wood with primary and secondary resource capture, which may lead to competition for domination of the territory (Grifith & Boddy, 1991).

1.4 Types of interaction

1.4.1 Gross interaction outcomes

Antagonistic interactions can result in: (1) replacement, where one fungus obtains territory of the opposing fungus; (2) deadlock, where neither fungus progresses into the territory of the other; (3) partial replacement when one fungus initially advances into the territory of the opponent but deadlock follows; (4) mutual replacement, where both fungi obtain some access into the territory occupied by the other (Boddy, 2000). It is important not to confuse physical over growth with replacement. However,

overgrowth can be occasionally an essential feature of antagonism, where some cord forming fungi that form deadlock with the antagonist on soil, are able to grow over the opposing mycelium to reach the organic resource from which the opponent is growing, with subsequent replacement of the opponent within the resource (Snajdr *et al*., 2011).

Most studies on interspecific interactions have been done by inoculating fungi onto agar, wood blocks, soil trays, logs, microscope slides, gradient plates or 3-dimensional columns of growth media. Interaction outcomes can be most easily seen on agar (Holmer *et al*., 1997; Donnelly & Boddy, 2001; Tordoff *et al*., 2006), but outcomes in different substrata are not always the same (Dowson *et al*., 1988; Wald *et al*., 2004). Furthermore the outcomes of interspecific interactions may vary depending on the microclimatic environment, the place of interaction (soil or wood), the amount of resources, and the presence of other fungi in the same resource (Dowson, Rayner & Boddy, 1988; Holmer & Stenlid, 1997; Boddy & Abdalla, 1998; Boddy, 2000). Interactions are brought about in a variety of different ways either at a distance and /or following mycelial contact.

1.4.2 Interaction at a distance

Chemical signalling probably plays a key role in fungal recognition systems. Aggressive interaction can be effected at distance by volatile organic compounds (VOCs), diffusible organic compounds (DOCs) before the fungi make physical contact (Boddy, 2000; Wheatley, 2002). A diversity of chemicals has been suggested as potential antagonistic DOCs, for example, aromatic compounds (Wheatley, 2002;

Wald *et al.*, 2004). VOCs comprise a range of chemicals, such as ketones, terpenes, aldehydes, aromatic compounds and alcohols, which alter qualitatively and/or quantitatively during interactions (Hynes *et al.* 2007; Evans *et al.*, 2008).

With some fungi, interactive responses begin before mycelial contact occurs. When mycelia of different species meet, the recognition of 'non-self' elicits responses in the area where the mycelia are in physical contact at the interaction zone. Diffusible and volatile organic compounds (DOCs and VOCs), extra-cellular enzymes and secondary metabolites may be produced, and also changes in the morphology of the mycelium may take place (Boddy 2000; Woodward & Boddy 2008). In addition, fungal metabolites play an aggressive role in the animal toxicity by producing toxins and in diseases of plants and insects, as well as in interspecific mycelial interactions (Gloer, 1995).

Though DOCs can have an inhibitory effect on some species, they can also stimulate the growth of other species (Heilmann-Clausen and Boddy, 2005). They have been revealed to affect foraging behaviour and spore germination in fungi, to dramatically change the mycelial morphology of *Hypholoma fasciculare*, and to increase ligninolytic enzyme production in *Marasius pallescens* (Rayner *et al.*, 1994; Heilmann-Clausen and Boddy, 2005; Ferreira-Gregorio *et al.*, 2006).

1.4.3 Following contact

Sometimes the growth of two mycelia toward each other does not show slowing or termination of extension, an obvious interaction only begins following contact (Boddy, 2000; Rothray *et al.*, 2010). The replacement interaction may start with lysis

ahead of the proceeding mycelium, overgrowth of mycelium due to immediate death of the weaker competitor or overgrowth by the whole mycelium with later death of the weaker antagonist. The winner in the overgrowth of replacement can be confirmed by making isolations (Crockatt *et al.*, 2008; Rotheray *et al.*, 2010). The colour of aerial mycelium may change and crystals and amorphous precipitates may form as well as production of aromatic compounds (Griffith *et al.*, 1994).

Parasitism, in which the hyphae of one fungus coil around those of another species, and then penetrate, is another form of interaction following contact. Enzymes and toxins may be produced by the fungus causing lysis of the host cell wall, or allowing penetration of it, allowing nutrients to be absorbed by the parasitic fungi (Vasquez-Gerciduenas *et al.*, 1998; Howell, 1998). For example, *Lenzites betulina* is a mycoparasite on *Trametes* species (Boddy, 2000; Boddy, 2001). Mycoparasitism is usually obvious macroscopically as growth of the parasite does not slow following contact with the host. Parasitism might be a modification of a phenomenon called hyphal interference, in which contact of the hypha of one species by that of another may lead to the death of the vulnerable one (Woodward & Boddy, 2008). For example, *Phlebia gigantea* interferes with *Heterobasidion annosum* (Ikediugwu *et al.*, 1970).

1.5 Production of VOCs

Fungi are known to produce a number of diffusible and volatile (DOCs and VOCs) chemicals, many of which have antibiotic properties that have been developed in industry and medicine. Many species produce a unique reproducible profile of

chemicals which can be used as a type of fingerprint for their identification (Scotter *et al.*, 2005). The production of particular chemicals can be used for the detection and identification of fungi present in buildings and stored food products and allows timely treatments if necessary. For example, toxigenic strains of *Fusarium* spp. produce tricodiene, which is a precursor to the mycotoxin tricothecene (Demyttenaere et *al.*, 2004).

Many of the volatile chemicals produced by fungi, have a variety of potential ecological roles, and foraging insects (Steiner *et al.*, 2007). They may can also induce defence responses in plants (Mendgen *et al.*, 2006; Splivallo *et al.*, 2007) and furthermore affect fungal development. During interactions between fungi different profiles of volatiles have been detected compared to those produced by individual species grown alone (Hynes *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; de Jong and Field, 1997; Jelen, 2002).

The nature of the interactions of volatile compounds with fungi and their impact on fungal growth at the molecular level is mostly unknown. However, exposure to VOCs may change the profile of pr oteins 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).

1.6 Ligninolytic enzymes

Saprotrophic wood-inhabiting basidomycetes are the most important decomposers of cellulose and lignin in dead organisms and, as such, have attracted considerable attention such as *Hypholoma fasciculare* and *Phanerochaete velutina* (Vetrovsky *et al.*, 2011). Wood decomposition involves lignin breakdown, and this is a multienzymatic process involving many phenoloxidising enzymes (Leonowicz *et al.*, 1999). More than 100 different enzymes have been isolated from fungi (Baldrian, 2006), with different species possessing different sets of enzymes. The most common enzymes, however, are manganese-peroxidase, laccase and lignin peroxidase, which are the most studied and mostly produced for wood decomposition by white rot fungi (Vares *et al.*, 1995).

There are three groups of enzymes that wood-rotting basidiomycete fungi produce to help them enter wood and guide the mycelium to more easily metabolized, carbohydrate constituents : (1) comprises enzymes that attack the wood directly and they consist of carbohydrate (cellulose, hemicellulose) and lignin degrading enzymes, (2) superoxide dismutase and glycoxal oxidase, which cooperate with the first group, but can not attack the wood alone; and (3) the feedback enzymes which combine metabolic chains during decomposition of wood, such as glucose 1-oxidase, aryl alcohol oxidases, pyrnose 2-oxidase, cellulobiose quinone oxidoreductases and cellobiose dehydrogenase. All of these enzymes can function in cooperation with each other or individually (Leonowicz *et al.*, 1999). Peroxidase and laccase may generate melanins as mediating oxidatives (Gianfreda *et al.*, 1999; Baldrian, 2006). These enzymes may also be concerned in the formation of defensive barrages or invasive hyphal cords as a morphogenetic effect (Griffith *et al.*, 1994).

1.6.1 Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are important multicopper oxidase enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates (diphenols, methoxy-substituted monophenols, aromatic amines) (Thurston, 1994). Higher plants and fungi, especially wood-rotting fungi, produce the highest amount of laccases, although bacteria can also produce laccases (Gianfreda *et al*., 1999). Laccases are involved in the decomposition of lignocellulose (Leonowicz *et al*., 1999), and have been detected in a number of fungi and most ligninolytic fungal species produce at least one laccase isozyme (Baldrian, 2006).

Because of the broad variety of their substrates, laccases are not easily described by their reducing substrates, which vary between laccases and overlap with the substrate range of another class of enzymes - monophenol mono-oxygenase tyrosinases, although laccases are not able to oxidise tyrosine (Baldrian, 2006).

Production of laccases is affected by nutrient availability. For example, extracellular laccase formation can be stimulated by a glucose-based culture medium and significant laccase formation by *Trametes pubescens* only starts when glucose is totally consumed in the medium, while the nitrogen source employed had an important effect on laccase synthesis (Galhaup *et al*., 2002). *Trametes versicolor* growth on wheat straw and beech wood led to an increase as high as 3.5-fold in extracellular laccase activity, in comparison with growth on glucose (Schlosser *et al*., 1997). *Pleurotus ostreatus* increased lignonlytic enzymes laccase and manganese

peroxidase, when lignocellulose was added to the soil growth medium (Snajdr & Baldrian, 2006).

Laccases can be used in various applications such as: removing toxic compounds from terrestrial and aquatic systems; treating beverages; as biosensors to estimate the amount of phenols in natural juices as an analytical tool (Gianfreda *et al.*, 1999).

1.6.2 Peroxidases

Peroxidases are oxidoreductases that utilise hydrogen peroxide (H_2O_2) to catalyse one-electron oxidation of a number of organic and inorganic substrates (Conesa *et al.*, 2002). The majority have a haeme as a prosthetic group and these can be divided into two groups: the plant/fungal and the mammalian peroxidase superfamilies. Plant and fungal peroxidases are divided further into three classes: Class I, intracellular peroxidases; Class II, extracellular fungal peroxidases, and Class III, extracellular plant peroxidases (Welinder, 1992). The most important peroxidases are manganese peroxidase (MnP) and lignin peroxidase (LiP) with other peroxidases playing less important roles in white-rot basidomycetes which are involved in lignin degradation (Conesa *et al*, 2002; Martinez *et al.*, 2005). MnP and LiP are found in a wide variety of basidiomycetes including *Trametes versicolor, Pleurotus spp.* and *Bjerkandera adusta* (Conesa *et al.*, 2002).

1.6.3 Localisation of lignolytic enzymes

Peroxidases and laccases are mainly located extracellularly because of the location of their substrates and high oxidative ability, which may relate to their physiological function (Baldrian, 2006). Laccase is cell wall-associated in *T. versicolor* and *P. ostreatus*, while MnP may be wall-associated in liquid cultures of *T. versicolor* (Valášková & Baldrian, 2006). Peroxidases can cause fast mass loss in birch wood and wheat straw (Valášková & Baldrian, 2006).

1.7 Effects of biotic and mycelial interactions on the activity of ligninolytic enzymes

Enzyme production can be affected by biotic factors and abiotic environment (see Section 18). Biotic interactions generally tend to stimulate enzyme production, and during interaction there are increases in the production of reactive oxygen species, phenoloxidases, laccases and sometimes ß-glucosidase (White & Boddy, 1992; Iakovlev & Stenlid, 2000; Iakovlev *et al.*, 2004; Baldrian, 2006). For example, the laccase activity of *Pleurotus ostreatus* and *Trametes versicolor* was increased after contact with soil fungi, yeast and bacteria and this increase reached 40-fold when interacting with a *Trichoderma* species (Baldrian, 2004). Laccase and peroxidase production abundance was also modulated during interactions between mycelia of the basidiomycetes *Marasmiellus troyanus* and *Marasmius pallescens.* When the liquid culture was shaken to mix the two fungi, the laccase and manganese peroxidase activity increased while the activity of lignin peroxidase was not detected (Gregorio *et al.*, 2006).

Enzyme activity was also compared during agar interactions between *Trametes versicolor* and other wood decay fungi where a huge increase in laccase and manganese peroxidase activities was seen at the interaction zone and enzyme activity declined as distance from the interaction zone increased (Hiscox *et al.*, 2010). In these interactions, the outcome of the interaction affected enzyme production. Enzyme production was highest in interactions where *Trametes versicolor* was replaced by the competitor, compared to when it won or where it replaced or deadlocked with the opponent (Hiscox *et al.*, 2010). Peroxidase activity was also higher during interactions when *Stereum hirsutum* was losing and replaced by other fungi (Peiris, 2009). It has also been observed that the production of enzymes during interactions varies depending on the species (Iakovlev & Stenlid, 2000).

Other enzyme activities are also affected by biotic environmental factors. For example, chitinase activity increased during interactions of *Hypholoma fasciculare*, *Resinicium bicolor* and *Coniophora arida*. This change in enzyme activity may also result in morphological changes or allow toxic compounds to enter the cell as chitinase can modify the cell wall (Lindahl & Finlay, 2006). The chitinous cell wall can be an important nitrogen source for other fungi that may colonise later, as wood-degrading fungi degrade their own cell walls and the hyphae of earlier colonisers (Patil *et al.*, 2000; Lindahl & Finlay, 2006).

1.8 Effects of abiotic stress on basidiomycete fungi

Levitt (1980) defined stress as ‘any environmental factor with the property of inducing metabolic adjustment (a physical or chemical change) irrespective of

whether the change is harmful or beneficial to the organism'. There are two aspects of coping with stress: (1) avoidance; which is a mechanism where organisms can decrease stress impact, and (2) tolerance**;** which allows organisms to endure the changing environments (Levitt, 1980). An organism's reaction to stress may be a combination of diverse tolerance and avoidance mechanisms. Abiotic stress factors that affect fungi include low or elevated water availability, elevated CO_2/ reduced O_2, extreme or variable temperature, lack of nutrients, exposure to heavy metal ions and excess light (Griffin, 1994; Boddy *et al.*, 2008).

Temperature and moisture (water potential) are two of the major factors affecting the rate of wood decomposition in temperate woodlands by basidiomycete fungi, such as *Trametes versicolor*, *Bjerkandra adusta*, *Stereum gausapatum*, *S. hirsutum*, *Vuilleminia comedens* and others (Boddy, 1983). Temperature can affect enzyme-catalysed reactions, and in turn affect the growth rate when fungi are exposed to higher or lower temperatures than optimium (Boddy *et al.*, 2008). Most fungi are called mesophiles, which grow well between 10ºC – 30ºC favouring intermediate temperatures. However some fungi have adapted to high (thermophiles, 20-50°C) or to low (psychrophiles 0-17°C) temperatures (Carlile *et al.*, 2000). Water potential is the relative ease and difficulty with which the fungus can obtain water from the medium (Boddy *et al.*, 1984). For example, in isolates of *Trichoderma viride* hyphal growth rate and conidial germination declined with decreasing water potential (Jackson *et al.*, 1991).

Other abiotic environmental factors, such as availability of nitrogen (see Section 1.7.3) and carbon source can also influence the fungus to produce lignolytic enzymes. Carbon sources, cellobiose and mannitol promoted the highest laccase activity of

Trametes versicolor, while glucose gave maximum manganese peroxidase and peroxidase activity (Mikiashvili *et al.*, 2005). Toxic abiotic stress also affects enzyme production: activity of laccase but not peroxidase increased after heavy metal exposure (Baldrian *et al.*, 2005)

Although stresses are combined in nature, in the following sections they are dealt with separately. Examples of methods of coping with stress are discussed including enzyme production, which may act as a defence mechanism against abiotic and biotic stress.

1.8.1 Heat stress

A number of physiological and ecological strategies can be used by fungi to protect themselves from the effect of high temperatures. These include: (1) effects on the growth rate and the physiology of the fungi such as affecting the fruiting (Boddy *et al.*, 1984; Gange *et al.*, 2007); and (2) Defense strategies such as production of heat shock proteins (HSPs) and changes in enzyme activity (Fink-Boots, 1999).

High temperature can stimulate enzyme activity such as extracellular and cellular peroxidases, superoxide dismutase and laccase (Griffin, 1994). Also, when high temperature is experienced as a heat shock it can result in a significant effect on the expression of HSP, hence HSPs are highly conserved and present in all cells of all organisms (eukaryotes and prokaryotes from bacteria to humans), and their expression increases in response to extreme conditions such as temperature or water potential etc. (Griffin, 1994; Wu, 1995; De Maio, 1999; Li and Srivastava, 2004) helping the cell to adjust to stressful conditions. Selected HSPs, are also known as chaperones which are

a ubiquitous class of proteins that play an important role in folding and unfolding of proteins and are able to protect other proteins from stress-induced denaturation. (Hightower and Hendershot, 1997; Gropper and Ludger, 2002).

High temperature can increase the production of enzymes in some wood decay species. For example, in *Abortiporus biennis* (55ºC) and *Trametes versicolor* (45ºC) laccase and peroxidase activity have been shown to increase in cultures following heat shock (Fink-Boots, 1999). Furthermore, in the fungus *Trametes modesta,* the highest laccase activity was shown at an incubation temperature of 50ºC (Nyanhongo *et al.,* 2002).

1.8.2 Cold stress

There are a range of physiological and ecological strategies eukaryotes use to protect themselves from the effect of low temperatures. These include: (1) avoiding cold by inhabiting niches that are protected from extremes of temperature to avoid tissue cooling (animals, humans); (2) synthesis of protective substances such as anti-freeze compounds to prevent the tissue fluids from freezing (fish, spiders, insects, etc.); (3) plants can form a liquid crystal structure to prevent formation of ice nuclei; and (4) modifying the composition of the lipid bilayer in membranes to be able to transfer from the gel state to liquid crystal state (Nazawa & Kasai, 1978; Crowe *et al*., 1984; Karow, 1991; Feofilova, 2000).

Fungi use both physiological and ecological mechanisms to avoid or tolerate low temperature, since their cellular osmotic balance can be affected by low temperature and can cause damage to the cell membrane (see Fig. 5.1 and chapter 5). For example,

low temperature decreased the mycelial extension rate in *Hypholoma fasciculare*, *Phanerochaete velutina*, *Phallus impudicus* and *Resinicium bicolor* and also resulted in changes in mycelial morphology, while a cold shock caused greater changes in morphology than constant cold (Allawi, 2011).

1.8.3 Nutrient stress

Nutrients are one of the most important factors that can affect the growth and metabolism of fungi. These nutrients consist of organic nutrients (simple energy sources such as simple sugars, e.g. glucose) to make the carbon skeleton for cellular synthesis, and inorganic sources (such as ammonium, nitrate ions, phosphate ions, calcium, potassium, magnesium or iron). Fungi can absorb simple nutrients from the surrounding environment through their cell membranes, but also can use enzymes to breakdown complex compounds (cellulose, lignin, hemicellulose) into simple nutrients that can be absorbed easily (Deacon, 2005). Nitrogen limitation was focused on in this thesis to see the effect on *Trametes versicolor* when using limited nitrogen and no nitrogen media.

The concentration and nature of the nitrogen sources are potential keys to regulating ligninolytic enzyme production by wood-rotting basidiomycetes (Galhaup *et al*., 2002; Mikiashvili *et al*., 2005), however there is no clear relationship between high or low nutrient availability and enzyme production across different species. For example, nitrogen-limited media conditions enhance the production of some enzymes such as laccase in some fungi, such as *Trametes pubescens*, *Pycnoporus cinnabarinus, Phlebia radiata* and *Pycnoporus sanguineus.,* The latter fungus was found repeatedly

to increase the laccase activity when exposed to limited nitrogen conditions (Gianfreda *et al*., 1999; Pointing *et al*., 2000; Galhaup *et al*., 2002). On the other hand, some fungi such as the white rot fungus *Bjerkandra sp*. strain BOS55, produced high amounts of MnP in the presence of excess nitrogen (Mester & Field, 1997).

Carbon availability is another nutritient that can influence enzyme production, for example laccase and Mn-peroxidase activities increased in *Pleurotus ostreatus* when grown on medium with high carbon content (Snajdr & Baldrian, 2006).

1.8.4 Osmotic pressure

Fungi sense the surrounding osmotic environment through cellular signaling pathways, and they respond to osmotic pressure by the following adaptations: (1) the production of compounds for protection such as glycerol, (2) reorganization of the cell wall biogenesis and the cytoskeleton, and (3) the biosynthesis of natural products such as mycotoxins (Duran, 2010). They can also respond by changes in their morphology or development. Thus fungi increase chances of survival by conidiation, enabling dispersal by wind to other more suitable environments (Rothschild and Mancinelli, 2001). Some fungi respond by a reduction or cessation of mycelial growth. For example, fungi tested did not grow or very slowly at -4.4 MPa water potential (Boddy, 1983). However, there are differences between species in their ability to cope with osmotic stress. For example, *Irpex* sp. grew better at lower water potentials (-7.0 MPa) than other fungi (Boddy, L. 1983; Mswaka and Magan, 1999).

1.9 Gene expression changes in mycelial fungi in response to biotic and abiotic factors

A combination of alterations in gene expression and enzyme activity in different conditions are likely to be responsible for changes in morphology and the production of volatile and diffusible compounds during fungal interactions and in response to abiotic stress.

As yet, our depth of understanding of the regulation of gene expression in basidiomycetes is restricted to a relatively small number of model species and a small number of genes, although the advent of new genome sequencing programmes (Adomas *et al*., 2006; Kulikova *et al.*, 2006) and high throughout transcriptomic analysis through new sequencing platforms (Parkinson *et al.*, 2002; Benson *et al.*, 2007) is likely to make important advances in this area in the coming years.

1.9.1 Alteration of ligninolytic enzyme transcript abundance in response to mycelial interactions and abiotic stress.

Effects of both abiotic and biotic stresses on ligninolytic enzyme production have been studied. Abiotic effects include nutrient source, water stress and toxic metals while biotic effects of most relevance to this thesis are those that relate to interactions between competing mycelia.

Differential expression of different laccase gene family members has been noted in several basidiomycete species. Two laccase genes (*lac1* and *lac2)* from *Lentinula*

edodes were differentially expressed under different media conditions, where *lac1* expression was much higher than *lac2* expression in a high nutrient glucose medium, but the expression of the two genes was similar in high nutrient media supplemented with cellulose, or sawdust (Zhao and Kwan, 1999). On the other hand, in *Trametes pubescens* glucose repressed expression of the laccase gene *lap2* (Galhaup *et al*., 2002). In *Pleurotus sajor-caju* two laccase genes were differentially expressed in response to high nitrogen (Soden and Dobson, 2001). Nitrogen and carbon source also affected laccase gene expression in the basidiomycete I-62 (CECT 20197) (an environmental isolate from the Polyporaceae family) where non-limited nitrogen cultures could increase transcript levels of *Lac1* and *Lac2* to 100 times than under limited-nitrogen conditions (Mansur *et al*., 1997). On the other hand, MnP gene expression (*mnp1* and *mnp2*) in *Phanerochaete chrysosporium* was increased in response to nitrogen levels (limited-nitrogen cultures) (Gettemy *et al*., 1998).

Ecophysiological factors (low temperature and water stress) can reduce fungal growth and consequently affect gene expression. Stress had an effect on gene expression of the *FUM1* gene (involved in fumonisin mycotoxin biosynthesis) in *Fusarium verticillioides* (Jurado *et al*., 2008). Water stress increased *FUM1* transcript levels and had an opposite effect on fungal growth.

Toxic compounds such as heavy metals also have an effect on the gene expression level of ligninolytic enzymes which can be considered as an important regulatory factor, with putative xenobiotic response elements occurring in the promoter regions of lignin peroxidase (LiP) and manganese peroxidase (MnP) genes of *P. chrysosporium* and *Pleurotus eryngii* (Kersten and Cullen, 2007). Laccase genes of *Trametes versicolor* can also be regulated by copper (Collins and Dobson, 1997).

Ligninolytic enzyme gene expression is also affected by biotic interactions. *Trametes versicolor* laccase expression was increased at the interaction zone between *T. versicolor* and competing mycelium of *Hypholoma fasciculare* compared to the interaction zone in a self pairing of *Trametes versicolor* (Hiscox *et al*., 2010). Changes in gene expression however were not as marked as changes in enzyme activity, suggesting the possibility that the changes are rapid and transient. Interaction between isolates of the same species but of different vegetative compatibility, can also up-regulate ligninolytic enzyme gene expression. Thus laccase expression was induced in vegetatively incompatible interactions in the white rot basidiomycete *Amylostereum areolatum* but not in compatible interactions (van der Nest *et al.,* 2011).

1.10 Global gene expression changes during interactions

Very few studies have used global transcriptomic approaches to study mycelial interactions between two fungi; most examples are between host and fungus. One type of interaction studied relates to gene expression changes during mycorrhizal interactions between plant and fungus. For example, over 1500 expressed sequence tags (ESTs) were generated from the ectomycorrhizal basidiomycetes *Laccaria bicolor* and *Pisolithus microcarpus* cDNA libraries (Peter *et al*., 2003), where a relatively small proportion (11%) of the unique transcripts in *L. bicolor* were found in *P. microcarpus.* Fungal transcripts from a model of an ectomycorrhizal relationship between a plant and a fungus: the interaction between *Castanea sativa* roots and the fungus *Pisolithus tinctorius* were identified by using cDNA microarray analysis by

using an *in vitro* interaction system. This study identified over 30 unique sequence tags (ESTs) that were differentially expressed.

In one study of gene expression changes in relation to interactions between two fungi transcripts were isolated by a rapid subtraction hybridisation approach and were used as markers for pre-identification of biocontrol strains during the interaction between *Rhizoctonia solani* and *Trichoderma harzianum*, offering the possibility to differentiate effective biocontrol isolates from intermediate or no biocontrol strains (Scherm *et al.*, 2009).

Gene expression changes associated with the interaction between the biocontrol agent *Trichoderma haematum* and the phytopathogen *Sclerotinia sclerotiorum* were studied by using subtractive hybridisation to identify nineteen genes (Carpenter *et al.*, 2005), showing a change in gene expression compared with the control *T. hamatum* alone. Some cDNA fragments were similar to fungal or bacterial genes, while some were completely novel. Genes of known function included those encoding; others were related to growth and nutrition (Carpenter *et al.*, 2005).

During mycoparasitism of *Heterbasidion annosum* by *Physisporinus sanguinolentus* mRNA differential display was used to identify different gene expression patterns, that were confirmed by semi-quantitative RT-PCR and real time RT-PCR. Twenty one unique genes were cloned and sequenced and showed differential gene expression. One of the induced genes showed high similarity to *Coprinus cinereus* recA/RAD5/homolog (rah1) which is essential for homologous recombination, DNA repair and stress responses (Iakovlev *et al.*, 2004).

One of the few detailed studies on global gene expression changes during inter-mycelial interactions is that of Eyre *et al.* (2010). More details of this study are presented in the introduction to Chapter 4 where results are presented on RT-PCR confirmation of the microarray data.

Project objectives

Mycelial interactions are very important for driving and maintaining decomposition, nutrient recycling, community structure and diversity (Boddy, 2000). Our knowledge of these interactions may help also to develop strategies to manipulate the outcomes of interactions which could be exploited for biocontrol, help understand how communities will be affected by climate change (abiotic stress), as well as contributing to the general body of knowledge about these fungi.

To understand some of these processes better, work has been focused on *T. versicolor*, to investigate changes in gene expression where it replaced, deadlocked with, or was replaced by a competitor (Eyre, 2007). *T. versicolor* is a useful model because it is a common species with intermediate combative ability (a late primary to early secondary coloniser), and thus displays a range of interaction outcomes with other species (Boddy, 1988; Boddy, 2000; Boddy, 2001). It is also easy to manipulate in culture.

There is little known about the chemical production during interspecific interactions between saprotrophic basidiomycetes and also the relationship between interaction

outcome and production of enzymes and metabolites, all of which needs to be explored further (Hynes *et al.*, 2007; Evans *et al.*, 2008). A better understanding of the chemicals, enzymes and genes involved in interactions, and how they are regulated, may help to improve our understanding of the fungi themselves (their ecological and biological characters).

The project objectives were to fill some of these gaps in our understanding of basidiomycete fungi by:

1 – A study of interspecific interactions on agar. This included a study of the qualitative morphological changes and outcomes of interactions, and also aimed to identify a species to study further that produces different outcomes when interacting with different species, which can replace, be replaced or deadlock with other species mycelium. Another objective was to determine a range of combinations of species that produce volatiles and can affect other species following on from the work on VOCs by Hynes *et al.* (2007) and by Evans *et al.*(2008) in the Cardiff Laboratory. This included a study of the effect of volatile compounds produced during different combinations of interactions of fungi on a third fungus.

2 – **A study of laccase enzyme activity in *Trametes versicolor*:** by qualitatively (activity staining) and quantitatively (enzyme assays) measuring changes in the activity of laccase produced by *T. versicolor* when affected by VOCs produced by interaction of two basidiomycete fungi on agar and on woodblocks or when affected by abiotic stress factors.

3 – **An investigation of gene expression in *T. versicolor.*** Genes identified in a microarray analysis of global *T. versicolor* gene expression during mycelial interactions (Eyre *et al.,* 2010) were tested to choose the right genes based on their expression as determined by microarray analysis or because of their putative function for further work with *T. versicolor* under stress. Expression of selected genes was tested in response to VOCs produced during interactions between itself and other selected wood-rotting basidiomycetes (*Stereum guasapatum, Hypholoma fasciculare* and *Bjerkandera adusta*) as well under abiotic stress. Another objective was to compare laccase enzyme activity and gene expression in *T. versicolor* mycelia alone under stress and/or under the effect of VOCs produced by interaction of two basidiomycete fungi, as well as comparing the resulting cloned sequences with *lacc* gene family tree to determine whether there was differential expression of gene family members.

Chapter 2: General Materials and Methods

2.1 Fungal Isolates and culturing

Different isolates of basidiomycetes and ascomycete species commonly found on Beech trees were used (Cardiff University Culture Collection). These fungi cover a range of different host species (Table 2.1). The species were maintained on 2% (w/v) malt agar (MA; 20 g l^{-1} Munton & Fison spray malt, 15 g L^{-1} Laboratory M agar No.2) incubated at 20° C in the dark. Cultures were routinely subcultured every 10 days or just before the colony margin reached the edge of the plate. Stock cultures of the studied species were maintained at 4° C on slant tubes of 2% MA. All the culturing work was done in a sterile laminar-flow hood.

Table 2.1. Details of the wood-inhabiting species used in the interactions on agar.

Ecological role	Species	Isolate Code	Host	Isolated by
Primary colonizer	*Eutypa spinosa*	Es 1	*Fagus sylvatica*	S.J. Hendry
Primary colonizer	*Stereum gausapatum*	Sg 1	*Quercus robur*	L. Boddy
Primary colonizer	*Vuilleminia comedens*	Vc 1	*Quercus robur* On dead branches of broad-leaf trees	L. Boddy
Heart rotters, primary decayer	*Fomes fomentarius*	JHC P855	*Fagus sylvatica* Birch,Beech,Sycamore	J. Heilmann-Clausen
Early secondary colonizer	*Trametes versicolor*	Cv D2	On dead wood of broad-leaf trees	L. Boddy
Secondary	*Resinicium bicolor*	LH-	Coniferous woodlands	L. Holmer

colonizer; cord-former		M6a	(occasionally angiosperm woodlands)	
Primary colonizer	*Trichoderma viride*		Ubiquitous	L. Thomas
Early secondary colonizer	*Stereum hirsutum*	Sh 1	*Quercus robur* (Oak)	L. Boddy
Late secondary colonizer; Cord-former	*Hypholoma fasciculare*	Hf DD4	Angiosperm wood and leaf litter	D.P. Donnelly
Late secondary colonizer	*Bjerkandera adusta*	Ba 1	*Fagus sylvatica* and other broad-leaf trees	L. Boddy

2.2 Extension rate measurements

2.2.1 Species growing alone or growing in Petri dishes on top of interacted two fungi (two-plate interaction method)

Fungi were cultured by inoculating 6 mm diameter agar plugs, cut from the growing margin of colonies with a Number 3 cork borer, centrally on 2% w/v MA and 0.5% w/v MA (Section 2.1). The plates were incubated face down, after sealing with Nescofilm®, at 20° C in plastic bags in the dark. Three replicates were used. Colony extension was measured daily, at the same time, across two diameters using vernier calipers. Measurements were taken until the colony was 1 cm away from the edge of the plate (Fig. 2.1).

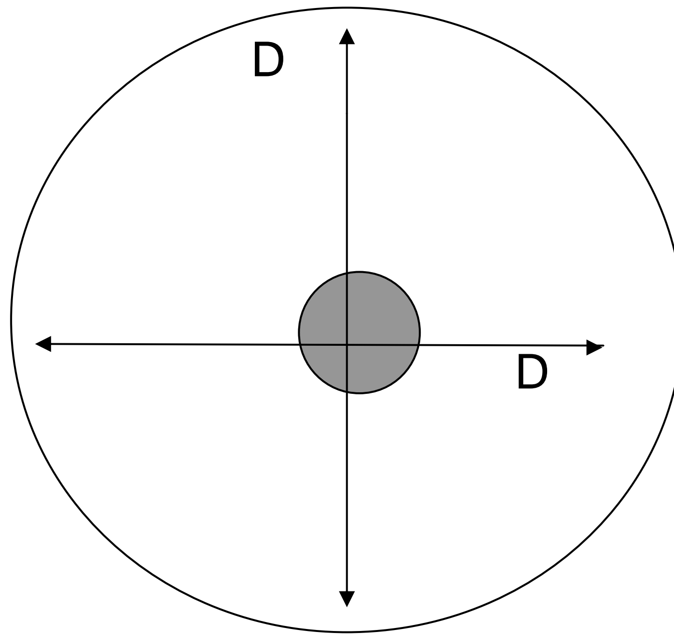


Fig. 2.1. The growth measurement positions diameters (D1 and D2).

2.3 Analysis of gene expression

2.3.1 Culturing the fungi for harvesting

Colonies were grown at 20°C on 2% MA (20g L^{-1} of agar) to facilitate harvesting mycelium from the surface of the agar. Paired interactions of *T. versicolor* with *S. guasapatum*, *B. adusta*, *H. fasciculare*, self pairing or *T. versicolor* alone were set up by inoculating 6 mm diam. plugs, cut from the actively growing mycelial margin, 30 mm apart (Fig 2.2). For pairings of *T. versicolor* vs *H. fasciculare*, the *H. fasciculare* plugs were plated 2 days prior to adding the opposing *T. versicolor* plugs, ensuring that opposing mycelia met at the centre of the plate. The mycelium was harvested aseptically from plates using a small spatula to skim the mycelium from the surface of the agar, flash frozen in liquid N_2 and transferred to -80 °C until required for extraction. 60 plates of each interaction were used for each extraction. Only an area

behind the interaction zone in interacting cultures, or just behind the culture margins for cultures grown alone, was harvested for extraction (Z in Fig. 2.2).

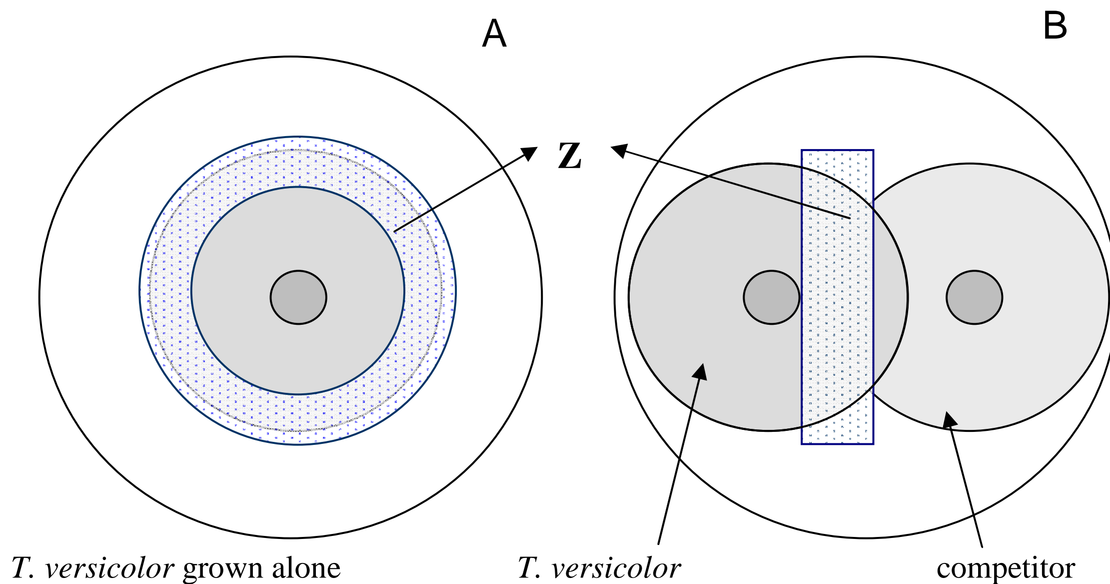


Fig. 2.2. The growth and harvesting of colonies when alone (A) and when interacted (B), harvesting zone (Z).

2.3.2 RNA extraction

Mycelium was ground to a powder in a cooled, autoclaved, pestle and mortar using liquid nitrogen. TRI-Reagent (2 ml) (Sigma-ALDRICH, Dorset, UK) was added to the mycelium with further grinding to obtain homogenous paste which included DNA, RNA and protein.

The mixture was then divided into two equal portions and transferred into two 1.5 ml sterile Eppendorf tubes, and allowed to stand at room temperature for 5 minutes. Then the tubes were centrifuged at 10500 g at 4° C for 10 minutes using the F2402H rotor in a Beckman Coulter™ Allegra™ 2IR Centrifuge (Beckman Coulter Ltd., High

Wycombe, UK). Clear supernatants were transferred to new 1.5 ml Eppendorf tubes where 200 µl of chloroform was added to each tube and then the tubes were vortexed for 15 seconds. Tubes were allowed to stand at room temperature for 5 minutes, and then centrifuged again at the same g and temperature for 15 minutes. The top layer (aqueous), which includes the RNA, was transferred to new 1.5 ml Eppendorf tubes and 0.5 ml isopropanol was added to each tube, mixed, and then left to stand at room temperature for 10 mins to precipitate the RNA. Tubes were then centrifuged again for 10 minutes, and carefully marked to identify the location of the pellet. Supernatants were removed and 1 ml of 75% ethanol added to the pellet and then tubes were vortexed for 15 seconds. Tubes were re-centrifuged, then the supernatant was removed and pellets allowed to dry in air (in a laminar flow cabinet) for 10-30 minutes. The dried pellet was then dissolved in 50 µl of sterile distilled water in each tube, then the contents of the two tubes were combined together to form 100 µl in total. The end product (10 µl) was checked for the presence of RNA by gel electrophoresis, the rest was stored at -80°C. Dedicated autoclaved disposable plasticware was used throughout the extraction to avoid RNA degradation by contaminating RNases.

2.3.3 Gel electrophoresis

Gel electrophoresis was performed by adding agarose (1% for RNA, 1.5-2% for DNA) to 1X TAE Buffer (50X TAE: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0), this was microwaved for 1 min and then cooled slightly before adding ethidium bromide (EtBr) (10 µg ml^{-1}). For RNA analysis, before pouring the gel into the tank (Mini Sub™ DNA cell, Biorad, Hemel Hempstead, UK)

the comb and tray were soaked in 0.1 M NaOH for 10 min and rinsed in sterile dH_2O to prevent degradation of RNA samples. Cooled agar was poured into the gel mould and allowed to set. Loading buffer (10x) (16.5 ml 150 mM Tris-HCl pH 7.6, 30 ml glycerol, 3.5 ml H_2O, bromophenol blue to desired colour) of 1/10 volume was mixed with the samples to run on the gel. A 1kb DNA ladder (500 ng) (Invitrogen, Paisley, UK) in 1x loading buffer was loaded alongside samples. Samples were run at roughly 110 V for 20 min. The gels were then viewed under UV light via a GeneGenius Bioimaging System (SynGene, Cambridge, UK) and images were captured via Genesnap 4.00.00 software (SynGene, Cambridge, UK).

2.3.4 DNase treatment of RNA samples

RNA concentration was checked using a spectrophotometer and 1-5 μg of it were added to form 16 μl with sterile distilled water, and then 2 μl of RQ1 DNase 10X buffer and 2 μl of RQ1 DNase (Promega, UK) were then added to make 20 μl. The reaction was incubated at 37°C for 30 minutes then 2 μl of RQ1 DNase stop solution was added to stop the reaction.

To inactivate the DNase the reaction was incubated at 65°C for 10 minutes. To test for full removal of genomic DNA, DNAase treated RNA was used as a control in the RT-PCR reaction (note that all the incubations were done in a PCR machine).

2.3.5 cDNA Synthesis

After treating the RNA to remove residual DNA, the samples were ready for cDNA synthesis. The RNA (19 µl) was pipetted into a 0.5 µl Eppendorf tube. Oligo (dt) 15 (Promega, UK) (500µl/ml) (Deoxy poly T primer which anneals to the poly A trail of RNA) 1 µl was added. Then the mixture was incubated at 70°C for 10 minutes and cooled at 4°C for another 10 minutes. Then 6 µl of 5x1st strand buffer (Promega, UK) 2 µl of 0.1 M DDT (dithiothreitol-reducing agent to stabilize the enzyme) and 1 µl of 10mM dNTPs were added before incubation at 42° C for 2 minutes. Then 1µl of reverse transcriptase enzyme (Promega, UK) was added and the mixture incubated at 42° C for 50 minutes. To stop the reaction the mixture was then incubated at 70°C for 15 minutes. cDNA was stored at - 80°C.

2.3.6 Design of gene-specific primers

Genes were selected based on their expression patterns from microarray analysis of mycelial interactions between *T. versicolor* and competing fungi (Table 2.2). Primer sequences were designed using an online program (Primer3; http://biotools.umassmed.edu/bioapps/primer3_www.cgi) using sequences previously obtained (Eyre, 2007).

Table 2.2 Specific primers of different genes from *T. versicolor*. Tv; *Trametes versicolor*, Sg; *Stereum gausapatum*, Hf; *Hypholoma fasciculare*, Bk; *Bjerkandera adusta*.

Primer	**Primer sequence (5' to 3')**	**No. bases**	**Specific for:**	**Annealing temperature (°C)**	**Product size**
FRA2-F FRA2-R	CGGTACTGTCTGCTGCGATA CGAACACACGAGTTCTTGGA	20 20	Tv/Sg(high), Tv/Bk (high)	60	230
F1D8-F F1D8-R	CGGAAAAAGAGCGAGGAGA CTCCTCGAGAAGTGCAAAGC	19 20	Tv/Bk (low), Tv/Hf (low)	60	207
F2A1-F F2A1-R	GGAGAGTGCGGTGACAATGAA GTGTGACTGTCATTGTCGGG	20 20	Tv/Hf (high)	60	160
FRA19-F FRA19-R	AGACTACCAGGACGGAACGA AATGGAAAGCATGGGAAGG	20 19	Tv/Sg (low)	60	154
Nox-F Nox-R	TCGGTTGGTTCCAGACTCTC TAGATGGCCTTCCAGTCAGG	20 20	Tv interacted with (Sg, Ba, Hf, Tv)	60	205
Lacc-F Lacc-R	CTTCAACGGCACCAACTTCTT GAAGTCGATGTGGCAGTGGAG	21 21	Tv alone	55	376
M13-F M13-R (Promega)	TCACACAGGAAACAGCTATGAC GTTTTCCCAGTCACGAC	22 17	Controls	50	variable
18S(EF4) 18S(fung5) 18S rRNA (Smit et al., 1999)	GGAAGGGRTGTATTTATTAG GTAAAAGTCCTGGTTCCCC	20 19	Controls	48	563

High= up regulated gene, low= down regulated gene.

2.3.7 PCR of cDNA (Semi-quantitative RT-PCR)

Reactions were set up as follows: 18.9 µl sterile distilled water was added to an Eppendorf tube, together with 2.5 µl buffer (X10) (Qiagen, Crawley, UK), 0.5 µl of 10mM dNTPs (Promega, UK) 1µl of each primer (10:90 µl sterile distilled water), 0.125 µl Hotstar enzyme (Qiagen) and 1 µl of the cDNA sample to a total of 25 µl. A master mixture was made up depending on the number of replicates to include common reagents.

Reactions were cycled in a Perkin Elmer 2700 thermocycler and the following programme: 94 ^{o}C for 15 min, {94 ^{o}C for 1 min, Tm for 1 min, 72 ^{o}C for 1 min} x30-35 cycles, 72 ^{o}C for 6 min then held at 20 ^{o}C. Tm for FRA2, F1D8, FRA19, Nox and F2A1 was 60° C, for 18S it was 48° C.

2.3.8 Normalization of the data

Normalisation was achieved by using 18S rRNA primers EF4 and fung5 (Smit *et al.*, 1999). Reactions were cycled as above. Three or more replicates for each primer set were all amplified using the same machine to avoid any variability due to the machine parameters. Products were analysed by agarose gel electrophoresis and PCR products quantified using the Gene Genius bioimaging system and GeneSnap software, (SynGene, Synoptics Ltd., Cambridge, UK). Product quantitation from the 18S target was used to normalise results for all the other primer sets. Cycle number was optimised and limited for each primer set and cDNA synthesis batch combination. This ensured that the reactions were in the exponential phase and therefore product

quantitation could be considered semi-quantitative with respect to message abundance. Although the cDNA was retro-transcribed using oligo (dT), sufficient amount of rRNA are also retro-transcribed due to the high A-T content of r RNA. This methodology has been used successfully to obtain semi-quantitative RT-PCR data for a range of experimental systems (Parfitt *et al*., 2004; Wagstaff *et al*., 2005; Orchard *et al*., 2005; Price *et al*., 2008).

