UNIVERSITY OF WESTMINSTER

WestminsterResearch

http://www.westminster.ac.uk/westminsterresearch

The physiological response of the white-rot fungus, *Schizophyllum commune* to *Trichoderma Viride*, during interspecific mycelial combat.

Victor Chinomso Ujor

School of Life Sciences

The physiological response of the white-rot Fungus, *Schizophyllum commune* to *Trichoderma Viride*, During interspecific mycelial combat.

A thesis submitted in partial fulfilment of the requirement of the degree of Doctor of Philosophy of the University of Westminster.

Victor Chinomso Ujor

April 2010

Abstract

Fungal species compete for space and nutrients in organic matter, resulting in strong morphological and biochemical reactions in the interacting mycelia. Interspecific mycelial interactions have attracted extensive studies because of their potential applications in biological control, bio-pulping, screening for novel bioactive metabolites and enhancement of extracellular enzyme production. Studies of interspecific mycelial combat have also contributed to the understanding of the structure and development of fungal communities. Although the behaviour of interacting mycelia has been understood, mainly at the morphological level, the biochemical aspects have yet to be fully elucidated. The main aim of this study was to endeavour to understand the underlying cellular and molecular response patterns and adaptations of the white-rot fungus, *Schizophyllum commune* to a highly antagonistic strain of *Trichoderma viride*, by correlating the expression patterns of metabolites, proteins and selected genes of *Schizophyllum commune* in response to the antagonist. The study also investigated the implication of oxidative damage in these response patterns. Microscopic examination of stained and unstained mycelia of *S. commune* confronted by the mycelia of *T. viride*, revealed cell wall lysis, protoplasmic degeneration, hyphal expansion and subsequent hyphal disintegration, hence, cell death in the mycelia of *S. commune*, after 7 days of mycelial contact. Metabolite patterns of both species near the interaction zone were profiled by HPLC and GC/MS, in comparison to their self-paired mycelia. Sugar alcohols, phenolic compounds and organic acids were up-regulated in the interacting mycelia of both species, while γ-aminobutyric acid, myo-inositol phosphate, pyridoxine and N-acetylglucosamine, were up-regulated in *S. commune* mycelia with a concurrent decrease in the levels of fatty acids detected in the latter. Expression patterns of selected genes of *S. commune* confronted by *T. viride* were investigated by RT-PCR, relative to patterns in its self-paired cultures. Genes encoding proteins involved in the synthesis of cell wall polymers, protein synthesis and protein quality control, signalling, and stress response were up-regulated. On the other hand, genes that code for proteins associated with glycolysis, nitrogen assimilation, membrane transport, mitochondrial ATP-synthetic machinery, and cellular multiplication/growth were down-regulated. Changes in protein

expression were profiled in the mycelia of both species paired against each other using 2-Dimensional gel electrophoresis, and differentially expressed proteins were identified by MALDI-TOF-MS/MS, following peptide fragmentation. Proteins involved in protein synthesis and assembly, unfolded protein response, response to cellular injury, synthesis of phenolic compounds, recycling of carbon and nitrogen were up-regulated in the confronted mycelial domain of *S. commune*. Proteins involved in glycolysis and heat shock response were predominantly down-regulated in the mycelia of *S. commune* paired against *T. viride*. Proteins associated with antagonism, cellular metabolism, glycolysis, and ATP generation and protein synthesis were up-regulated in the mycelia of *T. viride* interacting with *S. commune* with a decline in the detected levels of proteins involved in cytoskeleton organisation. Biochemical assays revealed increases in the activity levels of antioxidant enzymes, superoxide dismutase, catalase, succinic semialdehyde dehydrogenase, glucose-6-phosphate dehydrogenase and in the levels of indicators of oxidative stress and secondary metabolism, such as lipid peroxidation, protein carbonylation, superoxide anion and phenolic levels in the mycelia of *S. commune* paired against *T. viride*. Similarly, the activities and protein levels of phenol-oxidising enzymes, namely laccase and manganese peroxidase increased in the confronted mycelial domain of *S. commune*. Chitinase activity increased in mixed liquid cultures of both fungi. Protein, and gene expression patterns, in the confronted mycelia of *S. commune* suggest an increase in the flux through the protein synthetic machinery, possibly resulting in endoplasmic reticulum stress, which may have activated the unfolded protein response. These are strong indications of oxidative stress induction and switch of mycelial growth to secondary metabolism. There was little evidence of antagonism by *S. commune* towards *T. viride*, suggesting that the patterns reported herein, may be a response rather than an attack mechanism towards the latter.

Table of contents

List of Abbreviations

μl	microlitre
μM	micromolar
1D	1-Dimensional
2D	2-Dimensional
4-HNE	4-hydroxyalkenals
ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACP	Acyl carrier protein
AKDH	Alpha –ketoglutarate dehydrogenase
ATP	Adenosine triphosphate
BCKAD	branched-chain α-ketoacid dehydrogenase
BLAST	basic local alignment search tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
DMP	2,6-dimethoxyphenol
DNA	Deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
CP	Capping protein
DNPH	2,4-dinitrophenyl hydrazine
DIGE	Difference gel electrophoresis
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylnediaminetetraacetic acid

eIF	Elongation initiation factor
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GABA	Gamma-aminobutyric acid
G6PDH	Glucose-6-phosphate dehydrogenase
GC/MS	Gas Chromatography/Mass Spectrometry
GT	Glycosyltransferase
HPLC	High Performance Liquid Chromatography
HCL	Hydrochloric acid
HSP	Heat shock protein
IEF	Isoelectric focusing
IPG	Immobilised Ph gradient
kDa	Kilo dalton
LPO	Lipid peroxidation
MnP	Manganese peroxidase
mRNA	Messenger RNA
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MDA	Malondialaldehyde
MDH	Malate dehydrogenase
MudPIT	MultiDimensional Protein Identification Technology
NADH	Nicotonamide adnine dinucleotide
NCBI	National Center for Biotechnology Information

NADPH	Nicotonamide adnine dinucleotide phosphate
NBT	Nitrotetrazolium
OFR	Open reading frame
PDA	Potato dextrose agar
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PAL	Phenylalanine ammonia lyase
PD	Pyruvate decarboxylase
PPP	Pentose phosphate pathway
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RBB	Remazol Brilliant Blue
ROS	Reactive oxygen species
RT-PCR	Reverse transcribed polymerase chain reaction
rpm	Revolutions per minute
RP	Reverse-phase
SAGE	Serial Analysis of Gene Expression
SCX	Strong Cation Exchange
SSADH	Succinic semialdehyde dehydrogenase
SCSC	*Schizophyllum commune + Schizophyllum commune*
SCTR	*Schizophyllum commune + Trichoderma viride*
SDS-PAGE	Sodium dodecyl sulphate-polyacrilamide gel electrophoresis
SOD	Superoxide dismutase

TEMED	Tetrametylenediamine
TRSC	*Trichoderma viride + Schizophyllum commune*
TRTR	*Trichoderma viride + Trichoderma viride*
TIM	Triphosphate isomerase
TCA	Tricarboxylic acid
UV	Ultra violet
UPR	Unfolded protein response
VOCs	Volatile organic compounds
V	Volts
WB	Woronin body

List of figures

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

List of tables

CHAPTER 1

Introduction

1.1 Introduction

Decomposer fungi are capable of colonising a wide range of organic substrates in nature, resulting in the formation of communities of interacting species (Heilmann-Clausen & Boddy, 2005). Colonisation of a virgin resource such as wood often commences with the establishment of distinctive colonies of individual species, which spread with time, leading to overlap of mycelial fronts that consequently interact with one another (Boddy, 2000; Donnelly & Boddy, 2001). It is thought that fungal mycelia possess a "recognition" mechanism that allows them to detect and respond to non-self mycelia - inter-specific interaction, (Rayner, 1991; Rayner *et al*., 1995; Griffith *et al*., 1994 a, b & c) or incompatible mycelia of the same species - intra-specific interactions, (Saupe, 2000; Demethon *et al*., 2003; Pinan-Lucarré *et al*., 2007). This allows fungi to directly defend their territories and indirectly, restrict access to captured nutrients by the invading/opposing individuals of the same or opposing species (Rayner *et al*., 1995; Boddy, 1993).