2.3.9 Cloning

2.3.9.1 Purification of PCR products

A QIAquick Gel Extraction Kit (Qiagen) was used to extract PCR products. The bands were excised from the gel using a razor blade and the agarose slices transferred to 1.5 ml Eppendorf tubes, and weighed. Three volumes of buffer QG were added to one volume of gel (where 100 mg ~ 100 µl). Tubes were incubated for 10 min at 50 °C, or until the gel piece had completely dissolved. One gel volume of isopropanol was added to the sample and mixed well, and the mixture applied to a QIAquick spin column. This was centrifuged at 11,000 g for 1 min in an Eppendorf Minispin microcentrifuge (Eppendorf, Cambridge, UK), and then the supernatant was discarded. 750 µl of the washing Buffer PE was added to the column, and centrifuged for 1 min twice. The supernatant was discarded and the column centrifuged again at 11,000 g for 1 min to remove any traces of buffer PE. The column was then transferred to a fresh 1.5 ml Eppendorf tube, and 30 µl buffer EB (10mM Tris-CL, pH 8.5) was added to the DNA at the centre of the column membrane, left to stand for 1

min, and then centrifuged for 1 min. The eluted DNA was checked by running 5 µl on an agarose gel and the rest was kept at -20 °C until used for ligation.

2.3.9.2 Ligation into pGEMT-Easy vector

Ligation for each PCR product was set up as follows: 1 µl of each of (10x ligation buffer, (10-100 ng), plasmid pGEM T-Easy (Promega), T4 DNA ligase (Promega) and 7 µl of the purified PCR product to form 10 µl in total. The samples were then incubated overnight at 4 °C.

2.3.9.3 Transformation of *E.coli* DH5α competent cells

Escherichia coli DH5α cells in 100 µl (prepared house) were transferred from -80°C to be thawed on ice. DNA ligation (2 µl) was added, mixed, and then incubated for 20 min on ice. Heat shock was applied by placing the tubes containing the cells in a water bath for exactly 45 s at 42°C, and then transferred back for 2 min to ice. SOC medium (900 µl) (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20mM glucose) was added then the tubes were transferred to a 37°C shaking incubator (100 rpm) for 1 hr. The cells (200 µl) were streaked onto 9 cm non-vented Petri dishes of LB solid medium containing ampicillin (100 µg/ml) (Luria-Bertani medium; 1.0 g tryptone, 0.5g yeast extract, 1.0 g NaCl, 1.5 g agar, 100 ml distilled water), plates were incubated overnight (less than 18 hrs) at 37°C then transferred to 4°C.

2.3.9.4 Colony PCR for identification of positive colonies

PCR of positive colonies for positive clones was performed with the use of M13-F/R primers (within for the pGEM-T vector) and 1 µl of the bacterial cultures as a sample. This was done by inoculating a single well-separated colony in to a 1.5 ml Eppendorf tube containing 200 µl pf LB liquid medium (as above but without agar). Ten colonies were chosen randomly for each transformation, and then incubated at 37°C for 4-5 hrs with gentle shaking (100 rpm). The bacterial growth was then checked by odour. PCR reactions were set up as described in Section 2.3.7 using a Tm of 50°C. PCR products were analysed by gel electrophoresis to identify the positive cultures based on the size of bands. 10 µl of bacterial cultures were used to inoculate a 3 ml LB liquid medium and Ampicillin to be grown overnight at 37° C with shaking at 200 rpm for plasmid DNA purification.

2.3.9.5 Plasmid DNA purification

A QIAprep Spin Miniprep Kit (Qiagen) was used to purify and extract plasmid DNA. Cultures (3ml overnight cultures) were divided into 700 µl to be used as a stock (by adding 300 µl of glycerol) at -80°C, and the rest (2300 µl of the culture) were centrifuged in two Eppendorf tubes at 8.000 rpm for 3 min in an Eppendorf Minispin microcentrifuge (Eppendorf, Cambridge, UK). The supernatant was discarded, then 250 µl Buffer P1 containing RNase A was added and vortexed until the pellet was resuspended. Buffer P2 (Lysis Buffer) 250 µl was then added and mixed by inverting the tubes 4-6 times. N3 Buffer 350 µl was added immediately so as not to let the lysis reactions proceed for more than 5 mins and then mixed 4-6 times by inverting the

tubes. After being centrifuged at 11.000 g for 10 min, the supernatant was transferred into QIAprep Spin Columns, and centrifuged at 11.000 g for 45 s. The flow through was discarded then the column washed twice by adding 750 µl PE Buffer and centrifuged at 11.000 g for 45 s, each time discarding the flow through. The columns were centrifuged again for 1 min to remove any residual PE Buffer. The spin column then was transferred to a new 1.5 ml Eppendorf tube, and 50 µl pf EB Buffer (Tris-CL, pH8.5) was added to the centre of the QIAprep Spin column membrane. Columns were left to stand for 1 min then centrifuged for 1 min. A 5 µl sample of the end product was checked by gel electrophoresis for the presence of plasmid DNA, while the rest of the product was stored at -20° C until used.

2.3.9.6 Sequencing of plasmid DNA

The purified plasmid DNA was checked on 1% agarose gels then the DNA concentration was checked using a Nanodrop spectrophotometer (Thermo Scientific, USA). Samples were sent for sequencing to the Cardiff University Sequencing Service using M13-F primers.

2.4 Enzyme analysis (laccase)

Measurement of laccase activity was carried out by adding 50 µl of the sample extract (samples of growing mycelium taken by Number 8 cork borer from the margin of the colony and kept in 1 ml deionized water over night in 4°C with smooth shaking) into the wells of a Bioscreen C plate (100 well plate; Oy Growth Curves Ab Ltd., Finland). Citrate- phosphate buffer (150 µl, 100 mM citrate, 200 mM phosphate, pH 5.0) and

50 µl 0.08% (w/v) ABTS were added to the wells using a multichannel pipette then the plate was placed in a Bioscreen spectrophotometer (Oy Growth Curves Ab Ltd., Finland) to detect laccase activity by the formation of a green colour at 420 nm (Bourbonnais & Paice, 1990). The laccase activity was determined by using the molar extinction coefficient of ABTS (36,000 M cm^{-3}), where one unit of laccase activity was defined as the amount of enzyme releasing 1 µmol of product per min.

2.5 Statistical analysis

For each experiment (of each single gene expression in four interactions of *T. versicolor* (with *S. guasapatum*, *H. fasciculare*, *B. adusta* or *T. versicolor* self-pairing and *Tv* alone or during the test of *Tv* under stress) results were compared using one-way ANOVA, using an Anderson-Darling test for normally distributed residuals and also Leven's test for equal variances between groups. If significantly different ($P \leq 0.05$) then further tests were used (Tukey-Kramer) (a *posterior* test to explore differences between means), but if not then Kruskal-Wallis tests with post hoc Tukey's test or Mann-Whitney U-tests (if the data were non normally distributed) can be used. The tests were carried out in Minitab (v.15) and SPSS (v.16).

Chapter 3: Effect of VOCs produced during mycelial interaction on growth and enzyme production by a third fungus

3.1 Introduction

Aggressive mycelial interactions can be affected at a distance by production of volatile organic compounds (VOCs), diffusible chemicals or antibiotics before the fungi make physical contact (Boddy, 2000; Wheatly, 2002). VOCs produced by fungi cause spoilage of stored food, and in buildings have been implicated in Sick Building Syndrome (Ramin *et al.*, 2005; Wady *et al.*, 2005). VOCs can inhibit the extension rate of a fungus and its opponent, and sometimes even result in self-inhibition. Their production is often correlated with synthesis of pigments, lysis and change in mycelial morphology (Griffith *et al.*, 1994; Howell, 1998).

Fungi secrete secondary metabolites when colonising wood which may affect other species that are trying to colonize the occupied area. Competitors may be completely inhibited or have their growth slowed, some may not be affected at all, while growth of others may be stimulated by these chemicals. Furthermore, the chemicals produced may change in the presence of a probable competitor (Boddy, Frankland & Van West, 2008). There may be indirect effects of some chemicals, such as lowering the pH of the area and others may act as antibiotics (Boddy & Heilmann Clausen, 2008; Hynes et al., 2007; Woods, 1996; Griffith *et al.*, 1994b, c).

VOCs are produced by many fungi when growing alone (Ewen *et al.*, 2004; Wheatley, 2002). When mycelia interact quantities may increase and additional VOCs are often produced. In interactions between *Resinicium bicolor* and *Hypholoma*

fasiculare, 24 VOCs, predominantly sesquiterpenes, were detected. Of these four were detected in all treatments; 14 in interspecific interactions; four in monocultures of *R. bicolor,* and two in monocultures of *H. fasciculare* (Hynes *et al*., 2007). The six VOCs identified in monocultures all increased during interspecific interactions. Five volatile organic compounds were produced by *Trichoderma* spp (Wheatley *et al.*, 1997), and four (Acetone, 2-methyl-1-butanol, heptanal and octanal) were tested at a variety of concentrations against four wood inhabiting fungi. Over 80% of the tested fungi were affected by heptanal, but acetone had no affect, while only the highest concentrations of 2-methyl-1-butanol showed an effect.

GC and GC-MS analysis revealed that the major volatiles produced by fungi were linalool, citronella, geraniol and methoxyphenylacetate or drimenol depending on the culture nature (Kahlos *et al.*, 1994). The constituents of the culture medium and the age of the fungal colony can affect the inhibition of wood decay fungi by the VOCs (Bruce *et al*., 1996). The composition of the profile of VOCs produced by a fungus in nature varies slightly from that produced in artificial cultures where, for example, methyl *p*-methoxyphenylacetate was the main compound produced by *Gloeophyllum odoratum* (Kahlos *et al.*, 1994).

There were differences in VOC profiles of interacting wood decay fungi when growing on agar, in wood or across soil (Elerabei, Muller & Boddy, unpub.). Also the production of inhibitory VOCs was highly dependent on the particular competitor, the growth substratum and species of fungi during interactions (Bruce *et al*., 2002). VOCs, like the diffusible chemicals (DOCs) released from partially decomposed wood, may have a role as infochemicals during community development in decomposing wood (Heilmann-Clausen & Boddy, 2004). The VOCs and DOCs

produced during inter-specific mycelial interactions may not only affect the fungi generating them, and the opponent that triggered their production, but also other fungi in the vicinity. Some chemicals are specific to certain fungi, and can be used to detect the presence of specific fungi within, for example, hosts. The presence of, for example, tricodiene in stored crops indicates the presence of certain species of *Fusarium* that produce aflatoxin (Demyttenaere *et al*., 2004; Scotter *et al*., 2005).

The specific aims of the work described in this chapter were to determine:

(1) the effect of VOCs produced by interacting fungi on the growth of a third fungus on agar;

(2) spatial distribution of laccase and peroxidase production in interacting colonies of *Trametes versicolor* on agar;

(3) the effect of VOCs produced during interactions between fungi in wood blocks on the growth of a third fungus on agar;

(4) the effect of VOCs produced during interactions in wood blocks on laccase and ligninolytic activity of a test fungus on agar.

3.2 Materials and methods

3.2.1 The effect of VOCs produced by interacting fungi on the growth of a third fungus on agar

3.2.1.1 Effect of mycelial interaction between Hypholoma fasciculare *and* Resinicium bicolor *on nine wood decay fungal species*

Hypholoma fasciculare and *Resinicium bicolor* were paired against each other or against themselves on 0.5% MA for 13-14 d until they began to interact and produce pigments. A plate inoculated centrally with a test fungus (*Eutypa spinosa, Stereum gausapatum, Vuilleminia comedens, Fomes fomentarius, Trametes versicolor, Resinicium bicolor, Stereum hirsutum, Hypholoma fasciculare* or *Bjerkandera adusta*) was then taped above (Fig.3.1). Colony extent of the test species was measured daily across diameters perpendicular to each other using vernier calipers. The measurements were taken for 5-7 d for all the species, except *Fomes fomentarius*, which was measured for 15 d every 2 d, and at least 5-6 measurements were taken. Five replicates were made. Controls employed a test species above uninoculated agar. Plates were incubated at 20^{0}C in the dark.

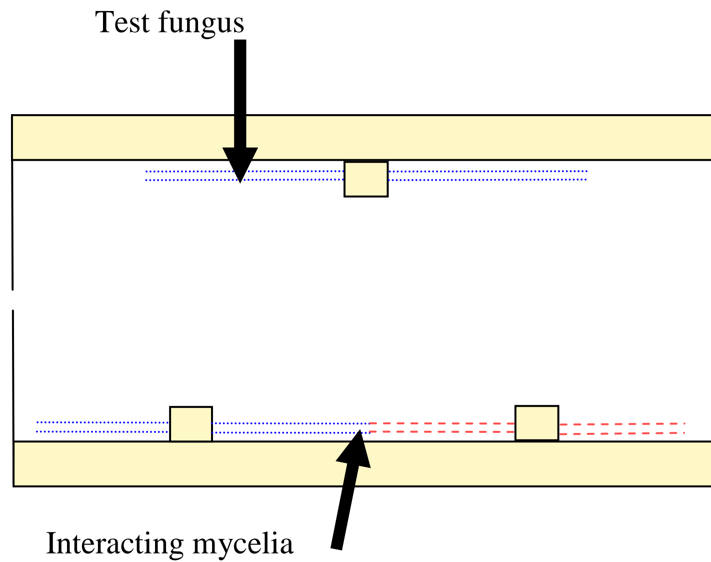


Fig.3.1. Two plates experiment, with mycelial interactions on the lower plate, and the test fungus on the upper plate.

3.2.1.2 The effect of mycelial interaction between *Trametes versicolor* ***and*** *Resinicium bicolor* ***on eight fungal species***

The above method (Section 3.2.1.1) was used in the same way with *Trametes versicolor* and *Resinicium bicolour* interacting with each other, and the extension rate (mm d^{-1}) measured for eight fungi (*Eutypa spinosa* , *Stereum gausapatum, Vuilleminia comedens, T. versicolor, R. bicolor, Stereum hirsutum, Hypholoma fasciculare* and *Bjerkandera adusta*).

3.2.1.3 The effect of mycelial interaction between *Stereum gausapatum* ***and four species***

The two-plate method described above (Section 3.2.1.1) was used to determine the effect of interaction of *Stereum gausapatum* with *Eutypa spinosa*, *Bjerkandera*

adusta, *Stereum hirsutum, Trametes versicolor* and *Vuilleminia comedens,* on extension rate of *E. spinosa*, *S. gausapatum, B. adusta*, *S. hirsutum, T. versicolor* and *V. comedens. S*elf-pairings and uninoculated agar were used as controls.

3.2.2 Effect of mycelial interaction between pairs of wood decay fungi on agar on extension rate of *Trametes versicolor*

Eutypa spinosa, Stereum gausapatum, Vuilleminia comedens, Fomes fomentarius, Trametes versicolor, Resinicium bicolor, Stereum hirsutum, Hypholoma fasciculare and *Bjerkandera adusta*, were paired against each other or against themselves on 0.5% MA for 13-14 d until they began to interact and produce pigments. A plate inoculated centrally with the test fungus *Trametes versicolor* was then taped above (Fig. 3.1). Colony extent of the test species was measured daily, across diameters perpendicular to each other using vernier calipers, for 5-6 d. Five replicates were made. Controls employed the test species above uninoculated agar. Plates were incubated at 20^0C in the dark.

3.2.3 The effect of VOCs produced during interaction between fungi in wood blocks on the extension rate of a third fungus on agar

3.2.3.1 Wood bock preparation

Wood, obtained freshly from beech (*Fagus sylvatica;* from Coed Cymru Hardwood Sawmill, Wentwood, UK) was cut into 2 x 2 x 1 cm blocks. Stained or knotted blocks were discarded, and the rest were stored at -18°C until needed. The wood blocks were soaked in de-ionized water overnight then sealed and autoclaved in double-wrapped

autoclave bags (in groups of 20 to 30 wood blocks) at 121°C for 30 mins and reautoclaved twice more then stored at -18°C until required. Blocks were autoclaved once more before use.

3.2.3.2 Preparation of fungal inocula

Eutypa spinosa, Stereum gausapatum, Vuilleminia comedens, Fomes fomentarius, Trametes versicolor, Resinicium bicolor, Stereum hirsutum, Hypholoma fasciculare, and *Bjerkandera adusta* were cultured on 2% malt extract agar (MEA: 20g L^{-1} malt, Munton and Fison, UK, 15 g L^{-1} lab M agar no.2) at 20°C in the dark in non-vented 9 cm diam. Petri dishes until the mycelium covered the surface of the plates. Each of the species were transferred as inocula to 2% MEA in 14 cm diam. Petri dishes sealed with Nescofilm® and incubated in the dark at 20°C. When the mycelium covered the whole surface of the plate, sterile wood blocks were added (20 per dish) and the plates returned to the incubator at 20°C in darkness for 2 months before use.

3.2.3.3 Experimental design

The two-plate method described in (Section 3.2.1.1) was used to determine the effect of interaction of two fungi on the extension rate and the enzyme production of *Trametes versicolor.* Two colonised wood blocks were joined together with rubber bands and attached centrally to the base of a sterile 9 cm diam Petri dish with a drop of agar medium, and left for 4 weeks to interact and produce VOCs. Afterwards, *T. versicolor* was inoculated centrally onto 0.5% malt extract agar plates and taped above the Petri dish containing the paired wood blocks (Fig.3.2) for 5 to 6 d. Colony

extent of the test species was measured daily, across diameters perpendicular to each other using vernier calipers.

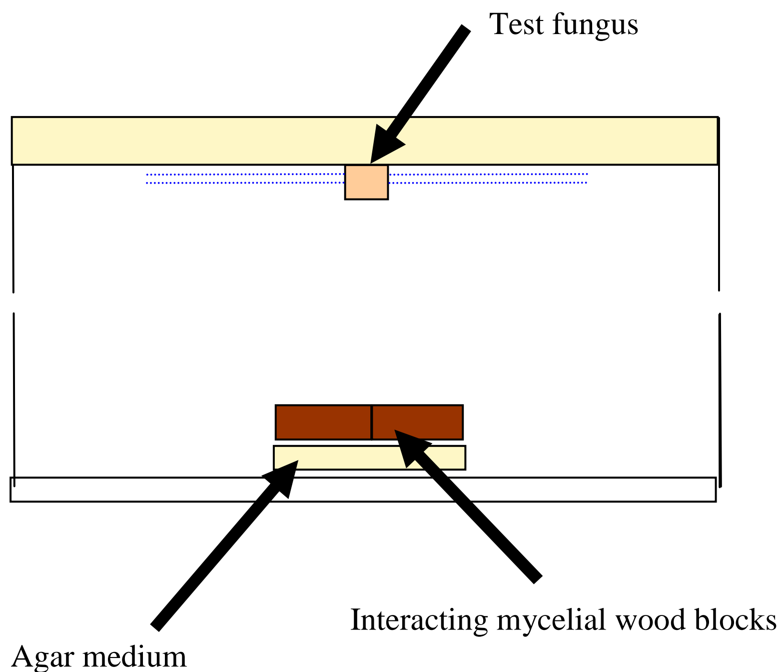


Fig.3.2. Two plates experiment, with mycelial interacting in wood blocks on the lower plate, while the upper plate contained the tested fungus.

3.2.4 The effect of mycelial interaction in wood blocks on laccase and ligninolytic activity of a test fungus on agar

3.2.4.1 Sample preparation for enzyme assays

T. versicolor cultures were set up as described previously in Section 3.3.3 on 16 ml 0.5% Malt Agar in 9 cm non-vented Petri dishes for 5 d then 12mm diam plugs were removed with a No.8 cork borer from the margin of the colonies. Plugs were transferred to 1.5 ml Eppendorf tubes, and 1 ml of deionised water was added. Tubes

were shaken gently overnight at 4 °C, and then 0.8 ml of extract was transferred to a new 1.5 ml Eppendorf tube and centrifuged (8000*g*) for 10 min at 4 °C to precipitate debris. The assays were carried out instantly while the extracts were kept at 4 °C. Five replicate plates were made and the data were normalised to the wet weight of agar added to mycelium extracted for each sample.

3.2.4.2 Laccase activity

Laccase activity was measured by the use of ABTS (as described in Section 2.4) following Bourbonnais & Paice (1990).

3.2.4.3 Manganese peroxidase activity

Manganese peroxidase activity was analysed in a succinate-lactate buffer (100 mM, pH 4.5), where 3-methyl-2-benzothiazoline-hydrazone hydrochloride (MBTH) and 3-(dimethyl amino)-benzoic acid (DMAB) were joined oxidatively by MnP action following Ngo & Lenhoff (1980). A purple colour was observed by using a spectrophotometer at 590 nm. Extracts (50 µl) and 200 µl substrate solution were added to wells of a Bioscreen II plate using a multichannel pipette. Plates were then directly transferred to a Bioscreen C II plate reader (Oy Growth Curves Ab Ltd., Finland). The substrate solution comprised 25 $MnSO_4$ and 5 mM hydrogen peroxide. The tests were done: (a) without manganese, where EDTA (2 mM,) was used to chelate Mn^{2+} present in the extract to allow detection of Mn^{2+}-independent

peroxidases; and (b) in the absence of hydrogen peroxide (H_2O_2) to detect activity of oxidases other than the peroxidases.

3.2.4.4 1,4-β-glucosidase activity

Microplates were used to assay using 1,4-β-glucosidase activity by using *p*-nitrophenyl-β-D-glucoside (pNPG, Glycosynth, UK) following Valášková *et al.* (2007). A reaction mixture comprised 40 µl sample and 160 µl 1.2 M pNPG in sodium acetate buffer (50 mM, pH 5.0) and the reaction mixtures were incubated at 40°C for 120 min. Sodium carbonate (0.5 M) was added to stop the reaction, and absorbance was read at 400 nm in a Dynex Revelation microplate reader (Dynex Technologies Ltd., Sussex, UK). Data were calculated using the molar extinction coefficient of *p*-nitrophenol (11,600 M cm^{-1}). The amount of enzyme discharging 1 µmol of *p*-nitrophenol per minute was defined as one unit of enzyme activity (U).

3.2.4.5 NAG and acid phosphatase activity

p-nitrophenyl N-acetyl-β-D-glucosaminide (pNPN; Glycosynth, UK) and *p*-nitrophenyl phosphate (pNPP; Glycosynth, UK) were used, respectively, in the method described in Section 3.2.4.4 to assay the activity of 1,4-N-acetylglucosaminidase (NAG) and phosphomonoesterase (acid phosphatase). Spectrophotometric measurements were made as for 1,4-β-glucosidase activity (Section 3.2.4.4).

3.2.5 Spatial distribution of laccase and peroxidase production in *Trametes versicolor* on agar

Trametes versicolor (TvD4) (Cardiff University Fungal Ecology Group culture collections) inoculated onto an agar plates (details below) was placed above a plate containing two fungi interacting on agar as in Section 3.2.2 or in woodblocks as in Section 3.2.3. Localisation of laccase activity was determined in 9 cm Petri dishes of 0.5% Malt Agar plus 250 mg l^{-1} 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) which is oxidised to a green/violet product in the presence of laccase (Steffen *et al.*, 2000). Photographs were taken with a Nikon Coolpix Camera at 6 d and 16 d.

Peroxidase activity was visualised on 2% Malt Agar in 9 cm Petri dishes flooded with 2.5 mM diaminobenzidine (DAB) in phosphate buffer (0.1 M, pH 6.9), and incubated for 30 min with gentle rotation following Silar (2005). The stain was drained off and the location of a red precipitate was observed after 1 h, 2h and one day. Control plates contained buffer only.

3.3 Results

3.3.1 The effect of VOCs produced by interacting fungi on the growth of a third fungus on agar

3.3.1.1 Effect of VOCs produced during mycelial interaction between Hypholoma fasciculare *and* Resinicium bicolor *on a third fungus*

Volatiles produced during the interaction between *H. fasciculare* and *R. bicolor* inhibited the extension rate of *T. versicolor, B. adusta, R. bicolor* and *F. fomentarius*, compared with controls. The self-pairing of *H. fasciculare* inhibited the extension rate of *S. gausapatum* but enhanced that of *T. versicolor,* while the self-pairing of *R. bicolor* inhibited the extension rate of *R. bicolor* and enhanced that of *B. adusta* (Table 3.1).

Table 3.1. Mean radial extension rate (mm d^{-1} ± SEM) of test fungi when grown above malt agar with interspecifically and self interacting mycelium of *Hypholoma fasiculare* and *Resinicium bicolor*.

Tested Fungi	Agar	Rb/Hf	Rb/Rb	Hf/Hf	Significant difference
Resinicium bicolor	4.45 ± 0.03	3.63± 0.05 ***	4.14 ± 0.04 ***	4.39 ± 0.03 ns	$F_{3,16}$ = 108.49, P<0.001
Bjerkandera adusta	9.41 ± 0.06	8.80 ± 0.10 ***	9.73± 0.06 ***	9.53 ± 0.08 ns	$F_{3,16}$ = 12.36, P>0.001
Fomes fomentarius	0.68 ± 0.01	0.62 ± 0.01 **	0.70 ± 0.01 ns	0.70± 0.01 ns	$F_{3,16}$ = 8.22, P<0.01
Trametes versicolor	6.39 ± 0.05	5.94 ± 0.03 ***	6.47± 0.03 ns	6.88 ± 0.05 ***	$F_{3,16}$ = 76.29, P<0.001
Stereum gausapatum	5.62 ±0.09	5.53±0.029 ns	5.59 ± 0.08 ns	5.16 ±0.06 ***	$F_{3,16}$ = 9.88, P<0.01
Eutypa spinosa	8.15 ± 0.11	7.10 ± 0.15 ns	8.22 ± 0.12 ns	7.37 ± 0.37 ns	$F_{3,16}$ = 3.29, P<0.05
Vuilleminia comedens	3.28 ± 0.09	3.19± 0.05 ns	3.17 ± 0.09 ns	3.30 ± 0.09 ns	$F_{3,16}$ = 0.48, P>0.05
Trichoderma viride	12.94 ±0.08	12.890±0.2 ns	12.49 ±0.06 ns	12.57±0.07 ns	$F_{3,16}$ = 3.68 , P<0.05
Hypholoma fasiculare	3.72 ± 0.08	3.80 ± 0.10 ns	3.59 ± 0.09 ns	3.42 ± 0.15 ns	$F_{3,16}$ = 4.38, P<0.05

Significance of difference compared with growth above uncolonised agar
(*, P≤0.05; **, P≤0.01;***, P≤0.001, ns, not significant).
Abbreviations: Rb, *Resinicium bicolor;* Hf, *Hypholoma fasciculare.*

3.3.1.2 The effect of volatile compounds produced during mycelial interaction between *Trametes versicolor* ***and*** *Resinicium bicolor* ***on a third fungus***

The VOCs that were produced during mycelial interaction between *Trametes versicolor* and *Resinicium bicolor* enhanced the extension rate of *B. adusta, V. comedens, R. bicolor* and *H. fasciculare* but inhibited that *S. hirsutum* compared with the controls grown above agar (Table 3.2). The self-pairing of *R. bicolor* enhanced the extension rate of six species - *S. gausapatum*, *B. adusta*, *V. comedens*, *R. bicolor H. fasciculare* and *S. hirsutum*, but inhibited *E. spinosa*. On the other hand, the self pairing of *T. versicolor* enhanced the growth rate of three species - *B. adusta*, *V. comedens* and *H. fasciculare*, but inhibited *S. hirsutum*, compared with the controls (Table 3.2).

Table 3.2. Mean radial extension rate (mm d^{-1} ± SEM) of test fungi when grown above malt agar with interspecifically and self interacting mycelium of *Resinicium bicolor* and *Trametes versicolor*.

Tested Fungi	Agar	Rb/Rb	Rb/Tv	Tv/Tv	Significant difference
Trametes versicolor	6.88 ± 0.05	6.85 ± 0.09 ns	6.35± 0.51 ns	6.82± 0.12 ns	$F_{3,16}$ = 0.96, P>0.05
Stereum gausapatum	5.62 ±0.09	5.96±0.16 *	5.44 ± 0.09 ns	5.55 ±0.07 ns	$F_{3,16}$ = 4.32, P<0.05
Eutypa spinosa	8.15 ± 0.11	5.80 ± 0.32 ***	7.73 ± 0.22 ns	8.62 ± 0.49 ns	$F_{3,16}$ = 15.15, P<0.001
Bjerkandera adusta	8.27 ± 0.03	10.25±0.25 ***	10.71± 0.19 ***	10.59 ± 0.10 ***	$F_{3,16}$ = 47.76, P<0.001
Vuilleminia comedens	1.77 ± 0.09	2.75± 0.11 ***	2.74 ± 0.05 ***	2.84 ± 0.10 ***	$F_{3,16}$ = 30.28, P<0.001
Resinicium bicolor	2.68± 0.09	3.55 ± 0.06 ***	3.50 ± 0.12 ***	3.74 ± 0.07 ***	$F_{3,16}$ = 27.76, P<0.001
Hypholoma fasiculare	2.37 ± 0.03	2.55 ± 0.05 **	2.64 ± 0.05 **	2.63 ± 0.04 **	$F_{3,16}$ = 7.18, P<0.01
Stereum hirsutum	6.33 ± 0.20	7.31 ± 0.21 ***	5.93 ± 0.22 ***	5.44± 0.10 ***	$F_{3,16}$ = 17.42, P<0.001

Significance of difference compared with growth above colonised agar. (*, P≤0.05; **, P≤0.01;***, P≤0.001; ns, not significant). **Abbreviations**: Rb, *Resinicium bicolor*; Tv, *Trametes versicolor.*

3.3.1.3 The effect of VOCs produced during interaction between** Stereum gausapatum **and four other species

The volatile compounds produced during all of the nine interspecific interactions or self-pairings tested inhibited the extension rate of *S. hirsutum,* while *E. spinosa* was inhibited by VOCs of four interactions (Es/Es, Sg/Es, Sg/Vc and Vc/Vc) (Table 3.3). *S. gausapatum* extension rate was enhanced by the VOCs effect of four interactions (Sg/Sg, Sg/Sh, Sh/Sh and Vc/Vc) while *T. versicolor* was enhance by three interactions (Es/Es, Sg/Sg and Sh/Sh). *B. adusta* was enhanced by four interactions (Sg/Sh, Sg/Vc, Sh/Sh and Vc/Vc) while it was inhibited by two interactions (Ba/Ba and Sg/Es), on the other hand *V. comedens* was inhibited by two interactions (Sg/Ba and Sg/Es) but it was enhanced by two interactions (Es/Es and Vc/Vc).

The VOCs of self-pairings of the tested species inhibited the extension rate of the same tested species in the case of: *B. adusta*, *E. spinosa* and *S. hirsutum,* but enhanced the extension rate of *V. comedens* and *S. gausapatum.* On the other hand, *T. versicolor* was only enhanced by three self pairings (Es/Es, Sg/Sg and Sh/Sh) and was not affected by the rest of the interactions.

Table 3.3. Mean radial extension rate (mm d^{-1} ± SEM) of test fungi when grown above agar interspecifically interacting mycelium and self pairings.

Tested Fungi	Agar controls	Ba/ Ba	Es/ Es	Sg/ Ba	Sg/ Es	Sg/ Sg	Sg/ Sh	Sg/ Vc	Sh/ Sh	Vc/ Vc	Significant difference
Stereum hirsutum	6.43± 0.14	5.10± 0.04 ***	5.40± 0.09 ***	5.41± 0.21 ***	4.91± 0.12 ***	5.65± 0.05 ***	5.15± 0.10 ***	4.85± 0.01 ***	5.91± 0.08 ***	5.38± 0.18 ***	$F_{9,40}$ = 17.52, P<0.001
Eutypa spinosa	9.14± 0.45	7.58± 0.24 ns	5.90± 0.52 ***	8.36± 0.19 ns	6.87± 0.37 ***	9.14± 0.33 ns	9.48± 0.12 ns	6.09± 0.17 ***	10.1± 0.48 ns	4.72± 1.44 ***	$F_{9,40}$ = 10.25, P<0.001
Stereum gausapatum	4.89± 0.06	4.91± 0.11 ns	5.07± 0.14 ns	5.10± 0.05 ns	5.13± 0.08 ns	5.45± 0.15 ***	5.36± 0.06 ***	5.09± 0.08	5.42± 0.07 ***	5.43± 0.07 ***	$F_{9,40}$ = 5.12, P<0.001
Trametes versicolor	6.59± 0.04	6.81± 0.11 ns	6.87± 0.07 **	6.73± 0.09 ns	6.77± 0.09 ns	7.06± 0.11 **	6.81± 0.06 ns	6.81± 0.05 ns	6.91± 0.10 **	6.58± 0.06 ns	$F_{11,48}$ = 3.31, P<0.01
Bjerkandera adusta	9.02± 0.07	7.51± 0.54 ***	9.59± 0.13 ns	8.75± 0.10 ns	8.25± 0.19 ***	9.62± 0.04 ns	9.76± 0.19 ***	9.76± 0.05 ***	9.78± 0.16 ***	9.69± 0.04 ***	$F_{9,40}$ = 14.29, P<0.001
Vuilleminia comedens	2.31± 0.01	2.43± 0.08 ns	2.99± 0.11 ***	1.55± 0.02 ***	2.06± 0.11 ***	2.50± 0.05 ns	2.38± 0.02 ns	2.34± 0.07 ns	2.40± 0.08 ns	2.66± 0.04 ***	$F_{9,40}$ = 32.55, P<0.001

Significance of difference compared with growth above uncolonised agar (*, P≤0.05; **, P≤0.01;***, P≤0.001; ns, not significant). **Abbreviations**: Es, *Eutypa spinosa;,* Ff, *Fomes fomentarius*; Vc, *Vuilleminia comedens*; Tv, *Trametes versicolor;* Ba, *Bjerkandera adusta*; Hf, *Hypholoma fasciculare*; Rb, *Resinicium bicolor*; Sg, *Stereum gausapatum.*

3.3.2 Effect of mycelial interaction between two fungi on agar on *Trametes versicolor* (its growth rate and laccase production)

Although there were significant differences in *T. versicolor* extension rate compared with the controls which were collected from previous experiments from table 3.1, 3.2, 3.3 and also form the work of Evans *et al*., 2008 to put together in Table 3.4 A and B. The laccase activity was not significantly different in any of the treatments used (Fig.3.3).

Table 3.4. (A, B) The double plate results of the effect of VOCs on the extension growth rate (mm d^{-1}) of *T. versicolor* only (taken from tables 3.1, 3.2, 3.3 and Evans *et al*., 2008).

(A)

Tested fungi	Agar	Rb/Hf	Rb/Rb	Hf4/Hf4
T. versicolor	6.39 ± 0.05	6.39 ± 0.05 ***	6.39 ± 0.05 ns	6.39 ± 0.05 ***
Tested fungi	**Agar**	**Rb/Rb**	**Rb/Tv**	**Tv/Tv**
T. versicolor	6.88 ± 0.05	6.85 ± 0.09 ns	6.35 ± 0. 51 ns	6.82 ± 0.12 ns

(B)

Tested fungi	Agar	Ba/Ba	Es/Es	Sg/Ba	Sg/Es	Sg/Sg	Sg/Sh	Sh/Sh
T. versicolor	6.59± 0.04	6.81 ± 0.11	6.87± 0.07 **	6.73 ± 0.09	6.77 ± 0.09	7.06 ± 0.11 **	6.81 ± 0.06	6.91 ± 0.10 **
Tested fungi	**Tv/Tv**	**Tv/Hf**	**Tv/Sg**	**Tv/Ba**	**Hf3/Hf3**	**Sg/Sg**	**Ba/Ba**	
T. versicolor	ns	ns	ns	sd	sd	sd	ns	

Significance of difference compared with growth above uncolonised agar (*, P≤0.05; **, P≤0.01;***, P≤0.001). **Abbreviations**: Es, *Eutypa spinosa;*, Tv, *Trametes versicolor ;* Ba, *Bjerkandera adusta*; Hf, *Hypholoma fasciculare*; Rb, *Resinicium bicolor*; Sg, *Stereum gausapatum* . ns = not significantly different, sd = significantly different, (results in red colour taken from Evans *et al*., 2008).

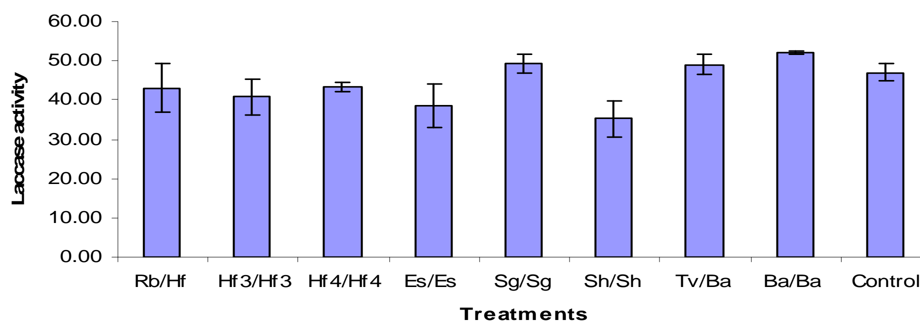


Fig. 3.3 The laccase activity of *T. versicolor* on 0.5% MA exposed to VOCs produced by mycelial interaction of two fungi on agar. There were no significant (P> 0.05) differences between treatments) as analyzed by ANOVA, $F_{11, 15}$ = 2.20. **Abbreviations**: Es, *Eutypa spinosa;* Tv, *Trametes versicolor;* Ba, *Bjerkandera adusta*; Hf, *Hypholoma fasciculare*; Sg, *Stereum gausapatum.*

3.3.3 The effect of VOCs produced during interaction in wood blocks on extension rate, enzyme activity of a test fungus on agar

There were significant differences in extension rates (mm d^{-1}) of *T. versicolor* compared with the control (Fig. 3.4). The extension growth rate of *T. versicolor* was higher than the controls in all treatments and it was the highest with the combinations Tv/Tv and Rb/Rb.

There were also some differences between the treatments in the laccase activity of *T. versicolor* but there were no significant differences (P< 0.01) from the control, it was the highest with the combination Ba/Ba and the lowest with Es/Es (Fig 3.5A).

Manganese peroxidase activity of *T. versicolor* was not significantly different (P> 0.05) between any treatments (Fig. 3.5B), there were however significant differences (P< 0.001) for the oxidase activity compared with the wood block controls (Fig. 3.5C). There was a highly significant increase with combinations of Ba/Ba, Tv/Tv, Hf3/Hf3, Tv/Sg, Sg/Sg, Hf4/Hf4 and Rb/Hf4 activity. On the other hand, there were no significant differences (P> 0.05) in 1,4-β-glucosidase activity (Fig 3.5D).

The -1, 4-β-poly-N-acetylglucose aminidase chitinase activity was the only enzyme activity that showed significant differences between all the treatments (P< 0.001) and the control apart from Sg/Sg. With all the other interactions there was a significant decrease in the activity (Fig 3.5E). In addition, the effect of Es/Es and Rb/Tv was significantly different to the affect of Sg/Sg. There were some significant differences (P<0.001) between treatments for the acid phosphate activity but not between treatments and the control, thus Sg/Sg showed the highest the acid phosphatase activity (Fig 3.5F), which was significantly higher than Tv/Ba, Rb/Tv, Ba/Ba, Tv/Sg,

Sg/Es and Sg/Ba. In contrast, Es/Es induced a significant lower activity than three other interactions (Tv/Tv, Hf3/Hf3 and Sg/Sg).

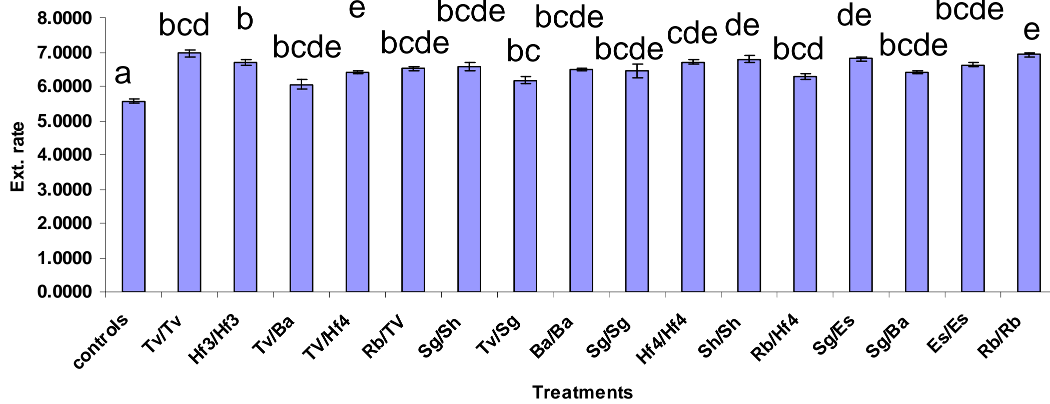


Fig. 3.4 The extension rate of *T. versicolor* when exposed to paired fungi on wood blocks, mean ± SE (n = 5), as analyzed by ANOVA, $F_{16, 66} = 10.147$, ($P < 0.001$).

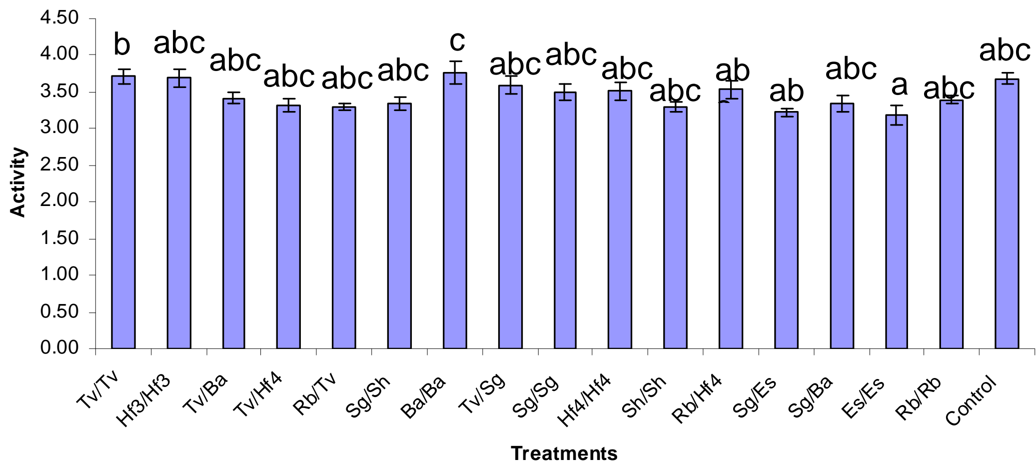


Fig. 3.5A Laccase activity of *T. versicolor* when exposed to paired fungi on wood blocks, mean ± SE (n = 3), as analyzed by ANOVA, $F_{16, 66} = 3.094$, ($P < 0.01$).

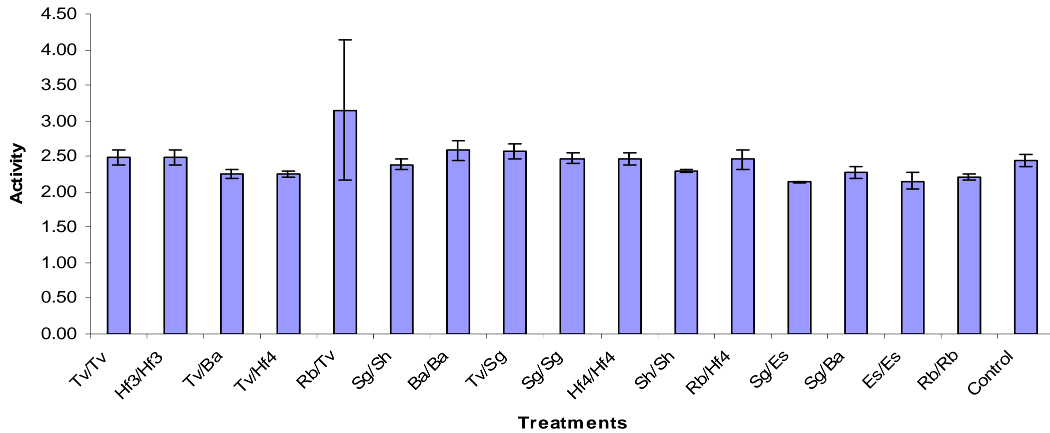


Fig. 3.5.B Manganese peroxidase activity of *T. versicolor* when exposed to paired fungi on wood blocks, mean ± SE (n = 3), as analyzed by ANOVA, $F_{16, 66} = 0.806$, (P> 0.05).

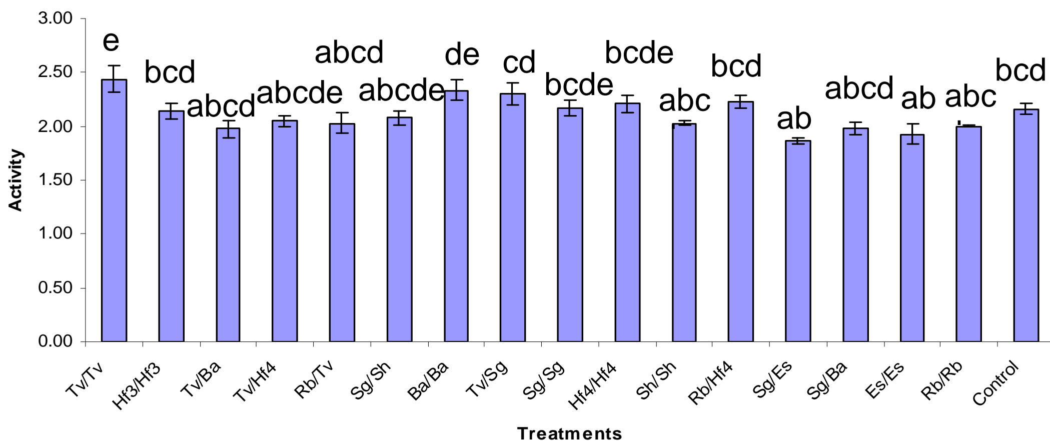


Fig. 3.5.C Oxidase activity of *T. versicolor* when exposed to paired fungi on wood blocks, mean ± SE (n = 3), as analyzed by ANOVA, $F_{16, 66} = 4.014$, (P< 0.001).