Fungal species may possess defence or attack mechanisms, or both, for responding to non-self and these mechanisms influence the reactions exhibited by fungal mycelia during non-self response. Based mainly on these defence/attack mechanisms, the outcome of interactions, between fungal mycelia may be classified as, combative/antagonistic, mutualistic or neutralistic (Cooke & Rayner, 1984).

Combative interspecific interactions among higher fungi are typified by protoplasmic degeneration, intra- and extracellular pigmentation, cessation of mycelial growth and sealing-off of mycelial front, marked increases in the secretion of phenol-oxidases and secondary metabolites, phase shifts-redistribution of mycelia and barrage formation (Boddy, 2000; Griffith *et al*., 1994 a, b & c; Rayner *et al*., 1995; Peiris *et al*., 2008; Peiris, 2009; Gregorio *et al*., 2006; Rayner *et al*., 1984; Sonnenbichler *et al*., 1994). These reactions are considered to be the after-effects of resistance to axial deformation by confronting mycelia (Rayner *et al*., 1995).

Following the work of Rayner and Todd (1979), fungus-fungus interactions have been studied for the past three decades. Earlier studies concentrated mainly on the structure and development of fungal communities among wood degraders (Rayner & Todd, 1979). Subsequent studies endeavoured to understand the morphology, chemistry and biochemistry of these interactions (Boddy, 1993, 1999, 2000; Griffith *et al*., 1994a, b & c; Sonnenbichler *et al*., 1989, 1993, 1994; Score *et al*., 1997; Baldrin, 2004; Gloer, 1995; Hynes *et al*., 2007; Gregorio *et al*., 2006; Rayner *et al*., 1994). The works of Griffith *et al*. (1994a & b) and Rayner *et al*. (1994) led to the suggestion that interspecific antagonistic interactions influence the synthesis of hydrophobic metabolites, which results in the sealing of mycelial boundaries between interacting mycelial fronts. Iakovlev *et al*. (2004) and Adomas *et al*. (2006) used molecular tools, namely mRNA differential display and macroarray technology respectively, to probe gene expression patterns in response to non-self mycelia. While the results of the former group showed the induction of secondary metabolism and oxidative stress in interacting mycelia, the results of the latter strongly implicated nutrient acquisition as the key mechanism employed by the more antagonistic species.

Combative interspecific interactions have been shown to hold promise as a screening tool for the efficacy of bio-control fungi against phytopathogenic and wood-rot fungi (Fokkema, 1973; Savoie & Mata, 1999; Rishbeth, 1971; Highley & Ricard, 1988; Bruce & Highley, 1991); for enhanced wood-pulping (Baldrin, 2004; Chi *et al*., 2007); and for optimizing enzyme production and screening for novel enzymes with an industrial bias (Zhang *et al*., 2006; Gregorio *et al*., 2006; Score *et al*., 1997; Freitag and Morrell, 1992). In addition to the modulation of metabolite expression (Peiris *et al*., 2007; Hynes *et al*., 2007; Rayner *et al*., 1994; Griffith *et al*., 1994b), combative interactions have been demonstrated to cause the production of compounds with novel chemical structures (Peiris, 2009). This suggests that interspecific mycelial interactions could be used in screening programmes for biologically active molecules. However, the biochemical and molecular basis of the strong physiological reactions associated with interspecific combative interactions have remained poorly understood.

1.2 Mechanisms of interspecific antagonistic interactions between higher fungi

Fungal species employ assorted mechanisms to respond to non-self mycelia. Mechanisms of interspecific interactions in fungi can be broadly classified into antagonism at a distance, hyphal interference, mycoparasitism and gross mycelial contact (Boddy, 2000). Antagonism at a distance is thought to be mediated by volatile/diffusible bioactive compounds or fungal waste products (Boddy, 2000; Sonnenbichler *et al.*, 1994). In some cases, mycelial degeneration in one of the interacting species is observed before contact, followed by overgrowth of the more adversely affected species (Boddy, 2000). However, mutual inhibition is often the outcome of antagonism at a distance (Boddy, 2000). This signifies an exchange of chemical signals between the paired species (Rayner *et al.*, 1984; Heilmann-Clausen & Boddy, 2005). The work of Heilmann-Clausen & Boddy (2005), demonstrated the existence of a metabolite-based passive defence mechanism in wood-rot fungi, where wood previously colonized by one fungus either promotes or inhibits the growth of other species, possibly contributing to the development of fungal communities in the field. For instance, they found that wood decayed by *Stereum hirsutum* caused reduced mycelial extension rate, delayed growth and in some cases, total inhibition of the majority of the species tested (Heilmann-Clausen & Boddy, 2005).

Hyphal interference is a programmed sequence of cytoplasmic degeneration characterized by granulation of cytoplasmic contents, vacuolation, loss of opacity and subsequent penetration of the degenerate hyphae by the invading species (Ikediugwu *et al.*, 1970; Ikediugwu, 1976; Behrendt & Blanchette, 2001; Boddy, 2000). This phenomenon is predominant amongst wood-decay fungi, and has been extensively studied in *Heterobasidion annosum*, because it is an economically important forest pathogen (Rayner & Boddy, 1988; Jefferies, 1997; Boddy, 2000). It has been suggested that hyphal interference is mediated by non-enzymic diffusible metabolite(s), which are actively secreted when non-self mycelia are detected at close range (Boddy, 2000). Furthermore, it can be mediated by both active and passive secretion of non-enzymic toxins in the presence and absence of confronting fungal mycelia respectively, as

reported in *Hypomyces aurantius* and *Phanerochaete magnoliae* (& Boddy, 1988; Jefferies, 1997; Boddy, 2000; Ainsworth *et al*., 1991).

1.2.1 Mycoparasitism

Mycoparasitism involves step-wise parasitizing of a fungus by another, involving host-directed mycelial growth, host recognition and attachment, pronounced synthesis and secretion of cell wall lytic enzymes and antibiotics, penetration and lysis of the host fungus (Markovich & Kononova, 2003). Some studies have demonstrated that host recognition is mediated by cell wall lectins (Jefferies, 1997; Chet *et al*., 1997; Inbar & Chet, 1994; Barak & Chet, 1986). Mycoparasites utilize host fungi as nutrients biotrophically or necrotrophically (Boddy, 2000). Examples of mycoparasitic wood-decay fungi include *Lenzites betulina*, which is parasitic on *Coriolus* species and *Pseudotrametes gibbosa*, which parasitizes *Bjernkandera* species (Rayner *et al*., 1995).

However, the most extensively studied mycoparasitic fungi are the members of the genus *Trichoderma*. *Trichoderma* species have been isolated worldwide from soil, decaying wood and other forms of plant-related organic materials (Zeilinger & Omann, 2007). The drivers of the mycoparasitic machinery of *Trichoderma* species include: cell wall hydrolytic enzymes - chitinases, proteases, glucanases and laminarases (Kubicek *et al*., 2001; Brunner *et al*., 2003; Cortes *et al*., 1998; Zeilinger *et al*., 1999; Howell, 2003; Elad *et al*., 1983); competitive edge - advanced growth and nutrient acquisition mechanisms (Chet, 1987; Hulme & Shields, 1970), and secretion of antifungal metabolites/peptides - peptaibols/mycotoxins (Dennis & Webster, 1971; Claydon *et al*., 1987; Schirmbrök *et al*., 1994; Lorito *et al*., 1996; Jaworski *et al*., 1999). Other mycoparasitic mechanisms of *Trichoderma* species include morphological adaptations such as coiling around the host and the development of antagonistic appressoria-like structures (Elad *et al*., 1983; Lu *et al*., 2004). All of these mechanisms often work synergistically for host damage and utilization of host components (Zeilinger & Omann, 2007).

According to Kubicek *et al*. (2001), the median lethal concentration (LC_{50}) values of *Trichoderma* chitinases are too high to explain mycoparasitism exclusively on the basis

of their action. However, this was clarified by the identification of antifungal peptides (peptaibols/mycotoxins) produced by *Trichoderma* species during interactions with host fungi (Kubicek *et al*., 2001). Peptaibols are linear oligopeptides, which form voltage-gated ion channels in lipid membranes thereby modifying membrane permeability (Kubicek *et al*., 2001; El Hajji *et al*., 1989). While chitinases reduce cell wall rigidity, peptaibol antibiotics impair the ability of the hyphae to repair cell wall damage by inhibiting the activities of membrane-bound synthases of the cell wall (figure 1.1) (Kubicek *et al*., 2001). Nonetheless, these factors do not exclude the participation of other mechanisms in the synergistic lysis of host cell wall.
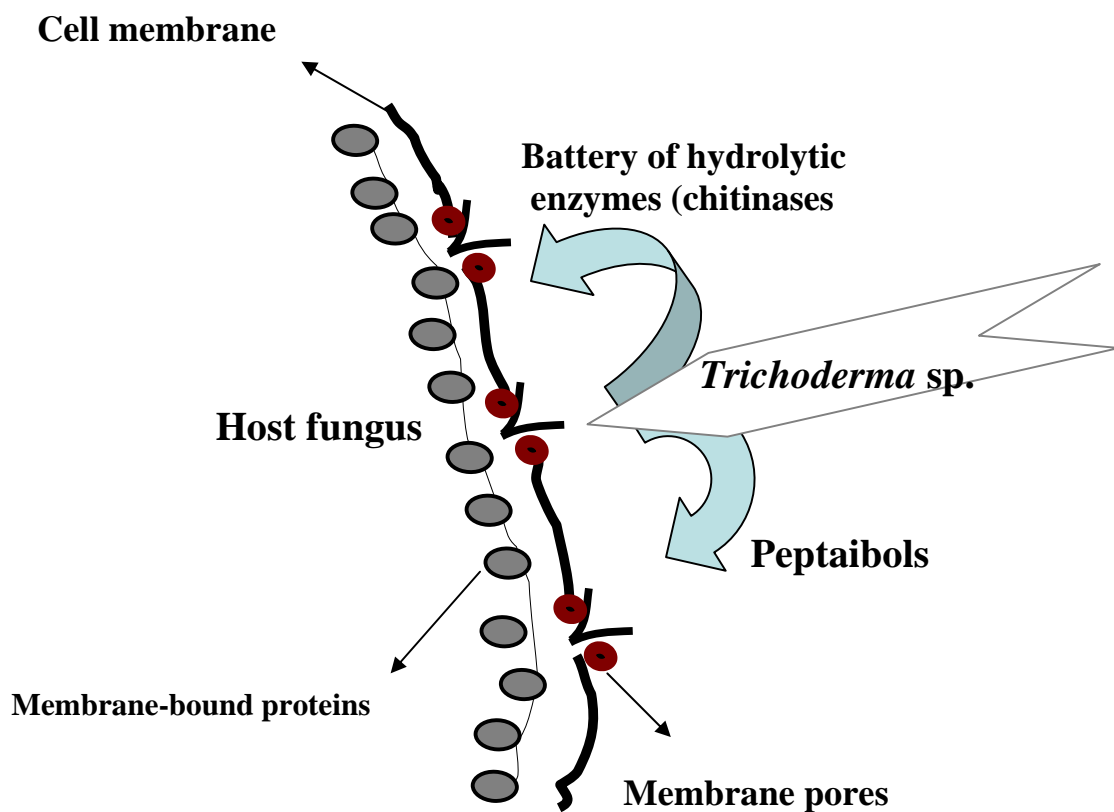
Fig 1.1 Hypothetical model of synergism between *Trichoderma* cell wall hydrolases and membrane disturbing compounds such as peptaibols employed in mycoparasitism (adapted from Kubicek *et al*., 2001).

The chitinases of *Trichoderma* species have been shown to be induced by stress conditions such as prolonged carbon and nitrogen starvation, acidic pH, cold temperature (4°C), high temperature (40°C), high osmotic pressure and the presence of ethanol (Mach *et al*., 1999; Margolles-Clark *et al*., 1996; Carsolio *et al*., 1994; Garcia *et al*., 1994). This suggests that contact with host fungi might impose physiological stress on the mycelia of *Trichoderma* species, eliciting the expression of the mycoparasitic machinery. The work of Inbar and Chet (1995) showed that *Trichoderma harzianum* produced different combinations of chitinase isozymes in response to different host/confronting species. This underscores the antagonistic adaptability of *Trichoderma* species, and could be attributed to variations in host cell wall composition and varying antagonistic pressures posed by different host species.

1.2.2 Gross mycelial contact

Gross mycelial contact occurs predominantly amongst wood decay fungi (Boddy, 2000; Webber & Hedger, 1986). As shown in figure 1.2, gross mycelial contact is characterized by morphogenetic and physiological changes, such as formation of mycelial barrages at the contact interface to stave off invasion, mycelial fans and cords, a shift in mycelial distribution, inter- and intracellular pigmentation, sealing-off of the mycelial front, release of hydrophobic metabolites and profound increase in the secretion of phenol-oxidases (Boddy, 2000; Griffith *et al*., 1994 a, b & c; Rayner *et al*., 1994; Gregorio *et al*., 2006; Peiris *et al*., 2007; Peiris, 2009). Incorporation of uncoupling agent, 2,4-dinitrophenol in the growth medium of unpaired fungal cultures, resulted in morphologies and metabolite profiles similar to interspecifically paired cultures (Griffith *et al*., 1994). However, addition of phenol-oxidase inhibitor to the unpaired cultures enhanced the release of metabolites (Griffith *et al*., 1994; Rayner, *et al*., 1994), suggesting that a relationship exists between mycelial morphologies, the extracellular chemistry and activity of phenol-oxidising enzymes during gross mycelial contact (Boddy, 2000; Rayner *et al*., 1995).

Outcomes of antagonistic interactions may be either replacement or deadlock (Boddy, 2000). Where the former occurs, one fungus invades the territory occupied by the other,

while in the latter, neither fungus is able to overgrow the other (Boddy, 2000; Owens, *et al.*, 1994). Partial replacement may occur, where one or both fungi make some initial incursion into the territories occupied by the other, followed by stalemate (Boddy, 2000). Replacement is largely influenced by the combative machinery available to one/both interacting species. This may include secretion of lytic enzymes, faster mycelial growth rate and more advanced nutrient acquisition mechanisms and/or production of biologically active compounds deleterious to the competitor(s). Repeated pairings between the same fungi do not always produce the same outcome (Boddy, 2000). For instance, pairing of *Peniophora lycii* with *Coriolus versicolor* in twenty replicates resulted in overgrowth of *P. lycii* by *C. versicolor* in two cases, while *P. lycii* replaced *C. versicolor* in the other pairings (Rayner *et al.*, 1995).

Gross mycelial interactions have been observed in leaf litter and soil between mycelial cord formers and fairy ring-forming basidiomycetes (figure 1.2) (Boddy, 2000). This manifests as brownish/yellowish pigmentation and lytic response in both cord formers and ring formers after contact (Boddy, 2000). Similar reactions have also been observed in woody materials evident as a variety of patterns of discolouration separated by interactions zone lines, which are often darker when decayed wood is sawed into sections (Boddy, 2000).