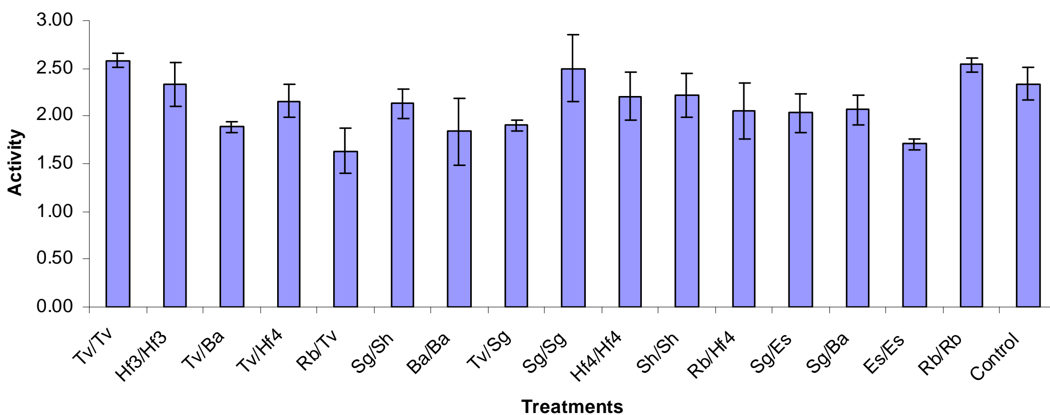


Fig. 3.5.D 1,4-β-glucosidase activity of *T. versicolor* when exposed to paired fungi on wood blocks, mean ± SE (n = 3), as analyzed by ANOVA, $F_{16, 66} = 1.790$, (P> 0.05).

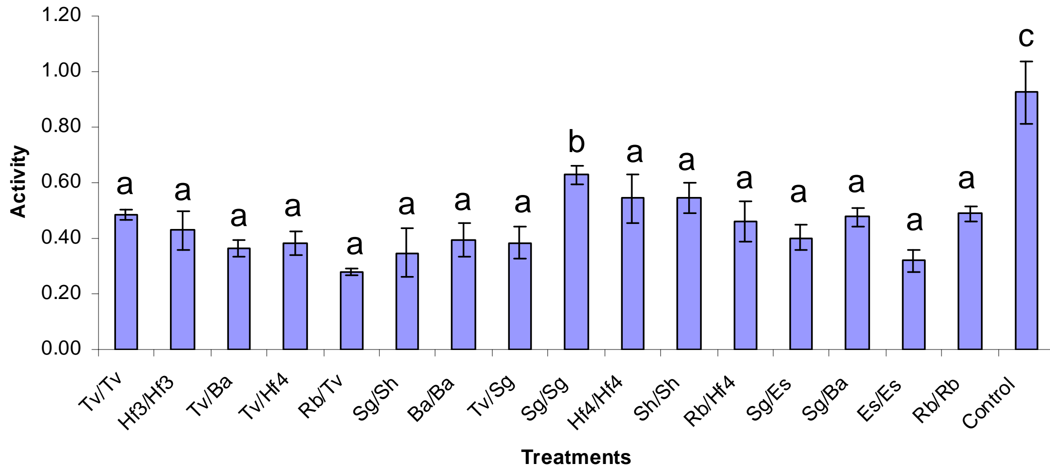


Fig. 3.5.E 1, 4-β-poly-N-acetylglucoseaminidase chitinase activity of *T. versicolor* when exposed to paired fungi on wood blocks, mean ± SE (n = 3), as analyzed by ANOVA, $F_{16, 66} = 6.169$, ($P < 0.001$).

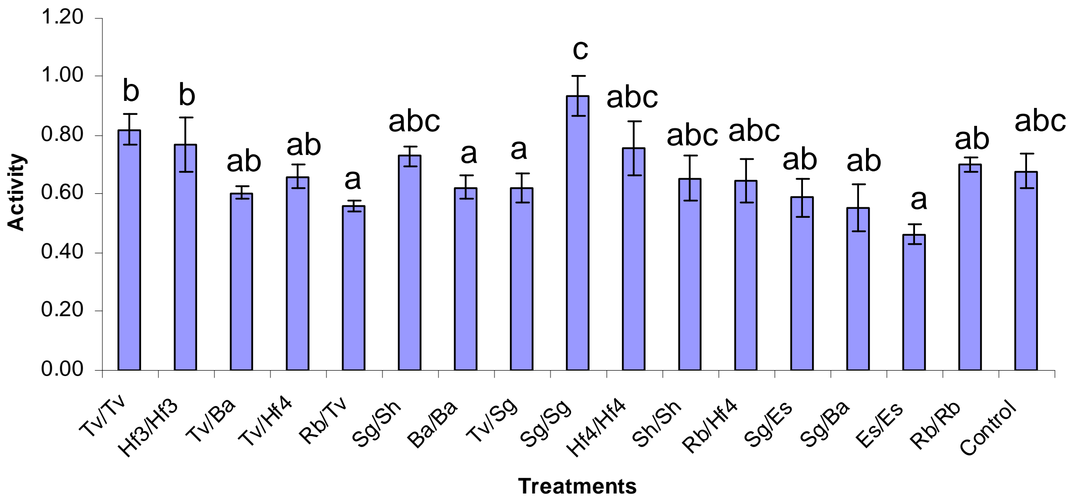


Fig. 3.5.F Acid phosphatase activity of *T. versicolor* when exposed to paired fungi on wood blocks, mean ± SE (n = 3), as analyzed by ANOVA, $F_{16, 66} = 3.396$, ($P < 0.001$).

Fig.3.5 The enzyme activities were measured from fungi grown on 0.5% MA exposed to the effect of VOCs produced from two fungi interacting on woodblocks.). **Abbreviations**: Es, *Eutypa spinosa;,* Ff, *Fomes fomentarius*; Vc, *Vuilleminia comedens*; Tv, *Trametes versicolor;* Ba, *Bjerkandera adusta*; Hf, *Hypholoma fasciculare*; Rb, *Resinicium bicolor*; Sg, *Stereum gausapatum.*

3.3.4 Spatial distribution of laccase and peroxidase production in *Trametes versicolor* on agar

There was some enzyme activity associated with early stages of *T. versicolor* growth at 6 d and this activity showed some clearing at the colony plug zone after 6 d which lead to complete clearing of the colour by 16 d. This clearing indicated that there was laccase production where the oxidation of ABTS turned the substratum colourless (Fig. 3.6). For all the treatments, ABTS gave a green colour with 0.5% MA and red colour 2% MA which may relate to the presence of more nutrients. With the peroxidase staining, more intense more colour developed with time especially near the plug of the colony which may indicate the production was at the early stage of growth as well (Fig 3.7).

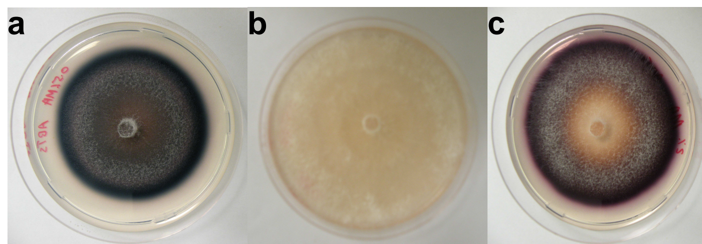


Fig.3.6 ABTS staining indicating the location of laccase activity of *T. versicolor* under the effect of VOCs (a) after 6 d on 0.5% MA, (b) after 16 d on 0.5%MA and (c) after 6 d on 2% MA.

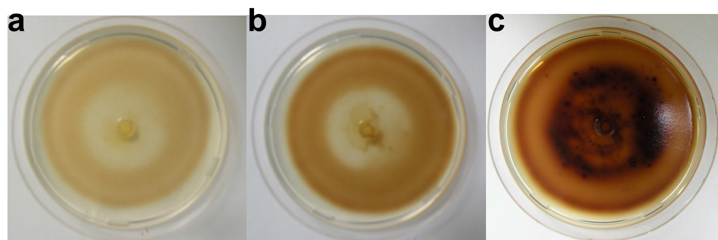


Fig.3.7 Peroxidase staining of *T. versicolor* under the effect of VOCs after 6 d of cultivation on 0.5% MA (a) after 1 hour, (b) after 3 hours and (c) after 1 d.

3.4 Discussion

3.4.1 Inhibition and stimulation of extension rate during paired interactions, and the outcomes

Outcomes were not always the same between replicates perhaps resulting from slight differences in the age of inoculum (Bruce *et al*., 1996), the timing of interaction, the medium of growth, and slight differences in environmental conditions such as temperature, pH and water potential etc, though every effort was made to standardize these. Outcomes can also be affected by the species involved and size of the starting mycelium (Stenlid, 1997, Holmer & Stenlid, 1997).

The fungal outcomes seem to vary depending on the resources and the nutrients they consume, and differences in outcomes are found depending on where the interaction is taking place (e.g. wood, soil and agar cultures) (Dowson, Rayner & Boddy, 1988; Wardle *et al.*, 1993; Holmer & Stenlid, 1997; Woods *et al.*, 2006). On the other hand other studies found that the fungal interaction outcomes of some species were similar when grown on wood and agar (Rayner & Boddy, 1988; Holmer *et al.*, 1997).

The tested species *Stereum gausapatum*, *Hypholoma fasciculare* and *Resinicium bicolor* showed diverse combative abilities, deadlocking, replacing or being replaced by other species. *Bjerkandera adusta* was the highest competitor that takes bigger space against the tested species, while *Eutypa spinosa* had the lowest combative ability. In this study, *H. fasciculare* showed dissimilar outcome to that reported by Boddy (1993) where it replaced *S. gausapatum,* while in this work they deadlocked.

Note, however, that different strains were used, and there can be considerable variation in combat ability of different strains (Rothery *et al*., 2009).

3.4.2 The effect of volatile organic compounds of paired plates

Wood-decay fungi produce volatile metabolites during mycelial interactions in agar and wood which act as infochemicals and antifungal volatile chemicals that could influence other fungi not in physical contact with these mycelia (Evans *et al*., 2008).

When mycelia interact additional VOCs are often produced (Hynes *et al*., 2007), more than those produced when fungi grow alone (Ewen *et al*., 2004; Wheatly 2002), and these compounds certainly affect the growth rate of some fungi.

Hynes *et al.* (2007) found that VOCs were produced following mycelial contact which agrees with the present study. Again, similar to the Hynes *et al* (2007) study, VOC production was correlated with pigment production. The results showed a distance signalling that may enhance or inhibit the extension rates in the top plate, which resulted as a response to the VOCs produced during the interaction of the fungi in the lower plate. For example, *Stereum hirsutum* was the species that was most affected by the VOCs by the decreasing in its extension growth rate, while the interacted *S. gausapatum vs. E. spinosa* were the combination that most affected a third fungus.

Extent of effects varied. For example, results also revealed that VOCs produced when *H. fasciculare* and *R. bicolor* interact clearly affected extension rate of other wood-

decay fungi, and the VOCs produced when *S. gausapatum* interacted with *E. spinosa, V. comedens, S. hirsutum, T. versicolor* and *B. adusta*, and also the self pairings significantly affected the extension rate of the other species, some were inhibited (e.g. *S. hirsutum,* while *S. gausapatum* extension rate was stimulated).

In the context of this recent work, it is clear that the mechanism of the effect of VOCs as an effect on the extension rate has not yet been fully investigated. Therefore, VOCs may lower the pH of the medium by interacting with the film layer of the moisture found at the surface of the medium which may lead to a change in the chemical composition of the medium (Boddy & Heilmann-Clausen, 2008) or they may act directly on the mycelium as a toxic compound to some fungi, as it has been shown to be toxic to some invertebrates (Stadler & Sterner, 1998). In conclusion, in nature community development may, therefore, be influenced not only by direct mycelial interactions, but also indirectly via products of fungal interaction, such as volatile or diffusible metabolites.

Future work will explore how widespread are the effects of interactions between *T. versicolor* and the species tested have. Further, gene expression during such interactions will be explored. The three pairings chosen for further work were: *T. versicolor* paired with *S. gausapatum*, which it replaces; *H. fasciculare* which it is replaced by; and *B. adusta* with which it deadlocks. This covers the spectrum of interaction outcomes, and will extend the work of Eyre (2008), who investigated these same combinations.

3.4.3 Effect of mycelial interaction between two fungi on agar on *Trametes versicolor* (its growth rate and laccase production)

Unlike the results of the effect of VOCs of two interacting fungi on agar on the growth extension rate of *T. versicolor* where it was significantly different, the results on its laccase activity was not significantly different which suggests that there was no correlation between the growth rate and the enzyme activity when the mycelium interactions took place on agar. Elerabei *et al*. (unpublished) found groups of VOCs produced during mycelial interactions on wood blocks that affected the growth of *T. versicolor*, and that gave the motivation to try to study the effect of VOCs produced during mycelial interaction on wood blocks on extension rate and enzyme activities of *T. versicolor*.

3.4.4 The effect of VOCs produced during interaction in wood blocks on extension rate, laccase and ligninolytic activity of a test fungus on agar

Other reports on direct interactions between fungi (Iaklovlev & Stenlid, 2000; Baldrian, 2004; Baldrian, 2006; Chi *et al*., 2007) stated that there are different levels of enzymes produced during direct interaction of two fungi. Since, the current experiments with fungi not in direct contact, but exposed to VOCs from interactions had not been done before, the results obtained here were compared to the results from studies with directly interacting fungi. The extension rate was affected by VOCs produced by interacting fungi grown either on woodblocks or agar. In contrast, laccase activity was affected by the VOCs produced by fungi interacting on woodblocks but not on agar. Hence, further enzymes were analyzed in the woodblock experiment. 1,4-β-glucosidase activity and manganese peroxidase activity were both not significantly different to the controls which suggest that the exposure to VOCs did

not affect the production of those enzymes. The results were significantly different to the controls in the rest of the enzyme activities which suggests that those VOCs produced during the interaction of those combinations enhanced or inhibited the production of those enzymes by *T. versicolor*.

3.4.5 Spatial distribution of laccase and peroxidase production in *Trametes versicolor* on agar

The staining of laccase and peroxidase were both generalized, and were more concentrated near the centre for the peroxidase (Silar, 2005), while more towards the margins of the mycelium of the *T. versicolor* colony for the laccase. The laccase colour started to clear with time in the centre of the colony indicating that the enzyme travels towards the growing mycelium, or no longer active in older mycelium. On the other hand, the laccase activity showed different staining colours when *T. versicolor* was cultivated on 0.5% MA (green colour) while it was purple colour on 2% MA which may indicate the effect of the concentrations of the nutrients in the medium on the production of those enzymes (Steffen *et al*., 2000).

3.4.6 Conclusions:

- Results confirm previous studies showing that production of VOCs by interacting fungi can affect growth of a third fungus.
- Subtle differences were revealed between different combinations of interacting fungi suggesting perhaps a different VOCs profile being produced.

- Enzyme production was not universally affected by the VOCs exposure: laccase was unaffected, whereas activity of other enzymes such as 1.4- β Glucosidase and oxidase were clearly affected by the VOCs and the effect differed depending on the interacting fungi and hence probably the VOCs composition.

Chapter 4: Gene expression during interspecific interactions of *Trametes versicolor*

4.1. Introduction

Biochemical and cellular changes occur during inter-mycelial interactions (Woodward *et al*., 1993; Griffith *et al*., 1994; Gloer, 1995; Bruce *et al*., 2000; Heilmann-Clausen and Boddy, 2005; Hynes *et al*., 2007; Woodward and Boddy, 2008). Although it would seem likely that they are accompanied by changes in gene expression few studies have used global transcriptomic approaches to study mycelial interactions. A few studies have investigated gene expression changes during mycorrhizal interactions (e.g. Peter *et al*., 2003; Deveau *et al*., 2008; Acioli-Santos *et al*., 2008) using microarray analysis. Other studies have used microarrays or related transcriptomic approaches to follow changes in gene expression during host-pathogen interactions between fungi and plant (Jakupovic *et al*., 2006) or mammalian (Steen *et al*., 2002) hosts. Yet other transcriptomic studies have focussed on fungal development such as the change between yeast and mycelial forms in *Paracoccidioides brasiliensis* and the development of fruit bodies in *Sordaria* (Nowrousian *et al*., 2005).

In a recent analysis of gene expression during interspecific interactions, microarrays were used to analyze the interaction between *T. versicolor* and three competitors *S. gausapatum* (which is replaced by *T. versicolor*), *B. adusta* (which results in deadlock) and *H. fasciculare* (which replaces *T. versicolor*) (Eyre *et al.*, 2010).

Trametes versicolor was selected as a model as it is a secondary coloniser and has an 'intermediate' combative ability (Chapela *et al*., 1988; Boddy & Heilmann-Clausen 2008). Thus it was possible to select competitors that lose territory, gain territory or form a deadlock when challenged with this species. A key aim of the work was to compare transcriptomic profiles during interactions which ultimately result in different outcomes to discover whether the same gene expression patterns are common or divergent (Eyre *et al.*, 2010).

Subtractive cDNA libraries were made to enrich for genes that are up-regulated when *T. versicolor* interacts with another saprotrophic basidiomycete, *Stereum gausapatum,* an interaction that results in the replacement of *S. gausapatum* by *T. versicolor.* ESTs (1824) were used for microarray analysis, and their expression compared during interaction with three different fungi: *Stereum gausapatum* (replaced by *T. versicolor), Bjerkandera adusta* (deadlock*)* and *Hypholoma fasciculare* (replaced *T. versicolor*). Expression of significantly more targets changed in the interaction between *T. versicolor* and *S. gausapatum* or *B. adusta* compared to *H. fasciculare* suggesting a relationship between interaction outcome and changes in gene expression (Eyre *et al.*, 2010).

The aim of the work reported in this chapter was to analyse expression of selected genes from the *T. versicolor* EST collection using semi-quantitative RT-PCR and compare expression to the array data. The genes were selected based on their expression as determined by microarray analysis or because of their putative function.

The first prep was to design suitable primers for RT-PCR and confirm their specificity by cloning and sequencing the PCR product. The primers were designed for use with semi-quantitative RT-PCR (Table 4.1).

The specific aims for the work described in this chapter were:

1. Selection of genes for analysis by RT-PCR and cloning of PCR products for confirmation of primer specificity.
2. Analysis of gene expression through RT-PCR in *Trametes versicolor* during interspecific mycelial interactions.

4.2. Materials and Methods

4.2.1 Primer design

As described in Section 2.3.6.

4.2.2 PCR amplification from cDNA

As described in Sections 2.3.1 to 2.3.5.

4.2.3 Cloning of PCR products for confirmation of primer specificity

Cloning and sequence analysis was done as described in Sections 2.3.10.1 to 2.3.10.5.

4.2.4 Analysis of gene expression through semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out as described in Sections 2.3.1 to 2.3.9, and the PCR primers used are listed in Table 4.1. One biological replicate and three technical replicates were used.

Table 4.1 Specific primers used to test extracted *T. versicolor* when interacted with opposing species.

Primer	Primer sequence (5' to 3')	No. bases	Annealing temperature (°C)	No. of cycles	Product size
FRA2-F	CGGTACTGTCTGCTGCGATA	20	60	32	230bp
FRA2-R	CGAACACACGAGTTCTTGGA	20			
F1D8-F	CGGAAAAAGAGCGAGGAGA	19	60	36	207bp
F1D8-R	CTCCTCGAGAAGTGCAAAGC	20			
F2A1-F	GGAGAGTGCGGTGACAATGAA	20	60	28	160bp
F2A1-R	GTGTGACTGTCATTGTCGGG	20			
FRA19-F	AGACTACCAGGACGGAACGA	20	60	23	154bp
FRA19-R	AATGGAAAGCATGGGAAGG	19			
Nox- F	TCGGTTGGTTCCAGACTCTC	20	60	32	205bp
Nox- R	TAGATGGCCTTCCAGTCAGG	20			

4.3 Results

4.3.1 Cloning of PCR products for confirmation of primer specify

Five genes were selected for PCR analysis (Table 4.2).

Table 4.2. Analysis of gene expression through semi-quantitative RT-PCR in *T. versicolor* during interspecific mycelial interactions.

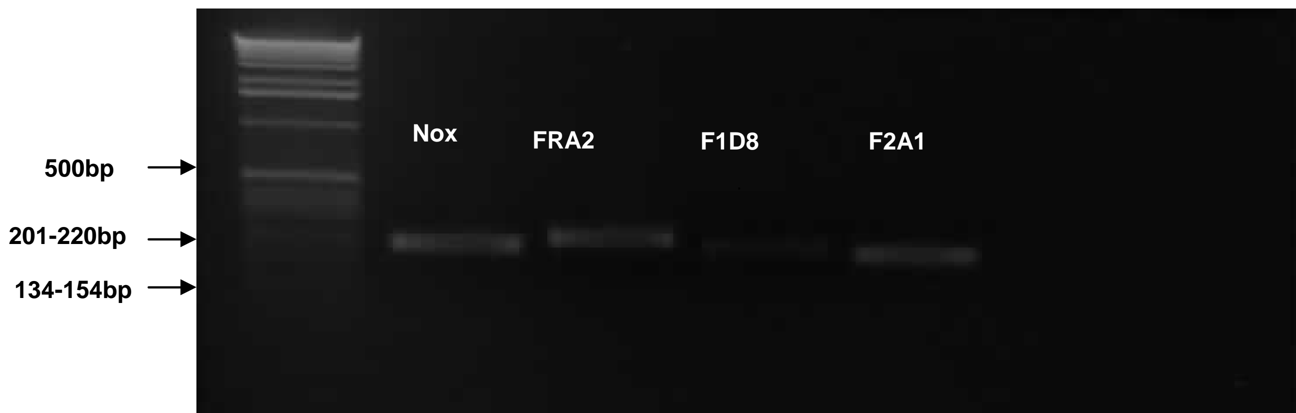
EST Code	**Clone ID**	**The number of Clone IDs**	**Cluster ID**	**Putative function**	**Expression on arrays (mean value)**		
					Tv/Sg	**Tv/Ba**	**Tv/Hf**
FRA2	F_10A03	6	TVC00203	unknown function	1.80	1.87	1.53
FRA19	R_10A01	6	TVC01061	Glycine-rich RNA binding protein.	0.49	0.77	0.7
F2A1	F_06A01	4	TVC00043	unknown function	0.78	0.996	1.73
F1D8	F_03D03	1	TVC00110	unknown function	0.37	0.33	0.49
Nox	R_08B09 R_07A07 R_03A07	1 1 1	TVC01049 TVC00960 TVC00679	NADPH oxidase (enzyme involved in ROS production)	1.01 0.72 0.86	1.37 1.18 0.97	1.65 0.95 1.10

Tv = *Trametes versicolor*, Sg = *Stereum gausapatum*, Ba = *Bjerkandera adusta* and Hf = *Hypholoma fasciculare*. Down-regulated and up-regulated genes from Eyre *et al.* (2010).

To verify that the products amplified by PCR using specific primers were in fact the genes analysed on the microarray, PCR products were cloned and sequenced. PCR products of *T. versicolor* were amplified with the specific primers for FRA19, FRA2,

F1D8, F1A2, Nox; as in Table 4.1; product sizes were as expected from the primer design (Fig. 4.1). PCR products were purified from the gel, then cloned into *E.coli* in the vector pGEM-T Easy and sequenced. One clone was sequenced for each PCR product cloned and sequences compared to the original EST clones. The results showed that all the products were identical to the sequences. Alignments of the sequences generated with original EST sequences from Eyre *et al* (2010) are shown in Appendix D.

(A)



(B)

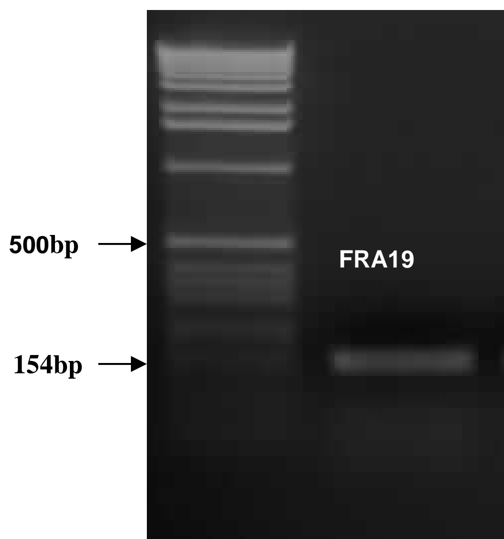


Figure 4.1 Ethidium bromide stained agarose gel of PCR products from: A, Nox, FRA2, F1D8 and F2A1; B, FRA19 primer pairs. Product sizes were as expected from the primer design in Table 4.1

4.3.2 Analysis of gene expression through semi-quantitative RT-PCR in *T. versicolor* during interspecific mycelial interactions

4.3.2.1 Nox gene expression

Three clusters identified from the ESTs (TVC01049, TVC00960 and TVC00679) (Table 4.2) each only comprising one target sequence, were homologous to a gene related to ROS production: NADPH oxidase. On the arrays, all three spots were stable in expression in all three interactions except TVC01049 in the *T. versicolor vs. H. fasciculare* (up-regulated by 1.65 fold) (Table 4.2). RT-PCR using primers designed to TVC00960 confirmed the array result that expression of this gene was unaffected by any of the three interactions with competitors tested (Fig 4.2).

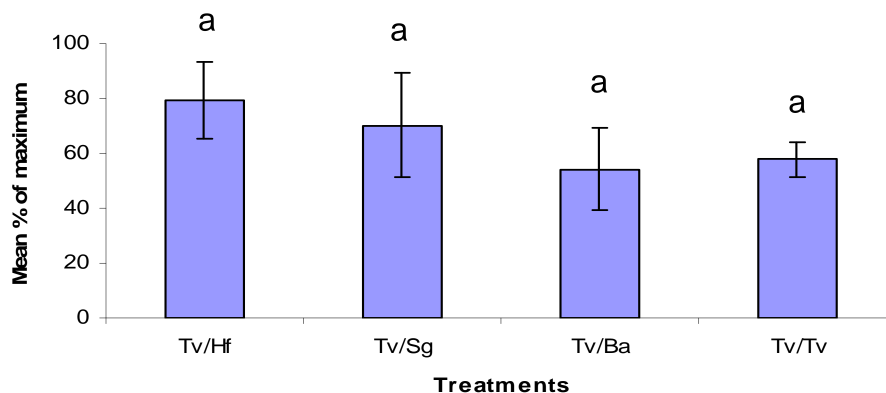


Fig. 4.2 Gene expression of Nox gene (TVC01049, TVC00960 and TVC00679).

Different letters above the histogram bars indicate significant difference (P>0.05) as analyzed by ANOVA, $F_{3,16}$ = 10.815, relative gene expression expressed as mean % of maximum value (Appendix E), error bars are SEM (n=3), Tv = *Trametes versicolor*, Sg = *Stereum gausapatum*, Ba = *Bjerkandera adusta* and Hf = *Hypholoma fasciculare*.

4.3.2.2 FRA2 gene expression

TVC00203 (FRA2) is of unknown function and is represented by just one target on the microarray (Table 4.2) and its mean expression ratio was close to one in all three interaction experiments (*T. versicolor vs. S. gausapatum*: 1.16±0.13, *T. versicolor vs. B. adusta*: 1.14±0.14, *T. versicolor vs. H fasciculare*:1.19±0.10). Semi-quantitative RT-PCR indicated a similar pattern (*T. versicolor vs. S. gausapatum*: 1.17± 0.29, *T. versicolor vs. B. adusta*: 1.10± 0.09, *T. versicolor vs. H fasciculare*: 1.43± 0.28). Thus, only in the interaction with *H. fasciculare* did expression rise above the 1.4 fold threshold used in the microarray analysis to define up or down regulations. The gene expression results showed no significant differences (P>0.05) between all the treatments (Fig. 4.3).

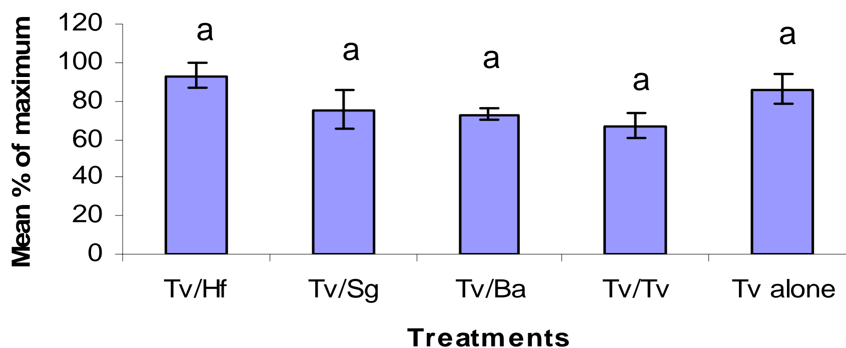


Fig. 4.3 Gene expression of FRA2 gene (TVC00203).

Different letters above the histogram bars indicate significant difference (P>0.05) as analyzed by ANOVA, $F_{4,10} = 2.127$, mean relative gene expression expressed as mean % of maximum value (Appendix E), error bars are SEM (n=3). Tv = *Trametes versicolor*, Sg = *Stereum gausapatum*, Ba = *Bjerkandera adusta* and Hf = *Hypholoma fasciculare*.

4.3.2.3 F1D8 gene expression

TVC00110 (F1D8) also represented just one target on the array (Table 4.2). Its sequence did not match any database sequences and is hence of unknown function. Its expression was down regulated in all three interaction experiments (*T. versicolor vs. S. gausapatum*: 0.37±0.07, *T. versicolor vs. B. adusta*: 0.35±0.02, *T. versicolor vs. H fasciculare*: 0.50±0.09) on the microarrays as the mean of the replicates of the regulated gene. Semi-quantitative RT-PCR confirmed this expression pattern for two of the interactions: with *S. gausapatum* and *B. adusta* (*T. versicolor vs. S. gausapatum*: 0.74± 0.01, *T. versicolor vs. B. adusta*: 0.72± 0.16) (Fig. 4.4). Unexpectedly, however, RT-PCR indicated an up-regulation of this gene in the interaction with *H. fasciculare* (*T. versicolor vs. H fasciculare*: 1.84± 0.44) (Fig.4.4). Although the up-regulation was only statistically significant (P<0.01) in relation to Tv grown alone, but not self-paired *T. versicolor*.

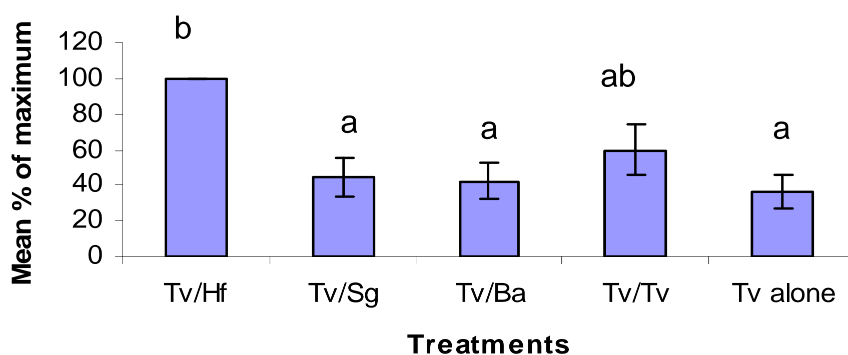


Fig. 4.4 Gene expression of F1D8 gene (TVC00110).

Different letters above the histogram bars indicate significant difference (P<0.01) as analyzed by ANOVA, $F_{4,10} = 6.621$, mean relative gene expression expressed as mean % of maximum value (Appendix E), error bars are SEM (n=3). Tv = *Trametes versicolor*, Sg = *Stereum gausapatum*, Ba = *Bjerkandera adusta* and Hf = *Hypholoma fasciculare*.

4.3.2.4 FRA19 gene expression

TVC01061 (FRA19) is homologous to glycine-rich RNA binding proteins and represents six targets on the array (Table 4.2). These targets were both up- and down-regulated on the array indicating that they may comprise a gene family and that their role is complex. They were highly represented amongst the up-regulated genes in all three experiments, however, the mean expression of this cluster on the microarrays indicated little change in response to interactions (*T. versicolor vs. S. gausapatum*: 1.26±0.22, *T. versicolor vs. B. adusta*: 0.90±0.10, *T. versicolor vs. H fasciculare*:1.00±0.10). This is not supported by the RT-PCR data where the specific sequence charted was significantly (P<0.001) down-regulated both in relation to *T. versicolor* grown alone and self-paired *T. versicolor* (Fig. 4.5, Table 4.2).

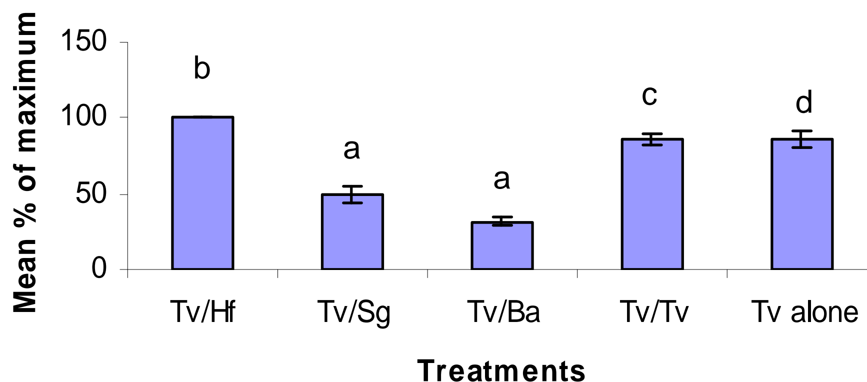


Fig. 4.5 Gene expression of FRA19 gene (TVC01061).

Different letters above the histogram bars indicate significant difference (P<0.001) as analyzed by ANOVA, $F_{4,10}$ = 260.429, mean relative gene expression expressed as mean % of maximum value (Appendix E), error bars are SEM (n=3). Tv = *Trametes versicolor*, Sg = *Stereum gausapatum*, Ba = *Bjerkandera adusta* and Hf = *Hypholoma fasciculare*.

4.3.2.5 F2A1 gene expression

The final cluster for which RT-PCR data were obtained was TVC00043 (F2A1). This cluster comprised four targets on the array (Table 4.2) and is of unknown function. Its microarray expression remained below the 1.4 fold threshold in all three interaction experiments (*T. versicolor vs. S. gausapatum*: 0.79±0.08, *T. versicolor vs. B. adusta*: 0.83±0.05, *T. versicolor vs. H fasciculare*: 1.29±0.21). This expression pattern was in agreement with the RT-PCR in one of the interaction experiments: *T. versicolor vs. S. gausapatum* (ratio: 0.77± 0.17). However, for *T. versicolor vs. H. fasciculare* (ratio: 1.16± 0.20) RT-PCR revealed a strong up-regulation both in relation to *T. versicolor* grown alone and self-paired *T. versicolor.* In contrast, RT-PCR revealed a down-regulation of the expression of this gene in the interaction with *B. adusta,* again in relation to both *T. versicolor* grown alone, and self-paired *T. versicolor* (ratio: 0.28±0.10) (Fig. 4.6, Table 4.2).

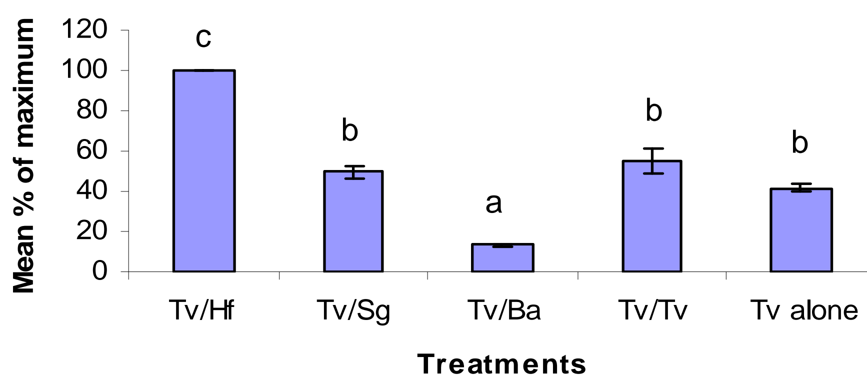


Fig. 4.6 Gene expression of F2A1 gene (TVC00043).

Different letters above the histogram bars indicate significant difference (P<0.001) as analyzed by ANOVA, $F_{4,10}$ = 29.282, mean relative gene expression expressed as mean % of maximum value, error bars are SEM (n=3). Tv = *Trametes versicolor*, Sg = *Stereum gausapatum*, Ba = *Bjerkandera adusta* and Hf = *Hypholoma fasciculare*.

4.4 Discussion

All the clones obtained using the PCR primers designed to the ESTs from Eyre *et al.* (2010) were identical to the EST sequences. This shows that the primers were sufficiently specific for use in expression analysis by semi-quantitative RT-PCR. Although only one biological replicate was used, the material was different to that used for the array analysis but essentially the two support each other. Thus, results that agree between the array and the RT-PCR constitute two biological replicates. The gene expression of the Nox gene was flat in all the treatments which suggests that other genes or enzymes affect superoxide generation shown at the interaction zone by staining (Eyre *et al.*, 2010).

Further genes were selected on the basis of their array expression as up-regulated or down-regulated genes. One of the difficulties in comparing microarray and RT-PCR data is that there may be differences in specificity. The microarray in this case used cDNA targets which are likely to hybridise to several members of a gene family, in contrast PCR primers were designed to specific gene sequences. It is therefore possible, in cases where there is a discrepancy between the RT-PCR and the array results, that different members of the gene family predominate in the array expression. One discrepancy was noted in the expression of F1D8 in the *Trametes versicolor* vs. *Hypholoma fasciculare* interaction; this difference may relate to the more global changes in gene expression noted as a result of interaction with *H. fasciculare* which differed from those elicited by the other two interactions. The up-regulation of F1D8 in the *Trametes versicolor* vs. *Hypholoma fasciculare* interaction was however only significant in relation to *Trametes versicolor* growing alone, suggesting that further

experiments may be required (Eyre *et al*., 2010) to provide conclusive evidence of this gene expression pattern (Rayner, 1991; Glass and Dementhon, 2006; Peiris *et al.*, 2008). The differences in expression between *Trametes versicolor* growing alone and self-paired *Trametes versicolor* are interesting and need further confirmation. Both FRA19 and F2A1 are members of a complex gene family in *T. versicolor* several members of which were represented on the array. The complexity of these genes may not be correctly reflected in the clustering of the sequences. Thus the gene family structure may explain the discrepancies noted between the array and RT-PCR results.

The relatively low levels of change detected on the array and verified by RT-PCR, indicate that further gene expression studies on mycelium closer to the interaction zone will be of interest, where big changes in enzyme activity are seen with staining (Steffen *et al*., 2000; Pointing *et al*., 2005; Silar, 2005; Eyre *et al*, 2010).

The major findings are:-

- The gene expression results by using RT-PCR confirmed the microarray's results.
- The RT-PCR with the use of designed primers confirmed that the gene expression of Nox gene was unaffected by any of the three interactions
- There were differences between the gene expression of *T. versicolor* when interacting with other fungi and when it was alone.

Future work could investigate more genes of functional significance or that represent interesting array patterns. Given that ROS are clearly involved in inter-mycelial interactions, this suggests a possible relationship to stress, where ROS levels are also

elevated (Henson *et al*., 1999; Silar, 2005; Jazek *et al*., 2006; Zhao *et al*., 2009). Therefore, it would be interesting to investigate the expression of genes when Tv mycelium is subjected to other forms of stress. Some results to address this question are presented in Chapter 5.

Chapter 5: The effect of abiotic stresses on growth, gene expression and laccase activity on *Trametes versicolor*

5.1 Introduction

Fungi respond to a variety of stresses including extreme and/or variable temperature, low water availability, elevated water, elevated CO_2 / reduced O_2, excess light, lack of nutrients and exposure to heavy metal ions (Griffin, 1994; Boddy *et al.*, 2008). Temperature is one of the most important stress factors and it influences fungi mainly through its effects on enzyme-catalysed reactions (Rayner & Boddy, 1988). Some wood decay fungi grow successfully over a variety of temperatures, matric and osmotic potentials allowing them decompose in conditions ranging from polar to tropical adding to their rapid wood decay ability in tropical environments (Mswaka & Magan, 1999), wood-rotting basidiomycetes are however mainly mesothermic with minimum, optimum and maximum temperatures of 5, 25 and 40°C (Rayner & Boddy, 1988).

Low temperatures cooling down to freezing will affect the cellular osmotic balance and can also lead to membrane damage (Feofilova *et al.*, 2000). Activity and growth of fungi at low temperatures may switch on ecological and or physiological mechanisms for survival (Fig 5.1). Ecological mechanisms include avoiding cold enviroments and spore germination. Physiological mechanisms resulting in cold tolerance include protection against osmotic effects through accumulation of polyols such as trehalose, found in both xerophilic and nonxerophilic fungi (Hallsworth & Magan, 1995, 1999). There are also increases in unsaturated membrane lipids and

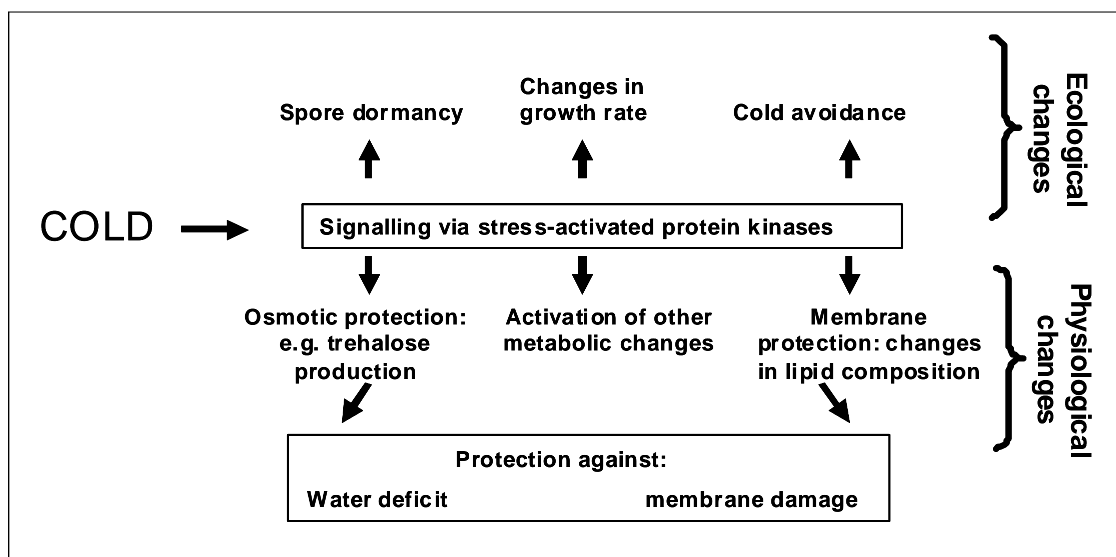


Fig. 5.1 Scheme of fungal responses to cold stress

secretion of antifreeze proteins and enzymes. For example, a psychrotrophic isolate of *Geomyces pannorum* grown at 5°C exhibited altered lipid composition compared with the same isolate grown at 15°C (Weinstein, 2000) and antifreeze proteins with epitopic homology to those of the Atlantic winter flounder were found in hyphae of *Sclerotinia borealis*, *Coprinus psychromorbidus* and *Typhula incarnata* (Newsterd *et al.*, 1994). Extracellular enzymes - proteases and acid phosphomonoesterase - were produced by *Hebeloma* strains in the arctic only at as low temperature as 6°C (Tibbett *et al.*, 1998; 1999).

Some proteins and transcripts induced in response to cold temperature (8°C) detected in *Metarhizium anisopliae* include those with sequence similarities to actin, NADPH quinone oxidoreductase, a thiamine biosynthesis protein and a yeast-like membrane protein (De Croos & Bidochka, 2001). Smith *et al.* (2010) presented an overview of diverse strategies used in the model yeasts *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Candida albicans*, to sense and transduce stress

signals to their respective stress-activated protein kinases. These signaling mechanisms appear to be highly conserved across fungal species, although the sensing mechanisms are more divergent. Smith *et al.* (2010) conclude that some of the differences in these signalling mechanisms may reflect adaptation to environmental conditions in different fungi.

Temperature changes, especially elevated temperatures, are also linked to the production of particular sets of proteins, such as the heat-shock proteins (Hightower & Hendershot, 1997; Higgins & Lilly, 1993; Gropper & Rensing, 1993). Melanin in dark septate hyphae may also protect these structures from extreme temperatures (Robinson, 2001).

Low nitrogen availability is also an important stress for fungi. Fungi can use a range of N sources. Nitrocellulose (N content above 12%) alongside amino acids or sole N was used as a nitrogen source for *Trametes versicolor*, *Pleurotus ostreatus* and *Trichoderma reesei,* and the nitrocellulose in the media decreased by 10%-22%. (Auer *et al*., 2005). Chitin can also be used as a nitrogen source by some fungi such as *Hypholoma capnoides*, *Resinicium bicolor* and *Coniophora arida* (Lindahl & Finlay, 2006). The ability of the fungi to use inorganic (nitrate, ammonium) and organic (amide, peptide, protein) nitrogen sources was determined in *Russula spp., Lactarius sp*., and *Amanita sp*. These fungi utilized ammonium and glutamine but had little ability to use other N sources (Sangtiean & Schmidt, 2002). Responses to nutrient stress can again be ecological or physiological. For example, *Phanerochaete velutina* shows an ecological response: it was insensitive to small organic resources but it rearranged its mycelial biomass just after it came across a new resource that was

significantly bigger than the food-base from which it extended (Dowson *et al*., 1986; Bolton, 1993; Bolton & Boddy, 1993).