Although laboratory media have been extensively used for the study of fungal interactions, according to Boddy (2000), caution ought to be exercised in the interpretation of results from agar-based interaction studies. This is because a wider range of factors influences interactions in natural substrata; hence, outcomes of interactions in nature would most likely vary from those on agar (Dowson *et al.*, 1988; Griffith & Boddy, 1991; Pearce, 1990). Dowson *et al.* (1988) showed that whereas *Hypholoma fasciculare* replaced *Steccherinum fimbriatum* under ambient conditions on agar, it deadlocked with it in soil and was replaced by the latter in wood. Water activity, gaseous regimes, and to a lesser extent temperature, have been shown to have a sizeable effect on the outcomes of interactions in agar cultures (Griffith & Boddy, 1991; Boddy *et al.*,

1985; Boddy *et al.*, 1987). However, it is worth mentioning that different species are affected differently (Boddy, 2000).
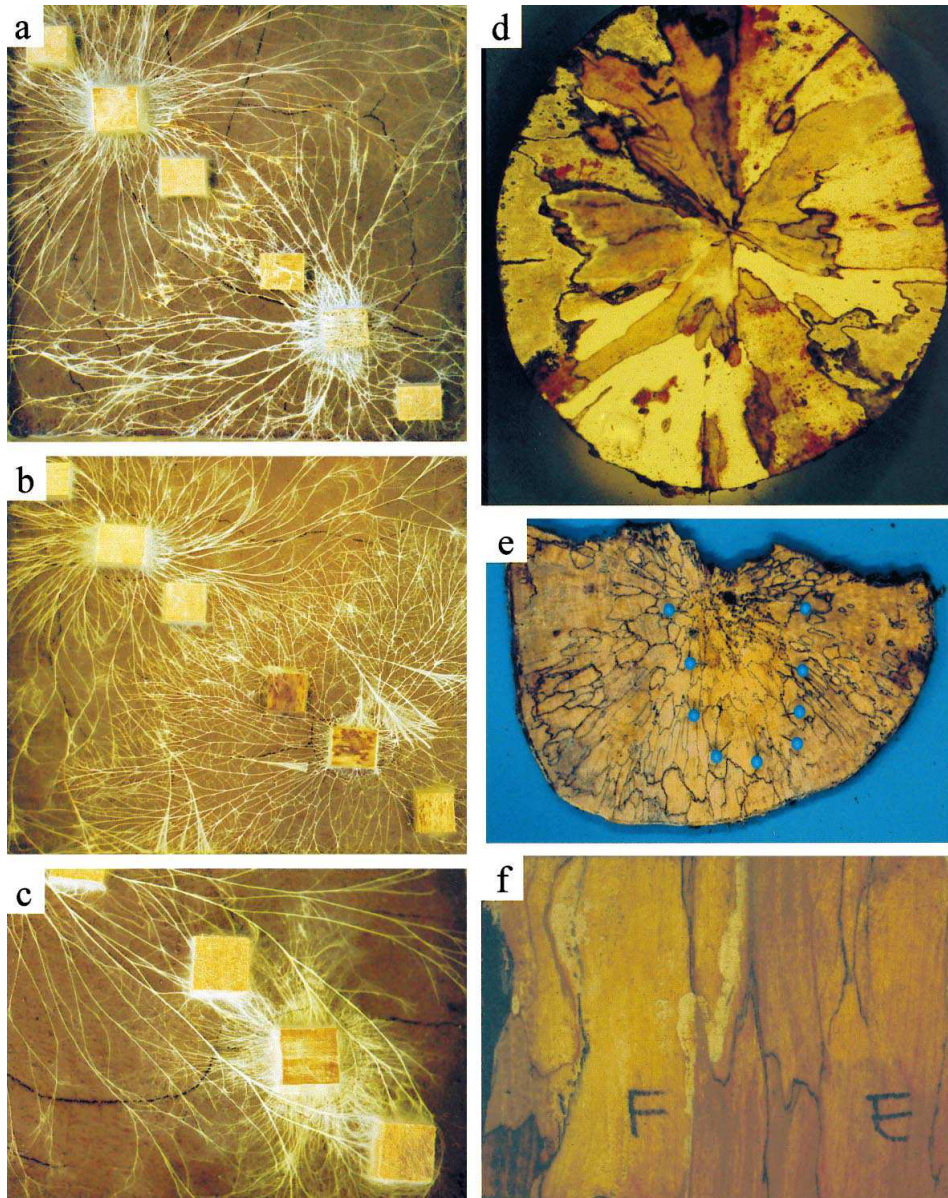
Figure 1.2 Macroscopic images of combative interactions in soil and wood. A-C: mycelial cord formers growing from wood block inocula on non-sterile soil at 12.5°C. D-F: transverse (d & e) and longitudinal (f) sections of sawed beech logs, four and a half years after felling, showing dark interaction zone lines demarcating different fungal individuals (adapted from Boddy, 2000).

1.3 The physiology, chemistry and biochemistry of combative interactions in fungi

1.3.1 The chemistry of combative interactions

Development of pigments in cultures of interspecifically paired fungi has been strongly attributed to increased synthesis and secretion of secondary metabolites (Rayner & Boddy, 1988; White & Boddy, 1992; Griffith *et al*., 1994 a, b & c; Rayner *et al*., 1994; Score *et al*., 1997; Peiris *et al*., 2008; Hynes *et al*., 2007; Peiris, 2009). Several workers demonstrated the increased expression of a wide range of secondary metabolites in paired fungal cultures (Ayer & Miao, 1993; Gloer, 1995; Humphris, 2001, Wheatly, 2002; Peiris *et al*., 2007; Peiris, 2009). Establishment of the fact that some of these metabolites exert antifungal activities, participate in pH regulation and adjustment of moisture content of the medium led to the assumption that they may play attack or defence roles during interactions (Woodward & Boddy, 2008).

Pigmentation during combative interactions is thought to be mainly an after effect of the interplay between phenolics and phenol-oxidase activities (Gregorio *et al*., 2006; Rayner *et al*., 1994; Griffith *et al*., 1994 a, b & c; Boddy, 2000; Peiris *et al*., 2008; Peiris, 2009). This assumption was strengthened by the work of Peiris *et al*. (2008), who reported a preponderance of metabolite peaks with mass spectra strongly indicating that they possess aromatic structures such as 1-methyl-3,5-dihydroxybenzene and 1,2-dihydroxyanthraquinone, potent inducers of laccase/manganese peroxidase, during combative interactions between *Stereum hirsutum* and its two competitors *Coprinus micaceus* and *Coprinus disseminatus*.

Furthermore, Hynes *et al*. (2007) reported synchronized detection of volatile organic compounds (VOCs) mainly sesquiterpenes (with benzene structures) and a quinolinium type compound and induction of pigment accumulation in paired cultures of *H. fasciculare* and *Resinicium bicolor*. These more recent studies lend more weight to the suggestion that interactions between phenolics and phenol-oxidases account for pigment production during mycelial conflict. Tsujiyama and Minami (2005) concluded that phenol-oxidizing enzymes play a central role in the detoxification of harmful compounds

during fungus-fungus interactions, as species with superior phenol-oxidase-secretory abilities in their study were more predominant during mycelial conflicts. Their assumption was reinforced by Hynes *et al*. (2007), who suggested that the outcomes of interactions may be greatly influenced by the ability of the interacting species to detoxify the opponent's "arsenal" of secondary metabolites.

Responses occurring before contact are likely to be mediated by volatile/diffusible compounds (Rayner *et al*., 1994; Rayner & Webster, 1985; Heilmann-Clausen & Boddy, 2005). This was well illustrated by the study of Heilmann-Clausen & Boddy (2005), who demonstrated the existence of a passive metabolite-based defence system in selected wood-rot fungi. However, Hynes *et al*. (2007) reported that VOCs production in paired cultures of *H. fasciculare* and *R. bicolor* was detected only after contact, negating possible existence of antagonism at a distance between these species. However, it is worth mentioning that the latter study was carried out in liquid culture, while Heilmann-Clausen & Boddy (2005) used a solid medium for their study. This variation in experimental design may have contributed to the discrepancy in the results of the two studies.

1.3.2 The enzymology of combative interactions

The ability of fungi to thrive in a broad range of environments/substrates is largely dependent on their highly developed enzyme secretory property. Although enzymes are central to the nutrient acquisition machinery of fungi, they also play accessory roles essential for survival. During intra- and interspecific interactions in fungi, enzymes have been strongly implicated in detoxification, attack/defence and nutrient acquisition functions that influence the outcome of competition (Savoie *et al*., 1998; Freitag & Morrell, 1991; White & Boddy, 1992; Rayner *et al*., 1994; Griffith *et al*., 1994a, b, & c; Score *et al*., 1997; Cortes *et al*., 1998; Zeilinger *et al*., 1999 ; Boddy, 2000; Iakovlev & Stenlid, 2000; Kubicek *et al*., 2001; Brunner *et al*., 2003; Tsujiyama & Minami, 2005; Adomas, *et al*., 2006; Gregorio *et al*., 2006; Chi *et al*., 2006; Zhang *et al*., 2006; Peiris, 2009). Phenol-oxidases are perhaps the most studied enzymes in connection with interspecific antagonistic interactions between fungal species (Freitag & Morrell, 1991;

White & Boddy, 2992; Rayner *et al*., 1994; Griffith *et al*., 1994a, b, & c; Score *et al*., 1997; Boddy, 2000; Iakovlev & Stenlid, 2000; Tsujiyama & Minami, 2005; Gregorio *et al*., 2006; Chi *et al*., 2006; Zhang *et al*., 2006; Peiris, 2009). Although they are known to be involved in the degradation of lignin and/or detoxification of products of lignin breakdown, they have also been shown to be involved in pigmentation and sporulation (Thurston, 1994). In addition, their ability to detoxify a wide range of xenobiotics *in vitro*, particularly those with the phenolic/aromatic structures with subsequent production of pigments/discolouration (Thurston, 1994; Reinhammer & Malstrom, 1981; Guillen, *et al*., 1994; Eggert *et al*., 1996) has strengthened the assumption that their major role during fungal interactions is to oxidise toxic compounds produced by the same or confronting species during mycelial conflict (Rayner *et al*., 1994; Griffith *et al*., 1994a, b, & c; White and Boddy, 1992; Tsujiyama and Minami, 2005; Gregorio *et al*., 2006).

Gregorio *et al*. (2006) reported a rapid rise in the activities of extracellular laccase and manganese peroxidase when liquid cultures of *Marasmiellus troyanus* and *Marasmius pallescens* were mixed. More interestingly, they also reported a more rapid increase in the activities of these enzymes when filter-sterilized culture broth of *M. pallescens* was added to growing cultures of *M. troyanus*. The authors ascribed this to phenol-oxidase inducers/substrates secreted into the culture broth by the former. Another interesting implication of this finding is that such compounds were secreted passively (without mixing or confrontation from another fungus) thus, supporting the work of Heilmann-Clausen and Boddy (2005) which demonstrated the existence of a passive defence mechanism in studied fungi. Freitag and Morrell (1991) also showed that laccase activity increased when liquid cultures of *Trametes versicolor* and *Trichoderma harzianum* were mixed. According to Tsujiyama and Minami (2005), phenol-oxidase activity was detected predominantly in the confrontation zones and underneath mycelia overgrowing another fungus during inter-specific combat involving *Pleurotus sotreatus*, *Trametes versicolor*, *Pynoporus occineus*, *Ganoderma applantum* and *Schizophyllum commune* on agar. In this study, species that were more efficient at phenol-oxidase secretion were more successful at overgrowing less efficient ones.

Cellulases, chitinase, proteases, and glucanases have been implicated in antagonistic interactions as well (Elad *et al*., 1982; Freitag and Morrell, 1991; Zeilinger *et al*., 1999; Kubicek *et al*., 2001; Brunner *et al*., 2003; Howell, 2003;). They are recruited mainly by mycoparasitic *Trichoderma* species for the lysis and subsequent utilization of their host's components. Proteolytic activity is a requirement for complete lysis of fungal cells (Scott & Schekman, 1980; Andrews *et al*., 1987). This is because the key components of fungal call wall, chitin and/or fibrils of β-glucan are embedded into a protein matrix (Wessels, 1986; Peberdy, 1990). In addition to the production of chitinases, *Trichoderma harzianum* also secretes an alkaline protease Prb1 among its mycoparasitic repertoire (Markovich & Kononova, 2003). Furthermore, there are indications that proteases produced by *Trichoderma* species may be involved in the inactivation of vital extracellular enzymes of their host species, especially those involved in nutrient hydrolysis and absorption (Elad, *et al*., 1999).

Adomas *et al*. (2006) studied gene expression in the biocontrol fungus *Phlebiopsis gigantea* as it overgrew the phytopathogen *Heterobasidion parviporum* in a plate confrontation assay. Profiling by cDNA macroarray showed the up-regulation of genes encoding intracellular enzymes involved in varied functions such as protein folding, stress response and virulence (cyclophilin, heat shock protein 90), toxin detoxification (cytochrome p450 mono-oxygenase) and nutrient metabolism (fructose biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, glutamine synthetase, endo-galacturonase). Other functions included, transport (vacuolar ATPase), protein degradation/stress response (ubiquitin) and ribosomal proteins (protein synthesis) (Adomas *et al*., 2006). The gene expression profile observed in this study (Adomas *et al*., 2006) suggests that the encoded enzymes may have been up-regulated in the invading fungus, thereby implicating stress response/tolerance, detoxification, transport, protein synthesis/folding, protein degradation and pronounced nutrient acquisition and processing in the antagonistic machinery of *P. gigantea*.

1.3.3 The physiological consequences of interspecific combative interactions

A switch of mycelial growth to secondary metabolism has been suggested as one of the major consequences of non-self fungus-fungus interactions (Rayner *et al*., 1994; Griffith *et al*., 1994a, b &c; Rayner *et al*., 1995; Rayner, 1997). Disruption of resource supply by nutrient limitation, impaired ability to absorb and/or process nutrients is an established cue for the inception of secondary metabolism (Rayner *et al*., 1995). Such disruption might result in drop in energy charge, with a corresponding rise in the levels of the secondary messenger, cAMP (Rayner *et al*., 1995). The ensuing physiological and metabolic effects include increased production of secondary metabolites such as phenolics, inhibition/disrutption of ATP synthesis, probably by uncoupling oxidative phosphorylation and electron transport with a subsequent rise in the levels of free radicals (Rayner *et al*., 1995). Lyr (1958; 1963) reported the evidence of reciprocal correlation between oxidative phosphorylation and the activity of phenoloxidases. This perhaps explains why the exposure of single fungal species to the uncoupling agent 2,4-dinitrophenol resulted in the same reactions as mycelia confronted by another species (Griffith *et al*., 1994; Rayner *et al*., 1994). Based on these observations, it could be inferred that non-self mycelial interactions have a significant impact on nutrient supply/uptake/processing, thereby stimulating a switch of mycelial growth to secondary metabolism, with pivotal effects on the energy generation machinery of the mitochondrion (Rayner *et al*., 1995).

These assumptions were supported by the work of Iakovlev *et al*. (2004) who, using differential display of mRNA, reported the repression of a gene encoding a fimbrin protein and a gene that codes for a mitochondrial import component in the mycelia of *Physisporinus sanguinolentus*, and up-regulation of a gene homologous to the *Coprinus cinereus* (*Coprinopsis cinerues) recA* in *Heterobasidion annosum* during antagonistic interactions between both species. Fimbrin protein is involved in the polarization of the actin cytoskeleton, hence, its repression in *P. sanguinolentus* was an indication of arrest of cell growth (Iakovlev *et al*., 2004). Furthermore, Iakovlev *et al*. (2004) suggested that the repression of a gene that codes for a protein involved in mitochondrial import could be associated with changes in mitochondrial energy machinery, synonymous with

secondary metabolism. *recA* codes for a multifunctional RAD51 protein (enzyme) which participates in DNA repair, stress response and homologous recombination (Halliwell & Gutteridge, 1989; Stassen *et al*., 1997; Game, 1993; Bishop, 1994; Nathan & Shiloh, 2000). In *C. cinereus* (Stassen *et al*., 1997) and *S. cerevisiae* (Mikosch *et al*., 2001) RAD51 is strongly induced by γ-irradiation, a potent inducer of hydroxyl radicals that cause severe damage to DNA (Iakovlev *et al*., 2004). Based on this, the authors attributed the up-regulation of this gene to possible increase in the production of free radicals during interspecific mycelial interactions. Other studies have implicated strong production of reactive oxygen species (ROS) in non-self fungus-fungus interactions (Baker & Orlandi, 1995; Li *et al*., 1995; Ruiz-Dueñas *et al*., 1999; Hammel, *et al*., 2002). It appears production of ROS is part of the stress exerted on interacting fungal mycelia, either as an aftermath of interspecific mycelial contact or as a result of the induction of secondary metabolism.