Stress factors have an effect on fungal metabolism and the fungi are able to regulate their biological activity to resist stresses (Fink-Boots *et al*., 1997). In particular, laccase activity increased in response to temperature stress in several basidiomycete species (Fink-Boots *et al.,* 1999). The focus of the current study is on the white-rot basidiomycete *Trametes versicolor,* which is a high laccase producer. Laccase also shows a large increase in activity during interactions, in *T. versicolor* (White & Boddy, 1992; Baldrian, 2004; Baldrian, 2006; Hiscox *et al*., 2010), and hence it was chosen for the experiments in this chapter. Hiscox *et al.* (2010) tested laccase activity and gene expression as a result of mycelial interactions and found that laccase transcript abundance was affected. Laccase is encoded by a gene family and a small scale study was also performed to determine whether members of the laccase gene family were differentially expressed during interactions, although results were not conclusive (Hiscox, 2010).

Given the parallel increases in laccase activity during interactions between competing fungi and also under stress, further questions were asked whether stress would affect gene expression more generally in the same way as was found during fungal interactions (Eyre *et al*., 2010 and Chapter 4).

The main aims of this study was to test the effects of single stress treatments on *Trametes versicolor* grown on solid medium and to determine the stresses applied could affect the tested fungus at the level of growth rate, and enzyme activity, as well

as the gene expression. Stresses imposed were high and low temperature, osmotic stress and low nitrogen source. Temperature stress was imposed both as a continuous treatment and as a short treatment. The latter was tested to compare results to a previous study performed in liquid culture (Fink-Boots *et al.*, 1999). The hypotheses tested were that:

(1) stress treatments affect extension rates;

(2) stress treatments induce an increase in laccase production;

(3) increase in laccase enzyme production is preceded by increases in transcripts;

(4) changes in laccase transcript levels are not the same for all gene family members;

(5) stress affects expression of other genes in the same way as interactions.

To test these hypotheses, the following parameters were investigated:

(1) extension rate under continuous stress;

(2) spatial distribution of laccase activity under continuous stress;

(3) Lacc, FRA19 and Nox gene expression continuous under stress;

(4) laccase activity under continuous stress;

(5) effect of heat and cold (continuous and shock) on the laccase enzyme activity;

(6) expression of the laccase gene family under continuous stress.

5.2 Materials and Methods

Experiments were conducted on the effect of abiotic stresses (temperatures, osmotic pressure and deficiency of nutrients) on *T. versicolor* to observe the effect of those stresses on extension rate, laccase activity and also gene expression. The gene expression was for three genes; laccase (Lacc) which degrade lignin, the gene

(FRA19) which gave the best results in testing the microarrays in chapter 4 which was down-regulating in all replicates and also it had a putative function and the Nox gene (NADPH oxidase) which is important in sharing the capacity to transport electrons across the plasma membrane and to generate superoxide and down-stream reactive oxygen species.

5.2.1 Culture media and conditions for *T. versicolor* under continuous stress

For longer-term temperature stress experiments, *T. versicolor* was cultured in separate experiments on 2% malt agar at 20°C (control), 5°C (low temperature stress) and 35°C (high temperature stress). In a separate experiment osmotic stress was imposed by adding, 0.3M KCl (22.367 g L^{-1}) to the 2% malt agar medium. In a further experiment nitrogen stress was applied by culturing the fungus on 20 g L^{-1} agar only; a cellophane layer was added, after sterilization (Appendix B) on top of the medium to ease harvesting the mycelium for RNA extraction for the gene expression experiments, while cellophane was not applied for the enzyme analysis experiments. Limited-nitrogen medium was used as described in Appendix B.

In all these experiments the fungus was cultured until the colony reached a diameter of approximately 62 mm. Thus times of incubation varied depending on the growth rate of the fungus under the different stress treatments (Table 5.1).

Table 5.1 incubation times under different stress treatments required to obtain cultures of the same diameter.

Treatment	**Days of growth**
20°C (control)	5
5°C,	12
35°C	6
KCl	11
agar only	10
Limited-Nitrogen (L.N)	5

The short term heat and cold shock experiments were done by growing the fungus on Fahraeus medium (Fahreus & Rainhammar, 1967, Appendix B). Note that when culturing for RNA extraction, the agar was 20 g instead of 15 g per litre. In this case mycelium was harvested after defined time points rather than relative to colony diameter, as described above.

5.2.2 Extension rate of *T. versicolor* under continuous stress

T. versicolor was grown alone, as described in Section 2.2.1 at 20°C for controls, KCl, agar only and limited nitrogen, 5°C as a low temperature and 35°C as a high temperature. Radial extension rate was determined by measuring across 2 diameters perpendicular to each other every day (at least six readings) until the colony reached the size of the control colonies as shown in Table 5.1.

5.2.3 Spatial distribution of laccase activity under continuous stress

Five *T. versicolor* replicates were cultured (Section 2.4), testing for the laccase presence by inclusion of ABTS in the medium and following the methods of Steffen *et al*. (2000) and Pointing (1999). Stresses were applied as described in Section 5.2.1. Photographs were taken with Nikon Coolpix Camera at different times depending on the size of the colony when it reached the size of the controls (20°C for 5d) (Fig. 5.3).

5.2.4 Gene expression under continuous stress

As described in Section 2.3. The mycelium was taken, by using a small spatula to skim the mycelium from the surface of the agar, only from the margin of the colony (Fig. 5.2).

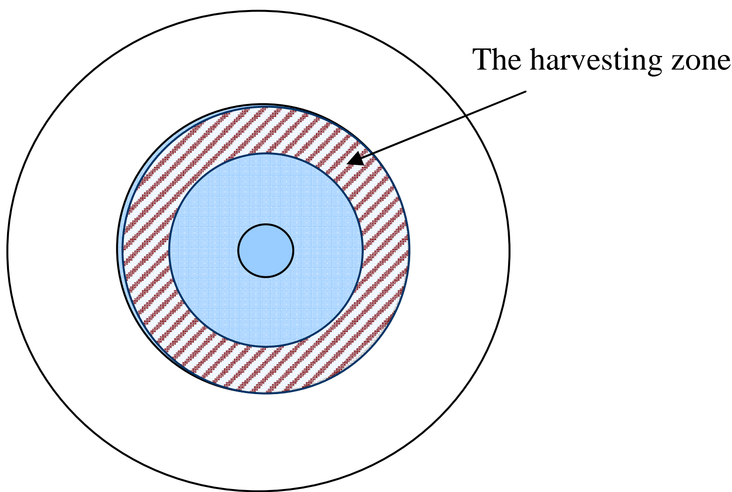


Fig. 5.2 *T. versicolor* grown alone for harvesting for molecular work (blue colour is the fungus colony; red colour is the harvesting zone).

RNA was extracted and cDNA synthesised as described in Section 2.3.2 - Section 2.3.5. Semi-quantitative RT-PCR was performed as described in Section 2.3.7 using primers listed in Section 2.3.6. Two biological replicates (each with three technical replicates) were used to measure expression of the *T. versicolor* genes.

5.2.5 Laccase activity of *Trametes versicolor* under continuous stress

Laccase activity was measured by using ABTS (as described in Section 2.4) following Bourbonnais & Paice (1990).

5.2.6 Expression of the laccase genes family under continuous stress

PCR products were amplified using laccase primers (as detailed in Section 2.3.6). Cloning and sequence analysis were done as described in Sections 2.3.10.1 - 2.3.10.5, then sequences were aligned using ClustalX 2.0.9.

5.2.7 Statistical Analysis

For the gene expression experiments, means of the percentage maximum, and for the of the laccase activity means, were compared using SPSS (v.16), where significant differences between the means were tested using one-way ANOVA (Post Hoc Multicomparisons, using Tukey's test where the significance level was at 0.05) and the resulting data were arranged into groups to show the significance of differences compared with the control, where each group was assigned a different alphabetical letter, where graph bars with the same letters are not significantly different.

5.2.9 Analysis of expression of the laccase gene family under stress

PCR products of *T. versicolor* under stress treatments (20, 5 and 35°C, KCl, Limited-N, agar only) were cloned (as described in Section 2.3.9.1 - 2.3.9.4). Plasmid DNA was extracted as described in Section 2.3.9.5 - Section 2.3.9.6, and sequences from five clones of each treatment were obtained (four for KCl treatment), this yielded a total of 29 sequences. Sequences were aligned using BioEdit (also BlastX 2.9.1) programs to make a dendrogram of them and to compare them with accessioned laccase sequences groups (γ, β and δ).

5.3 Results

5.3.1 Extension rate of *Trametes versicolor* under continuous stress

The extension rate of *T. versicolor* when exposed to the four stress treatments (5°C, 35°C, no nitrogen and KCl) was significantly ($P \leq 0.05$) decreased compared with the control (Fig. 5.3). Low nitrogen stress was the only treatment that did not significantly ($P > 0.05$) affect extension rate compared to the control (20°C).

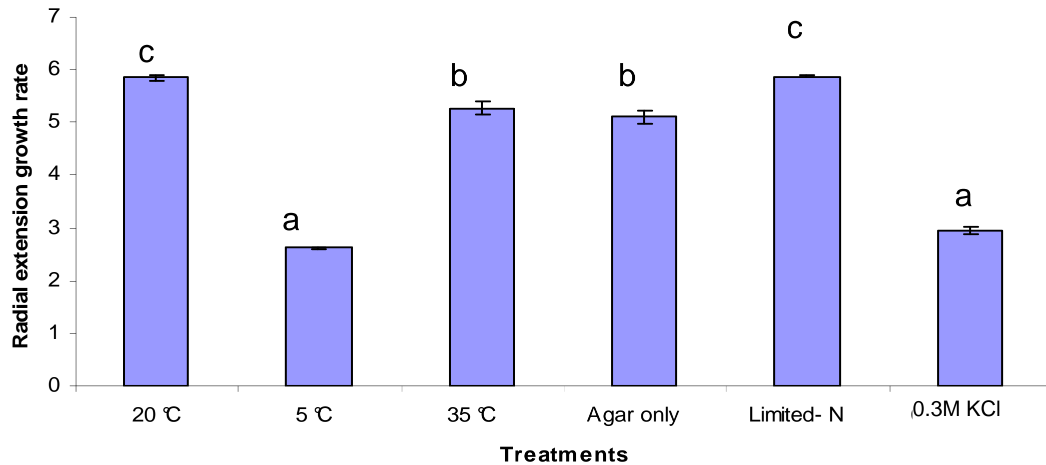


Fig. 5.3 Radial extension growth rate of *T. versicolor* under stress, mean ± SE (n = 5). Different letters indicate significant differences between means. Anova, $F_{5,24} = 326.79$, ($P \leq 0.05$).

5.3.2 Spatial distribution of laccase activity under continuous stress

The enzyme activity was detected by adding ABTS to the medium and was revealed by the appearance of purple colour with KCl, at 5°C, 35°C and also the controls

(20°C), while it appeared as a green colour for the limited-nitrogen and the agar only plates, which may be related to deficiency of nutrients in the media (Fig.5.4).

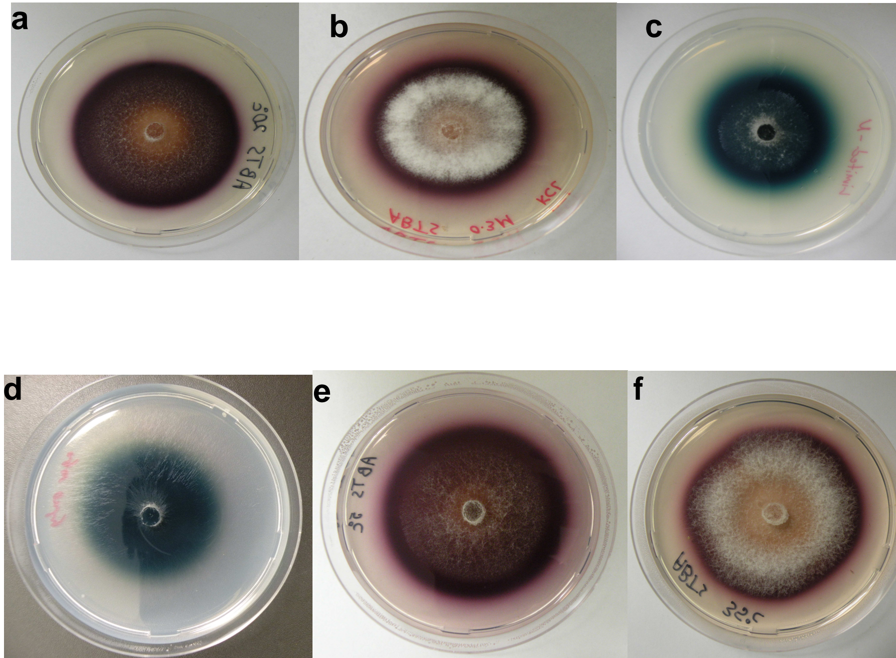


Fig. 5.4 Spatial distribution of laccase activity under stress **(a)** 20°C (5 d),**(b)** 0.3M KCl (11 d), **(c)** Limited-N (5 d), **(d)** agar only (10 d), **(e)** 5°C (12 d), **(f)** 35°C (6 d). ABTS medium.

5.3.3 Gene expression under continuous stress

5.3.3.1 Laccase gene expression

All the treatments resulted in significantly ($P \leq 0.05$) lower expression than the controls in the first replicate (Fig. 5.5), in the second replicate however the results were less clear: although expression was reduced when grown on low or no nitrogen media, and at elevated temperature, expression was not significantly ($P > 0.05$)

reduced at low temperature, and osmotic stress appeared to increase expression significantly ($P \leq 0.05$).

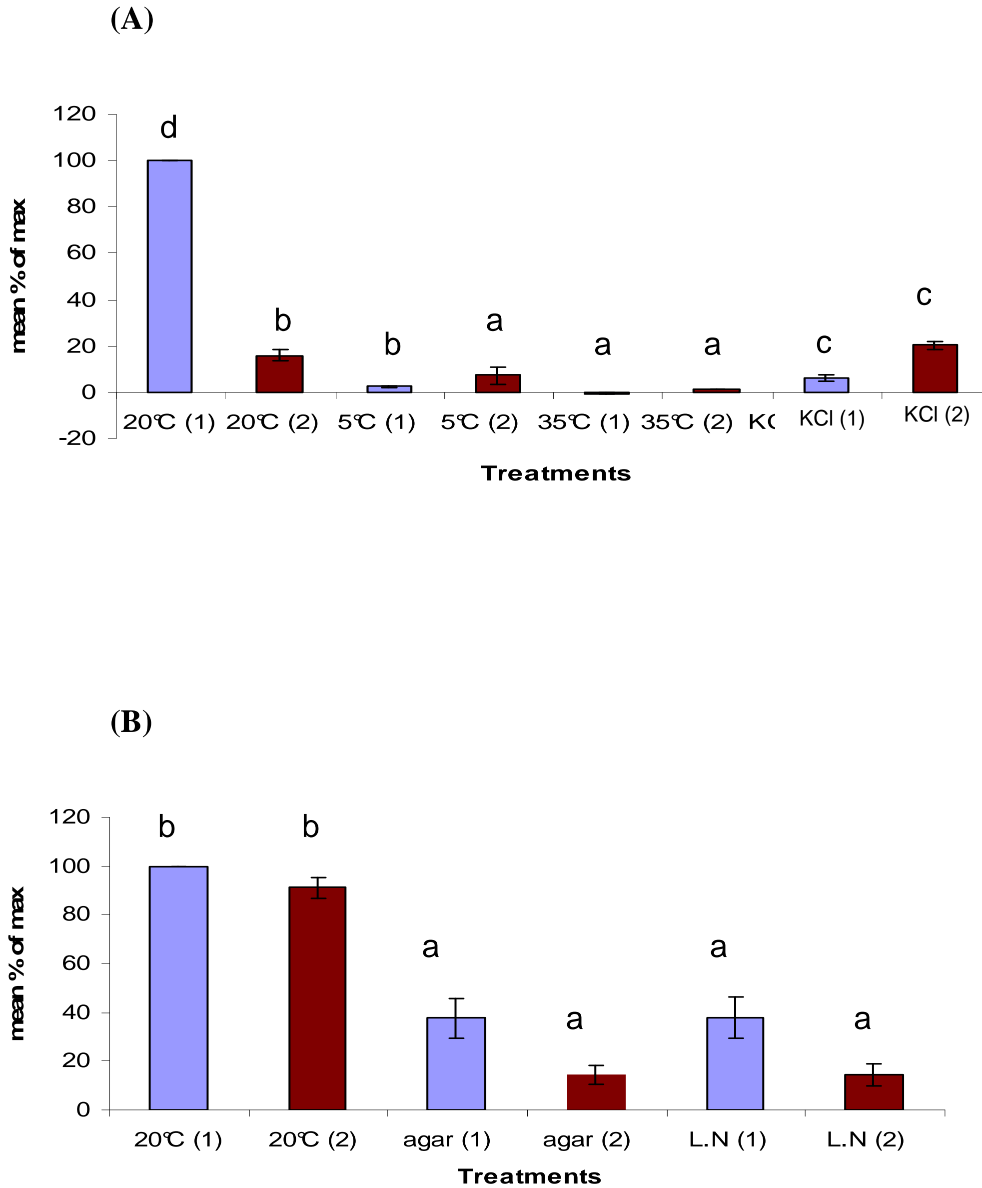


Fig. 5.5 *T. versicolor* Lacc gene expression under stress. **(A)** 5°C, 35°C, KCl, Anova, $F_{3,8} = 6242.658$, ($P \leq 0.05$); **(B)** low nitrogen (L.N), agar only (no nitrogen), Anova, $F_{2,6} = 108.823$, ($P \leq 0.05$); (1), (2) = two biological replicates. Mean ± SEM (n = 3). Different letters indicate significant differences between means.

5.3.3.2 FRA19 and Nox gene expression

The gene expression of *T. versicolor* FRA19 gene under stress was significantly ($P \leq 0.05$) lower than the control for the treatments of 5°C, 35°C and KCl (Fig.5.6.A) in both biological replicates, however, expression was only significantly ($P \leq 0.05$) lower under low nitrogen in one replicate and expression when grown without nitrogen source was unaffected in both biological replicates (Fig 5.6B).

Expression of the *T. versicolor* Nox gene was again reduced when the fungus was grown at 5°C, 35°C or with KCl in one replicate, but no significant differences to the control were detected in the second replicate for these treatments (Fig 5.7A). A lack of nitrogen source appeared to slightly stimulate Nox gene expression in one replicate, and low nitrogen reduced expression, however, again no significant ($P > 0.05$) changes in expression were detected in the second replicate (Fig 5.7B).

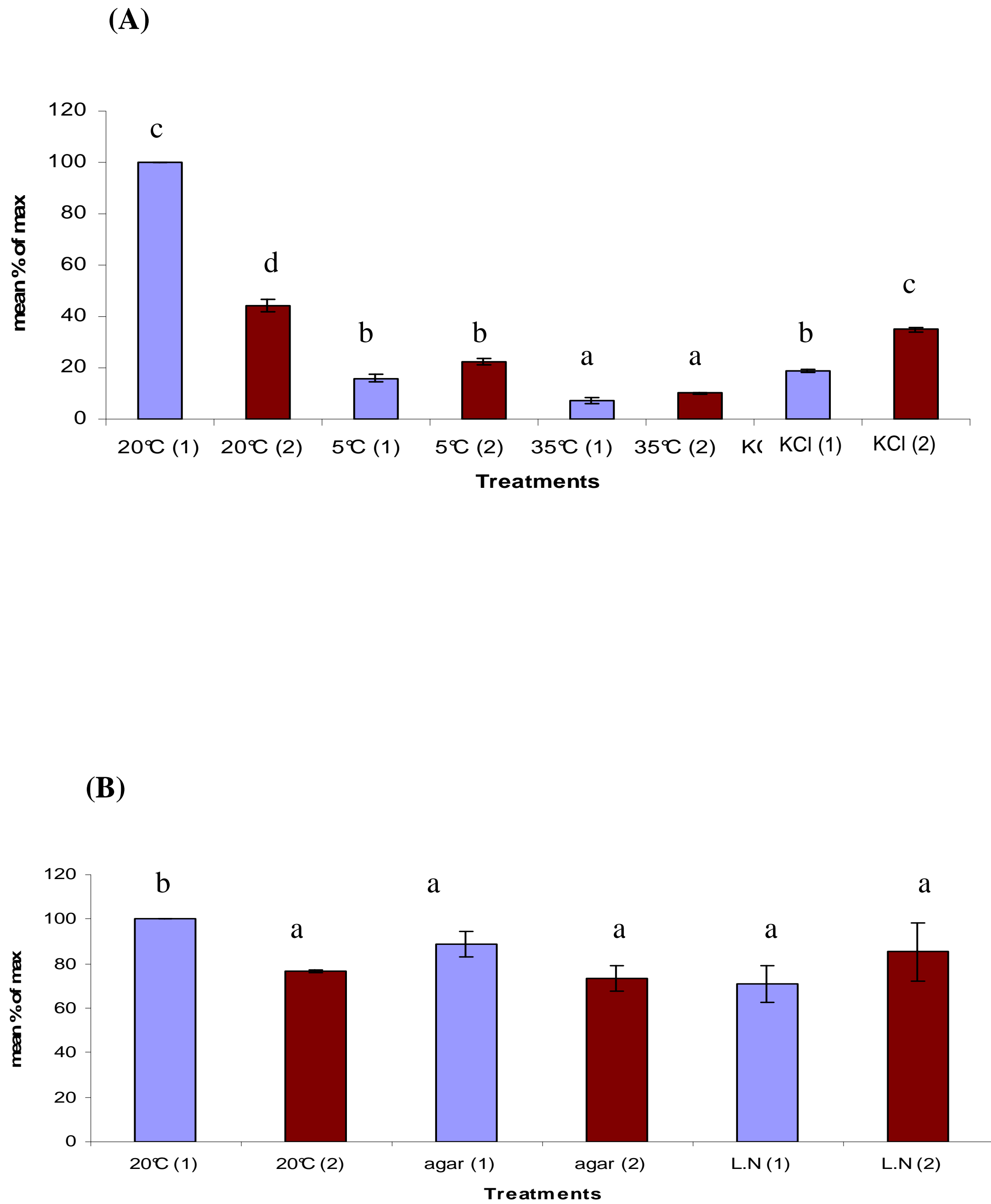


Fig. 5.6 FRA19 gene expression of *T. versicolor* under stress. **(A)** 5°C, 35°C, KCl, Anova, $F_{3,8} = 2101.221$, ($P \leq 0.05$); ; **(B)** low nitrogen (L.N), agar only, Anova, $F_{2,6} = 6.534$, ($P \leq 0.05$); (1), (2) = two biological replicates. Mean ± SEM (n = 3). Different letters indicate significant differences between means.

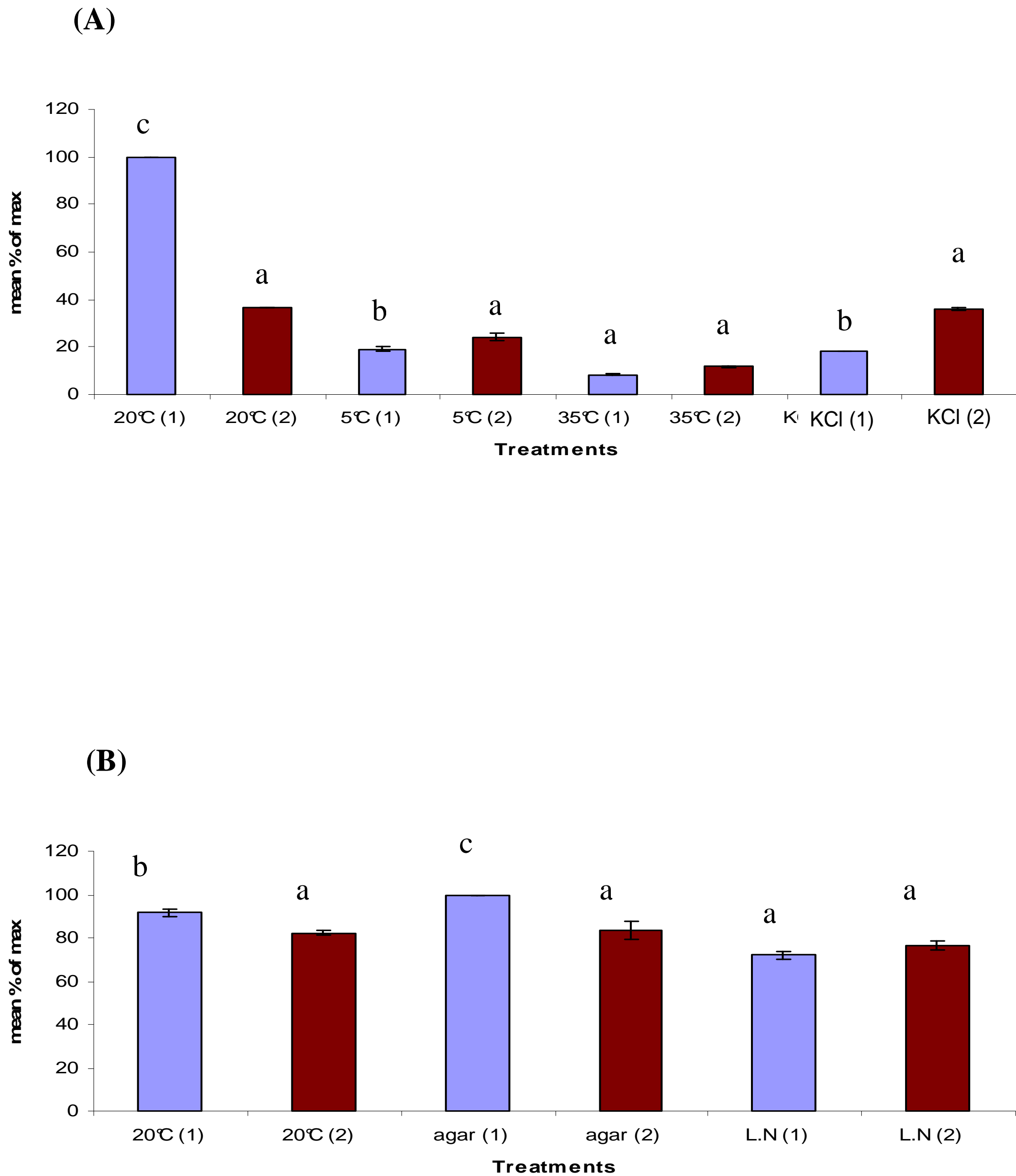


Fig. 5.7 Nox gene expression of *T. versicolor* under stress. **(A)** 5°C, 35°C, KCl, Anova, $F_{3,8} = 0.673$, $(P > 0.05)$;; **(B)** low nitrogen (L.N), agar only, Anova, $F_{2,6} = 84.249$, $(P \leq 0.05)$; (1), (2) = two biological replicates. Mean ± SEM (n = 3). Different letters indicate significant differences between means.

5.3.4 Laccase activity of *Trametes versicolor* under continuous stress

All the treatments except 5°C and growth with osmotic stress (KCl) resulted in a significant (P ≤ 0.05) decrease in the laccase activity compared with the control (Fig.5.8). The limited nitrogen and no nitrogen (agar only) treatments resulted in significant (P ≤ 0.05) difference decrease in laccase activity compared to the control.

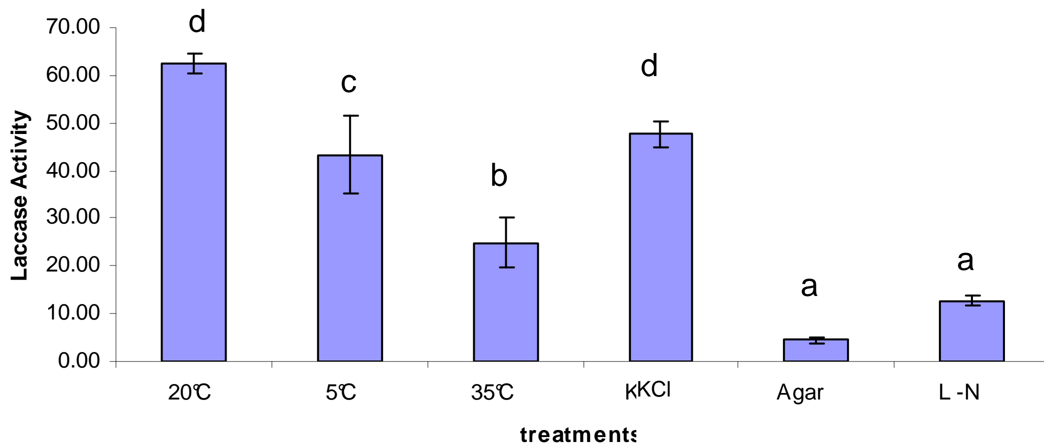


Fig.5.8 Laccase activity of *T. versicolor* under stress (5°C, 35°C, KCl, agar only, no nitrogen (L-N)), mean ± SEM (n = 3), Anova, $F_{5,12}$ = 27.187, (P ≤ 0.05);. Different letters indicate significant differences between means.

5.3.5 Expression of the laccase gene family under stress

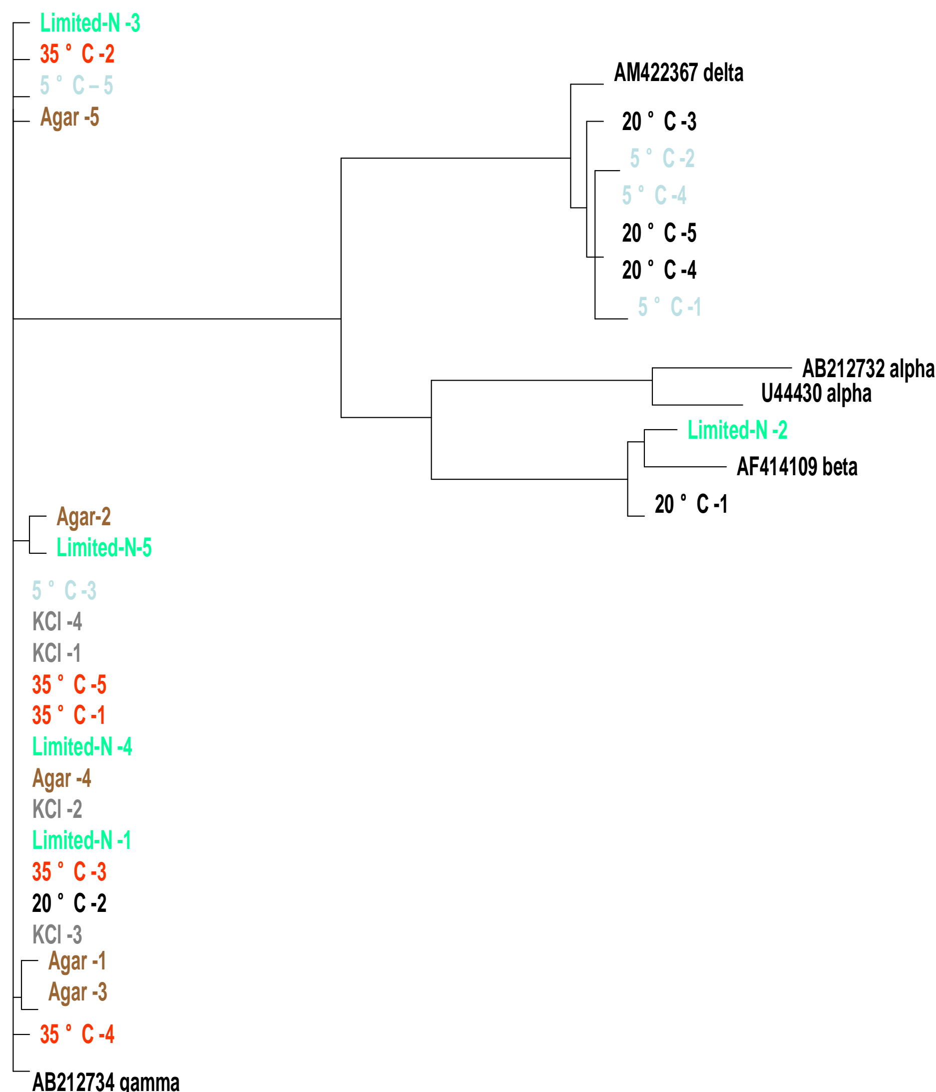


Fig.5.9 *T. versicolor* laccase gene family under stress in comparison with laccase groups (γ, β and δ).

The majority of the clones derived from the agar, 35°C and KCl treatments represented sequences belonging to the gamma laccase group (Fig. 5.9 and Table 5.2). Three sequences of 20°C and 5°C were most similar to the delta group, while three sequences of limited-N were homologous to the gamma group. One sequence of each of agar, Limited-N, 5°C and 35°C appeared to form a separate group.

Table 5.2 Matching sequences of *T. versicolor* under stress treatments with the laccase groups (γ, β and δ)

Treatments	**Replicates**	Laccase group
Agar	1	γ
	2	γ
	3	γ
	4	γ
	5	separate group
KCl	1	γ
	2	γ
	3	γ
	4	γ
Limited-N	1	γ
	2	β
	3	separate group
	4	γ
	5	γ
20°C	1	β
	2	γ
	3	δ
	4	δ
	5	δ
5°C	1	δ
	2	δ
	3	γ
	4	δ
	5	separate group
35°C	1	γ
	2	separate group
	3	γ
	4	γ
	5	γ

5.4 Discussion

The extension rate of *T. versicolor* under continuous stress showed that the treatments of 5°C and the KCl stress decreased the growth rate of *T. versicolor* mycelium but did not decrease the production of laccase enzyme. On the other hand, the increase in the extension rate (Fig. 5.2) of *T. versicolor* under stress was not a sign of increased laccase activity: the growth rate increased at 35°C, with agar only and under Limited-N while laccase enzyme activity was decreased compared with the control (Fig. 5.8).

The spatial distribution of laccase activity under stress showed that the decrease in laccase activity was reflected in a green colour in the medium as in the case of limited nitrogen and agar only (no nitrogen) plates, as the ABTS was oxidised to form a green colour product (Pointing *et al.* 2005), and the green staining colour was detected also when the nutrients in the malt agar were low (0.5% MA). The green colour was always associated with low nutrients and lower enzyme activity. These results have been validated in the Cardiff laboratory by other colleagues and they obtained similar results (Hunt, *et al.*, unpublished). In contrast, the purple colour represents higher enzyme activity, and when the colour starts to clear near the centre of the colony this indicates an even higher laccase activity.

Gene expression of Lacc, FRA19 and Nox genes all fell under temperature stress or osmotic stress (KCl) although for the laccase and Nox expression; responses were not always consistent between replicates. Under nitrogen stress laccase expression was consistently reduced, however, for FRA19 and NOX the results were less consistent between replicates or between the two low nitrogen treatments. The reduction in

laccase gene expression was surprising given the decrease seen in enzyme activity, which was the opposite with the work of Peiris (2009) where it showed increase in the enzyme activity. Velazquez-Cedeno *et al*. (2007) suggested that the lag between gene expression and enzyme activity is normal and they found small alterations in laccase transcript levels in interacting cultures of *P. ostreatus* in comparison to big changes in enzyme activity. Likewise a discrepancy between changes in gene expression and the accumulation of enzymes with time was also found in *T. versicolor* during mycelial interactions (Hiscox *et al*, 2010).

Sequences from PCR products of laccase transcripts were homologous to sequences from either γ, β or δ groups (Necochea *et al*., 2005), with exception of one sequence of each of agar, limited-N, 5°C and 35°C which were not strongly related to any of the allocated groups, and they may correspond to a new laccase gene. The sequences derived from the control 20°C treatments represented all three accessioned laccase sequence groups (γ, β and δ) which may suggest that *T. versicolor* produces all types of laccase during normal conditions. The over-representation of the γ group sequences in the temperature stress and osmotic stress treatments may however suggest that this group is differentially up-regulated under these stress conditions. To verify this in further investigations, the use of more replicates (about 20 for each treatment) will be required.

Chapter 6: Synthesis and future directions

This thesis started by investigating interspecific interactions between basidiomycetes (and some ascomycetes), given the important role of these interactions in the woodland ecosystem in modulating decay rate when these fungi colonise wood and also in affecting their role in nutrient recycling and mineralization (Boddy, 1993, 1999, 2000, 2001; Holmer & Stenlid, 1997; Wells, 2002). The lack of understanding about these interactions led to focus a part of my work on a model basidiomycete fungus *Trametes versicolor* which showed three different outcomes when interacting with other wood-rot basidiomycetes: it replaced (won) *Stereum gausapatum*, deadlocked (equal) with *Bjerkandra adusta* and it was replaced by *Hypholoma fasciculare* (lost).

The first aims were to relate the effect of VOCs released during interaction between two basidiomycetes on a third fungus (focusing especially on *T. versicolor*), monitoring effects on growth and enzyme production of the third fungus. This was addressed in Chapter 3. Other aims were to analyse expression of selected genes from the *T. versicolor* EST collection using semi-quantitative RT-PCR and compare expression to the array data (Chapter 4). Further aims were to analyse effects on enzyme production and gene expression when *T. versicolor* was exposed to abiotic stress conditions (in Chapter 5).

6.1 Effect on growth rate

Q1. Do VOCs from fungal interactions affect growth rate in the same way as stress factors? Mechanisms?

Growth of *T. versicolor,* as with other fungi can be affected by abiotic factors, including temperature, pH, osmotic pressure, nutrients etc. Biotic factors such as interactions with other fungi and grazing of invertebrates on fungi (Tordoff *et al*., 2006; Rotheray *et al*., 2008; Crowther *et al*., 2011) will also affect growth. Of particular interest in this study was the effect on growth elicited by VOCs produced by other interacting fungi. Furthermore, abiotic factors will also affect the interaction outcomes (Boddy, 1983; Boswell *et al*., 2007), thus forming a complex network of effects on the growth of the fungus.

The effect of VOCs produced by two interacting fungi on a third fungus (*T. versicolor*) was variable. Some interactions increased the *T. versicolor* growth rate such as Rb/Hf, Hf/Hf, Sg/Sg, Es/Es, Sh/Sh and Tv/Ba on agar (where Rb, *Resinicium bicolor; Hf, Hypholoma fasciculare; Sg, Stereum gausapatum; Ba, Bjerkandera adusta; Es, Eutypa spinosa; Sh, Stereum hirsutum)* which was in agreement with the results of Evans *et al*. (2008). There was however no significant effect on laccase activity. Interactions on wood blocks all increased growth rate compared with the controls, while there was an increase in laccase activity for only some interactions: the Tv/Tv and Ba/Ba increased growth rate whereas there was decrease in response to Es/Es and Sg/Es interactions.

On the other hand, for *T. versicolor* under abiotic stress it was interesting to see that extension rate at 35°C, and limited-nitrogen was not significantly different from 0.3M KCl (-1.344 MPa) nor 5°C treatments. The effects on enzyme activity correlated with growth rate for the treatments of 35°C, agar only and limited-nitrogen (Table 6.1).

Table 6.1 A comparison of growth rate and laccase activity of *T. versicolor* under stress (abiotic stress of high/low temperature, osmotic pressure of KCL, no nitrogen, limited-nitrogen)

Treatments	Growth rate	Laccase activity
20°C	Control	Control
5°C	↓	0
35°C	↓ 0	↓
KCl	↓	0
Agar only	↓	↓
Limited-nitrogen	↓ 0	↓

↓ = decrease, 0 = no significant difference, ↓ 0 = there was a decrease but it was not significant

The results of this study were as expected: other studies also showed that stress has an important affect on fungi. For example high temperature (45°C) could arrest the growth of some fungi such as *Schizophyllum commune* (Higgins and Lilly, 1993). Other fungi could however grow at temperatures as low as 5°C although this is a stressful temperature which made *Trichoderma viride* a good candidate to be used for biological control at low temperatures (Jackson *et al*., 1991). Gahlaup *et al*. (2002) suggested that high N levels are important for laccase formation which agree with the current study, while Eggert *et al*. (1992) found that limited N conditions resulted in high laccase activity. Fungi can make osmotic adjustments but high concentrations of KCl can be toxic resulting in death of the fungus (Hallsworth and Magan, 1999). In the current study

trial experiments exposing the fungus to different concentrations of KCl were carried out to select a concentration for *T. versicolor* that imposed stress, but was not lethal. In the environment, of course, fungi are exposed to multiple stresses simultaneously and the combination of several stresses may elicit more effects on fungi than the single stresses alone. An example of this approach is a study of the effect of different temperatures and water availability on the growth rate of three entomogenous fungi on a medium modified with KCl (Hallsworth & Magan, 1999) which helped to establish their environmental limits.

Q2. What is the effect of ecology and environmental conditions on growth rate, morphology and enzyme production of fungi?

There are two main categories for fungal competition: (1) Primary resource capture; and (2) Secondary resource capture (Boddy, 2000). Primary resource capture occurs with pioneer species that can colonise an uncolonised resource being latently present within functional sapwood, or colonising freshly fallen wood via spores (Boddy, 2001). These colonisers are able to defend their territory for a limited time until secondary colonisers grow and replace them using antagonistic mechanisms, or following major changes in the abiotic environment (Griffith & Boddy, 1991). Saprotrophic fungi from mid- to late-successional stages are thought to have higher enzyme activity (laccase) than earlier stage fungi (Iakovlev & Stenlid, 2000). For example, some of the fungi that been used in this study such as *S. gausapatum* is a primary colonizer, but it had similar levels of laccase

production during growth alone compared to the late-stage cord-forming species such as *H. fasciculare*. In contrast, *T. versicolor* which is a late primary to early secondary coloniser had much higher levels of laccase production than *H. fasciculare* (Boddy, 2001; Hiscox *et al*., 2011), despite the fact that *H. fasciculare* had the ability to replace and win *T. versicolor*'s territory with its lower laccase activity. This may suggest that laccase production is not closely linked to defense, although it is also possible that the laboratory conditions under which laccase activity was being measured were different to those operating in the environment. When fungal mycelium of different species meet they interact and then changes occur in the mycelial morphology, extracellular enzyme production and also the production of secondary metabolites (Boddy, 2000, Woodward & Boddy, 2008). The self-pairing of *T. versicolor* enhanced the extension rate of *R. bicolor* mediated by VOCs (Table 3.2), while the self-pairing of *R. bicolor* did not affect the extension rate of *T. versicolor* (Table 3.1). On the other hand, *T. versicolor* (early secondary colonizer) could replace *R. bicolor* (secondary colonizer cord-former) by direct contact when they interacted with each other on agar plates (Table A.7), indicating that interactions vary depending on proximity, this is presumably due to different mechanisms: production of enzymes probably allowed *T. versicolor* to win the confrontation when in close proximity, while the VOCs of *T. versicolor* enhanced the growth of *R. bicolor* in the absence of direct contact.

The present study also showed that the changes in the abiotic environment could affect fungal morphology. When *T. versicolor* was exposed to abiotic stressful conditions the mycelium changed markedly in morphology: it showed light growth at 5°C or no

nitrogen, and dense fluffy growth at 35°C or on KCl medium, while it was only slightly affected when the fungus was grown on a limited nitrogen medium (photos in Appendix H). When the fungi are exposed to certain forms of stress in nature such as high or low temperatures, they produce high amounts of ROS (reactive oxygen species) which could damage the proteins. To avoid such damage fungi would change the activity of the antioxidant enzymes, such as superoxide dismutase. For example, fluctuations in environmental temperature can result increased activity of laccase and also the formation of the fungal fruit-bodies (Dighton, 2003, Cairney, 2005). Since 1978 the climate change has affected fruiting phenology in Europe (studied in the UK and Norway) (Gange *et al.*, 2007; Kauserud *et al.*, 2010). In the UK the average first fruiting date has become significantly earlier which correlated with elevated August temperatures and later fruiting with both elevated August temperature and October rainfall, while average last fruiting date has become significantly later. These climate changes are not only important in extending the duration of fungal fruit body production, but also affects decomposition. Many fungi also now fruit in spring indicating that they are now active for longer periods including winter (Gange *et al.*, 2007).

Increased temperatures may lead to increased decomposition by fungi, and consequent release of more carbon dioxide from the soil. On the other hand, also as temperatures increase the nitrogen levels in the soil may increase, and the high nitrogen tends to suppress decomposition rates of wood, that would lead to less carbon dioxide evolution. Global warming can increase the litter decomposition rates only if there is sufficient soil moisture. Such environmental changes can repress the transcription of genes coding for enzymes (degrading lignin, cellulose, hemicellulose, and chitin) (Kellner &Vandenbol,

2010). Environmental change can also affect the outcome of mycelial interactions, which in turn may affect decay rate, depending on whether favored species decay litter more or less rapidly. Microorganisms are very sensitive to climate changes but their responses in field situations are still not well studied.

Future Directions

1. Clearly it is more meaningful to use a medium that yields results close to those that would happen in the natural environment. The use of agar cultures in the laboratory is ecologically unrealistic although it has several advantages such as ease of use, and gives results in a short time. It is more realistic to use woody tissues such as wood blocks to study fungal interactions and VOC production, although it is relatively more complicated to set up interactions and interpret outcomes on wood blocks (Wheatly *et al*., 1997, Bruce *et al*., 2000; Mikiashvili *et al*., 2005). For example although there were a wide range of VOCs produced and identified during interactions of the studied fungi on agar (Hynes *et al*., 2007; Evans *et al*., 2008), VOC profiles were more complex when the interactions took place on wood blocks, which suggests the presence of specific inducers. Thus future work needs address this issue with more experiments using more realistic media such as wood blocks.

2. Work on the abiotic stress also needs to move towards more realistic experiments such as those reported by Hallsworth & Magan, (1999), where interactions between stresses

are examined and limits on growth and cumulative effects on interactions are defined. Again work needs to move away from the use of agar to more realistic substrates where possible. Combinations of VOCs and stress experiments could give interesting results. This could be done by setting up double plate cultures with the interaction of two fungi on the lower plate to produce VOCs, while stress conditions could be applied to the studied fungus in the top plate, such as changing the osmotic pressure or nutrient status. As discussed above, this is of relevance to the natural environment where fungi are subject to multiple stresses simultaneously, for example combining temperature with osmotic pressure to see the effect of both factors on fungi, has been applied to rock-inhabiting meristematic fungi (Sterflinger, 1998).

By defining the limits and interactions between different stresses on fungal growth these more realistic approaches may begin to probe the underlying ecological mechanisms and constraints that define community development in these fungi.

6.2. Enzyme Activity

Q1. Is the effect on *T. versicolor* laccase activity of interactions and abiotic stress the same or different?

Data presented in Table 6.2 show that the laccase activity was variable; while it was not affected by the VOCs in any of the interactions tested when interaction was on agar, it was affected by VOCs produced by some interactions on wood blocks. Laccase activity was positively affected by VOCs when these resulted from Ba/Ba, Tv/Tv self pairing

interactions on wood blocks. The effect on laccase activity of VOCs produced by the interaction of Es/Sg, or with the self-pairing of Es on wood blocks, was however decreased, which suggests the presence of specific inducers related to the wood blocks.

Laccase activity also decreased when the *T. versicolor* was under limited nitrogen stress. This is in contrast with the work of Pointing *et al*. (2000) who found that the production of laccase of *Pycnoporus sanguineus* was 50-old increased in the presence of high carbon and low nitrogen medium. Laccase activity was also decreased at low temperature which disagrees with the results of Fink-Boots (1999). On the other hand, it was also negatively affected by the high temperature stress of 35°C which agrees with the results of Fink-Boots (1999).

Table 6.2. The laccase activity in both experiments of *T versicolor* under stress and under VOCs (interactions).

Treatments	**Stress**	**Interactions (agar)**	**Interactions (wood blocks)**
20°C [1]	Controls		
5°C [1]	0		
35°C [1]	↓ ↓		
KCl [1]	0		
Agar only [1]	↓ ↓		
Limited-N [1]	↓ ↓		
Rb/Hf [2]		0	0

Hf3/Hf3 [2]		0	0
Hf4/Hf4 [2]		0	0
Es/Es [2]		0	↓ 0
Sg/Sg [2]		0	0
Sh/Sh[2]		0	0
Tv/Ba [2]		0	0
Ba/Ba [2]		0	↑ 0
Tv/Tv [2]		0	↑ 0
Tv/Hf4 [2]		0	0
Rb/Tv [2]		0	0
Sg/Sh [2]		0	0
Tv/Sg [2]		0	0
Sg/Es [2]		0	↓ 0
Sg/Ba [2]		0	0
Rb/Rb [2]		0	0
Controls [2]		Controls	Controls

(↓ ↓) decrease, (0) not significantly difference, (↑ 0) there was a increase but it was not significant, (↓ 0) there was a decrease but it was not significant
1 = *T. versicolor* under stress.
2 = *T. versicolor* exposed to VOCs from interacting fungi.

Staining for enzyme activity showed localisation of the activity, which was localised mainly at the interaction zone of *T. versicolor* with other fungi and it showed lower activity in other areas of mycelium (Hiscox *et al*., 2010). Similar results were found

between *H. fasciculare* and *Peniophora lycii* (Rayner *et al*., 1994). In the current study, *T. versicolor* under stress (limited nitrogen and agar only (no nitrogen)) showed a decrease in laccase activity reflected by production of a green colour in the medium plates, as the ABTS was oxidised to form a green colour product (Pointing *et al.* 2005). The green colour was always associated with low nutrients and lower enzyme activity; these results have been validated by Hunt *et al*. (unpublished results Cardiff laboratory) who obtained similar results with different experiments.

From the work presented here a difference does emerge between abiotic stress and VOC signaling in that in all cases where abiotic stress was imposed, laccase activity decreased, whereas some VOC signaling was stimulatory.

Future Directions

1- To define better the role of laccase activity in response to abiotic stress and VOCs, it will be helpful to explore further the expression patterns of the different members of the laccase gene family in *Trametes versicolor.* To do this much larger experiments will be required designing gene specific primers. This will be facilitated by the recent publication of the *T. versicolor* genome sequence (http://genome.jgi-psf.org/Trave1/Trave1.info.html). Using combinations of stresses and VOCs derived from other interacting fungi may begin to define the role of different laccase genes and provide insights into their function during mycelial interactions and in response to abiotic stress.

2- In the experiment of *T. versicolor* under stress only one enzyme (laccase) was analysed, analysis of other enzymes would expand the knowledge about the enzymes produced, and activity when the fungus is exposed to such stresses.

6.3. Gene expression

Q1. Are the effects on the gene expression of FRA19, Nox and Lacc during interactions and stress the same or different?

Comparing gene expression between the two experiments (Table 6.3), Nox gene expression was mainly negatively or not affected by either stress treatments or VOCs derived from interactions. Nox expression was positively affected in one stress treatment when there was no nitrogen in the medium, although only in one replicate. FRA19 gene expression was negatively affected by almost all the stress treatments (temperature, osmotic pressure, limited nitrogen) and also by VOCs from two of the interactions: Tv/Sg, and Tv/Ba.. The effect on Lacc gene expression was variable. Most abiotic stress treatments had a negative or inconsistent effect, however, laccase expression was consistently down-regulated by high temperature and the lack of nutrients. Hiscox *et al*. (2010) showed that the interaction Tv/Hf was the only one tested that had a positive effect on laccase gene expression.

So no clear pattern emerged for any of the three genes analysed relating to their expression under abiotic stress or VOCs derived from other interacting fungi. This suggests a complex response to these stimuli, worthy of further investigation.

Table 6.3. The gene expression (up/down) of two biological replicates of the genes FRA19, Nox and Lacc of the fungus *T. versicolor* under the VOCs and stress effects.

Treatments	**FRA19**	**Lacc**	**Nox**
stress			
20 °C	Control	Control	Control
5 °C	-, -	-,+	-,=
35 °C	-, -	-,-	-,=
KCl	-, -	-,+	-,=
Agar only	=, =	-,-	+,=
Limited-N	-,=	-,-	-,=
Interactions			
Tv/Hf	+	+	=
Tv/Sg	-	=	=
Tv/Ba	-	=	=
Tv/Tv	0	0	0

(+) increase, (-) decrease, (=) not significantly difference, (0) control. Red colour results from Hiscox *et al*. (2010).

Q2. Why were there differences between replicates?

The culture conditions were kept as consistent as possible during the whole experiment, using the same medium and the same incubation temperature, and at the end of the treatment period all mycelia were extracted and PCR amplified at the same time, so any differences in the biological replicates must be due to uncontrollable factors during culturing or after the mycelium was harvested. Another possibility suggested by Eyre *et al* (2010), is that changes in expression may be dynamic and rapid such that it may be difficult to ensure that mycelia are harvested at exactly the same point in their growth.

Q3. What is the relationship between gene expression and enzyme activity in the study?

There was a fairly clear relationship between the abiotic stresses (high/low temperature, KCl, limited nitrogen, no nitrogen) on *T. versicolor* laccase activity and gene expression (Table 6.4). In all cases where enzyme activity was down regulated this was consistent with a down-regulation in gene expression. In the two treatments where there was no apparent effect on laccase activity, the results from the gene expression study were inconsistent between replicates. Galhaup *et al*. (2002) found that *Trametes pubescens* can up-regulate expression of genes encoding laccase and manganese peroxidase when it was exposed to high carbon and nitrogen, which are consistent with the present study. The agreement here between activity and expression contrasts with the studies on the effects elicited when *T. versicolor* interacted with other white-rot fungi. In this case there was a

large increase in laccase activity, while the gene expression change was not dramatic (Hiscox *et al*., 2010).

Table 6.4. The relationship between enzyme activity and *lacc* gene expression (two biological replicates) of *T. versicolor* under stress.

Treatments	**Laccase activity**	***Lacc* gene expression**
20 ^{o}C	Control	Control
5 ^{o}C	=	-,+
35 ^{o}C	-	-,-
KCl	=	-,+
Agar only	-	-,-
Limited-N	-	-,-

(+) increase, (-) decrease, (=) not significantly difference, (0) control.

Q4. What are the effects of stress and VOCs, produced during interactions, on laccase gene family expression?

Laccase genes can be classed into groups called γ, β or δ, and differential expression of different laccase gene family members has been noted in several basidiomycete species (Necochea *et al*., 2005). Most sequences from RT-PCR products of laccase transcripts from *T. versicolor* exposed to different abiotic stress treatments were homologous to

sequences from either γ, β or δ groups. It was found that the sequences derived from the control 20°C treatments represented all three laccase sequence groups (γ, β and δ) which may suggest that *T. versicolor* produces all types of laccase during normal conditions. The over-representation of the γ group sequences in the temperature stress and osmotic stress treatments may however suggest that this group is differentially up-regulated under these stress conditions. The one sequence of each of agar, limited-N, 5°C and 35°C which was not closely related to any of the published groups, may suggest that they correspond to a new laccase gene. Further sequence data are however required to confirm this. In addition, the study conducted here was small and greater numbers of sequences need to be analysed to confirm whether there really is a differential expression of the laccase gene family under abiotic stress.

A similar study of the laccase gene family in *T. versicolor* during mycelial interactions on agar was performed to determine whether members of the laccase gene family were differentially expressed during interactions, although results were not conclusive (Hiscox *et al.*, 2010). The use of more replicates will be required to verify further investigations. It would be interesting to know whether there is a specific association of particular laccase genes with specific conditions. Differential expression of laccase genes has been reported in other species. For example, Zhao & Kwan (1999) recognised two laccase genes (*lac1* and *lac2)* from *Lentinula edodes* that were expressed under different media conditions, where *lac1* expression was much higher than *lac2* expression in a high nutrient glucose medium. Also, it was found that three laccase genes from a *Trametes spp.* environmental isolate were differentially expressed (Mansur *et al.*, 1998).

Future directions

1- Nox encodes NADPH oxidase, an enzyme that is associated with ROS production (Gessler *et al*., 2007), and is associated with cell death. It would be interesting to know the extent of cell death occurring during interactions or when the fungus was under stress. This could be detected by the use of stains such as the combination of Sytox green, which can differentiate the dead and living cells, and Calcofluor white which stains all tissues (Green *et al*., 2000).

2- The use of more microarrays or new sequencing techniques such as deep sequencing (Hoen *et al*., 2008; Battk and Niesett, 2011; Ahmadian *et al*., 2006), will be important in revealing more clearly global changes in gene expression of fungi under both biotic and abiotic stress and under combined stresses which is clearly needed to unravel the complex expression patterns seen here.

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Appendix (A)

Preliminary experiments

A.1.Material and Methods

A.1.1 The effect of aeration and VOCs produced during interspecific mycelial interaction on a third fungus

Two sets of two-plate interaction method as described in section (3.2.1.1) were used to test the extension rate of the fungi *Stereum gausapatum* and *Fomes fomentarius,* cultured on 0.5% MA in 5 replicates, one set were opened and measured every day to examine the effect of aeration on the VOCs presence or absence and whether it can be affected by opening, the other set were opened and measured on day 2 and day 5 only. The cultures were incubated at 20^0C.

A.1.2. Species growing alone at two different malt concentrations

Nine Fungi (*Eutypa spinosa* , *Stereum gausapatum, Vuilleminia comedens, Fomes fomentarius, Trametes versicolor, Resinicium bicolor, Trichoderma viride, Stereum hirsutum, Hypholoma fasciculare, Bjerkandera adusta*) were cultured by inoculating 6 mm diam agar plugs, cut from the growing margin of colonies with a number 3 cork borer, centrally on 2% w/v MA as described in section (2.2.1) and 0.5% w/v MA (MA; 5 g l^{-1} Munton & Fison spray malt, 15 g l^{-1} lab M agar No.2). The plates were incubated face downwards, after sealing with Nescofilm, at 20^0C in plastic bags in the dark. Three replicates were used. Colony extension rate was measured daily, at the

same time, across two diameters using Vernier Callipers. Measurements were taken until the colony was 1 cm away from the edge of the plate.

A.1.3. Effect of nutrient concentration on the extension rate of fungi

The method described in section (3.2.1.1) was used as a preliminary method for estimating the effect of different concentrations of medium on the production of different volatiles, which may affect the growth of the tested fungus *Stereum gausapatum* and *Stereum hirsutum.* Five replicates of the two-plate interaction were set up on two different nutrient treatments at 0.5% and 2% MA cultures. Cultures were incubated in dark at 20^0C.

A.1.4. Extension rate measurements

Inhibition and stimulation of extension rate during interactions of five basidiomycetes with Hf, Rb and Sg, and their outcomes

This experiment was used to see the fungi that can produce pigments as a sign of producing VOCs, and the species *Hypholoma fasciculare* and *Resinicium bicolor* were chosen from the work of Julie Hynes 2007, while the fungus Sg was one of the best ones that can produce pigments. *H. fasciculare, R. bicolour* and *Stereum gausapatum* were paired against five different species of wood decay fungi (*Eutypa spinosa*, *Fomes fomentarius, Vuilleminia comedens*, *Trametes versicolor* and *Bjerkandera adusta*) and also the self pairing of *Hypholoma fasciculare, Resinicium bicolor* and *Stereum gausapatum* by inoculating 6 mm diameter agar plugs on 2% MA, cut from the actively growing margins of the colonies, 3 cm apart. 3-4 replicate plates of each pairing were made, sealed with Nescofilm, and then incubated in the dark at 20^0C. Controls were the self pairings. The extension rate was measured as diameter divided

by two, towards and away from each other, and the measurements were determined daily. Colonies grown alone were used as the controls and its two diameters measurements divided by four to have one radial measurement (Fig. A.1). Progress of interaction was recorded using digital cameras (**Nikon** Coolpix 5700 8x zoom ED 5.0 mega pixels and **Sony** Cyber-short DSC-P71, 3.2 mega pixels) after 7, 11, 17, 23, 28, 36, 43 and 50 days.

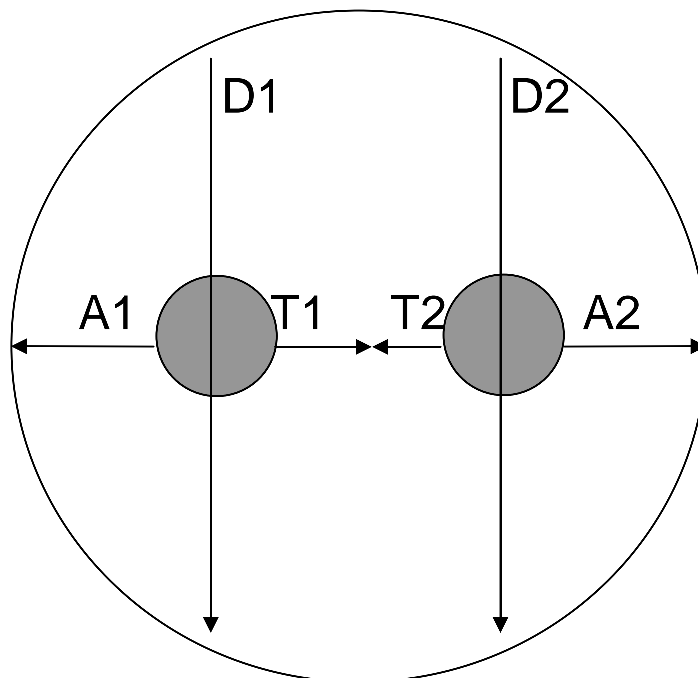


Fig.A.1. The growth measurement positions .Towards opponent (T1 and T2), away from opponent (A1 and A2) and diameters (D1 and D2) divided by 2.

A.2. Results

A.2.1. the effect of aeration and VOCs produced during interspecific mycelial interaction on a third fungus

When the plates were opened every day (1, 2, 3, 4, 5 d) there was no significant difference (P>0.05) between the extension rates of *S. gausapatum* and *F. fomentarius* growing above interactions of *R. bicolor* vs *H. fasiculare*, or the self pairings of *R.*

bicolor and *H. fasiculare* compared with the controls. By contrast, when plates were aerated by opening only after 2d and 5d, the extension rate of both *S. gausapatum* and *F. fomentarius* increased above self-pairings of *R. bicolor*, and that of Sg also increased above the self pairings of *H. fasiculare* (Table A.1).

Table A.1. Mean radial extension rate (mm d^{-1} ± SEM) when grown above interspecifically interacting mycelium on malt agar.

Fungi	**Agar**	**Rb/Hf**	**Rb/Rb**	**Hf/Hf**
Stereum gausapatum **(d 1,2,3,4,5)**	5.41± 0.07	5.34 ±0.02 ns	5.33± 0.03 ns	5.38± 0.05 ns
Stereum gausapatum **(d 2,5)**	4.90± 0.03	5.16 ±0.08 ns	5.41±0.04 ***	5.54± 0.11 ***
Fomes fomentarius **(d 1,2,3,4,5)**	1.05± 0.02	0.95 ±0.02 ns	1.0 ± 0.02 ns	1.0 ± 0.031 ns
Fomes fomentarius **(d 2,5)**	0.89± 0.03	0.89 ±0.04 ns	1.23± 0.07 **	0.89± 0.08 ns

Significance of difference compared with growth above agar. (*, P ≤ 0.05;**, P ≤ 0.01;***, P ≤ 0.001; ns, not significant)

A.2.2. Species grown alone at two different malt concentrations

Five species grew significantly faster on 2% MA than on 0.05% MA (*Trametes versicolor, Stereum gausapatum, Eutypa spinosa, Vuilleminia comedens and Trichoderma viride*), three were unaffected (*Bjerkandera adusta, Resinicium bicolor, and Fomes fomentarius*, while *Hypholoma fasciculare* grew more rapidly on 0.5% MA (Table A.2).

Table A.2. Mean extension rate (mm d^{-1} ± SEM) when grown on 0.5%MA and 2%MA (Two-sample t Test in Minitab)

Tested fungi	0.5%MA	2%MA	Significant difference
Trametes versicolor	11.965 ± 0.090	12.858 ± 0.084	** T_{43} = -7.24, P<0.01
Stereum gausapatum	12.983 ± 0.094	13.555 ± 0.11	* T_{43} = -4.04, P<0.05
Eutypa spinosa	12.24 ± 1.3	17.07 ± 0.62	* T_{43} = -3.44, P<0.05
Bjerkandera adusta	17.8917 ± 0.040	17.8633 ± 0.024	ns T_{43} = 0.61, P>0.05
Vuilleminia comedens	3.5150 ± 0.055	4.482 ± 0.24	* T_{43} = -3.89, P<0.05
Resinicium bicolor	6.098 ± 0.061	6.2867 ± 0.055	ns T_{43} = -2.29, P>0.05
Hypholoma fasiculare	5.503 ± 0.11	4.653 ± 0.15	** T_{43} = 4.74, P<0.01
Fomes fomentarius	2.0 ± 0.085	2.053 ± 0.064	ns T_{43} = -0.25, P>0.05
Trichoderma viride	25.817 ± 0.22	27.375 ± 0.44	* T_{43} = -3.44, P<0.05

Significance of difference (*, P≤0.05; **, P≤0.01;***, P≤0.001; ns, not significant)

A.2.3. Effect of nutrients concentration on the extension rate of fungi

Extension rate of *S. gausapatum* and *S. hirsutum* was inhibited when grown above self pairings of *H. fasiculare* in both nutrient concentrations 2% and 0.5% MA, while *S. hirsutum* was inhibited when grown above the mycelial interaction of *R. bicolor* and *H. fasciculare* on 2%MA, and above the self pairings *R. bicolor* on 0.5%MA. (Table A.3)

Table A.3. Mean radial extension rate (mm d^{-1} ± SEM) of *Stereum gausapatum* and *Stereum hirsutum* on 0.5% and 2% malt agar when grown above interacting mycelium.

Fungi	Agar	Rb/Hf	Rb/Rb	Hf/Hf	Significant difference
***Stereum gausapatum* (2%MA)**	7.04 ± 0.21	6.70 ± 0.16 ns	6.70 ± 0.26 ns	6.004 ± 0.14 ***	$F_{3,16}$ = 4.87, P<0.05
***Stereum gausapatum* (0.5%MA)**	5.65 ± 0.16	5.76 ± 0.06 ns	5.48 ± 0.10 ns	5.01 ± 0.12 ***	$F_{3,16}$ = 0.83, P>0.05
***Stereum hirsutum* (2%MA)**	8.38± 0.34	7.68 ± 0.14 **	7.98 ± 0.09 ns	7.05± 0.19 **	$F_{3,16}$ = 6.97, P<0.01
***Stereum hirsutum* (0.5%MA)**	5.65± 0.16	5.76 ± 0.06 ns	5.48 ± 0.01 ***	5.17 ± 0.12 ***	$F_{3,16}$ = 13.94, P<0.001

Significance of difference compared with growth above agar. (*, P ≤ 0.05;**, P ≤ 0.01;***, P ≤ 0.001; ns, not significant). **Abbreviations**: Rb, *Resinicium bicolor* ; Hf, *Hypholoma fasciculare.*

A.2.4. Extension rate measurements

Inhibition and stimulation of extension during interactions of five basidiomycetes with *H. fasciculare, R. bicolor* or *S. gausapatum*

Extension rates toward *H. fasciculare* decreased dramatically compared to growth alone in the case of *E. spinosa*, and *B. adusta,* and was reduced significantly but less dramatically for a further three species *(T. versicolor, H. fasciculare* and *F. fomentarius* (Table A.4). In the case of two species (*S. gausapatum* and *R. bicolor*) there was no significant difference, whereas the extension rate of *Vuilleminia comedens* actually increased. The diameter of the colonies followed the same trend as the growth towards *H. fasciculare* in four species (*E. spinosa, F. fomentarius, S. gausapatum* and *R. bicolor*).In four species (*V. comedens*, *H. fasciculare, B. adusta* and *T. versicolor*) there was an increase in the diameter compared to growth alone whereas the growth towards the paired species was reduced. Growth away from the paired fungus de-

creased for three species (*E. spinosa, F. fomentarius* and *B. adusta*), was not significantly different for three species (*S. gausapatum, R. bicolor* and *T. versicolor*), and increased for the remaining two species (*V. comedens* and *H. fasciculare*).

Table A.4. The mean radial extension rate (mm d^{-1} ± SEM) of fungi when paired against ***Hypholoma fasciculare***.

FUNGI	Alone	Away	Diameter	Toward
Eutypa spinosa	8.54 ± 0.31	6.63 ± 0.47 ***	6.35 ± 0.63 ***	0.97 ± 0.104 ***
Fomes fomentarius	1.01 ± 0.02	0.78 ± 0.021 ***	0.76 ± 0.007 ***	0.77 ± 0.040 ***
Stereum gausapatum	6.78 ± 0.05	5.08 ± 0.46 ns	6.21 ± 0.34 ns	5.57 ± 0.47 ns
Resinicium bicolor	3.14 ± 0.03	3.12 ± 0.12 ns	3.50 ± 0.07 ns	3.20 ± 0.13 ns
Vuilleminia comedens	2.24 ± 0.12	3.03 ± 0.10 ***	3.63 ± 0.03 ***	2.94 ± 0.07 ***
Hypholoma fasciculare	2.32 ± 0.073	2.58 ± 0.05 ***	2.56 ± 0.02 ***	2.11 ± 0.09 ***
Bjerkandera adusta	8.93 ± 0.01	7.80 ± 0.70 ***	9.31 ± 0.19 ***	4.30 ± 0.16 ***
Trametes versicolor	6.23 ± 0.042	6.39 ± 0.07 ns	7.17 ± 0.10 ***	5.23 ± 0.33 ***

Significance of difference compared with growing alone. (*, $P \leq 0.05$;**, $P \leq 0.01$;***, $P \leq 0.001$; ns, not significant)

The extension rates away from opponent for three species (*E. spinosa, B. adusta* and *F. fomentarius*) were decreased when interacted with *R. bicolor*, was not significantly different for three species (*T. versicolor, S. gausapatum and V. comedens),* whereas *R. bicolor* extension rate increased towards self compared with growing alone (Table A.5). The diameter of the colonies showed increase in three fungi (*T. versicolor, R. bicolor* and *V. comedens),* and decrease in *(F. fomentarius and E. spinosa),* while *B. adusta* and *S. gausapatum* was not significantly different. The extension rate toward the paired fungus decreased in five species (*E. spinosa, V. comedens, S. gausapatum,*

B. adusta and F. fomentarius), increased in *T. versicolor* but was not significant for *R. bicolor* against itself.

Table A.5. The mean radial extension rate (mm d $^{-1}$ ± SEM) of fungi when paired against ***Resinicium bicolor***.

FUNGI	Alone	Away	Diameter	Toward
Eutypa spinosa	8.54 ± 0.31	5.48 ± 0.27 ***	6.11 ± 0.28 ***	3.17 ± 0.09 ***
Vuilleminia comedens	2.24 ± 0.12	2.11 ± 0.07 ns	2.85 ± 0.23 **	1.55 ± 0.15 **
Resinicium bicolor	3.14 ± 0.03	3.49 ± 0.01 *	3.48 ± 0.05 *	3.05 ± 0.20 ns
Stereum gausapatum	6.78 ± 0.05	6.46 ± 0.16 ns	6.76 ± 0.13 ns	4.20 ± 0.51 ***
Trametes versicolor	6.43 ± 0.04	6.23 ± 0.21 ns	7.29 ± 0.03 **	6.78 ± 0.17 **
Bjerkandera adusta	8.93 ± 0.01	7.43 ± 0.41 ***	8.95 ± 0.08 ns	4.95 ± 0.63 ***
Fomes fomentarius	1.01 ± 0.02	0.74 ± 0.03 ***	0.67 ± 0.01 ***	0.70 ± 0.04 ***

Significance of difference compared with growing alone. (*, $P \leq 0.05$;**, $P \leq 0.01$;***, $P \leq 0.001$; ns, not significant)

The extension rate of *H. fasciculare, R. bicolor* and *S. hirsutum* did not alter significantly in any direction, when paired with *S. gausapatum* compared with growth alone (Table A.6), *E. spinosa* and *H. fasciculare* extended significantly more slowly in all directions when paired with *S. gausapatum,* while *B. adusta* and *T. versicolor* extension increased significantly more rapidly along the tested fungus.

Table A.6. The Mean radial extension rate (mm d $^{-1}$ ± SEM) of fungi when paired against ***Stereum gausapatum***.

FUNGI	Alone	Away	Diameter	Toward
Hypholoma fasciculare	2.33 ± 0.07	2.40 ± 0.18 ns	2.43 ± 0.06 ns	2.13 ± 0.27 ns
Resinicium bicolor	3.14 ± 0.03	3.39 ± 0.06 ns	3.46 ± 0.08 ns	3.47 ± 0.15 ns
Eutypa spinosa	8.54 ± 0.31	5.98 ± 0.40 ***	7.24 ± 0.35 ***	2.63 ± 0.28 ***

Fomes fomentarius	1.01± 0.02	0.36 ± 0.05 ***	0.54 ± 0.01 ***	0.62 ± 0.09 ***
Trametes versicolor	6.43 ± 0.42	6.30 ± 0.17 ns	6.86 ± 0.03 ***	2.10 ± 0.17 ***
Stereum gausapatum	6.78 ± 0.05	5.53 ± 0.57 ***	5.95 ± 0.24 ***	2.17 ± 0.18 ***
Vuilleminia comedens	2.24 ± 0.12	2.74 ± 0.05 ns	2.52 ± 0.03 ns	2.20 ± 0.36 ns
Bjerkandera adusta	8.93 ± 0.01	6.87 ± 0.54 ***	8.81 ± 0.13 ***	5.13 ± 0.84 ***
Stereum hirsutum	6.79 ± 0.11	6.43 ± 0.39 ns	6.63 ± 0.12 ns	6.94 ± 0.29 ns

Significance of difference compared with growing alone. (*, P ≤ 0.05;**, P ≤ 0.01;***, P ≤ 0.001; ns, not significant)

Overall, the extension rate of only two species: *E. spinosa and F. fomentarius* was decreased in all three directions (growth of one radial of diameter, growth away and growth towards) when paired against the three test species (*H. fasciculare, R. bicolor and S. gausapatum*). In general also, growth toward the paired species was more likely to be reduced than increased. Only one pairing resulted in increased growth rate for the paired species in all three growth directions, (*V. comedens* versus *H. fasciculare*).

A.2.5. Interaction outcomes

H. fasciculare and *R. bicolor* deadlocked with *F. fomentarius, V. comedens, S. gausapatum* and with each other, while *S. gausapatum* deadlocked with *H. fasiculare* and *R. bicolor* and with *F. fomentarius* (Table A.7). On the other hand *B. adusta* totally replaced *H. fasiculare* and partially replaced *R. bicolor* and *S. gausapatum. E. spinosa* was totally replaced by *H. fasiculare* and *S. gausapatum*, and partially replaced by *R. bicolor*. *T. versicolor* was totally replaced by *H. fasiculare*, but it replaced *R. bicolor* and *S. gausapatum.*

As the interaction zone became denser, pigments at the line between the two

colonies were produced (Figs. A.2, A.3). In self pairings the two colonies could not be distinguished from each other (Fig. A.4).

Table A.7. Outcomes of interactions between species of *Hypholoma fasciculare, Resinicium bicolour* and *Stereum gausapatum* and five other wood decay fungi.

Opponent ---------------- Fungi	**Es**	**Ff**	**Vc**	**Tv**	**Ba**	**Hf**	**Rb**	**Sg**
***Hypholoma fasciculare* (Hf)**	R	D	D	R	r	-	D	D
***Resinicium bicolor* (R)**	PR	D	D	r	pr	D	-	D
***Stereum gausapatum* (S)**	R	D	R	r	pr	D	D	-

Abbreviation: **D**, deadlock; **R**, replacement of Hf, Rb and Sg by the opponent; **PR**, partial replacement of Hf, Rb and Sg by the opponent; **r**, replacement of opponent; **pr**, partial replacement of opponent.
Abbreviations: Es, *Eutypa spinosa;* Ff;, *Fomes fomentarius*; Vc, *Vuilleminia comedens*; Tv, *Trametes versicolor*; Ba, *Bjerkandera adusta*; Hf; *Hypholoma fasciculare*; Rb; *Resinicium bicolour*; Sg, *Stereum gausapatum* .

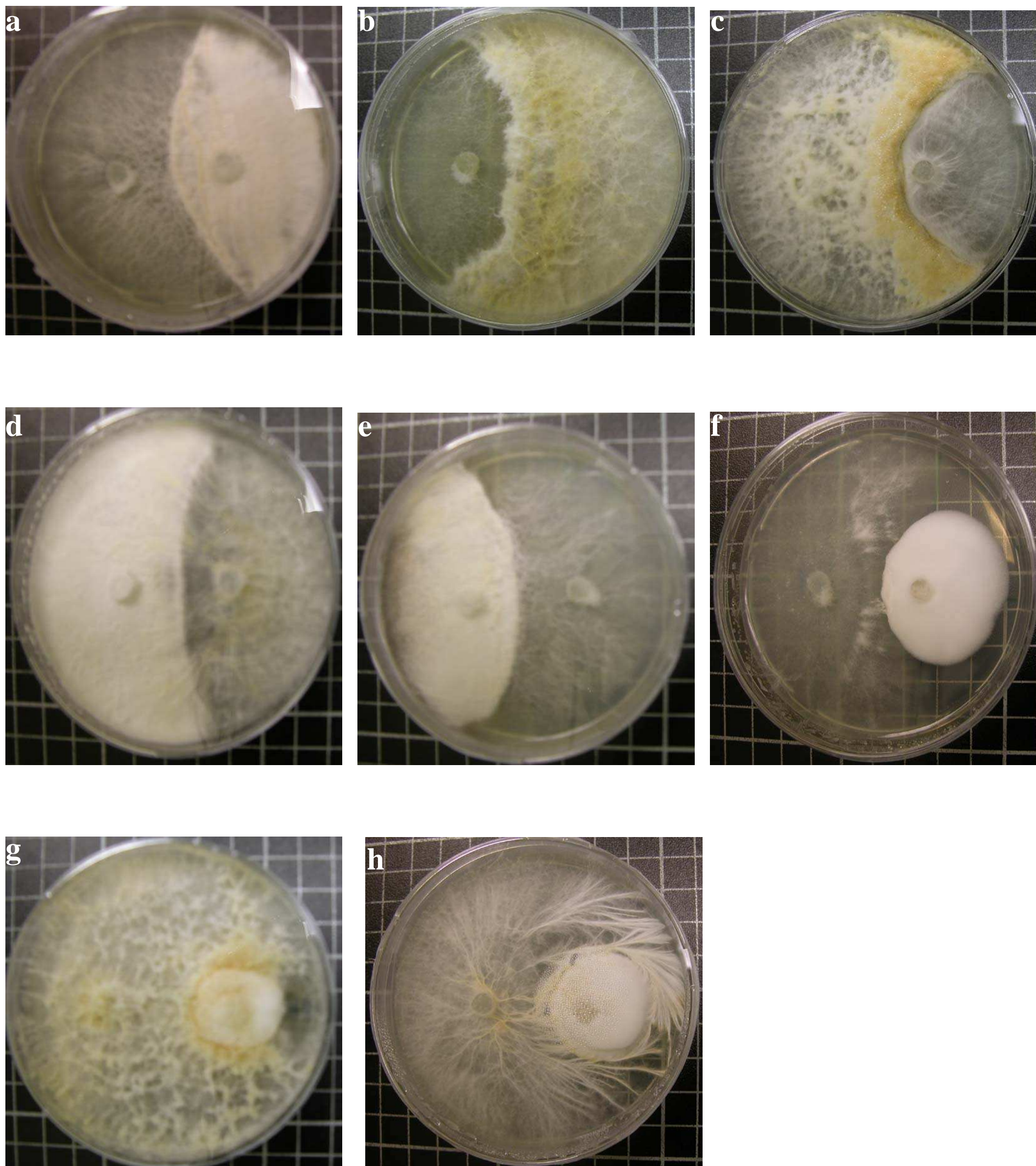


Fig. A.2. Deadlock. Pairing interactions of the speceies that showed deadlock result. a) Rb vs Hf , b) Rb vs Sg* , c) Sg* vs Hf , d) Vc vs Hf, e) Vc vs Rb , f) Rb vs Ff , g) Sg* vs Ff , h) Hf vs Ff . * = some deadluck were compined by the presence of pigments for Sg.

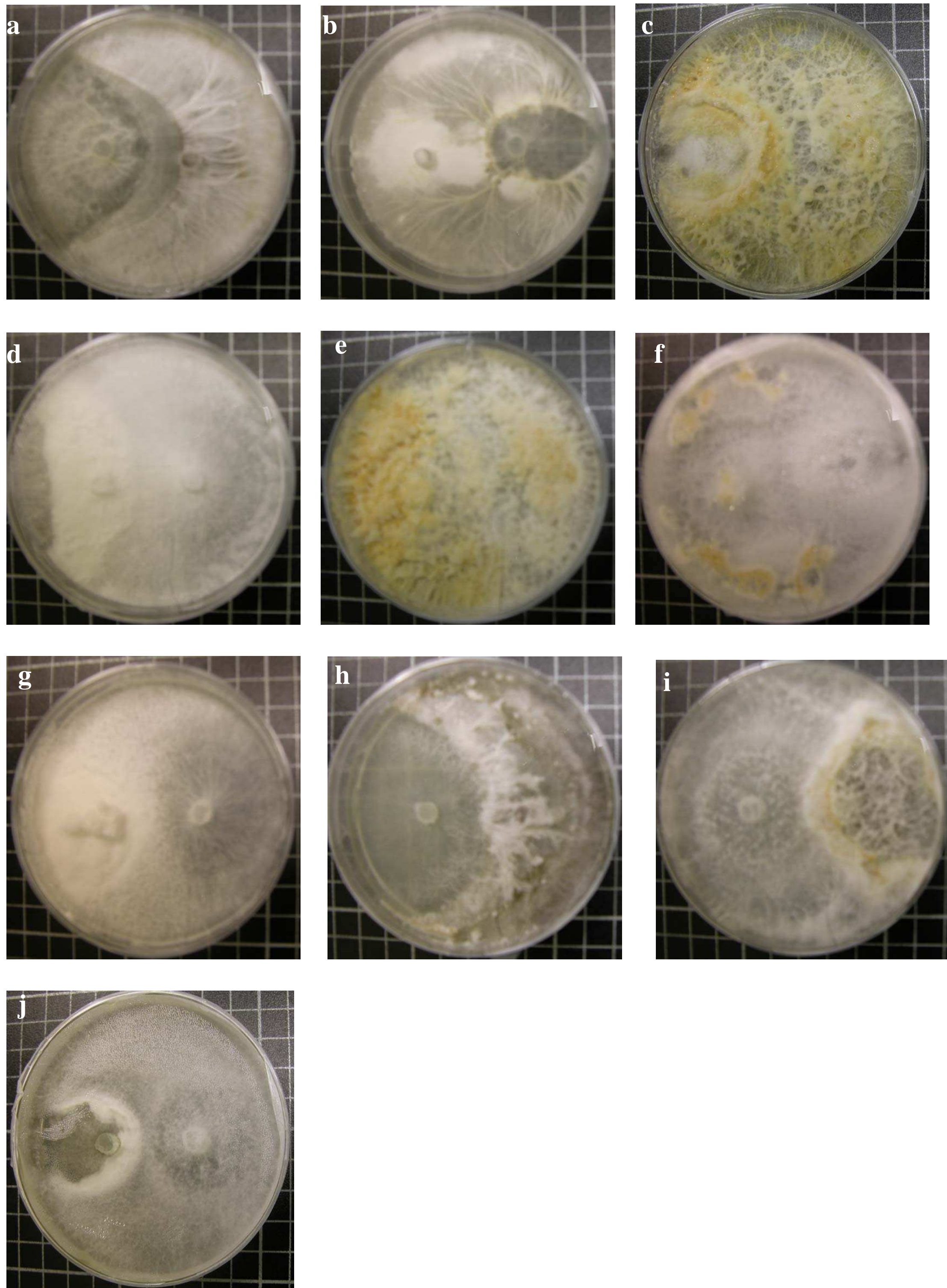


Fig. A.3. Replacement . Pairing interaction of different species that showed total replacement with the other species a) Hf* vs Es , b) Tv vs Hf* , c) Vc vs Sg* , d) Sg vs Tv* , e) Sg* vs Es , f) Ba* vs Hf , g) Rb vs Tv* , partial replacement h) Rb* vs Es , i)Ba* vs Sg , j) Rb vs ba*.
* = the winner fungus.

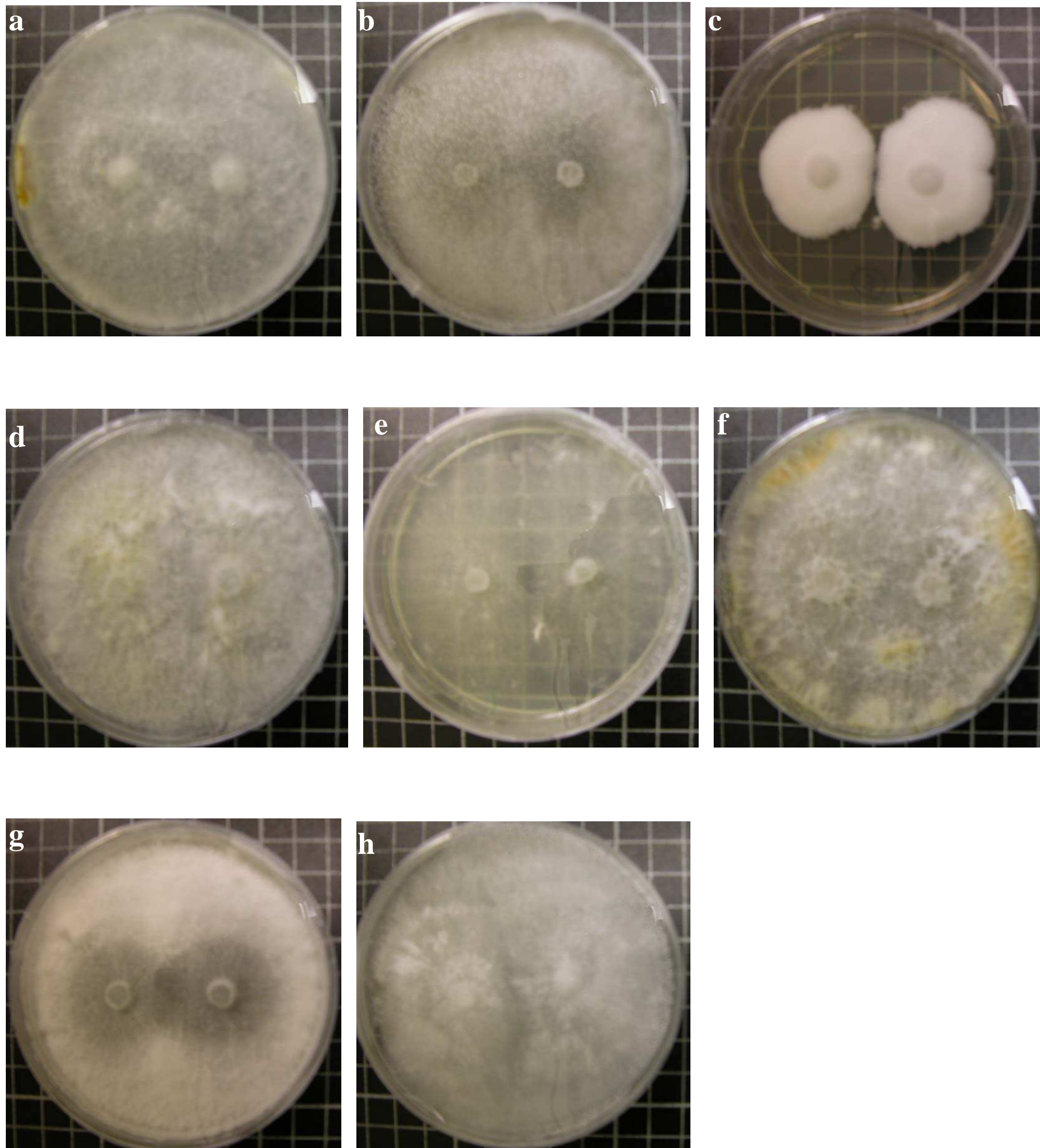


Fig. A.4. Self-pairings. Pairing interactions of the same species.
a) *Bjerkandera adusta* , b) *Eutypa spinosa* , c) *Fomes fomentarius* , d) *Hypholoma fasiculare* , e) *Resinicium bicolor* , f) *Stereum gausapatum* , g) *Trametes versicolor* , h) *Vuilleminia comedens*.

A.3.Discussions

The preliminary experiments revealed that fungi extended more rapidly on 2% Malt Agar than on 0.5% Malt Agar, but the latter medium was chosen for further experiments as 2% MA is much richer in nutrients than wood. Though there were slight differences in extension rate depending on whether plates were aerated daily or less frequently, measurements were made while the plates were closed during the whole experiment to keep the VOCs inside the plates resulting in more obvious effect on the fungi.

There were also differences in the extension rates between experiments, which may have been caused by slight differences in temperatures between incubators, and unknown aspects of fungus physiological state. Variations in microbial growth conditions can be affected by change in both the nature and quantity of VOCs (Wheatley, 2002). Production of VOCs can itself vary depending on the nutrient content of the medium on which the fungi are growing (Demyttenaere *et al*., 2004; Wheatley *et al*., 1997). The distance between the two inocula may yield different VOCs as mycelia that are older may produce different VOCs during interactions.

Outcomes were not always the same between replicates perhaps resulting from slight differences in the age of inoculum (Bruce *et al*., 1996), the timing of interaction, the medium of growth, and the slight different in environmental conditions such as temperature, pH and water potential etc. While every effort was made to standardize these, differences may still have contributed to differences in outcome of interactions.

Outcomes can also be affected by the species involved and size of the starting mycelium (Stenlid, 1997, Holmer & Stenlid, 1997).

The fungal outcomes seems to vary depending on the resources and the nutrients they consume, and differences in outcomes are found depending on where the interaction is taking place, e.g. wood, soil and agar cultures (Dowson, Rayner & Boddy, 1988; Wardle *et al.*, 1993; Holmer & Stenlid, 1997; Woods *et al.*, 2006). On the other hand other studies found that the fungal interaction outcomes of some species were similar when grown on wood and agar (Rayner & Boddy, 1988; Holmer *et al.*, 1997).