1.4 Application aspects of interspecific antagonistic fungal interactions

1.4.1 Screening for novel metabolites, and increasing the production of known active secondary metabolites

Fungi, including the higher fungi have demonstrated abilities to produce a broad range of metabolites with potentials for application as antimicrobial, antitumour, and antiviral agents as well as agrochemicals (Anke, 1995; Yun *et al*., 2002; Chu, 2003; Rosa *et al*., 2005; Silberboth, 2005; Kettering, 2005). For instance, ethyl acetate extract from *Oudemansiella canarii* grown in malt extract exhibited strong activity against tumour cells and trypanothine reductase from *Trypanosoma cruzi*, with very low activity against peripheral blood mononuclear cells from healthy adult males and females, suggesting low toxicity to normal human cells (Rosa *et al*., 2005). The illudins, a group of fungal, predominantly basidiomycete sesquiterpenes, have been studied extensively for several decades for their antitumour and antibacterial properties (Anchel *et al*., 1950). Gonzalez Del Val *et al*. (2003) isolated novel illudin-like compounds, although appreciably cytotoxic, showed high antibacterial activity from ten basidiomycetes grown on a rice-based solid medium. Twenty percent of the isolated compounds in the study above were strongly active against methicillin resistant *Staphylococcus aureus*. In addition, pilatin, a

marasmane derivative isolated from fermentations of *Flagellocypha pilatii* exhibited antifungal and antibacterial activities, at concentrations as low as 5-50 µg/ml (Anke, 1989; Kupka *et al*., 1983). Furthermore, some fungal sesquiterpenes have been shown to exhibit a level of antifungal activity (Florianowicz, 2000; Abraham, 2001; Roy *et al*., 2003), and VOCs from fungi have been considered as potential wood preservatives (Schoeman *et al*., 1996; Wheatley *et al*., 1997).

With the declining availability of antimicrobials and potent agrochemicals and the promises of microbial metabolites as antitumour agents, against a backdrop of poor results from synthetic chemistry-driven screening approaches, the near limitless wealth of natural products remain strongly attractive in the search for novel therapeutic agents and agrochemicals (Anke, 1989). More importantly, the barely screened higher fungi hold high promises as sources of novel exploitable chemical structures (Anke, 1989). However, from an economic standpoint, the slow growth rate and low titres of metabolites from higher fungi have made them less appealing for screening projects so far. Nevertheless, certain growth conditions and stress related responses can enhance the synthesis and secretion of metabolites by basidiomycetes and fungi of other classes. Studies of fungal interactions over the last three decades have revealed the induction of secondary metabolites and volatile organic compounds; (A) to defend colonised territories without the presence of competing species, (B) in response to confronting species before contact and (C) during gross mycelial contact. These three levels of metabolite secretion elucidated via studies of antagonistic interactions could contribute to the screening for novel secondary metabolites with commercial potential.

The inhibition of mycelial extension by wood pre-decayed by *S. hirsutum* (Heilmann-Clause & Boddy, 2005) is a strong indication of the secretion of secondary metabolites with potential for drug development. More importantly, the stress arising from interspecific confrontations before (exchange of chemical signals) and after (induction of secondary metabolism) contact has been shown to modulate fungal physiological machinery with consequent increase in the secretion of metabolites for offensive/defensive purposes (Gloer, 1995; Hynes *et al*., 2007; Strunz *et al*., 1972).

Apparently, these reactions could mitigate the economic cost of higher fungi-based screening, from the slow growth rate of higher fungi and could increase the levels of metabolite secretion for further analytical work. Although, industrial applications of interspecific interactions for full-blown metabolite production remain subject to further scrutiny, they have potentials for the discovery of novel compounds/structures, which could be taken through further drug development in combination with synthetic chemistry.

For instance, Whyte *et al*. (1996) identified novel isocoumarins and their derivatives in combination with a known compound (roridin E) as the main active ingredients behind the strong antagonistic properties of *Cercophora areolata*. More importantly, these compounds also exhibited strong activities against pathogens such as, *Candida albicans*, *Bacillus subtilis* and *Staphylococcus aureus*. Furthermore, studies of interspecific interactions between coprophilous fungi led to the identification of antifungal metabolites responsible for the inhibition of potential invading species (Webber, 1988; Gloer & Trunkenbrod, 1988; Alfatafta & Gloer, 1994). It has been shown that *H. annosum* up-regulates secondary metabolites when paired against the antagonist *Gleophyllum abientineum*, some of which have been shown to be active against bacterial and fungal species (Sonnenbichler *et al*., 1989). Evans (2000) and Gregorio (2007) also reported the induction of bioactive metabolites during interactions involving *Marasmius pallescens*, *Marasmiellus troyanus* and *Oudemansiella canarii* in liquid cultures. Peiris (2009) demonstrated the induction of a metabolite 2,3-dihydroxy-6-methyl benzoic acid; with a potentially novel structure similar to orselinic acid, in cultures of *S. hirsutum* confronted by *C. disseminatus*. Although this compound did not exert any antimicrobial activities against the tested bacteria and fungi, its production during interspecific combat underscores the potential of interspecific mycelial combat for the discovery of novels chemical structures, which may find some direct applications or serve as scaffolds for drug/agrochemical development.

1.4.2 Biological control

Increased concern over the environmental hazards of chemical biocides coupled with the restrictive legislation associated with their use in recent years has lead to a radical shift to the use of more environmentally friendly microorganisms in place of chemical preservatives (Bruce & Highley, 1991). Fungi including *Trichoderma* species, *Phlebiopsis gigantea*, and *Gliocladium virens* have shown strong antagonistic properties against phytopathogenic and wood decay fungi, making them prime candidates for the control of plant diseases and rot in commercial logging (Bruce *et al*., 1995; Boddy, 2000; Kubicek *et al*., 2001; Adomas *et al*., 2006;). A strain of *P. gigantea* isolated by Korhonen (1993) is commercially used in the Scandinavia (Rostop®, product of Verdera Oy, Espoo, Finland), Poland (PG IBL® from Biofood s.c., Walcz, Poland) and Great Britain (PG® suspension made by Forest Research Surrey, UK) for the control of *Heterobasidion* species in spruce and pine stumps (Adomas *et al*., 2006).

Species of *Trichoderma* are applied commercially as biocontrol agents against phytopathogenic fungi (Chet, 1987; Harman & Björkman, 1998; Chet *et al*., 1998). *Trichoderma* species have also been explored for the prevention of wood decay with varying degrees of protection achieved, but not in all cases (Highley & Ricard, 1988; Bruce & Highley, 1991; Bruce *et al*., 1995; Boddy, 2000). *Trichoderma* species have also been suggested for the control of dry rot in buildings caused by *Serpula lacrimans* (Score *et al*., 1998).

Hypholoma australe and *Phanerochaete filamentosa* have been shown to be successful against *Armillaria luteobubalina* plant infection in Australia (Pearce *et al*., 1995). *Bjerkandera adusta*, *Hypholoma fasciculare*, *Resinicium bicolor*, and *Hirschioporus abietinus* all exhibit strong antagonistic properties indicating that they could be employed for biological control (Boddy, 2000).

1.4.3 Enhanced enzyme production

Interspecific antagonistic interactions in fungi have been repeatedly shown to enhance the production of extracellular enzymes such as chitinase, xylanase, glucanases, cellulase,

and phenol-oxidases sometimes to levels induced by enzyme substrates (Freitag & Morrell, 1991; White & Boddy, 1992; Rayner *et al*., 1994; Griffith *et al*., 1994a, b, & c; Score *et al*., 1997; Zeilinger *et al*., 1999 ; Boddy, 2000; Kubicek *et al*., 2001; Tsujiyama & Minami, 2005; Gregorio *et al*., 2006; Zhang *et al*., 2006; Peiris, 2009). This suggests that combinatorial fungal culturing might be employed as a cheap traditional means of increasing enzyme production. Lignocellulolytic enzymes attract an appreciable degree of research attention for applications in bioremediation, food, paper and textile industries and wine making among several uses (Savoie *et al*., 1998; Hatvani *et al*., 2002; Chi *et al*., 2006). In view of this, cheap methods of enzyme production would drive down the cost of production as well as harness the applications of these enzymes in biotechnology.