The tested species *Stereum gausapatum*, *Hypholoma fasciculare* and *Resinicium bicolor* showed diverse combative abilities, deadlocking, replacing or being replaced by other species, while *Bjerkandera adusta* was the highest competitor against the tested species, *Eutypa spinosa* had the lowest combative ability. *H. fasciculare* produced cords when grown against opponents but does not produce them when grown alone on agar medium. In this study *H. fasciculare* showed dissimilar outcome to that reported by Boddy (1993) where it replaced *S. gausapatum,* while in this work they deadlocked. Note, however, that different strains were used, and there can be considerable variation in combat ability of different strain (Rothery *et al*., 2009)

Deadlock was the final outcome of these interspecific competitors, therefore, a clear distinction can be seen between interacted species, such inhibition at a distance frequently follows mycelial contact involves diffusible compounds that may be evidenced by pigment production (Griffith & Rayner 1994), whereas *S. gausapatum* produced orange pigmentation during interactions and was either replaced by or dead-

locked with other species. In another study (Rayner and Boddy 1988) it was also able to replace *E. spinosa* and *Vuilleminia comedens*, while the interaction between *H. fasiculare* and *R. bicolor* showed production of yellow pigment (Hynes *et al*., 2007). This secretion of pigments may occur as a result of a change in metabolism or as a result of chemical stress (Griffith *et al*., 1994).

Appendix (B)

Media used

B.1. Fahraeus medium

Glucose, puriss. (Kebo) …………………… 20 g
L-Asaragine, purum (Merch) ……………… 2.5 g
D,L-Phenylalanine, puriss. (Fluka) ……..... 0.15 g
Adenine (Nutr. Bio.-Corp.) ……………..... 0.0275 g
Thiamine-HCL …………………………….... 50 µg
KH2PO$_4$ ……………………………….... 1.0 g
Na2HPO$_4$.2H_2O ………………………….... 0.1 g
$MgSO_4.7H_2O$ .... 0.5 g
$CaCl_2$ .... 0.01 g
$FeSO_4.7H_2O$ .... 0.01 g
$MnSO_4.4H_2O$ 0.001g
$ZnSO_4.7H_2O$ .... 0.001 g
$CuSO_4.5H_2O$ .... 0.002 g
Distilled water .... 1000 ml
pH about 5.0 after sterilisation.

B.2. Kirk medium (N- limited, tartrate medium, Tien Kirk 1988)

<u>Mineral elixer</u>

$MgSO_4 . 7H_2O$ ……………….. 3,00 g/L
$MnSO_4 . H_2O$ ………………… 0,05 g/L
NaCl …………………………. 1,00 g/L
$FeSO_4 . 7H_2O$ ………………… 0,10 g/L
$CoCl_2 . 6H_2O$ ………………… 0,10 g/L
$CaCl_2 . 2H_2O$ ………………… 0,10 g/L
$ZnSO_4 . 7H_2O$ ……………… 0,10 g/L
$CuSO_4 . 5H_2O$ ………………. 0,01 g/L

<u>Basal III</u>

Ammonium tartrate ………….. 2 g/L
KH2PO$_4$ ……………………. 20 g/L
$MgSO_4 . 7H_2O$ ……………… 5 g/L
$CaCl_2 . 2H_2O$ ………………. 1 g/L
Mineral elixer ……………… 100 ml/L

<u>Complete medium</u>

D – glucose ………………… 10 g/L
Sodium tartrate ……………... 2,3 g/L adjust pH to 4,2 after addition
Basal III ……………………. 100 ml/L
pH ………………………… 4,5 adjust using 5M HCl
Agar ……………………….. 20-30 g/L

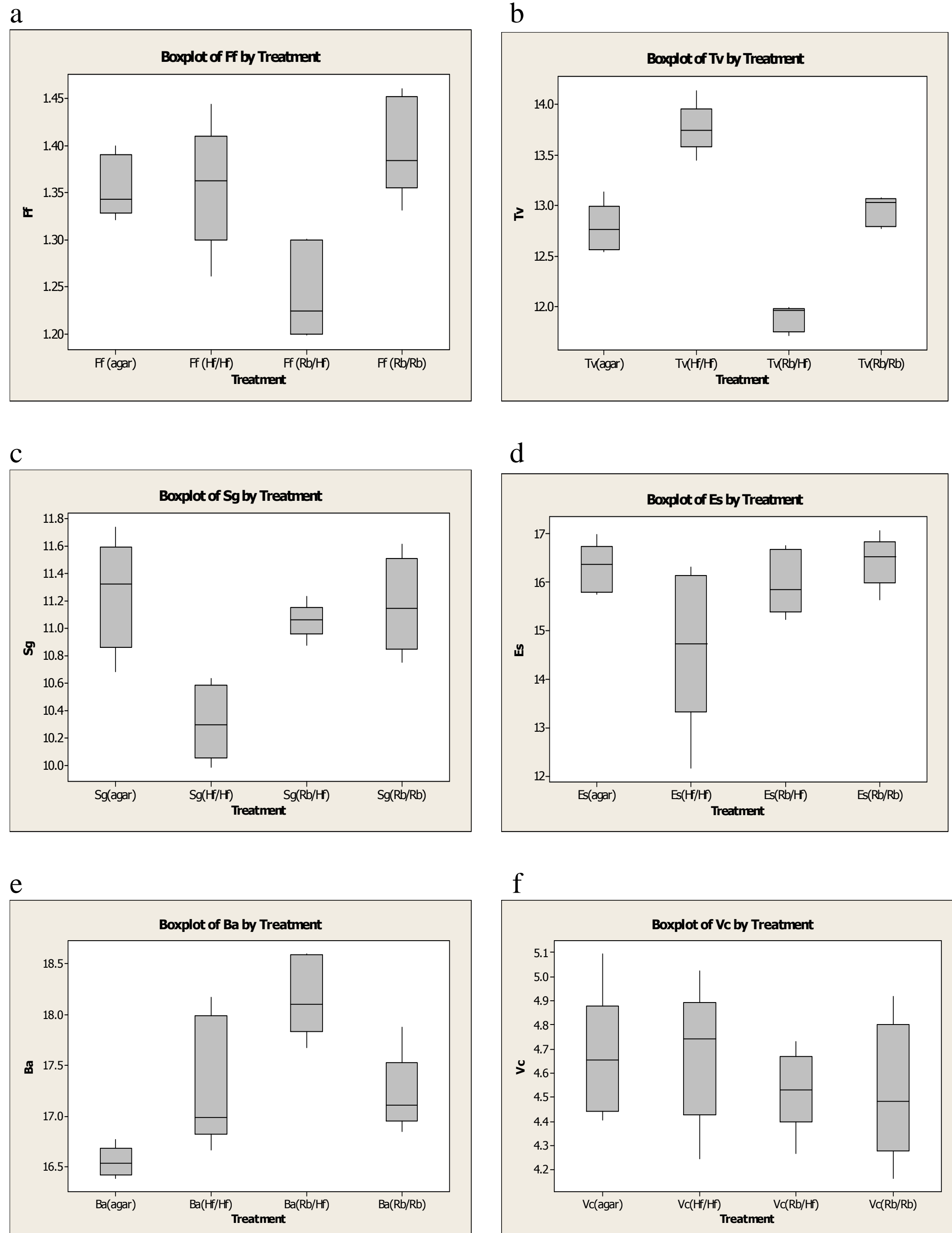
B.3. Agar Only

Agar …………. 20 g/L
H_2O …………. 1 L

Autoclave cellophane strips to loosen plasticizer. When cool enough to handle remove plasticizer by rubbing between finger and thumb. Put in a beaker of water to prevent drying at this stage, autoclave again to use sterilized. Cellophane put on top of the agar plate by sterilized handle.

Appendix (C)

Statistical figures and analysis



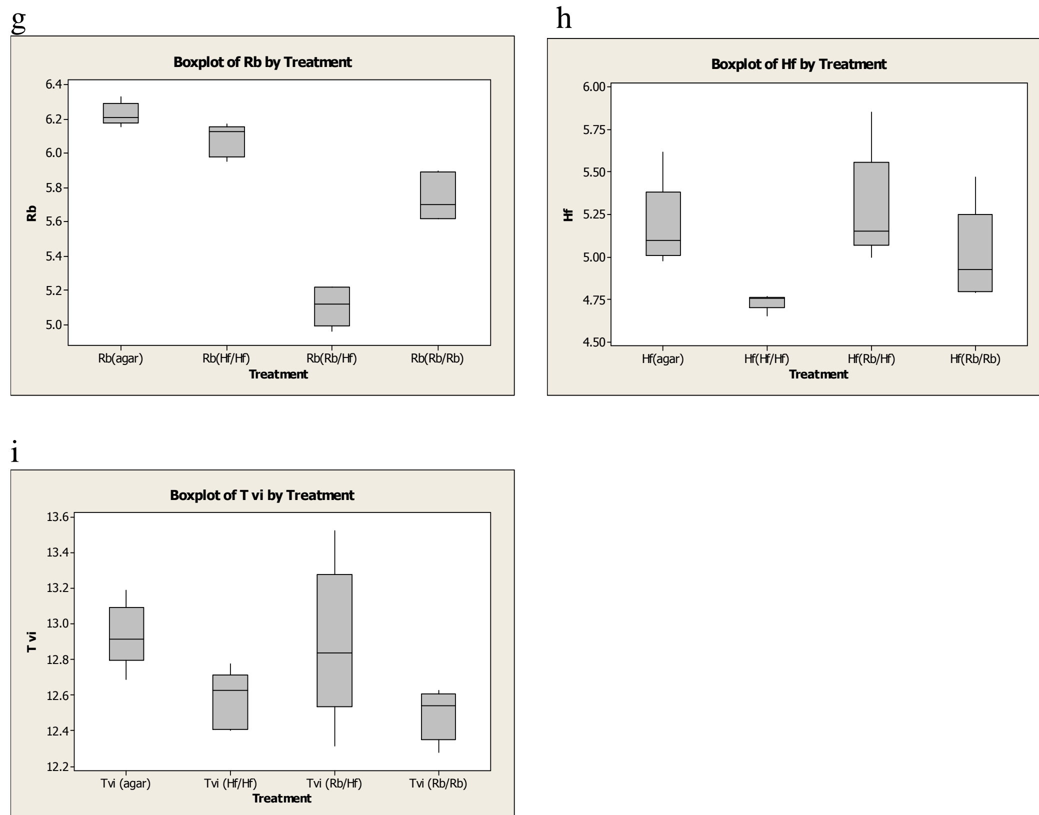
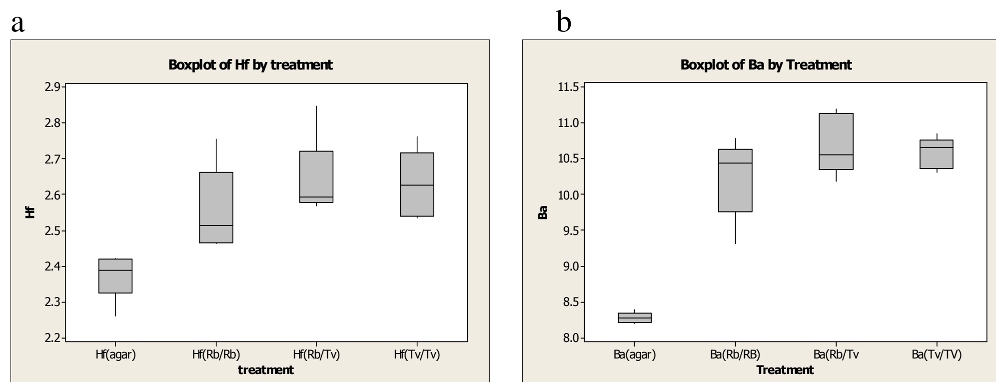


Fig.C.1. The effect of VOCs produced by interactions of *Hypholoma fasciculare* and *Resinisium bicolor* compared with controls: a, *Fomes fomentarius*; b, *Trametes versicolor*; c, *Stereum gausapatum*; d, *Eutypa spinosa*; e, *Bjerkandera adusta*; f, *Vuilleminia comedens*; g, *Resinicium bicolor*; h, *Hypholoma fasciculare* and i, *Trichoderma viride*.



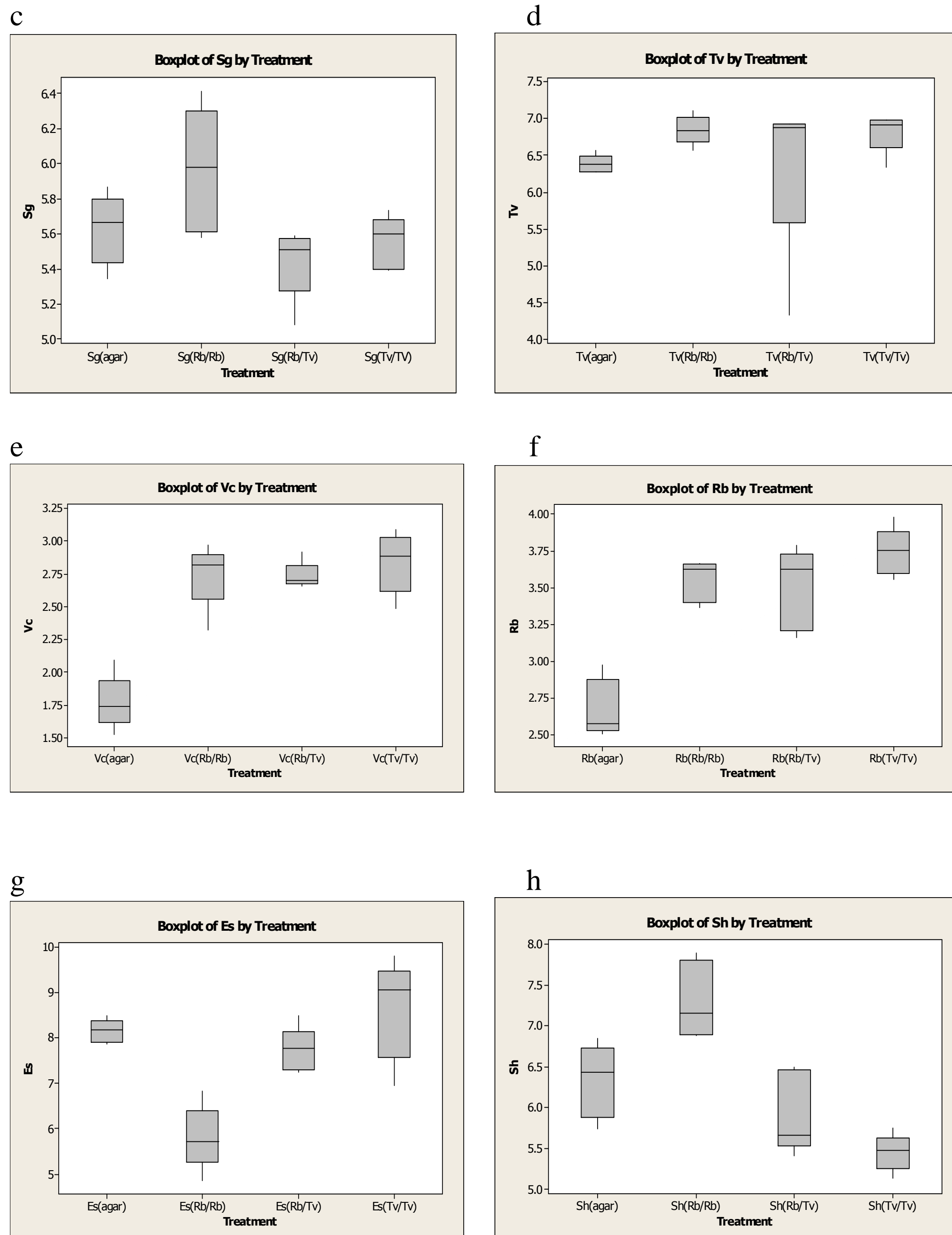


Fig.C.2. The effect of VOCs produced by interactions of *Trametes versicolor* and *Resinisium bicolor* compared with controls: a, , *Hypholoma fasciculare*; b, *Bjerkandera adusta; c, Stereum gausapatum;* d, *Trametes versicolor*; e, *Vuilleminia comedens*; f, *Resinicium bicolor*; g, *Eutypa spinosa* and h, *Stereum hirsutum*.

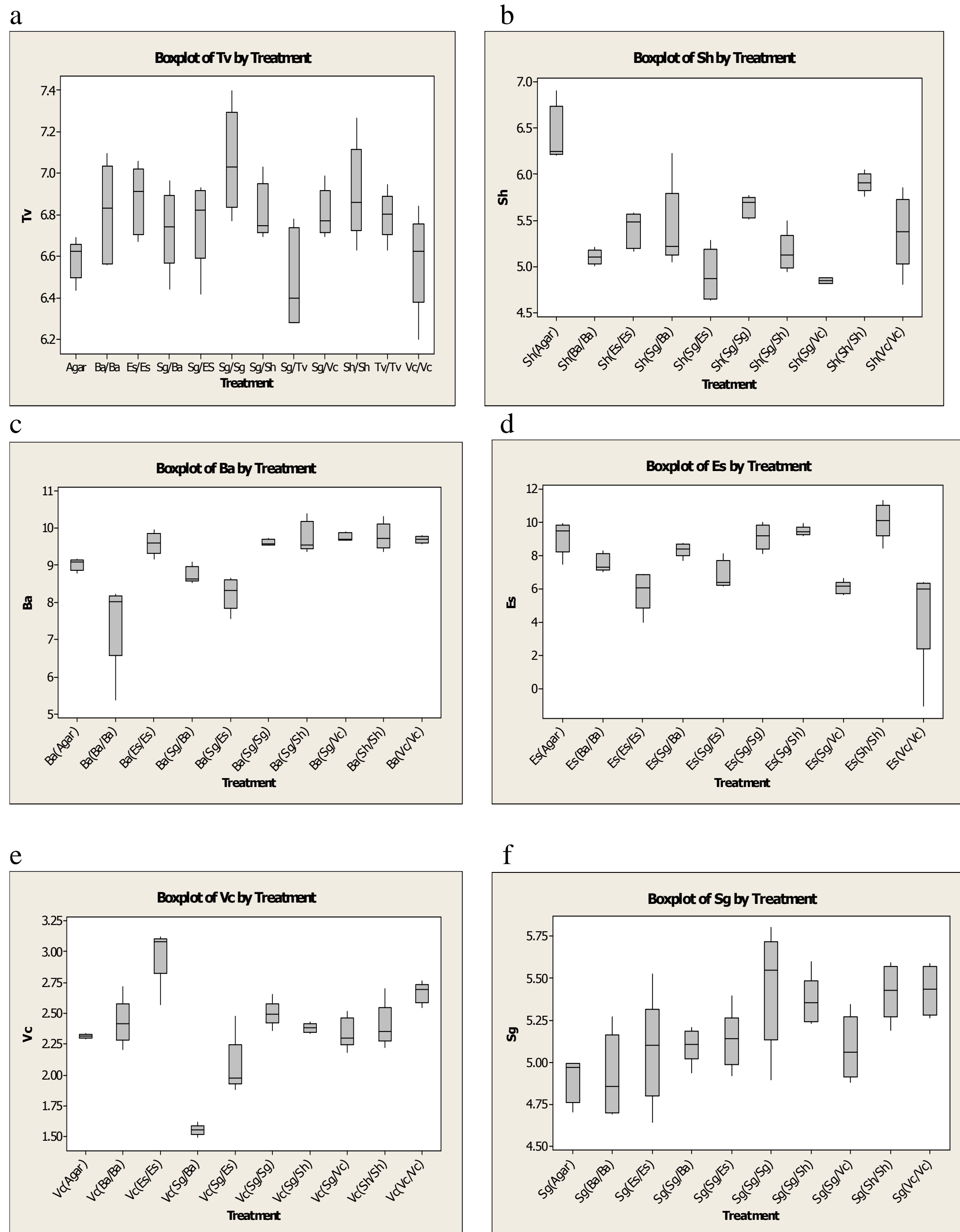


Fig.C.3. The effect of VOCs produced by *Stereum gausapatum* when interacted with *Bjerkandera adusta, Eutypa spinosa, Stereum hirsutum* and *Vuilleminia comedens* compared with controls on: a, *T. versicolor;* b, *S. hirsutum;* c, *B. adusta;* d, *E. spinosa;* e, *V. comedens* and f, *Stereum gausapatum*.

Table C.1. ANOVA analysis (Tukey 95%) for laccase activity of *T. versicolor* under stress (agar only, limited-N, 35°C, 5°C, KCl and 20°C)

Tukey HSD

Treatments	N	A	b	c	d
agar	3	4.4233			
LN	3	12.6900	12.6900		
35°C	3		24.8267	24.8267	
5°C	3			43.3400	43.3400
KCl	3				47.6567
20°C	3				62.4533
Sig.		.745	.392	.082	.069

One-way ANOVA, $F_{5,12} = 27.187$, $P<0.001$

Table C.2. ANOVA analysis (Tukey 95%) for radial extension rate of *T. versicolor* under stress (agar only, limited-N, 35°C, 5°C, KCl and 20°C)

Tukey HSD

treatments	N	a	b	c
5°C	5	2.6120		
KCl	5	2.9493		
agar	5		5.1077	
35°C	5		5.2670	
20°C	5			5.8590
LN	5			5.8720
Sig.		.066	.727	1.000

One-way ANOVA, $F_{5,24} = 326.79$, $P<0.001$

Table C.3. ANOVA analysis (Tukey 95%) for laccase activity of *T. versicolor* under stress of VOCs produced by mycelial interaction of two fungi on agar

Tukey HSD

Treatments	N	a
Sh/Sh	3	35.1800
Es/Es	3	38.4200
Hf3/Hf3	3	40.7600
Rb/Hf	3	42.9333
Hf4/Hf4	3	43.3567
Controls	3	47.0533
Tv/Ba	3	48.9867
Sg/Sg	3	49.3300
Ba/Ba	3	52.0600
Sig.		.100

One-way ANOVA, $F_{8,18} = 2.144$, $P>0.05$

Table C.4. ANOVA analysis (Tukey 95%) for Manganese peroxidase activity of *T. versicolor* on 0.5% MA under the effect of VOCs produced during interacting two fungi on woodblocks.

Wood VOCs

Tukey HSD

Treatments	N	a
Sg/Es	4	2.1350
Es/Es	4	2.1500
Rb/Rb	5	2.2100
Tv/Ba	5	2.2460
Tv/Hf4	5	2.2560
Sg/Ba	5	2.2740
Sh/Sh	5	2.2960
Sg/Sh	5	2.3880
Controls	5	2.4320
Rb/Hf4	5	2.4540
Hf4/Hf4	5	2.4620
Sg/Sg	5	2.4700
Hf3/Hf3	5	2.4800
Tv/Tv	5	2.4820
Tv/Sg	5	2.5680
Ba/Ba	5	2.5840
Woodblock Controls	7	3.1343
Rb/Tv	5	3.1500
Sig.		.309

One-way ANOVA, $F_{16,66} = 6.204$, $P<0.001$

Table C.5. ANOVA analysis (Tukey 95%) for laccase activity of *T. versicolor* under the stress of VOCs produced by the interaction of two fungi on wood blocks

Tukey HSD

Treatments	N	A	b	c	d	e
Woodblock controls	7	1.7314				
Sg/Es	4	1.8700	1.8700			
Es/Es	4	1.9250	1.9250	1.9250		
Tv/Ba	5	1.9740	1.9740	1.9740	1.9740	
Sg/Ba	5	1.9820	1.9820	1.9820	1.9820	
Rb/Rb	5	2.0000	2.0000	2.0000	2.0000	
Rb/Tv	5	2.0300	2.0300	2.0300	2.0300	
Sh/Sh	5	2.0300	2.0300	2.0300	2.0300	
Tv/Hf3	5	2.0480	2.0480	2.0480	2.0480	2.0480
Sg/Sh	5	2.0780	2.0780	2.0780	2.0780	2.0780
Hf3/Hf3	5		2.1440	2.1440	2.1440	2.1440
Controls	5		2.1600	2.1600	2.1600	2.1600
Sg/Sg	5		2.1720	2.1720	2.1720	2.1720
Hf4/Hf4	5		2.2100	2.2100	2.2100	2.2100
Rb/Hf4	5		2.2280	2.2280	2.2280	2.2280
Tv/Sg	5			2.3020	2.3020	2.3020
Ba/Ba	5				2.3280	2.3280
Tv/Tv	5					2.4340
Sig.		.155	.121	.078	.132	.062

One-way ANOVA, $F_{16,66} = 3.094$, $P<0.001$

Table C.6. ANOVA analysis (Tukey 95%) for FRA2 gene expression of *T. versicolor* interactions with Sg, Ba, Hf and Tv

Stat for FRA2

Tukey HSD

Treatments	N	a
Tv/Tv	3	67.0133
Tv/Ba	3	72.9885
Tv/Sg	3	75.1311
Tv alone	3	85.9037
Tv/Hf	3	93.1240
Sig.		.152

One-way ANOVA, $F_{4,10} = 2.127$, $P>0.05$

Table C.7. ANOVA analysis (Tukey 95%) for F2A1 gene expression of *T. versicolor* interactions with Sg, Ba, Hf and Tv

Tukey HSD

Treatments	N	a	b	c
Tv/Ba	3	17.0695		
Tv/Sg	3		48.4165	
Tv/Tv	3		66.3083	
TV/Hf	3		73.2451	
Tv alone	3			100.0000
Sig.		1.000	.069	1.000

One-way ANOVA, $F_{4,10} = 29.282$, $P<0.001$

Table C.8. ANOVA analysis (Tukey 95%) for Nox gene expression of *T. versicolor* interactions with Sg, Ba, Hf and Tv

Tukey HSD

Treatments	N	a
Tv/Ba	6	54.1966
Tv/Tv	6	57.7479
Tv/Sg	6	70.1435
Tv/Hf	6	79.3239
Sig.		.291

One-way ANOVA, $F_{3,8} = 10.815$, $P<0.01$

Table C.9. ANOVA analysis (Tukey 95%) for FRA19 gene expression of *T. versicolor* interactions with Sg, Ba, Hf and Tv

Tukey HSD

Treatment	N	a	b	c	d
Tv/Ba	3	18.6980			
Tv/Sg	3	24.1009			
Tv/HF	3		39.0858		
Tv/Tv	3			67.2339	
Tv alone	3				100.0000
Sig.		.412	1.000	1.000	1.000

One-way ANOVA, $F_{4,10} = 260.429$, $P<0.001$

Table C.10. ANOVA analysis (Tukey 95%) for F1D8 gene expression of *T. versicolor* interactions with Sg, Ba, Hf and Tv

Tukey HSD

Treatment	N	a	b
Tv alone	3	36.6338	
Tv/Ba	3	42.0146	
Tv/Sg	3	44.5772	
Tv/Tv	3	59.9804	59.9804
Tv/HF	3		100.0000
Sig.		.502	.102

One-way ANOVA, $F_{4,10} = 6.621$, $P<0.01$

Appendix (D)

Sequences

Fig. D1. PCR products compared with the sequencing results for ((FRA19, FRA2, F1D8, F1A2, Nox) for Tv/Tv interactions.

```
                      10        20        30        40        50        60        70
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1D8          ACGGAAAAAGAGCGAGGAGAAGCCCTCCTCATGATAATGGTTTTATCGTATACTGCCCTAATATCACTCC
F1D8 reverse  ACTAGTGATTAGCGAGGAGAAGCCCCCCTCATGATAATGGTTTTATCGTATACTGCCCTAATATCACTCC

                      80        90       100       110       120       130       140
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1D8          ACTCTCGGTTCTGCTCTCCTGCATTCCCAAGCGGCAATAATGGTGTGCACACCTCGAAGACTTGCTGTGT
F1D8 reverse  ACTCTCGGTTCTGCTCTCCTGCATTCCCAAGCGGCAATAATGGTGTGCACACCTCGAAGACTTGCTGTGT

                     150       160       170       180       190       200
              ....|....|....|....|....|....|....|....|....|....|....|....|....|...
F1D8          GGCTGCTACAGTAAGTCGCTCCAGGTGGGTGGTTCGCACATGAGCGTTGCTTTGCACTTCTCGAGGAG
F1D8 reverse  GGCTGCTACAGTAAGTCGCTCCAGGTGGGTGGTTCGCACATGAACGTTGCTTTGCACTTCTCGAGGAG
```

```
                10        20        30        40        50        60        70
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F2A1    GGAGAGTGCGGTGACAATGAAGGAGGCGCGAAGAACTCATGAGTTTGTAGTGAAGCACAACCAGAAGGTT
F2A1+   GGAGAGTGCGGTGACAATGAAGGAGGCTATGGTCGACCGCGAAGAACTCATGAAGGAGCGGGCGCAGTTT

                80        90       100       110       120       130       140
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F2A1    TTCGAGGCTGAATTCGCTATGTGCGAGCATTGATCGAGTATATACCTACCTCTGTGTTATACGTAGTGGT
F2A1+   GTAGTGAAGCACAACCAGAAGGTTTTCGAGGCTGAATTCGCTATGTGCGAGCATTGATCGAGTATATACC

               150       160       170       180
        ....|....|....|....|....|....|....|....|....|
F2A1    CGTGTGACTGTCATTGTCGGG
F2A1+   TACCTCTGTGTTATACGTAGTGGTCGTGTGACTGTCATTGTCGGG
```

```
                      10        20        30        40        50        60        70
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FRA2          CGGTACTGTCTGCTGCGATATTGGCAAAGAATGCGCCGAGAGGCTAATCGCGGAAGGATAGGGGTATACT
FRA2 reverse  CGGTACTGTCTGCTGCGATATTGGCAAAGAATGCGCCGAGAGGCTAATCGCGGAAGGATAGGGGTATACT

                      80        90       100       110       120       130       140
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FRA2          TACCACAAATCTAGAGCTTTCCGTTTTCTAGGGACGGTCGTTCGACATAGTGTATCAGCGATTCAAGATT
FRA2 reverse  TACCACAAATCTAGAGCTTTCCGTTTTCTAGGGACGGTCGTTCGACATAGTGTATCAGCGATTCAAGATT

                     150       160       170       180       190       200       210
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FRA2          GTGTCCCTTGAGCTTCTTTTCTACGATAACATTTGCCTGACATGCTGTAATTAAACCAGGATGTGCTTAA
FRA2 reverse  GTGTCCCTTGAGCTTCTTTTCTACGATAACATTTGCCTGACATGCTGTAATTAAACCAGGATGTGCTTAA

                     220       230
              ....|....|....|....|
FRA2          TCCAAGAACTCGTGTGTTCG
FRA2 reverse  TCCAAGAACTCGTGTGTTCG
```

```
                 10        20        30        40        50        60        70
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FRA19    CGAACACACGAGTTCTTGGATTAAGCACATCCTGGTTTAATTACAGCATGTCAGGCAAATGTTATCGTAG
FRA19    AGACTACCAGGACGGAACGACATGTGCTACCAGTAAGGAATGGCAGTGGGGAAGGAGTGTTGCGGATGG
```

```
                 80        90       100       110       120       130       140
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FRA19    AAAAGAAGCTCAAGGGACACAATCTTGAATCGCTGATACACTATGTCGAACGACCGTCCCTAGAAAACGG
FRA19    GGGGTCGAGCAAGAAATACGATCAGATGACAGGAGAAACGGAAATGGCGCTGTGGATAGGGCCGGAATGG

                150       160       170       180       190       200       210
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FRA19    AAAGCTCTAGATTTGTGGTAAGTATACCCCTCTCGGCGCATTCTTTGCCAATATCGCAGCAGACAGTACC
FRA19    AAAGCATGGGAAGG

         .
FRA19    G
FRA19
```

```
                 10        20        30        40        50        60        70
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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Nox+     TCGGTTGGTTCCAGACTCTCCTCCAGGAGGTCGAGGCTGCACAGGCCGACCCCAACTTCCTGCGCATCAA

                 80        90       100       110       120       130       140
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Nox      CATCTACCTCACGCAGAAGATCAGCGAGGACATGCTCTGGAACATCGCCGTCAACGACGCGGGCGCGGAG
Nox+     CATCTACCTTACGCAGAAGATCAGCGAGGACATGCTCTGGAACATCGCCGTCAACGACGCGGGCGCGGAG

                150       160       170       180       190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|
Nox      TACGACCCGCTTACGCTCCTCCGCACTCGTACCATGTTCGGTCGCCCTGACTGGAAGGCCATCTA
Nox+     TACGACCCGCTTACGCTCCTCCGCACTCGTACCATGTTCGGTCGCCCTGACTGGAAGGCCACTAA
```

Sequences details of *T. versicolor* under stress

The sequences of the laccase gene groups (alpha, beta, delta and gamma), and all the full 5 replicates of the sequences of laccase gene under stress that been used in the sequences alignments.

>AB212732(alpha)
cttcaacg gcaccaactt ctttatcaac aacgcgtctt tcacaccacc gacagtcccc gtgctcctcc agatcctgag cggtgcgcag
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1201 gagatcacgc tgcccgcgac cgcactagcc ccaggcgcgc cgcacccctt ccacctgcac
1261 ggtcacgcct tcgcggtcgt ccgcagcgca ggcagcacta cgtataatta caacgacccg
1321 atcttccgcg acgtcgtgag caccggcacg cccgccgcgg gcgacaacgt cacgatccgc
1381 ttccagacgg acaaccccgg gccgtggttc ctccactgcc acatcgactt

>U44430(alpha)
cttca acggcaccaa cttcttcatc aacaacgcga ctttcacgcc gccgaccgtc ccggtactcc tccagattct gagcggtgcg
1321 cagaccgcac aagacctgct ccctgcaggc tctgtctacc cgctcccggc ccactccacc
1381 atcgagatca cgctgcccgc gaccgccttg gccccgggtg caccgcaccc cttccacctg
1441 cacggtcacg ccttcgcggt cgttcgcagc gcggggagca ccacgtataa ctacaacgac
1501 ccgatcttcc gcgacgtcgt gagcacgggc acgcccgccg cgggcgacaa cgtcacgatc
1561 cgcttccaga cggacaaccc cgggccgtgg ttcctccact gccacatcga

>AF414109(beta)
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1201 atcgagatct ccttcccggc gaccgccgcc gcccccggtg cgcccccaccc nttccacttg
1261 cacgggcacg cgttcgcggt cgtccgcagc gccggcagca cggtntacaa ctacgacaac
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1381 cgcttccgca ccgacaaccc cggcccgtgg ttcctccact gccacatcga

>AM422387(delta)
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1201 cgcggccgac ctcctcccca gcggcagtct cttcgcgctc ccgtccaact cgacgatcga
1261 gatctcgttc cccatcaccg cgacgaacgc gcccggcgcg ccgcatccct tccacttgca
1321 cggtcacacc ttctccatcg ttcgtaccgc cggcagcacg gatacgaact tcgtcaaccc
1381 cgtccgccgc gacgtcgtga acaccggtac cgccggcgac aacgtcacca tccgcttcac
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>AB212734(gamma)
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1141 ttgagcggtg cgcaggatgc ggcgagcctg ctccccaacg gcagtgtcta cacactccct
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1261 cccggtgctc cgcacccgtt ccatctccac ggtcacactt tctcggtggt gcgcagcgcc
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>agar2
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TCTCGTTCCCCATCATCACCACCGACGGTGCTCTGAACGCGCCCGGTGCTC
CGCACCCGTTCCATCTCCACGGTCACACTTTCTCGGTGGTGCGCAGCGCCG

GGAGCTCGACCTTCAACTACGCCAACCCAGTGCGCCGGGACACCGTCAGT
ACTGGTAACTCTGGCGACAACGTCACTATCCGCTTCACGACCGACAACCC
GGGCCCATGGTTCCTCCACTGCCACATCGACTAA

>agar3
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TCTCGTTCCCCATCATCACCACCGACGGTGCTCTGAACGCGCCCGGTGCTC
CGCACCCGTTCCATCTCCACGGTCACACTTTCTCGGTGGTGCGCAGCGCCG
GGAGCTCGACCTTCGACTACGCCAACCCAGTGCGCCGGGACACCGTCAGT
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GGGCCCATGGTTCCTCCACTGCCACATCGACTTC

>agar4
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TGCTCCCCAACGGCAGTGTCTACACACTCCCTTCCAACTCGACTATTGAGA
TCTCGTTCCCCATCATCACCACCGACGGTGCTCTGAACGCGCCCGGTGCTC
CGCACCCGTTCCATCTCCACGGTCACACTTTCTCGGTGGTGCGCAGCGCCG
GGAGCTCGACCTTCAACTACGCCAACCCAGTGCGCCGGGACACCGTCAGT
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GGGCCCATGGTTCCTCCACTGCCNCATNGANTTC

>agar5
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TGCTCCCCAACGGCAGTGTCTACACACTCCCTTCCAACTCGACTATTGAGA
TCTCGTTCCCCATCATCACCACCGACGGTGCTCTGAACGCGCCCGGTGCTC
CGCACCCGTTCCATCTCCACGGTCACACTTTCTCGGTGGTGCGCAGCGCCG
GGAGCTCGACCTTCAACTACGCCAACCCAGTGCGCCGGGACACCGTCAGT
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>KCL1
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>KCL2
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>KCL3
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TCTCGTTCCCCATCATCACCACCGACGGTGCTCTGAACGCGCCCGGTGCTC
CGCACCCGTTCCATCTCCACGGTCACACTTTCTCGGTGGTGCGCAGCGCCG
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>KCL4
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>LN1
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>LN2
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CATCGACTTC

>20C2
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>20C3
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>20C4
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>35C1
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>5C1
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CTCGTTCCCCATCACCGCGACGAACGCGCCCGGCGCGCCGCATCCCTTCC
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>5C3
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>5C4
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CTCGTTCCCCATCACCGCGACGAACGCGCCCGGCGCGCCGCATCCCTTCC
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>5C5
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ACTGGTAACTCTGGCGACAACGTCACTATCCGCTTCACGACCGACAACCC
GGGCCCATGGTTCCTCCACTGCCACATCGACTTC

Design of gene-specific primers (See 2.3.6)

i.e.: F1D8 gene

Forward Oligo: CGGAAAAAGAGCGAGGAGA
Reverse Oligo: CTCCTCGAGAAGTGCAAAGC

>Tv_sshF_03D03 TVC00110_1

TACGACATCGCCCCTAGGGCGGAGGACCGGAAAAAGAGCGAGGAGAAGCCCTCCTCA

TGATAATGGTTTTATCGTATACTGCCCTAATATCACTCCACTCTCGGTTCTGCTCTC

CTGCATTCCCAAGCGGCAATAATGGTGTGCACACCTCGAAGACTTGCTGTGTGGCTG

CTACAGTAAGTCGCTCCAGGTGGGTGGTTCGCACATGAGCGTTGCTTTGCACTTCTC

GAGGAGATGAGGGAAAGAGTGAAATGGTTTATAAACGGAGCTGGT

Note: GCTTTGCACTTCTCGAGGAG= the reverse complement of CTCCTCGAGAAGTGCAAAGC

(primer3_results.cgi 0.4.0 modified for WI)

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any    3' seq
LEFT PRIMER         48   21   60.17   47.62  6.00  2.00 GCCCTCCTCAT-
GATAATGGTT
RIGHT PRIMER       234   20   60.28   55.00  8.00  2.00 CTCCTCGA-
GAAGTGCAAAGC
SEQUENCE SIZE: 273
INCLUDED REGION SIZE: 273

PRODUCT SIZE: 187, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

    1 TACGACATCGCCCCTAGGGCGGAGGACCGGAAAAAGAGCGAGGAGAAGCCCTCCTCATGA
                                                     >>>>>>>>>>>>>

   61 TAATGGTTTTATCGTATACTGCCCTAATATCACTCCACTCTCGGTTCTGCTCTCCTGCAT
      >>>>>>>>

  121 TCCCAAGCGGCAATAATGGTGTGCACACCTCGAAGACTTGCTGTGTGGCTGCTACAGTAA


  181 GTCGCTCCAGGTGGGTGGTTCGCACATGAGCGTTGCTTTGCACTTCTCGAGGAGATGAGG
                                        <<<<<<<<<<<<<<<<<<<<

  241 GAAAGAGTGAAATGGTTTATAAACGGAGCTGGT


KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                    start  len      tm     gc%   any    3' seq

3 LEFT PRIMER          28   19   60.60   52.63  2.00  0.00 CGGAAAAA-
GAGCGAGGAGA
   RIGHT PRIMER       234   20   60.28   55.00  8.00  2.00 CTCCTCGA-
GAAGTGCAAAGC
   PRODUCT SIZE: 207, PAIR ANY COMPL: 7.00, PAIR 3' COMPL: 0.00
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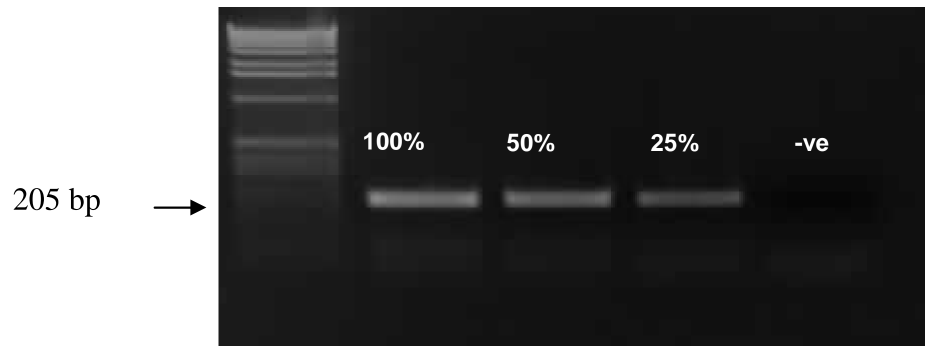
(primer3_results.cgi 0.4.0 modified for WI)

Appendix (E)

Optimisation of semi-quantitative RT-PCR

The optimization of the Nox gene under stress at 60C for 30 cycles.

205 bp →



(A) Image of gel electrophoresis of dilutions of the Nox gene, dilutions of a mixture of cDNA samples (100, 50, 25%) were amplified by RT-PCR using *NOX* primers and quantified (see methods section 2.3.8)

Intensity of gel fragments was quantified

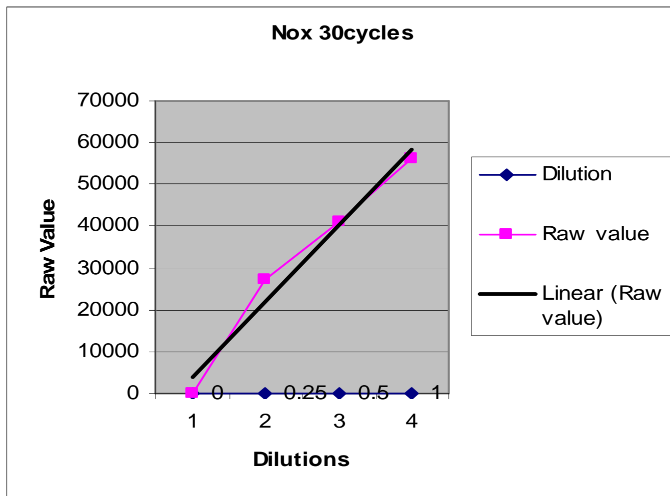
30 cycles

Spot blot results

Dilutions	Raw vol.	Raw vol. - (-ve)
100%	119160	56170
50%	104241	41251
25%	90286	27296
-ve	62990	0

Dilutions	Raw value
0	0
0.25	27296
0.5	41251
1	56170

(B) the molecular weight of the bands in A



(C) The excel result of the spot blot results, band intensity (product amount) was plotted against the dilution to check that a linear relationship was obtained at this cycle number

Appendix (F)

Gel images of RNA and cDNA extractions

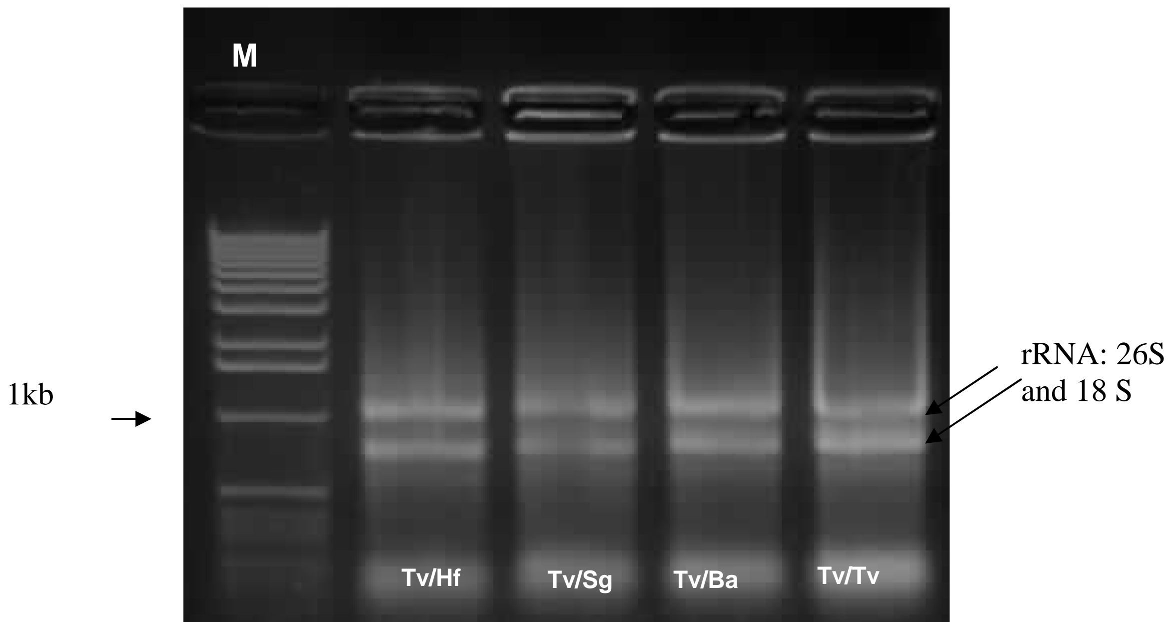


Fig.E.1. RNA extracted from the mycelium of *T. versicolor* when interacted with; *H. fasciculare* (Tv/Hf), lane 2 *S. gausapatum* (Tv/Sg)„ lane 3 *Bjerkandera adusta*(Tv/Ba)„ and lane 4 self pairing with *T. versicolor*(Tv/Tv), . M = 1 Kb ladder ***(Section 2.3.2 RNA extraction)***.