The study of Gregorio *et al*. (2006) showed that factors in filter-sterilized broth of *Marasmius pallescens* elicited increased laccase and manganese peroxidase activities in liquid cultures of *Marasmiellus troyanus*. It is likely that the reported increase in activity might be as a result of increase enzyme synthesis and secretion. Furthermore, such factors where identified could serve as cheaper elicitors for enzyme production compared to more expensive synthetic inducers (substrates). Similarly, high performance liquid chromatography-purified low molecular weight molecules from the cell wall of host fungi have been shown to increase the secretion of chitinolytic and related enzymes of *Trichoderma* species in liquid cultures than purified chitin and glucan monomers/oligomers (Kubicek *et al*., 2001). Zhang *et al*. (2006) reported significant increase in laccase activity and protein levels when liquid cultures of the white-rot fungus *Trametes* sp. AH28-2 and a biocontrol fungus *Trichoderma* sp. ZH1 were mixed. Laccase activity and levels remained significantly high for 15 days after mixing. Interestingly, mixing both cultures resulted in the induction of a novel 64kDa laccase isozyme (LacC), with a broad substrate range, typical of laccases, but showed thermal stability by retaining activity at 60°C and a wide pH range of 4.0 – 8.0; attributes that make it more amenable to industrial application (Zhang *et al*., 2006).

1.4.4 Wood pulping for paper production (biopulping)

Wood pulping is traditionally carried out by the incubation of wood chips with a single selected fungal species for about two weeks (Akhtar *et al*., 1998). The duration hence, cost of this traditional procedure warrants the need for more cost-effective methods with high-energy savings and improved lignin removal, resulting in paper with improved fibrous characteristics (Chi *et al*., 2006). Co-fungal cultures (biopulping) have been suggested as a cheaper alternative to the existing method (Hatakka *et al*., 2001; Chi *et al*., 2006). This stems from the vast body of evidence demonstrating that fungal competition often leads to improved lignin degradation (Sundman and Näse, 1972; Asiegbu *et al*., 1996) and increased secretion of phenol-oxidising enzymes (Baldrian, 2004; Iakovlev & Stenlid, 2000; White & Boddy, 1992; Score *et al*., 1997). Chi *et al*. (2006) showed that co-culturing involving *Ceriporiopsis submervispora*, *Physisporinus rivulosus*, *Phanerochaete chrysosporium* and *Pleurotus ostreatus* in different combinations resulted in altered patterns of lignin degradation with increased removal of more recalcitrant part of lignin although there was no significant improvement in total lignin removal. However, this study suggests that combinations of fungal cultures might enhance the removal of recalcitrant components of lignin thereby enhancing the quality of the resulting paper at lower cost (shorter time).

1.5 Aims of the investigation

Despite extensive studies of antagonistic interactions in fungi, the underlying molecular and cellular mechanisms and adaptations to non-self mycelia are still subject to better understanding, thereby requiring further investigations. The major aim of this study was to endeavour to understand the underlying cellular and molecular response patterns and adaptations of a white-rot, fungus *Schizophyllum commune* to a highly antagonistic strain of *Trichoderma viride*, by correlating the expression patterns of metabolites, proteins and selected genes in response to the antagonist. The study also investigated the implication of oxidative damage in these response patterns. *S. commune*, which has been studied extensively, was chosen a model white-rot basidiomycete for this study, given that expressed sequence tags (ESTs) and some full-length gene sequences of this white-rot fungus are available on the National Centre for Biotechnology Information (NCBI)

database. This particularly allowed the design of specific primers for reverse-transcription-polymerase chains reaction (RT-PCR). In addition, white-rot fungi have been reported to pose greater resistance to Trichoderma species particularly in the field (Bruce & Highley, 1991). In view of this, the relative resistance of white-rot fungi to *Trichoderma* species informed the choice of *S. commune*, in the bid to further understand the biochemical/molecular processes that may be responsible for such resistance. On the other hand, *T. viride* was chosen because of the strong antagonistic properties of *Trichoderma* species. The study was divided into the following sections:

1. Macro- and microscopic investigation of the morphological changes in the mycelia of *S. commune* confronted by *T. viride*.
2. Establishment of changes (up-/down-regulation) in the metabolomes of both species paired against each other in comparison to their self-paired cultures.
3. To make comparative measurements of indicators of the induction of oxidative stress and secondary metabolism in *S. commune* paired against *T. viride* relative self-paired mycelia of *S. commune*.
4. To make comparative measurements of the activities/levels of selected extra- and intracellular enzymes of *S. commune* interacting with *T. viride* and in its self-paired cultures.
5. Investigation of the mRNA levels of selected genes of *S. commune* interacting with *T. viride* in comparison to expression levels in self-paired and unpaired mycelia of the former.
6. Comparative profiling of the proteomes of both fungi in response to each other relative to their self-paired cultures.
7. Analytical correlation of morphological changes, levels of oxidative stress indicators, levels and activities of selected enzymes; protein, gene and metabolite expression patterns.

It is anticipated that data from the different aspects of this work (1-7), would shed more light on the cellular and molecular aspects of the behaviour of fungi during antagonistic interactions, particularly in response to a highly antagonistic competitor.

CHAPTER 2

Morphological changes in the mycelia of
S.   commune **confronted by** *T. viride*

2.1 Introduction

The early stages of fungal colonisation of organic materials are often characterised by the development of distinct domains occupied by individuals of fungal species (Boddy, 2000). However, mycelial extension over a prolonged period of colonisation results in overlap between domains, which leads to mycelial interactions between the colonising species (Boddy, 2000). Contact between different species on the same substrate triggers profound changes in mycelial morphology, and these changes very often promote resource capture, attack, or defence against the opposing species. An example of morphological change during fungus-fungus interaction is hyphal interference. A programmed string of cytoplasmic disintegration and subsequent cell death in the mycelia of interacting species occur during hyphal interference (Boddy, 2000). This is mainly evident in interactions involving basidiomycetes, and has been most extensively studied between *Heterobasidion annosum* and *Phlebiopsis gigantea* because of the potential of the latter for biocontrol of *H. annosum* (Rayner & Boddy, 1988; Boddy, 2000). It is thought that non-enzymic diffusible metabolites or toxins mediate hyphal interference, as indicated in the case of interactions involving *Hypomyces aurantius*, which produces non-enzymic toxins in both the presence and absence of confronting species (Boddy, 2000).

Mycoparasitism is another aspect of fungal interspecific interactions during which strong morphological changes occur in the antagonist. This is typified by profuse hyphal extensions towards host mycelia, development of specialised parasitic structures such as appressoria, and aggressive coiling around host hyphae. *Trichoderma* species are the most studied mycoparasites because of their potential in the biological control of plant pathogens (Howell, 1987; Boddy, 2000; Chet, 1987).

Fungus-fungus interactions can also take the form of gross mycelial contact predominantly among wood-decaying fungi, and are characterised by pronounced morphological changes (figure 2.1) (Boddy, 2000). For instance, on rich laboratory agar media, morphological modifications following mycelial contact often take the shape of profuse development of dense aerial hyphae, which form a tuft at the interaction interface

most commonly referred to as "barrage" (figure 2.1) (Boddy, 2000; Griffith *et al*., 1994; Peiris *et al*., 2007; Adomas *et al*., 2006; Peiris, 2009). Barrages serve as physical barrier against invasion by the opposing fungal species (Boddy, 2000). Other morphological changes associated with gross mycelial contact include the formation of invasive mycelial fronts, mycelial fans, and linear structures – cords and rhizomorphic structures (Boddy, 2000). Gross mycelial contact can lead to a redistribution of mycelium, such that mycelial density decreases within a domain away from the contact interface (Boddy, 2000). This leaves a fungus prone to invasion by the opposing species during interaction if the barrage is surmounted, and this phenomenon has been observed in decaying wood (Boddy, 2000).

Mycelial contact is most commonly accompanied by pigmentation resulting in yellowish/brownish to dark discolouration of mycelia, which spreads inwards from the contact interface into the domain occupied by individual species (Rayner & Webber, 1984; Rayner & Boddy, 1988; Rayner *et al*., 1994; Peiris *et al*., 2007; Gregorio *et al*., 2006; Peiris, 2009). Other morphological changes elicited by interspecific mycelial combat include vacuolation, swelling of hyphal filaments and protoplasmic degeneration; pronounced invagination of plasmalemma, loss of recognisable mitochondria, and appearance of dense intracellular streak-like aggregates within the cytoplasm (Gregorio *et al*., 2006; Iakovlev & Stenlid, 2000; Ikediugwu, 1976).