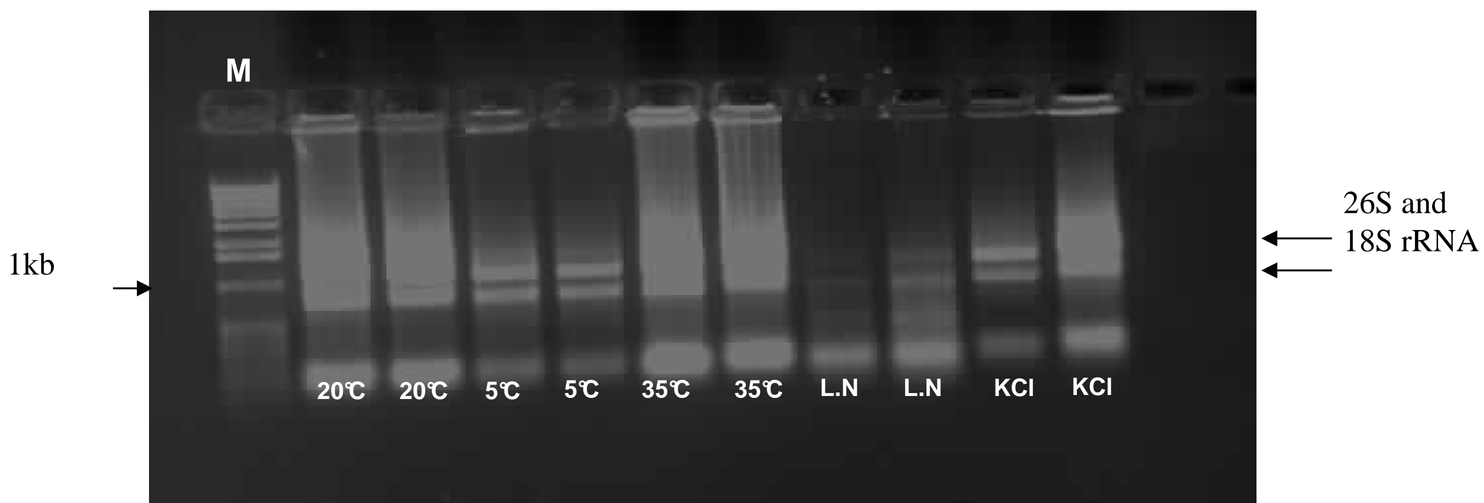


Fig.E.2. RNA extracted from the mycelium of *T. versicolor* under stress at different temperatures: 5, 20, 35 °C) low m=nitrogen (LN), and KCl.. M = 10 µl 1 Kb ladder (10 ng µl^{-1}). ***(Section 2.3.2 RNA extraction)***.

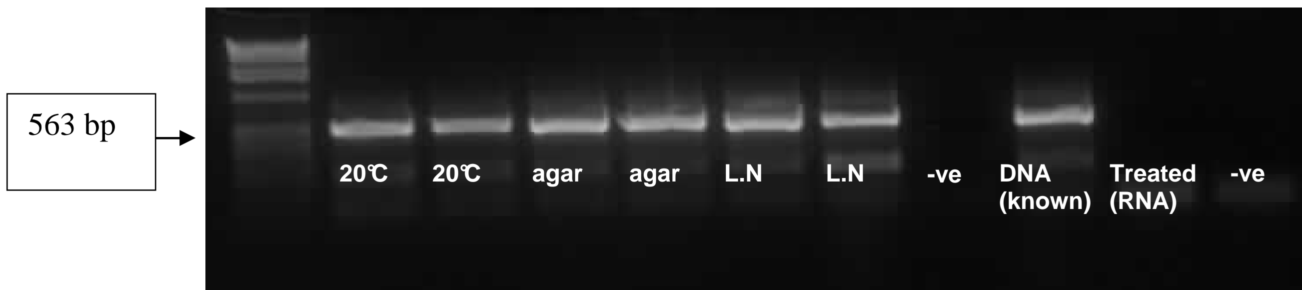


Fig.E.3. RT-PCR amplification with 18S primers of cDNA extracted from *T. versicolor* for the stress experiment for 20° C, agar only and Limited-N (LN). M=1Kb ladder (Invitrogen). ***(Section 2.3.4 DNase treatment of RNA, 2.3.5 cDNA Synthesis)***.

Example for Normalization of the gene expression data (See methods in section 2.3.8)

Three replicates needed

Stress 18 S

Replicate.1

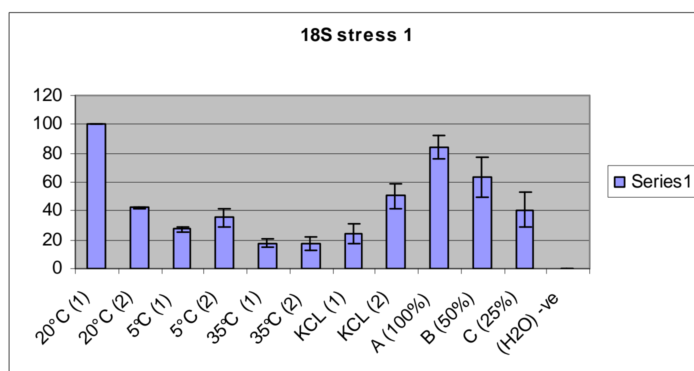
Treatments	Raw vol.	raw vol - (-ve)	% of max
20°C (1)	**65132**	15997	100
20℃ (2)	**56008**	6873	42.96431
5℃ (1)	**53407**	4272	26.70501
5℃ (2)	**52739**	3604	22.52922
35℃ (1)	**51020**	1885	11.78346
35℃ (2)	**50447**	1312	8.201538
KCL (1)	**50952**	1817	11.35838
KCL (2)	**54522**	5387	33.67506
A (100%)	**60359**	11224	70.16316
B (50%)	**54906**	5771	36.07551
C (25%)	**51842**	2707	16.92192
(H2O) -ve	**49135**	0	0

Results collected in excel file

Stress 18 S

	Rep.1	**Rep.2**	**Rep.3**	mean		SD	SE
Treatments	% of max	% of max	% of max		18S factor		
20°C (1)	100	100	100	100	1	0	0
20℃ (2)	42.96431	41.30129	42.26831	42.17797	0.42178	0.835181	0.482192
5℃ (1)	26.70501	24.94858	30.01472	27.22277	0.272228	2.572453	1.485206
5℃ (2)	22.52922	38.11302	45.06208	35.23478	0.352348	11.53888	6.661973
35℃ (1)	11.78346	20.88882	20.18049	17.61759	0.176176	5.064902	2.924223
35℃ (2)	8.201538	21.23525	23.25269	17.56316	0.175632	8.169913	4.716901

KCL (1)	11.35838	33.89629	27.52176	24.25881	0.242588	11.61785	6.707569
KCL (2)	33.67506	61.86532	56.48682	50.67573	0.506757	14.9666	8.640972
A (100%)	70.16316	97.66428	85.9511	84.59285	0.845928	13.80079	7.967887
B (50%)	36.07551	83.8205	70.59012	63.49538	0.634954	24.65051	14.23198
C (25%)	16.92192	55.75133	49.88479	40.85268	0.408527	20.9312	12.08463
(H2O) -ve	0	0	0	0	0	0	0



Stress FRA19

Rep.1

Treatments	Raw vol.	raw vol - (-ve)	18S factor	normalizing	% of max
20°C (1)	41371	18319	1	18319	100
20°C (2)	44562	21510	0.42178	9072.481	49.52498
5°C (1)	35849	12797	0.272228	3483.698	19.01685
5°C (2)	36178	13126	0.352348	4624.917	25.24656
35°C (1)	32559	9507	0.176176	1674.904	9.142989
35°C (2)	34434	11382	0.175632	1999.039	10.91238
KCL (1)	36294	13242	0.242588	3212.352	17.53563
KCL (2)	36239	13187	0.506757	6682.609	36.47912
A (100%)	38288				
B (50%)	34187				
C (25%)	31005				
(H2O) -ve	23052				

Stress FRA19

	Rep.1	Rep.2	Rep.3	mean	SD	SE
Treatments	% of max	% of max	% of max			
20°C (1)	100	100	100	100	0	0
20°C (2)	49.52498	41.79612	41.79612	44.3724	4.46226	2.576287
5°C (1)	19.01685	14.52072	14.52072	16.01943	2.595844	1.498711
5°C (2)	25.24656	20.95442	20.95442	22.38513	2.478066	1.430712
35°C (1)	9.142989	6.270586	6.270586	7.228054	1.658383	0.957468
35°C (2)	10.91238	9.677351	9.677351	10.08903	0.713044	0.411676
KCL (1)	17.53563	19.43753	19.43753	18.80356	1.098064	0.633967
KCL (2)	36.47912	34.12989	34.12989	34.91296	1.356327	0.783076
A (100%)						
B (50%)						
C (25%)						
(H2O) -ve						

Statistics:

% 0f max of 18S = (present raw vol – (-ve) / biggest value of raw vol – (-ve)) * 100

Mean = ∑ % of max of Rep. / no. of Rep.

18S factor = present mean / biggest mean

SD = STDEV (∑ % of max of Rep.)

SE = SD/ SQRT (n); n = number of Rep.

Normalizing = raw vol- (-ve) * 18S Factor

% of max of the gene = (present normalizing / biggest normalizing) * 100

Making slops for the extension rate (= SLOPE (B!: B!, $A!: $A!))

(A)

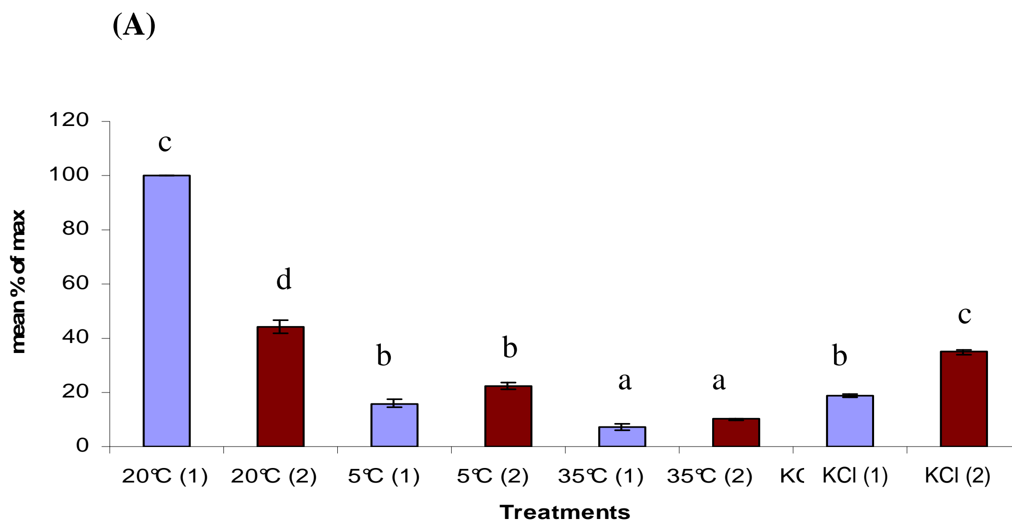


Fig.5.5 FRA19 gene expression of *T. versicolor* under stress. **(A)** 5 °C, 35 °C, KCl; mean ± SEM (n = 3). Different letters indicate significant differences between means (P ≤ 0.05).

The treatments and the mean % of max were taken to SPSS statistical program to test the significant of differences between these treatments means with Anova and Tukey tests.

Stress FRA19

	mean of % of max
20°C (1)	100
20°C (1)	100
20°C (1)	100
5℃ (1)	19.01685
5℃ (1)	14.52072
5℃ (1)	14.52072
35℃ (1)	9.142989
35℃ (1)	6.270586
35℃ (1)	6.270586
KCL (1)	17.53563
KCL (1)	19.43753
KCL (1)	19.43753
20℃ (2)	49.52498
20℃ (2)	41.79612
20℃ (2)	41.79612
5℃ (2)	25.24656
5℃ (2)	20.95442
5℃ (2)	20.95442
35℃ (2)	10.91238
35℃ (2)	9.677351
35℃ (2)	9.677351
KCL (2)	36.47912
KCL (2)	34.12989
KCL (2)	34.12989

SPSS results

Tukey HSD

Treatments	N	Subset for alpha = 0.05		
		a	b	c
35°C	3	7.2281		
5°C	3		16.0194	
KCl	3		18.8036	
20°C	3			100.0000
Sig.		1.000	.236	1.000

Tukey HSD

Treatments	N	Subset for alpha = 0.05			
		a	b	c	d
35°C	3	10.0890			
5°C	3		22.3851		
KCl	3			34.9130	
20°C	3				44.3724
Sig.		1.000	1.000	1.000	1.000

Appendix (G)

Cloning of PCR products

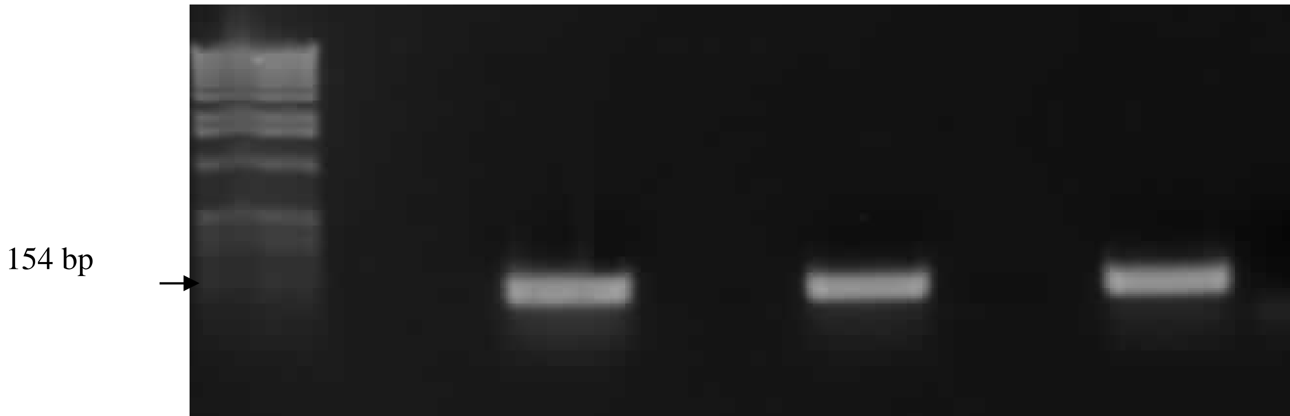


Fig.G.1. PCR products of FRA19 gene amplified from cDNA, (32 cycles) for purification and cloning, ***(Section 2.3.10.1 Purification of cDNA)***.

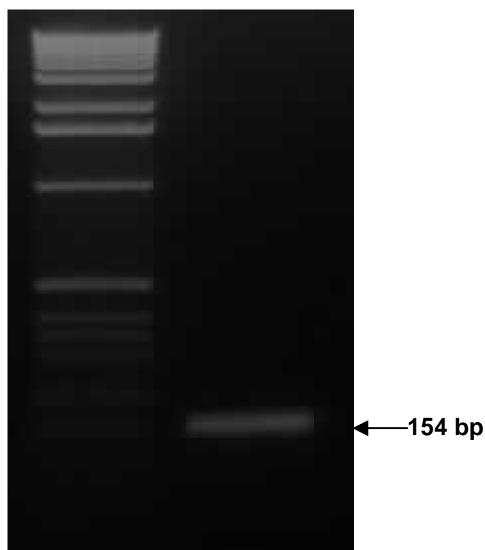


Fig.G.2. Purified PCR product of FRA19 gene fragment ***(Section 2.3.10.2 Ligation into pGEMT-Easy vector)***.

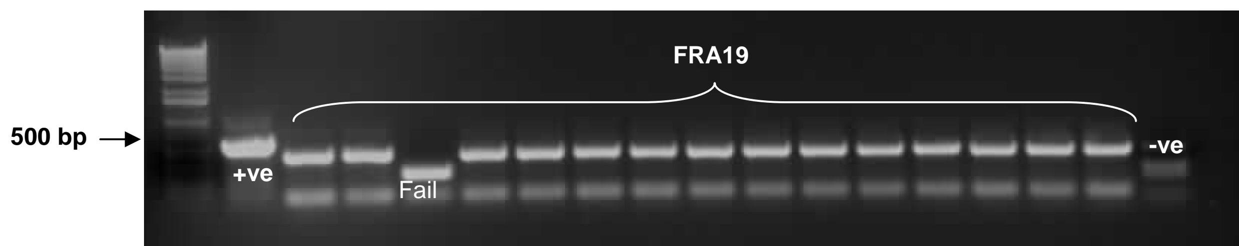


Fig.G.3. Colony PCR for the gene FRA19 amplified with M13F and R primers, +ve: plasmid control, -ve control: H_2O, All the FRA19 colonies amplified a fragment of the expected size of approx. 500bp whereas one (Fail) amplified a smaller fragment indicating an empty vector ***(Section 2.3.10.3 Transformation of E.coli DH5α competent cells)***.

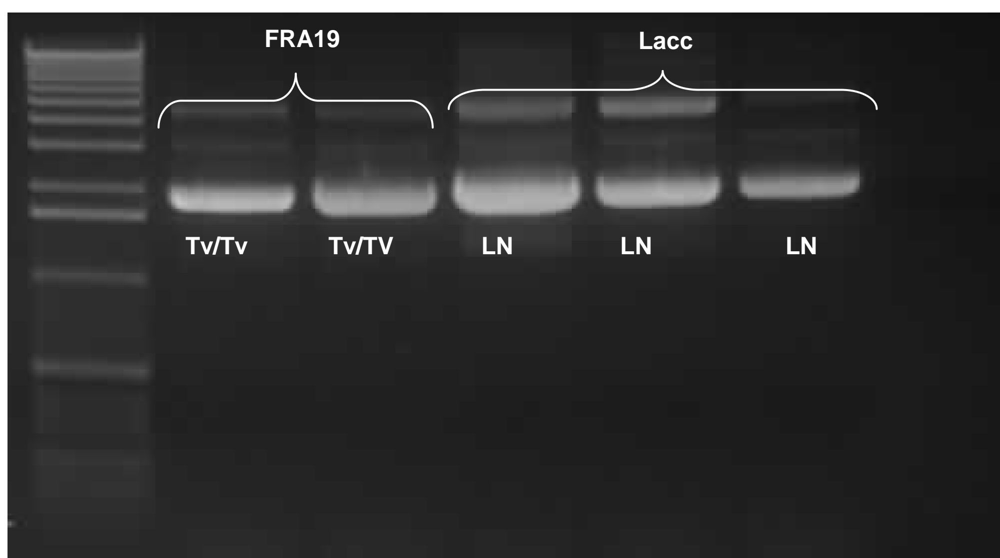
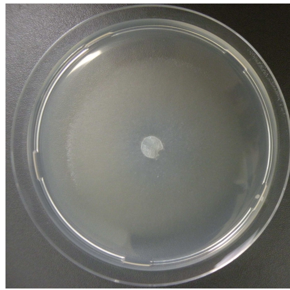
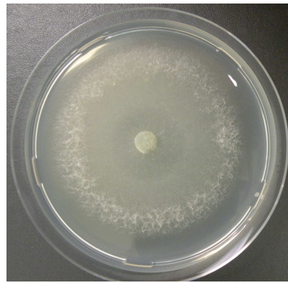


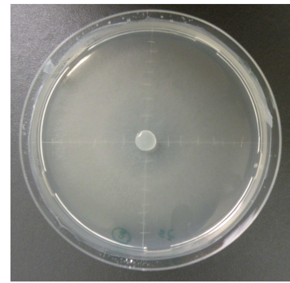
Fig.G.4. Miniprep DNA preparation of plasmid containing FRA19 gene,fragment lanes 1 and 2 FRA19, 3 4 and 5 are from Lacc gene for LN (limited-N), ***(Section 2.3.10.4 Plasmid DNA purification)***.

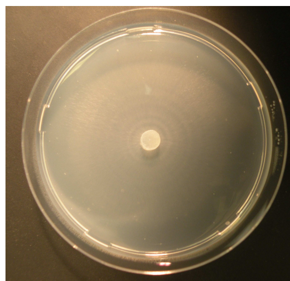
Appendix (H)

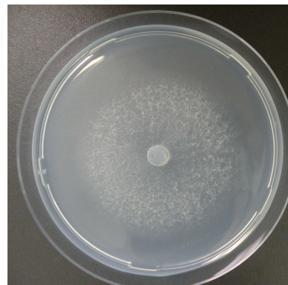
Morphology of *T. versicolor* under stress

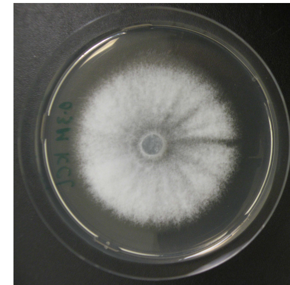

20°C (5 d)
'normal growth'

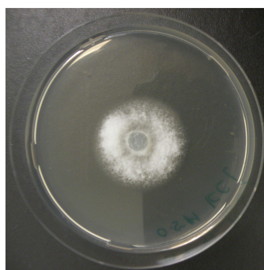

35°C (6 d)
'fluffy growth'

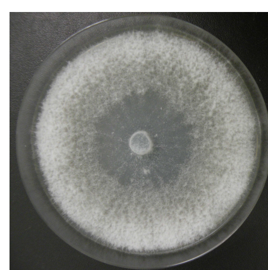

5°C (12 d)
'light growth'


Agar only (7 d)
'very light growth'


Limited-N (5 d)
'nearly normal growth'


0.3M KCL (11 d)
'affected, very fluffy growth'


0.5M KCL (11 d)
'super stressed'


0.1M KCL (11 d)
'not really affected but fluffy growth'

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Microarray analysis of differential gene expression elicited in *Trametes versicolor* during interspecific mycelial interactions

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ABSTRACT

Trametes versicolor is an important white rot fungus of both industrial and ecological interest. Saprotrophic basidiomycetes are the major decomposition agents in woodland ecosystems, and rarely form monospecific populations, therefore interspecific mycelial interactions continually occur. Interactions have different outcomes including replacement of one species by the other or deadlock. We have made subtractive cDNA libraries to enrich for genes that are expressed when *T. versicolor* interacts with another saprotrophic basidiomycete, *Stereum gausapatum*, an interaction that results in the replacement of the latter. Expressed sequence tags (ESTs) (1920) were used for microarray analysis, and their expression compared during interaction with three different fungi: *S. gausapatum* (replaced by *T. versicolor*), *Bjerkandera adusta* (deadlock) and *Hypholoma fasciculare* (replaced *T. versicolor*). Expression of significantly more probes changed in the interaction between *T. versicolor* and *S. gausapatum* or *B. adusta* compared to *H. fasciculare*, suggesting a relationship between interaction outcome and changes in gene expression.

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Introduction

Interspecific interactions between saprotrophic basidiomycetes are a key feature of their ecology, affecting substratum colonisation and access to nutrients (Boddy 2000; Woodward & Boddy 2008). This in turn affects the succession of species and substratum decay rate (Boddy & Heilmann-Clausen 2008). When mycelia of different species meet, the recognition of 'non-self' induces responses both in the area where the mycelia are in physical contact, known as the interaction zone, and elsewhere in the mycelium. This response includes changes in morphology of the mycelium, and the production of extra-cellular enzymes and secondary metabolites (Griffith *et al.* 1994; Rayner *et al.* 1994; Boddy 2000; Woodward & Boddy 2008). For example, volatile organic compounds comprise a diverse range of chemical classes, including alcohols, aldehydes, ketones, terpenes, and aromatic compounds, which alter qualitatively and/or quantitatively during interactions (Hynes *et al.* 2007; Evans *et al.* 2008). Production of reactive oxygen species (ROS), phenoloxidases, laccases and sometimes β-glucosidase increases during interactions (Freitag & Morrell 1992; White & Boddy 1992; Lang *et al.* 1997; Iakovlev & Stenlid 2000; Baldrian 2006). Spatio-temporal patterns of production of enzymes during interactions vary depending on species (Iakovlev & Stenlid 2000).

Although it would seem likely that such biochemical and cellular changes are accompanied by changes in gene expression, few studies have used global transcriptomic approaches to study mycelial interactions. Differential display was used to identify 21 genes whose expression changed in the

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interaction between the wood decomposing basidiomycete *Physisporinus sanguinolentus* and the conifer pathogen *Heterobasidion annosum*, one of which was related to DNA repair and stress responses (Iakovlev *et al.* 2004). A few studies have investigated gene expression changes during mycorrhizal fungal interactions with plants (e.g. Peter *et al.* 2003; Acioli-Santos *et al.* 2008; Deveau *et al.* 2008) using microarray analysis. Other studies have used microarrays or related transcriptomic approaches to follow changes in gene expression during host–pathogen interactions between fungi and plant (Jakupović *et al.* 2006) or mammalian (Steen *et al.* 2002) hosts. Yet other transcriptomic studies have focussed on fungal development such as the change between yeast and mycelial forms in *Paracoccidioides brasiliensis* (Felipe *et al.* 2005) and the development of fruit bodies in *Sordaria* (Nowrousian *et al.* 2005).

The aim of this work was to investigate changes in gene expression elicited as a result of interspecific mycelial interactions between saprophytic basidiomycetes. *Trametes versicolor* was selected as our model for this work as it is a secondary coloniser and has an 'intermediate' combative ability (Chapela *et al.* 1988; Boddy & Heilmann-Clausen 2008). This allowed us to select competitors that lose territory, gain territory or form a deadlock when challenged with this species. We were particularly interested in comparing transcriptomic profiles during interactions which ultimately result in different outcomes to discover whether the same gene expression patterns are common or divergent. Since the genome of *T. versicolor* is not yet available, it was necessary first to construct cDNA libraries and sequence sufficient ESTs to interpret the array results. Here we report on 1155 sequenced ESTs from *T. versicolor* expressed just behind the interaction zone. We also show that interactions with competitors resulting in deadlock or invasion of the competitor's territory elicit a different set of transcripts to an interaction where *T. versicolor* loses territory.

Materials and methods

Strains and culture conditions

Strains of *Trametes versicolor* (TvD2), *Stereum gausapatum* (Sg1), *Hypholoma fasciculare* (HfGTWV2) and *Bjerkandera adusta* (MA313), from Cardiff University culture collection, were maintained on 2 % (w/v) malt agar (MA; 20 g Munton and 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. Cultures were routinely subcultured every 10 d or just before the colony margin reached the edge of the plate. Stock cultures were maintained on 2 % MA slopes at 4 °C. Paired interactions were set up by inoculating 6 mm diam. plugs, cut from the actively growing mycelial margin, 30 mm apart (Fig 1). For pairings of *T. versicolor vs. Hypholoma fasciculare*, the *H. fasciculare* plugs were plated 2 d prior to adding the opposing *T. versicolor* plugs, ensuring that opposing mycelia met at the centre of the plate.

RNA extraction

Colonies were grown for 5 d and mycelium was harvested aseptically from plates using a small spatula to skim the mycelium from the surface of the agar. Mycelium was ground to a powder in liquid nitrogen using a mortar and pestle, RNA extractions were performed using 2 ml TRI Reagent (Sigma–Aldrich, Dorset, UK) according to the manufacturer's instructions. Residual genomic DNA was removed using RQ1 DNase (Promega, Southampton, UK). Absence of DNA contamination was checked by inclusion of a no enzyme control reaction in the subsequent cDNA synthesis and checked by reverse transcribed-PCR (RT-PCR) using species-specific primers (data not shown).

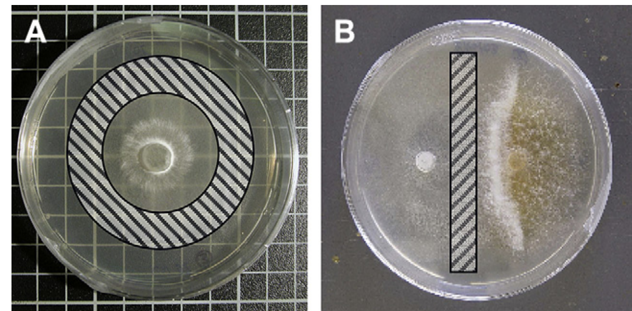

Fig 1 – Areas used for construction of the subtracted libraries (cross-hatched) from (A) *T. versicolor* growing alone, (B) *T. versicolor* interacting with *S. gausapatum*.

Subtracted library construction and EST analysis

Two subtracted libraries were made, one forward and one reverse to enrich respectively for mRNAs that are up- or down-regulated in mycelia collected from *Trametes versicolor* interacting with *Stereum gausapatum* compared to *T. versicolor* growing alone. For those plates inoculated with *T. versicolor* alone, mycelium was harvested from a circular zone 1 cm wide around the growing margin of a 5 d old colonies of approx. 60 mm diameter (Fig 1A). For interaction plates of *T. versicolor vs. S. gausapatum*, inoculated plates were grown for 5 d and harvested at a stage at which the mycelia of the two colonies had just met. Mycelium was taken from just behind the *T. versicolor* margin at the interaction zone, to ensure that no *S. gausapatum* mycelium was collected (Fig 1B). In this interaction, *T. versicolor* replaced *S. gausapatum* and hence it is possible to obtain material close to the interaction zone without risk of accidentally including mycelium from the competitor species. To verify that RNA was not contaminated with *S. gausapatum* RNA, cDNA was synthesised from a small aliquot of the RNA and RT-PCR performed with *S. gausapatum* specific primers (see below for details; Supplementary Fig 1). Approximately 0.5 μg total RNA was used for cDNA synthesis using a Clontech SMART cDNA synthesis (Palo Alto, USA) kit. PCR cycle number for second strand synthesis was optimised to ensure amplification in the exponential phase and required 20–29 cycles depending on the template. Ligation efficiency was tested using primers designed to *T. versicolor* β-tubulin. Subtraction of the ds cDNA was performed using PCR-Select cDNA subtraction (Clontech, St-Germain-en Laye, France) according to the manufacturer's protocols. The final amplification step was carried out over 12 cycles. PCR products were cleaned using a Qiaquick PCR purification kit (Qiagen, Crawley, UK), and cloned into pGEM-T Easy (Promega, Southampton, UK). Ligations were transformed into

high efficiency XL 10-Gold ultracompetent cells (Stratagene Ltd., Cambridge, UK). Individual colonies were picked manually, grown as liquid cultures in 96-well plates and stored as glycerol stocks at −80 °C.

For sequencing, inserts were PCR amplified using M13 forward and reverse primers from the glycerol stocks, and purified using a Millipore Montage Multiscreen μ96 plate (Millipore, Billerica, MA, USA, LSKM PCR10). Purified colony PCR products were single pass sequenced using an ABI3730 sequencer and M13F primer (GTTTTCCCAGTCACGACGTTG) at the Edinburgh Sequencing Facility. Sequences were stripped of contaminating vector sequence, then the Partigene software pipeline (Parkinson *et al.* 2004) was used to process EST sequences to create a database of non-redundant sequence objects (putative genes). Functions were assigned using BLASTX (NCBI) based on homology to sequences in the GCG database or the Concordia Fungal Genomic sequence database (https://fungalgenomics.concordia.ca/home/) and only ascribed where the *e*-value was below 5×10^{-3} or (in a few cases) where visual inspection of the sequence alignments indicated a clear match.

Design of species-specific primers

DNA was extracted from mycelium of *Stereum gausapatum*, *Hypholoma fasciculare* and *Bjerkandera adusta* as described in Parfitt *et al.* (2005), and amplified using conserved primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) that amplify an approximately 800 bp fragment of the ITS region. PCR products were gel purified and sequenced, and sequences aligned using BioEdit. Species-specific primers amplifying an approximately 500 bp fragment were selected from divergent regions of the alignment and tested on cDNA from all four species to check for specificity (data not shown). Primer sequences were: *S. gausapatum* (SgauF), GCGGGGGTCTCTTCGTTA (used with ITS4), *H. fasciculare* (HfF), CACCTTTTGTAGACCTGGATT and (HfR), AGTGCTATAAACGGCAAATAG, and *B. adusta* (BkF), GTTCGCGCACTTGTAGGT and (BkR), ACACTAGAATACCC TCCACA.

Printing microarrays

PCR products (1920) were purified as above and checked by gel electrophoresis. 50–100 ng of each PCR reaction in 5 μl was added to 5 μl of dimethyl sulfoxide (DMSO) in 384-well plates using a Multiprobe®II HT EX (PerkinElmer LAS (UK) Ltd.) robot, then spotted as a 12 × 4 metagrid of 12 × 12 subgrids onto UltraGAPS II (Gamma Amino Propyl Silane, Corning, NY, USA) coated slides using 120 μm solid pins on a robotic Flexys array printer (Genomic Solutions, Ltd., Cambridgeshire, UK). Two Lucida Microarray ScoreCard (Amersham Plc., Buckinghamshire, UK) control spots were printed in the top left hand corner of each subgrid. The scorecard consisted of different types of control: calibration, ratio, and negative control. In addition a β-tubulin clone shown to be invariant during the suppression subtractive hybridization (SSH) protocol was used as a gene control spot. Each control was spotted a total of four times on each microarray slide: in two separate subgrids and twice within each subgrid. Slides were air-dried for 12 h, baked at 80 °C for 2 h and then UV cross-linked with an autocrosslink cross-linker (Stratagene, Amsterdam, The Netherlands).

Labelling of RNA for hybridisation to the arrays

RNA was extracted as described above from the region just behind the interaction zone of *Trametes versicolor* paired against *Stereum gausapatum*, *Hypholoma fasciculare*, *Bjerkandera adusta* or *T. versicolor* growing alone. Each RNA extraction replicate used material derived from 30–50 plates. Three to five biological replicates were used for each of the three interaction experiments. RNA was DNase treated using RQ1 DNase (Promega, Southampton, UK) and checked for the presence of contaminating RNA from the paired species. A sample of the RNA was reverse transcribed using M-MLV RNase H^- Reverse Transcriptase (Promega) and PCR amplified using species-specific primers for the three interacting species (as described above), using ITS1/ITS4 as a positive control (data not shown). Once checked, RNA was purified using a Qiagen RNeasy kit (Qiagen, Crawley, UK). A Universal ScoreCard mRNA spike (1 μl of either test or reference; Amersham Biosciences, Buckinghamshire, UK) was added to the total RNA (10 μg) which was then reverse transcribed using Superscript II (Invitrogen, Paisley, UK) incorporating aa-dUTP. The cDNA was post-labelled using Cy3 and Cy5 dyes (Amersham Biosciences, Buckinghamshire, UK). Unincorporated Cy dyes were removed using CyScribe GFX purification columns (Amersham Biosciences, Buckinghamshire, UK). Each pair of labelled cDNA populations was concentrated using an Eppendorf Concentrator 5301 (Jencons PLS, Leighton Buzzard, UK) speedvac at 60 °C in the dark. cDNAs were resuspended in HPLC grade water (Chromasolv, Sigma–Aldrich, Dorset, UK) containing poly A (5 μM, Sigma–Aldrich, Dorset, UK).

Hybridisation of microarray slides

Slides were blocked in 5× saline sodium citrate (SSC), 0.1 % sodium dodecyl sulphate (SDS), 1 % w/v bovine serum albumin (BSA) for 45 min at 42 °C followed by four washes in sterile filtered water and dried with compressed air. For hybridisation the labelled cDNA was denatured in a heat block at 95 °C for 3 min and then immediately placed on ice. An equal volume of hybridisation buffer (50 % formamide, 10× SSC, 0.2 % SDS) was added to the labelled cDNA and mixed by pipetting. The probe (40 μl) was pipetted onto a new untreated microscope slide and the blocked microarray was placed face down on to this slide resulting in a sandwich. The array sandwich was hybridised in a humidity chamber at 42 °C overnight. Following hybridisation, slides were separated whilst immersed in 1× SSC, 0.2 % SDS at room temperature followed by a 10 min wash at 55 °C and five rapid washes in the same solution. They were then transferred to 0.1× SSC, 0.1 % SDS at 55 °C for three 10 min washes. Slides were then transferred to 0.1× SSC for 1 min at room temperature, dipped five times and then transferred to a further wash with 0.1× SSC for 1 min. Following this final wash the slide was removed and dried using compressed air.

Analysis of microarray slides

Microarrays derived from the subtracted libraries enriched for *Trametes versicolor* genes up- or down-regulated during interaction with *Stereum gausapatum* were used in three different

experiments. In each case transcripts expressed by *T. versicolor* growing alone were compared to those derived from just behind an interaction zone. Interactions with three different saprotrophic basidiomycetes were tested: *S. gausapatum*, *Bjerkandera adusta* and *Hypholoma fasciculare*.

Microarray slides were scanned using a ScanArray™Express HT microarray scanner (Perkin Elmer Precisely, MA, USA) and the accompanying ScanArray™ Express software at 543 nm (Cy3) and 633 nm (Cy5) with a resolution of 5 μm. Imagene version 5 software (BioDiscovery, El Segundo, CA, USA) was used to quantify scanned images. Spot quality labelling (flags) were defined for empty spots with a signal strength threshold of 1.0. The median signal intensity across each spot and the median background intensity were calculated in both channels, and these data were exported into GeneSpring (Version GX 7.3: Agilent Technologies UK Ltd., Cheshire, UK). Signal intensity was calculated for each spot by subtracting the local background signal from the spot signal for both channels, giving the background-corrected spot intensity. Three replicates of the array were printed on each slide and three slides were used for each experiment each with an independent biological replicate probe. Thus, there were three biological replicates each with three technical replicates making nine data points per spot on the array. Box plots were produced for the raw data within each array and compared between slides; poor slides were removed from the dataset prior to normalisation.

Normalisations were performed manually, using GeneSpring and LimmaGUI. LimmaGUI is a graphical user interface for linear modelling of 2-colour spotted microarray experiments (Wettenhall & Smyth 2004). Global normalisation was performed manually. GeneSpring was used for median polishing and inspecting invariant genes. The Lucidea internal scorecard calibration controls (CC1–CC10) were used to construct calibration curves for each slide, and ratio controls were used to assess the raw values of the experimental ratios and to verify normalisations. LimmaGUI was used for Lowess and print-tip Lowess analyses. The effects of each normalisation were assessed by inspection of box plots, MA plots and principal component analysis (PCA) plots of the array data (not shown).

Following hybridisation, analysis of the variation between individual print tips with LimmaGUI showed some variability between subgrids printed by different print tips. Data normalised using print-tip Lowess resulted in box plots of slides with very narrow interquartile ranges for *M* values (average ratio) and much of the variation of the raw data was removed. Thus, data were not subjected to normalisation using print-tip Lowess. Data were instead normalised in two steps: (i) Per gene – divide by control channel, i.e. the test signal for each spot divided by the corresponding control signal; (ii) Per chip – normalised to the 50th percentile, i.e. the data distributions for each array slide were aligned by their medians. Box plots of the data normalised with this method (Supplementary Fig 2A) showed similar distributions between samples within and between experiments, and the medians were aligned. The expression profiles of the β-tubulin invariant gene control spots (Supplementary Fig 2B) after normalisation confirmed that the normalisation had not altered the data distribution adversely.

Gene lists were generated by filtering on expression level for 1.4 fold changes up and down in at least two out of three arrays for each experiment. Data were also filtered on confidence using statistical differences between groups using a parametric test (*t* test), at 90 % ($p = 0.1$) confidence levels using the Benjamini and Hochberg false discovery rate. Significant differences between experiments were tested by 1-way ANOVA ($p < 0.05$).

Semi-quantitative RT-PCR

Primers were designed and checked using Oligoanalyzer 3.1 (http://www.idtdna.com/ANALYZER/Applications/OligoAnalyzer/Default.aspx) to five of the EST sequences: FRA2 from cluster TVC00203 (FRA2-F: CGGTACTGTCTGCTGCGATA, FRA2-R: CGAACACACGAGTTCTTGGA); F1D8 from cluster TVC00110 (F1D8-F: CGGAAAAAGAGCGAGGAGA, F1D8-R: CTCCTCGAG AAGTGCAAAGC); F2A1 from cluster TVC00043 (F2A1-F: GGAG AGTGCGGTGACAATGAA, F2A1-R: GTGTGACTGTCATTGTC GGG); FRA19 from cluster TVC01061 (FRA19-F: AGACTACCAGG ACGGAACGA, FRA19-R: AATGGAAAGCATGGGAAGG); Tvcat from cluster TVC00832: (Tvcat-F: AAC ATC CTC GAC CTG ACG AA, Tvcat-R: GAG AAG AGA CGC GAC TGG AG) and Nox from cluster TVC00679 (Nox-F: TCGGTTGGTTCCAGACTCTC and Nox-R: TAGATGGCCTTCCAGTCAGG). Reactions were cycled in a Perkin Elmer 2700 thermocycler using Hotstar Taq polymerase (QIAGEN, Crawley, UK) and the following programme: 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 for FRA2, F1D8, FRA19, Nox and F2A1 was 60 °C, and for Tvcat 55 °C.

Normalisation was performed to 18S rRNA using primers EF4: GGA AGG GRT GTA TTT ATT AG and fung5: GTA AAA GTC CTG GTT CCC C (Smit *et al.* 1999). Reactions were cycled as above. Three or more replicates for each primer set were amplified using the same machine to avoid any variability due to the machine parameters. Products were analysed by agarose gel electrophoresis, and PCR products quantified using the Gene Genius bioimaging system and GeneSnap software (SynGene, Synoptics Ltd., Cambridge, UK). Product quantitation from the 18S target was used to normalise results for all the other primer sets. Cycle number was optimised and limited for each primer set and cDNA synthesis batch combination. This ensured that the reactions were in the exponential phase and therefore product quantitation could be considered semi-quantitative with respect to message abundance. Specific cycle number is not reported here as it was optimised independently for each batch of cDNA. Dilution series of the cDNA were included in every PCR run and results only accepted where a linear response was obtained. This methodology has been used successfully to obtain semi-quantitative RT-PCR data for a range of experimental systems (Parfitt *et al.* 2004; Orchard *et al.* 2005; Wagstaff *et al.* 2005; Price *et al.* 2008).

Staining cultures with NBT to reveal superoxide production patterns

Interactions between *Trametes versicolor* and *Stereum gausapatum*, *Bjerkandera adusta* or *Hypholoma fasciculare*, and self-pairings of these species, were set up in triplicate as previously

described on 2 % MA in 9 cm Petri dishes. Plates were destructively stained for superoxide production at 5, 8 and 11 d following the method of Silar (2005). Plates bearing mycelium were flooded with 10 ml of 2.5 mM nitro blue tetrazolium (NBT) in 5 mM *n*-morpholino propane sulfonate-NaOH (MOPS) at pH 7.6, and incubated for 30 min at room temperature with gentle rotation to ensure equal coverage of the plate by the stain solution. The liquid was removed and plates incubated at room temperature for a further 30 min to 24 h. Control plates were flooded with 5 mM MOPS only. Photographs were taken with a Nikon Coolpix camera to show colour development 1 h, 4 h, and 24 h after application of the stain. No further development of purple colouration was observed after 4 h.

Results and discussion

Involvement of ROS in interactions between Trametes versicolor *and three competitors*

Three possible outcomes were elicited from *T. versicolor* when paired against competitors (Fig 2). *Stereum gausapatum* was replaced by *T. versicolor* (Fig 2A), there was deadlock with *Bjerkandera adusta* (Fig 2B), and *T. versicolor* was replaced by *Hypholoma fasciculare* (Fig 2C). Staining for superoxide revealed significant presence of ROS at the interaction zone (Fig 3), suggesting that ROS may play a role in all three of these interactions. Although the ways in which the two species sense each other and the signalling events that occur during interactions are unclear, H_2O_2 has previously been implicated as a signalling molecule together with at least one MAPK cascade (Silar 2005). EST and microarray analysis were used to further investigate processes occurring at interaction zones.

Construction of Trametes versicolor *subtracted libraries and analysis of ESTs*

A total of 8 499 clones were recovered from two subtractive libraries enriching for genes that are up- or down-regulated between *T. versicolor* growing alone and a region close to the interaction zone between *T. versicolor* and *Stereum gausapatum* (Fig 1B). Approximately equal numbers of clones were recovered from the subtractions: 3 939 from the forward subtraction, and 4 560 from the reverse subtraction.

Sequencing of equal numbers of clones (960) from each *T. versicolor* SSH library yielded 1 155 sequences, once poor and short sequences had been removed (Genebank Accession numbers are listed in Supplementary Table 1). Cluster analysis of the sequences produced 190 clusters and 483 singletons resulting in a total of 673 unique cluster IDs. Redundancy of the EST library was calculated to be 28 % (number of ESTs in clusters/total number of ESTs; Sterky *et al.* 1998). Therefore, any new sequence would have a 28 % chance of already being represented in the collection. However, there may be further redundancy due to non-overlapping fragments of the same gene. Out of 183 sequences where functions could be assigned (Supplementary Table 1), the largest cluster represents a putative septin (26 sequences) followed by a 40S ribosomal protein, and a glycoside hydrolase protein and four clusters putatively assigned to a glycine-rich binding protein (Table 1). Cytochrome c oxidase was also represented several times (15 sequences in four different clusters). Two transporters were also in this highly represented class of genes: an ABC transporter and a sugar transporter. Other sequences represented by clusters of three or more members included genes related to nitrogen metabolism, cell wall biosynthesis, ubiquitin-dependent proteolysis and redox reactions.

Based on GO annotations and broad functional classes, ESTs were assigned to 27 functional classes (Table 2). Overall biosynthetic processes accounted for 19 % of the ESTs. Lipid biosynthesis (7 %) and protein synthesis (7 %) were the two largest classes within this category and a further 2 % related to glycolipid biosynthesis. Another 9 % of ESTs were ribosomal proteins, presumed to be involved in protein synthesis. In contrast, 24 % were assigned to functions related to metabolism and proteolysis including proteins related to electron transport, carbohydrate metabolism, nucleic acid breakdown and several genes linked with ubiquitin-dependent proteolysis. Expression of genes related to biosynthesis and metabolism is consistent with the production of mounds of aerial mycelia and active growth found at mycelial interactions. Carbohydrate and nitrogen metabolism-associated genes may be related to nutrition since radio-isotope studies have shown that an interacting fungus can take up carbon from the opponent mycelium in the interaction zone (Wells & Boddy 2002). These genes may also be involved in the remobilisation of nutrients from dying cells as cell death often occurs at the

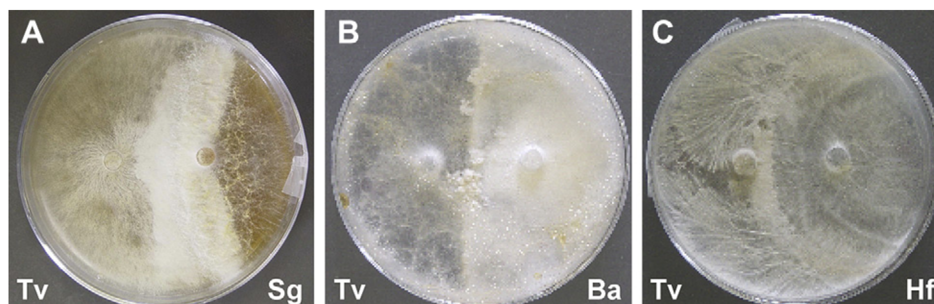

Fig 2 – Interactions on malt agar between *T. versicolor* (Tv, left of Petri dish) and three other saprotrophic basidiomycetes: (A) *Stereum gausapatum* (Sg), (B) *Bjerkandera adusta* (Ba), and (C) *Hypholoma fasciculare* (Hf). *Trametes versicolor* is replacing *S. gausapatum* in (A), deadlocking in (B) and being replaced in (C).

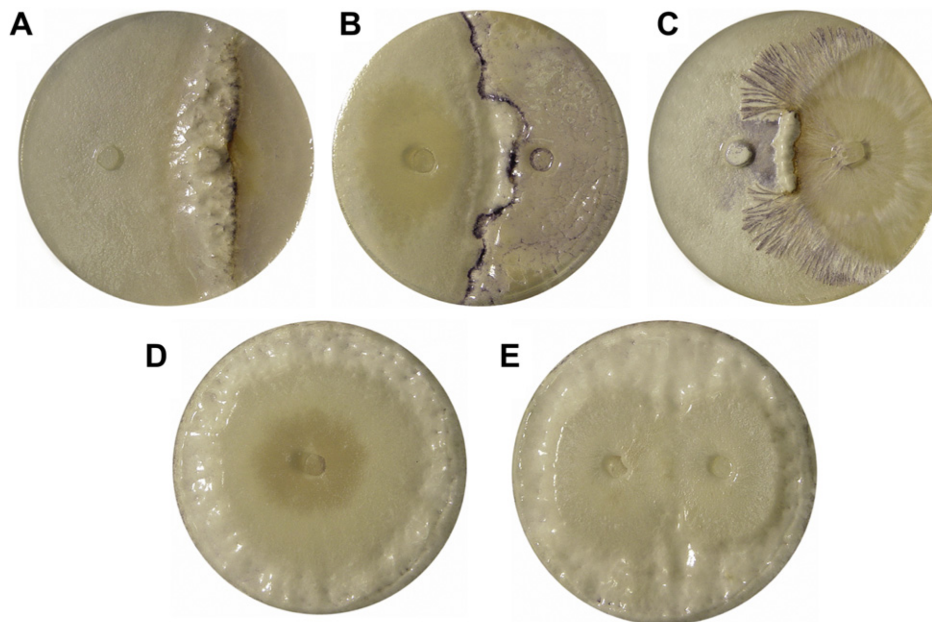

Fig 3 – Interactions on malt agar between *T. versicolor vs.* three competitors, stained with NBT to reveal production of ROS: (A) *Stereum gausapatum*, (B) *Bjerkandera adusta*, and (C) *Hypholoma fasciculare* 11 d after inoculation, *T. versicolor* is on the left in each case. (D) *T. versicolor* growing alone, (E) *T. versicolor* paired with itself.

interaction front (Silar 2005). Fungal mycelia grow at their tips, which involves rapid synthesis of lipid membranes and an extensive vesicle transport system, tightly controlled by cytoskeletal elements. Membrane associated functions (transporters, vesicle transport, and membrane proteins) accounted for 10 % of the ESTs, and another 2 % were cytoskeleton associated genes. Nuclear division is also tightly controlled so that the ratio of nuclei to cytoplasm is maintained.

Table 1 – EST clusters containing three or more sequences for which putative functions could be assigned by comparison to the databases.

Cluster ID	No. of sequences	Putative function	Functional class
TVC00017	27	Cytokinesis-related protein	Cell division
TVC00039	9	40S ribosomal protein S17-B	Ribosomal
TVC00574	9	Glycoside hydrolase family 13 protein	Carbohydrate metabolism
TVC00079	8	Glycine-rich RNA binding protein	RNA binding
TVC00748	8	Glycine-rich RNA binding protein	RNA binding
TVC01061	6	Glycine-rich RNA binding protein	RNA binding
TVC00032	5	Oxidoreductase	Redox
TVC00108	4	Glycine-rich RNA binding protein	RNA binding
TVC00689	5	Cytochrome c oxidase subunit 1	Electron transport
TVC00710	5	60S ribosomal protein L20A	Ribosomal
TVC00016	4	DNA binding protein SART-1	DNA binding
TVC00018	4	ABC transporter	Transporter
TVC00101	4	Cytochrome c oxidase subunit 1	Electron transport
TVC00675	4	Pre-mRNA-splicing factor 38B	RNA binding
TVC00004	3	Cytokinesis-related protein	Cell division
TVC00038	3	Glycine-rich RNA binding protein	RNA binding
TVC00047	3	Cytochrome c oxidase subunit 1	Electron transport
TVC00068	3	Cytochrome P450	Cytochrome P450
TVC00100	3	Sugar transporter	Transporter
TVC00144	3	Cytochrome c oxidase subunit 1	Electron transport
TVC00166	3	Cytokinesis-related protein	Cell division
TVC00300	3	Ubiquitin activating enzyme	Proteolysis
TVC00582	3	60S ribosomal protein L38	Ribosomal
TVC00603	3	Alpha-ketoglutarate dependent xanthine dioxygenase	Nitrogen metabolism
TVC00655	3	1,3-beta-glucan synthase	Cell wall biosynthesis

Table 2 – Functional analysis of ESTs from the subtractive libraries.

Functional class	No./class	% in class
Ribosomal	17	9
Electron transport	15	8
RNA binding	13	7
Protein synthesis	12	7
Cell division	11	6
Lipid biosynthesis	11	6
Signalling	10	5
Transporter	9	5
Metabolism	8	4
Metal ion homeostasis	8	4
Vesicle transport	8	4
Carbohydrate metabolism	7	4
Protein folding	7	4
Proteolysis	7	4
Endonuclease	5	3
ROS	5	3
Biosynthesis	4	2
Redox	4	2
Transcription	4	2
Cytoskeleton	3	2
Glycolipid biosynthesis	3	2
Amino acid biosynthesis	2	1
Cytochrome P450	2	1
DNA binding	2	1
Membrane protein	2	1
Nitrogen metabolism	2	1
Apoptosis	1	1

Cell division was clearly active in the zone behind the interaction front represented by 6 % of the ESTs. Nucleic acid binding and transcription-related ESTs comprised 10 % of the sequences including nine ESTs homologous to glycine-rich RNA binding proteins, although none of the ESTs were potential transcription factors. This is not unusual in this size of EST collection due to their low level of expression (Holland 2002).

Stress/protective responses were also active in this zone with 8 % of ESTs encoding genes related to ROS, protein folding or cytochrome P450. This class included two ESTs encoding nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, important in many systems for the generation of ROS (Bokoch & Knaus 2003; Lara-Ortíz et al. 2003). Only one EST was related to apoptosis: this encodes a homologue of CLPTM1 which belongs to a gene family including members involved in DNA-crosslinking-induced apoptosis (Yamamoto et al. 2001). 5 % of our ESTs were related to signalling processes including two receptor-like proteins and, interestingly, a heterokaryon incompatibility associated (HET-domain) protein. HET-domain proteins are important in mediating vegetative incompatibility reactions in many if not all fungal species (Glass & Dementhon 2006). Furthermore, autophagic-type cell death was induced when the HET-domain from a *Podospora anserina* gene was over-expressed (Paoletti & Clavé 2007) implicating the HET-domain as a mediator of autophagic-type cell death by vegetative incompatibility. Thus, the EST collection indicates a mycelium active in both biosynthetic and metabolic processes where signalling and molecule transport are also important but where a few cell-death related genes are represented. It is likely that the majority of the ESTs may not be specific for the events of the fungal interaction but reflect the high levels of growth and coordinated activity which take place in this zone. It is important to note that the region chosen for the EST isolation is well behind the interaction zone: it seems likely that the pattern of gene expression would change considerably closer to the interaction front.

Analysis of microarray data

Overall unexpectedly few probes were up- or down-regulated by more than two-fold in any of the interacting mycelia (Table 3), perhaps indicating that in this region of the mycelium transcriptional changes are either relatively minor compared to the fungus growing alone, or that a dynamic situation exists in which changes are transient.

Gene expression in the interaction between Trametes versicolor vs. Stereum gausapatum

Expression of 30 % of the probes on the microarray was up- or down-regulated as a result of the *T. versicolor vs. S. gausapatum* interaction (Table 3). Of these, 348 were up-regulated by 1.4-fold in at least two of three *T. versicolor vs. S. gausapatum* arrays, but fewer (42) were up-regulated by two-fold. However, within these up-regulated probe lists, two thirds (232) of the 1.4 up-regulated spots were clones from the reverse subtracted library (Table 3) indicating that the subtraction had not been very effective. The same pattern was observed for the down-regulated probes, with 328 down-regulated by at least 1.4 fold, but of these only one third (100) derived from the reverse subtracted library.

The highest proportions of the up-regulated genes for which it was possible to assign putative functions were ribosomal genes, and glycine-rich RNA binding proteins (Table 4). Glycine-rich RNA binding proteins are involved in post-transcriptional regulation including processing of mRNA and rRNA, RNA export, and RNA stability (Lunde et al. 2007). They have not been studied extensively in fungi, but in plants their expression is associated with several environmental stresses such as wounding, dehydration, and fungal infection (Sachetto-Martins et al. 2000), and it is possible that they are associated with the stress created by interspecific interactions.

Table 3 – Summary of the numbers of genes identified for each pairing combination when filtered on expression level.

Pairing		1.4 up		2 up		1.4 down		2 down	
TvSg	F	348	116	42	18	328	228	77	50
	R		232		24		100		27
TvHf	F	107	46	3	3	131	75	14	13
	R		61		0		56		1
TvBa	F	322	110	25	7	283	143	22	20
	R		212		18		140		2

F, forward subtracted library clones; R, reverse subtracted library clones; TvSg, *T. versicolor vs. S. gausapatum*; TvHf, *T. versicolor vs. H. fasciculare*; TvBa, *T. versicolor vs. B. adusta*.

Table 4 – Microarray probes for which putative functions can be assigned, that are up- or down-regulated by at least 1.4 fold in the interactions between *T. versicolor vs. S. gausapatum*, *B. adusta* or *H. fasciculare*.

Clone ID	Cluster ID	Putative function	Fold change			Up or down in all 3 interactions
			Tv/Sg	Tv/Ba	Tv/Hf	
Tv_sshF_07B01	TVC00300	Ubiquitin activating enzyme		1.89	1.61	
Tv_sshR_01B04	TVC00573	LAGLIDADG endonuclease		1.83	1.53	
Tv_sshR_01B09	TVC00577	Aminomethyltransferase, mitochondrial precursor			0.7	
Tv_sshR_03F04	TVC00079	Glycine-rich RNA binding protein		1.87	1.57	
Tv_sshR_03H01	TVC00675	Pre-mRNA-splicing factor 38B			0.61	
Tv_sshR_03H07	TVC00675	Pre-mRNA-splicing factor 38B		0.69	0.66	
Tv_sshR_04E09	TVC00786	Bud site selection-related protein			1.99	
Tv_sshR_04G06	TVC00802	Putative cytochrome oxidase assembly protein			1.55	
Tv_sshR_06D09	TVC00917	Mitochondrial intermediate peptidase			0.61	
Tv_sshR_06F01	TVC00748	Glycine-rich RNA binding protein			1.58	
Tv_sshR_06F08	TVC00936	GrpE domain chaperone protein		1.44	1.83	
Tv_sshR_08B07	TVC01047	GIY endonuclease		1.55	1.65	
Tv_sshR_08D02	TVC01061	Glycine-rich RNA binding protein		0.59	0.55	
Tv_sshR_09A02	TVC00778	Hypothetical iron permease			1.69	
Tv_sshR_09E02	TVC00573	LAGLIDADG endonuclease			1.97	
Tv_sshR_10D01	TVC01211	40S ribosomal protein mrp4		0.67	0.55	
Tv_sshR_02F09	TVC00574	Glycoside hydrolase family 13 protein		2.02		
Tv_sshR_03B07	TVC00689	Cytochrome c oxidase subunit 1		2.01		
Tv_sshF_10E11	TVC00144	Cytochrome c oxidase subunit 1		2		
Tv_sshF_10B02	TVC00428	Iron ion homeostasis-related protein		1.99		
Tv_sshF_03G06	TVC00137	Cytokinesis-related protein		1.87		
Tv_sshR_10E05	TVC00695	60S ribosomal protein L38		1.81		
Tv_sshR_08E06	TVC01073	ZIP-like iron-zinc transporter		1.77		
Tv_sshR_06E07	TVC00923	Retinal short-chain dehydrogenase/ reductase-like protein		1.77		
Tv_sshR_01A05	TVC00567	Phosphoglucomutase		1.68		
Tv_sshR_02A04	TVC00079	Glycine-rich RNA binding protein		1.68		
Tv_sshF_05C03	TVC00025	Cytosine–purine permease		1.68		
Tv_sshF_06F12	TVC00100	Sugar transporter		1.66		
Tv_sshR_09B10	TVC01123	60S ribosomal protein L39 (L46)		1.66		
Tv_sshF_07F06	TVC00337	Polyadenylate-binding protein 2		1.63		
Tv_sshR_10A04	TVC01187	Cytochrome c oxidase subunit 1		1.61		
Tv_sshR_06A02	TVC00884	Catalase		1.6		
Tv_sshR_02B07	TVC00624	Elongation factor 1-alpha		1.59		
Tv_sshR_08B07	TVC01047	GIY endonuclease		1.55	1.65	
Tv_sshF_07B09	TVC00306	Heterokaryon incompatibility protein (HET);		1.51		
Tv_sshR_09A07	TVC01110	Membrane protein of unknown function		1.5		
Tv_sshR_06E02	TVC00920	LAGLIDADG endonuclease		1.42		
Tv_sshR_01D02	TVC00574	Glycoside hydrolase family 13 protein		0.41		
Tv_sshR_10H11	TVC00144	Cytochrome c oxidase subunit 1		0.49		
Tv_sshR_08D08	TVC01061	Glycine-rich RNA binding protein		0.5		
Tv_sshR_06H08	TVC00832	Candidate catalase		0.6		
Tv_sshR_05D04	TVC00841	Ornithine-oxo-acid aminotransferase		0.61		
Tv_sshR_03H09	TVC00689	Cytochrome c oxidase subunit 1		0.62		
Tv_sshF_05H01	TVC00032	Oxidoreductase		0.63		
Tv_sshR_09D09	TVC01061	Glycine-rich RNA binding protein		0.64		
Tv_sshR_06D06	TVC00079	Glycine-rich RNA binding protein		0.64		
Tv_sshF_08H07	TVC00137	Cytokinesis-related protein		0.64		
Tv_sshR_06H06	TVC00951	Cell division control/GTP binding protein		0.64		
Tv_sshF_06H09	TVC00282	U3 snoRNP-associated protein Rrp9 (predicted)		0.64		
Tv_sshF_05H06	TVC00224	Translationally-controlled tumour protein		0.65		
Tv_sshF_05D05	TVC00017	Cytokinesis-related protein		0.65		
Tv_sshF_07D04	TVC00318	Mitogen-activated protein kinase 1		0.66		
Tv_sshF_10D05	TVC00039	40S ribosomal protein S17-B		0.66		
Tv_sshF_04D01	TVC00170	Iron ion homeostasis-related protein		0.68		
Tv_sshR_01D07	TVC00574	Glycoside hydrolase family 13 protein	4.34			
Tv_sshF_03D07	TVC00017	Cytokinesis-related protein	3.75		0.42	
Tv_sshR_03H02	TVC00710	60S ribosomal protein L20A	2.69			
Tv_sshF_08D12	TVC00345	Iron ion homeostasis-related protein	2.64			
Tv_sshR_02G03	TVC00663	Cleft lip and palate associated transmembrane protein	2.55	2.21		
Tv_sshR_09D02	TVC00640	Aldehyde reductase I (ARI), putative	2.53		0.49	

(*continued on next page*)

Table 4 – (continued)

Clone ID	Cluster ID	Putative function	Fold change			Up or down in all 3 interactions
			Tv/Sg	Tv/Ba	Tv/Hf	
Tv_sshR_10B08	TVC01200	AP-2 adaptor complex subunit mu, putative	2.43	1.94		
Tv_sshF_02C10	TVC00032	Oxidoreductase	2.41	1.65		
Tv_sshR_06D05	TVC00914	40S ribosomal protein S4	2.35			
Tv_sshR_10C12	TVC01210	Coatomer protein	2.31			
Tv_sshF_10H08	TVC00016	DNA binding protein SART-1, putative	2.16	0.59		
Tv_sshR_09D11	TVC01145	Eukaryotic translation elongation factor 2	2.11	0.55		
Tv_sshF_08G09	TVC00408	Glycine-rich RNA binding protein	2.07	1.67	1.78	*
Tv_sshF_10B08	TVC00432	Glutamate-cysteine ligase	2.04	1.97		
Tv_sshR_07F02	TVC01001	Phosphopyruvate hydratase	2.01	2.04	1.79	*
Tv_sshF_11F07	TVC00300	Ubiquitin activating enzyme	1.97	1.67	1.7	*
Tv_sshR_06C04	TVC00902	Alpha-1,2-mannosyltransferase	1.97	1.83		
Tv_sshF_11G02	TVC00016	DNA binding protein SART-1, putative	1.96	1.83		
Tv_sshR_02F03	TVC00655	1,3-beta-glucan synthase	1.95	2.43		
Tv_sshF_11G10	TVC00551	Syntaxin-like protein	1.94			
Tv_sshR_10B06	TVC01061	Glycine-rich RNA binding protein	1.92		1.63	
Tv_sshF_04G12	TVC00079	Glycine-rich RNA binding protein	1.91	1.77	2.03	*
Tv_sshR_06B07	TVC00895	Cyclophilin 1	1.89	1.8		
Tv_sshR_07F08	TVC00748	Glycine-rich RNA binding protein	1.88			
Tv_sshF_06C06	TVC00247	60S ribosomal protein L6	1.84			
Tv_sshF_10D11	TVC00017	Cytokinesis-related protein	1.84	0.62		
Tv_sshR_04G05	TVC00801	Delta 9-fatty acid desaturase protein	1.84	1.77		
Tv_sshF_04H08	TVC00204	Integral membrane protein	1.82			
Tv_sshR_06G08	TVC00944	40S ribosomal protein S21	1.82	1.75		
Tv_sshF_10H01	TVC00485	Mitogen-activated protein kinase 1	1.8		0.54	
Tv_sshR_03G05	TVC00710	60S ribosomal protein L20A	1.78	1.76		
Tv_sshR_07D05	TVC00748	Glycine-rich RNA binding protein	1.77			
Tv_sshR_01B07	TVC00575	GTP-binding protein Ypt1	1.76			
Tv_sshR_01H01	TVC00603	Alpha-ketoglutarate dependent xanthine dioxygenase	1.74			
Tv_sshR_04D09	TVC00775	Serine/threonine kinase receptor associated protein	1.74			
Tv_sshR_04C06	TVC00710	60S ribosomal protein L20A	1.73	1.73		
Tv_sshF_04F09	TVC00017	Cytokinesis-related protein	1.72	1.87		
Tv_sshR_04G02	TVC00799	60S ribosomal protein L17/L23	1.71	1.65		
Tv_sshR_07F06	TVC00655	1,3-beta-glucan synthase	1.7	1.61		
Tv_sshF_10H10	TVC00202	Peroxiredoxin	1.68	0.56		
Tv_sshR_10F08	TVC00748	Glycine-rich RNA binding protein	1.68	1.74	1.8	*
Tv_sshR_04B05	TVC00748	Glycine-rich RNA binding protein	1.67	2.2	1.59	*
Tv_sshR_02D02	TVC00638	GrpE domain chaperone protein	1.64			
Tv_sshR_02D05	TVC00640	Aldehyde reductase I (ARI), putative	1.63			
Tv_sshR_04D02	TVC00769	Steroid 5-alpha-reductase	1.63	0.63		
Tv_sshR_09B07	TVC01120	Stearoyl-CoA 9-desaturase	1.6			
Tv_sshR_01F03	TVC00574	Glycoside hydrolase family 13 protein	1.59	1.72		
Tv_sshR_05C04	TVC00832	Candidate catalase	1.58			
Tv_sshR_03C02	TVC00695	60S ribosomal protein L38	1.56			
Tv_sshF_02H10	TVC00079	Glycine-rich RNA binding protein	1.55			
Tv_sshF_05B04	TVC00016	DNA binding protein SART-1, putative	1.54	1.48		
Tv_sshF_11G05	TVC00017	Cytokinesis-related protein	1.54			
Tv_sshR_08C07	TVC01008	ZIP-like iron-zinc transporter	1.54		1.64	
Tv_sshR_03H08	TVC00740	Threonyl-tRNA synthetase	1.52	0.61		
Tv_sshR_07G09	TVC01018	Family S53 protease-like protein	1.52			
Tv_sshR_05C07	TVC00834	Pre-mRNA-splicing factor	1.47			
Tv_sshR_02D04	TVC00639	Acyltransferase	1.44			
Tv_sshR_08D07	TVC01065	Glycine-rich RNA binding protein	1.43	0.67		
Tv_sshF_03G10	TVC00017	Cytokinesis-related protein	0.7			
Tv_sshR_06H01	TVC00948	Ubiquinol-cytochrome c reductase complex 14 kDa protein	0.7	0.6		
Tv_sshF_05E06	TVC00018	ABC transporter	0.69			
Tv_sshR_01E02	TVC00590	Cytochrome c oxidase subunit 1	0.68			
Tv_sshF_08A06	TVC00100	Sugar transporter	0.67			
Tv_sshR_03A09	TVC00101	Cytochrome c oxidase subunit 1	0.66			
Tv_sshF_04A10	TVC00039	40S ribosomal protein S17-B	0.65			
Tv_sshF_04E03	TVC00017	Cytokinesis-related protein	0.64			
Tv_sshF_02B06	TVC00017	Cytokinesis-related protein	0.63			
Tv_sshF_02E08	TVC00047	Cytochrome c oxidase subunit 1	0.63			
Tv_sshR_05D01	TVC00838	Aldo/keto reductase family oxidoreductase	0.63		0.49	
Tv_sshF_03F05	TVC00017	Cytokinesis-related protein	0.61			

Table 4 – (*continued*)

Clone ID	Cluster ID	Putative function	Fold change			Up or down in all 3 interactions
			Tv/Sg	Tv/Ba	Tv/Hf	
Tv_sshF_06E06	TVC00262	Caprolactone hydrolase	0.61			
Tv_sshF_08A07	TVC00364	rRNA (adenine-N6,N6-)-dimethyltransferase	0.61			
Tv_sshF_02B04	TVC00016	DNA binding protein SART-1, putative	0.6			
Tv_sshF_03H07	TVC00144	Cytochrome c oxidase subunit 1	0.6	0.6		
Tv_sshF_04F05	TVC00017	Cytokinesis-related protein	0.59			
Tv_sshF_05B08	TVC00018	ABC transporter	0.59			
Tv_sshF_02A04	TVC00004	Cytokinesis-related protein	0.58			
Tv_sshF_08E11	TVC00068	Cytochrome P450	0.57			
Tv_sshF_11D01	TVC00518	Phosphomannomutase	0.57	0.48	0.53	*
Tv_sshF_05A04	TVC00004	Cytokinesis-related protein	0.56			
Tv_sshF_05H10	TVC00079	Glycine-rich RNA binding protein	0.56			
Tv_sshR_03A03	TVC00032	Oxidoreductase	0.56			
Tv_sshR_09A08	TVC00748	Glycine-rich RNA binding protein	0.56			
Tv_sshF_04G02	TVC00017	Cytokinesis-related protein	0.55		0.63	
Tv_sshF_10B04	TVC00017	Cytokinesis-related protein	0.55	0.61		
Tv_sshR_05E07	TVC00655	1,3-beta-glucan synthase	0.55	0.68		
Tv_sshF_03C10	TVC00017	Cytokinesis-related protein	0.54			
Tv_sshF_05G07	TVC00068	Cytochrome P450	0.54			
Tv_sshF_08E07	TVC00395	Vacuole fusion, non-autophagic-related protein	0.54		0.6	
Tv_sshR_02E09	TVC00650	Lanosterol 14-alpha-demethylase	0.54			
Tv_sshF_02B08	TVC00018	ABC transporter	0.53			
Tv_sshF_02F04	TVC00017	Cytokinesis-related protein	0.51			
Tv_sshF_02H01	TVC00032	Oxidoreductase	0.51		0.56	
Tv_sshF_06C07	TVC00101	Cytochrome c oxidase subunit 1	0.51	0.62		
Tv_sshR_10A01	TVC01061	Glycine-rich RNA binding protein	0.5		0.56	
Tv_sshR_10H12	TVC01230	Delta 9-fatty acid desaturase protein	0.5	0.62		
Tv_sshF_03C03	TVC00100	Sugar transporter	0.47	0.62	0.62	*
Tv_sshR_04E01	TVC00779	Co-chaperone	0.47	0.6	1.56	
Tv_sshF_10E12	TVC00333	Similar to eukaryotic peptide chain release factor GTP-binding subunit	0.46	0.62		
Tv_sshR_08E05	TVC00079	Glycine-rich RNA binding protein	0.46	0.65		
Tv_sshF_02D05	TVC00017	Cytokinesis-related protein	0.45	0.52	0.56	*
Tv_sshR_04A01	TVC00743	GIY endonuclease	0.45	0.6		
Tv_sshR_08E01	TVC01069	60S ribosomal protein L27	0.45	0.6		
Tv_sshF_02C03	TVC00025	Cytosine–purine permease	0.44	0.54	0.54	*
Tv_sshF_02D03	TVC00025	Cytosine–purine permease	0.44	0.49	0.54	*
Tv_sshF_03D01	TVC00108	Glycine-rich RNA binding protein	0.44	0.46	0.43	*
Tv_sshR_01E03	TVC00574	Glycoside hydrolase family 13 protein	0.44			
Tv_sshR_03E01	TVC00712	60S ribosomal protein L19	0.44	0.62		
Tv_sshR_07E01	TVC00990	Delta 9-fatty acid desaturase protein	0.44		1.84	
Tv_sshF_02D07	TVC00039	40S ribosomal protein S17-B	0.42	0.35	0.49	*
Tv_sshF_11E11	TVC00079	Glycine-rich RNA binding protein	0.42	0.59		
Tv_sshF_07E12	TVC00333	Similar to eukaryotic peptide chain release factor GTP-binding subunit	0.41	0.38		
Tv_sshF_11E12	TVC00534	Actin-related protein Arp4p	0.38	0.55		
Tv_sshR_04E02	TVC00743	GIY endonuclease	0.38	0.61		
Tv_sshF_03A01	TVC00047	Cytochrome c oxidase subunit 1	0.37	0.49	0.46	*

Three probes encoded enzymes involved in carbohydrate metabolism including an α-amylase family (glycoside hydrolase family 13) gene and a 1,3-beta-glucan synthase. Glycoside hydrolase family 13 is a family of starch modifying enzymes comprising around 30 different specificities, including isoamylases, glucan synthases, and pullunases (Machovic & Janecek 2008). Recent studies have indicated that some GH13 family enzymes in fungi may be involved in the synthesis or modification of alpha glucan in the fungal cell wall rather than starch degradation (Yuan *et al.* 2008). In filamentous fungi such as Aspergillus, for instance, glycosylhydrolases may be important for plasticising the cell wall during conidial swelling, hyphal branching or hyphal anastomosis (Latgé 2007). Three other genes were also represented by multiple probes, amongst these up-regulated genes were: a putative septin, a DNA-binding protein and an aldehyde reductase. In *Saccharomyces cerevisiae* septins anchor cell cycle regulatory proteins at the bud neck during cell division. However, in filamentous fungi, they play a wider role in hyphal growth: determining hyphal branch points (Helfer & Gladfelter 2006), and maintaining cell polarity and hyphal morphogenesis (Warenda & Konopka 2002). During hyphal interactions they may therefore be contributing to the interaction zone hyphal proliferation. The DNA-binding protein is related to SART-1, known as a tumour antigen, involved

in cell cycle arrest (Wilkinson *et al.* 2004) and thought to be involved in RNA-splicing in yeast (Makarov *et al.* 2002). Also of note was the up-regulation of an apoptosis related gene: a cleft lip and palate associated transmembrane protein (CLPT protein). Two probes related to signalling were also up-regulated: a serine/threonine kinase receptor associated protein and a MAP kinase. MAPK signalling cascades are associated with stress responses in many fungi (Zhao *et al.* 2007) and have also been implicated in interspecific interactions between plant and animal pathogens and their hosts (Bahn *et al.* 2007; Eaton *et al.* 2008).

The largest group of 12 down-regulated probes in the *T. versicolor vs. S. gausapatum* interaction all belonged to one cluster encoding a putative septin (Table 4), six probes belonging to four different clusters encoded subunit 1 of cytochrome c oxidase. Glycine-rich RNA binding proteins were also well-represented in this class, as were transporters: an ABC transporter, a sugar transporter, and a cytosine–purine permease. Ribosomal proteins were also well-represented amongst the down-regulated genes.

It is difficult to derive a clear picture from this pattern of expression, however, it would seem that both the putative septin and the glycine-rich RNA binding proteins were both up- and down-regulated. In the case of the septins this may reflect the participation of different members of a gene family which in yeast comprises seven members with varying functions (Douglas *et al.* 2005). Changes in transcription of the glycine-rich proteins are perhaps important in regulating other genes and possibly, as already mentioned, in responding to stressful conditions. Cell wall biosynthesis and starch breakdown-related probes were generally up-regulated, whereas cell division was largely down-regulated. There also appeared to be more signalling and regulatory genes (receptors, MAP kinases, DNA binding proteins) represented amongst the up-regulated genes.

Gene expression in the interaction between Trametes versicolor vs. Bjerkandera adusta

About the same number of the probes (605) changed in expression by more than 1.4 fold when *T. versicolor* was challenged with *B. adusta* compared to the interaction with *Stereum gausapatum* discussed above: of these 322 were up-regulated and 283 were down-regulated (Table 3). Of the up-regulated probes, just under half (48 %) were shared with those up-regulated when *T. versicolor* was challenged with *S. gausapatum*, and a similar proportion (41 %) of down-regulated probes were shared between these two interactions (Fig 4). Broadly similar genes were up-regulated during this interaction compared to the interaction with *S. gausapatum* (Table 4). Ribosomal genes and glycine-rich RNA binding proteins were again well-represented amongst the up-regulated probes. Genes related to carbohydrate metabolism also formed a prominent group including those related to glycolysis, starch breakdown, and cell wall biosynthesis. The CLPT protein was also up-regulated in this interaction by over two-fold. Two genes related to signalling were up-regulated, including the heterokaryon incompatibility protein discussed above. Of the down-regulated genes, RNA binding proteins were again highly represented, including the glycine-rich RNA binding proteins. In addition, as for the interaction with *S. gausapatum*, cell division-related genes were down-regulated including probes homologous to a putative septin gene but also a cell division control/GTP-binding protein. Subunit 1 of cytochrome c oxidase was also down-regulated. As for the interaction with *S. gausapatum*, it would seem that cell division processes are down-regulated whereas carbohydrate metabolism is up-regulated, although a completely clear picture does not emerge due to some overlap in functional classes between the up- and down-regulated genes. This is probably, at least in part, due to difficulties in assigning sequences to specific members of gene families as discussed above.

Gene expression in the interaction between Trametes versicolor vs. Hypholoma fasciculare

A much smaller number of probes changed in expression in response to *H. fasciculare*: 107 were up-regulated by more than 1.4 fold and 131 were down-regulated (Table 3). Of the up-regulated probes, a smaller proportion (30 %) was common to the interaction with *Stereum gausapatum* compared to the 41 % shared by interactions with *Bjerkandera adusta*. However, proportionately more down-regulated probes were shared between interactions with *S. gausapatum* (50 %) and *H. fasciculare*, a similar proportion to those shared between interactions

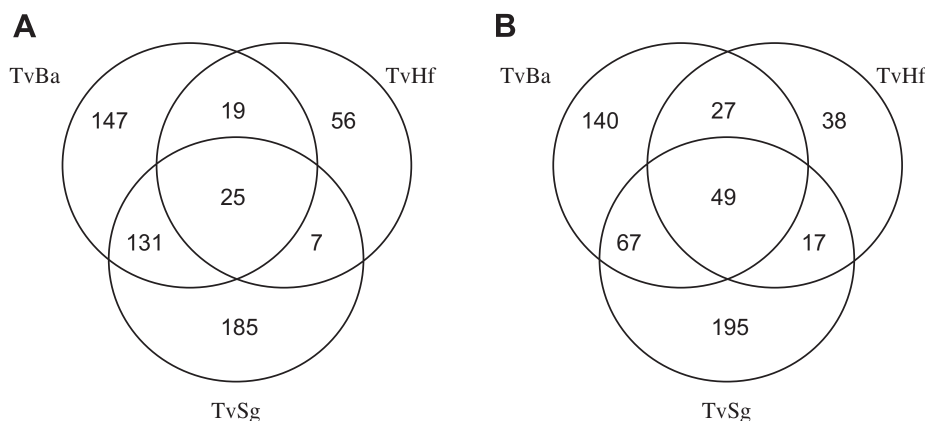

Fig 4 – Venn diagrams showing (A) up-regulated and (B) down-regulated genes for all three interactions, filtered on 1.4 fold change. TvSg = *T. versicolor vs. S. gausapatum*; TvHf = *T. versicolor vs. H. fasciculare*; TvBa = *T. versicolor vs. B. adusta*.

with *B. adusta* (58 %) (Fig 4). Putative functions could be assigned to 20 up-regulated and 22 down-regulated genes in the interactions between *T. versicolor* and *H. fasciculare* (Table 4). Although these genes belong to similar functional classes to those found with the other interaction experiments, the representation of the classes differed. Thus, although the major class of up-regulated genes was still RNA binding proteins, three probes homologous to two different endonucleases were up-regulated in the interaction with *H. fasciculare*. In addition, carbohydrate metabolism-related genes did not form a major functional class of up-regulated genes. The major functional classes of down-regulated genes were more similar to the other two interaction experiments with RNA binding proteins highly represented, as well as cell division-related genes, and transporters although carbohydrate metabolism-related genes were a larger proportion of down-regulated genes than in the other two interactions. The microarray results indicate that the gene expression changes behind the interaction front with *H. fasciculare* differed from those behind interaction fronts with the other two competitors in that fewer genes changed in expression, and the pattern of genes changing in expression was subtly different.

Comparison of gene expression elicited by the interaction between Trametes versicolor *and all three competitors*

The expression of a small number of probes was up-regulated (25) or down-regulated (49) consistently in all three interactions (Fig 4). Four of the up-regulated probes and one of the down-regulated probes were homologous to glycine-rich RNA binding proteins (Table 4). One of the remaining two up-regulated probes, for which a function could be assigned, was homologous to an ubiquitin activating enzyme. Ubiquitin-mediated proteolysis is an important regulator in all eukaryotes for removing unwanted proteins (Aguilar & Wendland 2003) and has been implicated in the response of *T. versicolor* to nitrogen deprivation (Staszczak 2008). In other basidiomycetes this process is involved in a wide range of developmental functions including fruit body formation (Miyazaki *et al.* 2005) and meiosis (Koshiyama *et al.* 2006) as well as stress responses to heat (Higgins & Lilly 1993) and UV (Taupp *et al.* 2008).

Analysis of gene expression of selected ESTs by semi-quantitative RT-PCR

Two genes were selected on the basis of functional interest from the EST collection for semi-quantitative RT-PCR analysis (Fig 5) in that they are related to ROS processes. Three clusters identified from the ESTs (TVC01049, TVC00960 and TVC00679) each only comprising one probe sequence, were homologous to a gene related to ROS production: NADPH oxidase. On the arrays all three probes were stable in expression in all three interactions except TVC01049 in the *Trametes versicolor vs. Hypholoma fasciculare* (up-regulated by 1.65 fold). RT-PCR using primers designed to TVC00960 confirmed the array result that expression of this gene was essentially unaffected by any of the three interactions with competitors tested (Fig 5).

A further two clusters (TVC00832 and TVC00884) representing three spots on the microarray showed homology to catalases, enzymes that remove ROS by the breakdown of hydrogen peroxide. On the arrays, catalase expression was slightly up-regulated in all three interactions (Fig 5A) but only in the *T. versicolor vs. Stereum gausapatum* interaction zone was expression above the 1.4 fold cut off (1.44 fold $\pm$ 0.12SE). This slight increase was also seen in the RT-PCR (using primers designed to TVC00884), for *T. versicolor vs. S. gausapatum* and *T. versicolor vs. Bjerkandera adusta* but not for *T. versicolor vs. H. fasciculare*, where expression was below that found in the *T. versicolor* self-paired control (Fig 5B). Thus, gene expression of catalase was confirmed to be relatively unchanged by interactions with a competitor in this region of the mycelium. These results suggest that either other genes related to ROS play a more significant role at the interaction zone, or regulation of ROS production is post-transcriptional.

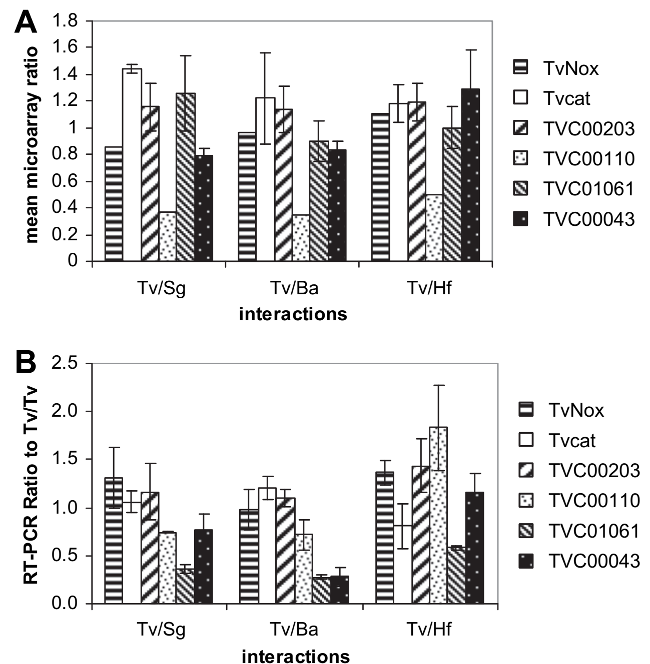

Fig 5 – Expression of six ESTs: TvNox (TVC00679), Tvcat (TVC00884), TVC01192, TVC00110, TVC1061 and TVC00043. (A) Microarray means of spots in cluster (±SEM, where clusters contained more than one spot), (B) semi-quantitative RT-PCR (mean ± SEM, $n \geq 3$). Tv/Sg = *T. versicolor vs. S. gausapatum*; Tv/Ba = *T. versicolor vs. B. adusta*; Tv/Hf = *T. versicolor vs. H. fasciculare*; Tv/Tv = *T. versicolor* self-pairing.

Four further array probes were chosen on the basis of their expression patterns on the array. TVC00203 is a gene of unknown function represented by six probes on the microarray and its mean expression ratio was close to one in all three interaction experiments (*T. versicolor vs. S. gausapatum*: 1.16 $\pm$ 0.18, *T. versicolor vs. B. adusta*: 1.14 $\pm$ 0.18, *T. versicolor vs. H. fasciculare*: 1.19 $\pm$ 0.14). Semi-quantitative RT-PCR indicated a similar pattern (*T. versicolor vs. S. gausapatum*: 1.17 $\pm$ 0.29, *T. versicolor vs. B. adusta*: 1.10 $\pm$ 0.09, *T. versicolor vs. H. fasciculare*: 1.43 $\pm$ 0.28). Thus, only in the interaction with *H. fasciculare* did expression rise marginally above the 1.4 fold threshold. TVC00110, represented by just one probe on the array, is also of unknown

function. Its expression was down-regulated in all three interaction experiments (*T. versicolor vs. S. gausapatum*: 0.37 ± 0.07, *T. versicolor vs. B. adusta*: 0.35 ± 0.02, *T. versicolor vs. H fasciculare*: 0.50 ± 0.09) on the microarrays. Semi-quantitative RT-PCR confirmed this expression pattern for two of the interactions: with *S. gausapatum* and *B. adusta* (*T. versicolor vs. S. gausapatum*: 0.74 ± 0.01, *T. versicolor vs. B. adusta*: 0.72 ± 0.16). Unexpectedly, however, RT-PCR indicated an up-regulation of this gene in the interaction with *H. fasciculare* (*T. versicolor vs. H. fasciculare*: 1.84 ± 0.44).

TVC01061 is homologous to glycine-rich RNA binding proteins and represented six probes on the array. These probes were both up- and down-regulated on the array, indicating that they may comprise a gene family and that their role is complex. They were highly represented amongst the up-regulated genes in all three experiments, however, the mean expression of this cluster on the microarrays indicated little change in response to interactions (*T. versicolor vs. S. gausapatum*: 1.26 ± 0.22, *T. versicolor vs. B. adusta*: 0.90 ± 0.10, *T. versicolor vs. H fasciculare*: 1.00 ± 0.10). This was not supported by the RT-PCR data where the specific sequence charted was down-regulated in all three experiments.

The final cluster for which RT-PCR data were obtained was TVC00043. This cluster comprised four probes on the array, is of unknown function, and its microarray expression remained below the 1.4 fold threshold in all three interaction experiments (*T. versicolor vs. S. gausapatum*: 0.79 ± 0.08, *T. versicolor vs. B. adusta*: 0.83 ± 0.05, *T. versicolor vs. H. fasciculare*: 1.29 ± 0.21). This expression pattern was in agreement with the RT-PCR in two of the interaction experiments: *T. versicolor vs. S. gausapatum* (ratio: 0.77 ± 0.17) and *T. versicolor vs. H. fasciculare* (ratio: 1.16 ± 0.20), but the RT-PCR revealed a strong down-regulation in the interaction with *B. adusta* (ratio: 0.28 ± 0.10).

Most of the RT-PCR results (12 out of 18) confirmed the array data. However a few differences in expression were noted above. This is likely to be due to two main factors. Firstly the microarray used cDNA probes which were likely to hybridise to several members of a gene family, in contrast PCR primers were designed to specific gene sequences. It is therefore possible that in specific interactions different members of a gene family predominated. This subtlety of expression may have important functions in fine-tuning mycelial responses and is currently being investigated.

Another aspect of this work is the dynamic nature of mycelial responses. The robustness of our experimental approach was tested by using biological replicates for the arrays and RT-PCR. However small changes in spatial or temporal expression will affect the results, and may be important in regulating progression of the interaction outcome. Again this is an area under investigation.

Concluding remarks

This work has highlighted the use of microarrays to investigate changes in gene expression during interactions between competing fungi. The relatively low levels of change detected on the array and verified by RT-PCR, coupled with the large changes in enzyme activity and morphology, indicate that further gene expression studies on mycelium closer to the interaction zone will be of interest. Fungal interactions represent a very dynamic system in which changes may occur rapidly and transiently. An analysis of the temporal and spatial gene expression in regions close to the interaction zone will be much easier now that specific probes can be studied. The results also give a strong indication that gene expression changes in response to competitors may depend on the competitor species, and/or final outcome of the interaction though clearly this requires further investigation. In particular, the pattern of gene expression elicited by an interaction in which *Trametes versicolor* was overgrown differed substantially to expression patterns seen in interactions where *T. versicolor* reached a deadlock or overgrew the competitor.

The picture presented by the EST analysis of gene activity behind the interaction zone suggests substantial biosynthetic activity presumably associated with formation of the barrage structures, but also metabolism which may represent activities such as remobilisation of nutrients from damaged cells or changes in cellular structure. Of particular interest was the high proportion of genes with putative functions in cytokinesis and as glycine-rich proteins, and the identification of signalling and regulatory genes which merit further study.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.funbio.2010.05.006.

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