Morphological changes arising from interspecific mycelial contact vary according to the interacting species, depending on their antagonistic properties. In this chapter, morphological changes in the mycelia of *S. commune* resulting from confrontation by *T. viride* are presented. Both species were paired on potato dextrose agar (PDA) for a maximum of 7 days and observed both macroscopically and microscopically. Stained and unstained preparations were viewed by phase contrast and light microscopy. For stained preparations, Nile Red, Congo Red and Evans Blue stains were used for sample staining before microscopy. In addition, Calcium Ionophore a potent transporter of calcium across membranes was incorporated in PDA prior to inoculation of self-interacting *S. commune*

cultures to assay for possible effect of calcium influx on pigmentation and perhaps laccase secretion.
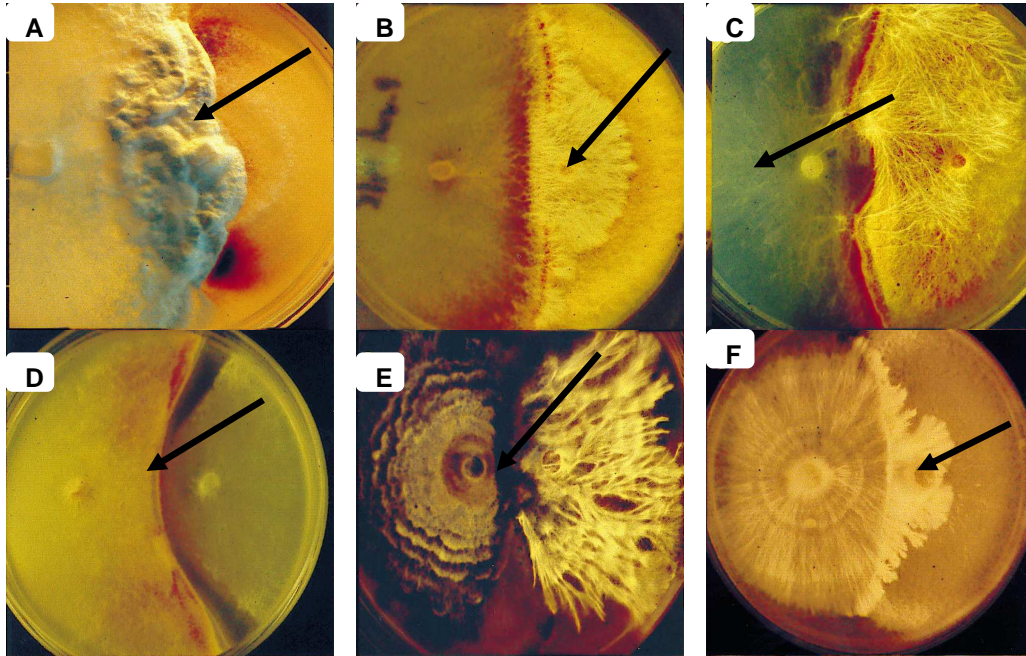
Figure 2.1 Agar plate images showing macroscopic morphological changes associated with interspecific mycelial interactions in fungi (adapted from Boddy, 2000). A: development of invasive mycelial front by *Phanerochaete velutina* replacing *Stereum hirsutum* at 25 °C; B: formation of mycelial fans by *P. velutina* replacing *S. hirsutum* at 10 °C; C: reduction of mycelial density by *Hypholoma fasciculare* replacing *Coriolus versicolor*; D: Complete replacement of *Armillaria gallica* by the ascomycete, *Xylaria hypoxylon*; E: mycelial interference of *P velutina* by *Resinicium bicolor* on soil; F: replacement of *S. hirsutum* by *Psathyrella hydrophillum*.

2.2 Materials and Methods

2.2.1 Agar plate interaction assay

S. commune was obtained from the culture collection of the School of Life Sciences, University of Westminster. *T. viride* was isolated from straw materials collected from a garden in Surrey (UK). Polymerase chain reaction using primers for the

5.8SrRNA/28SrRNA gene of *T. viride* was used to confirm the identity of the isolate (see VI Appendix). Self and non-self interaction assays were set up on PDA in 9 cm diameter Petri dishes following slight modifications of the methods described by Iakovlev and Stenlid (2000) and Iakovlev *et al*. (2004). 0.5 cm diameter mycelial plugs cut off behind the tip of extending mycelia of pre-grown cultures were used for inoculation. *S. commune* was inoculated 4 days prior to the inoculation of *T. viride* because of the latter's very fast growth rate. Mycelial plugs of *T. viride* were placed 35 mm away from the spreading mycelia of *S. commune* on day 4 (after the inoculation of *S. commune*) (figure 2.2). All cultures were incubated upside-down in the dark at 28°C. The sample zones represent points 10 mm from the interaction interface from where samples were taken for staining before microscopy, for metabolite, ribonucleic acid (RNA) and protein extraction (chapters 3, 6 and 7 respectively). The same agar plate assay protocol was used for subsequent experiments. For biochemical assays, RNA extraction and proteomics (Chapters 4, 5, 6 and 7 respectively), multiple agar plate assays (20 plates each, for test and control cultures), were set up for each experiment out of which 3 or 5 (depending on the experiment) of the most uniform plates, based on the reactions patterns on agar, were selected as replicas for analyses. For metabolomic experiments, 10 replicas were selected for each of test and control batches.

2.2.2 Incorporation of calcium ionophore in self-paired cultures of *S. commune*

Calcium ionophore A23187 (Sigma-Aldrich, UK) was incorporated in PDA prior to the inoculation of *S. commune* (self-paired) to a final concentration of 6 μM. Calcium ionophore A23187 was pre-dissolved in dimethyl sulfoxide before addition to and sterilization of PDA. An equal amount of DMSO was added to control cultures.
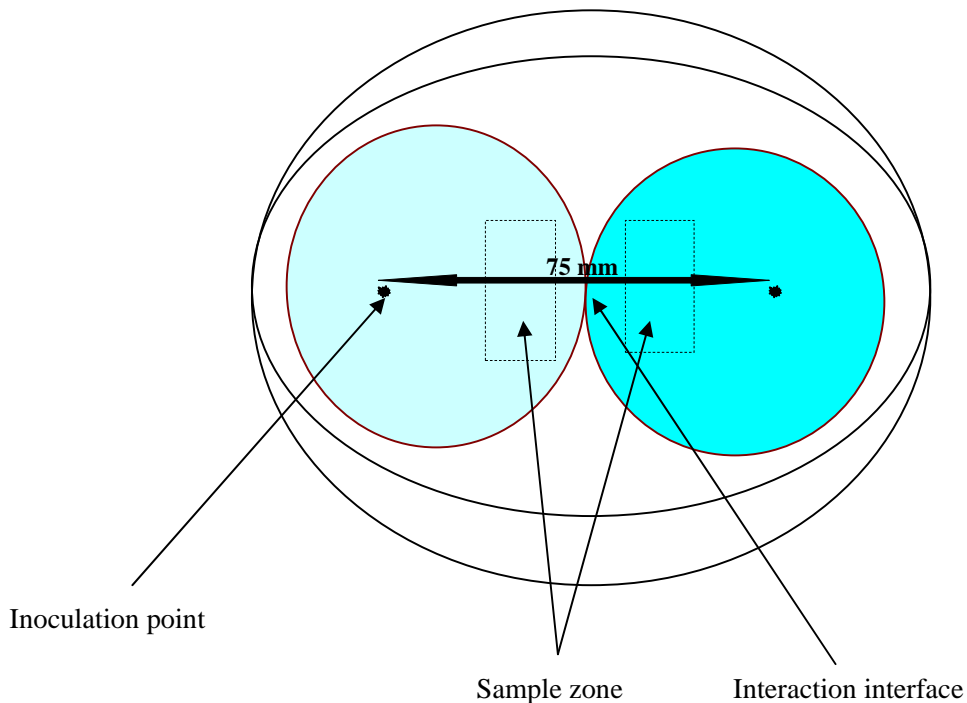
Figure 2.2 Schematic representation of an agar plate interaction assay between self and non-self cultures of *S. commune* and *T. viride* in 9 cm Petri dishes.

2.2.3 Microscopy

Both stained and unstained preparations covered with cover slip were viewed with a x100 (oil immersion) objective lens on a Leica DM microscope (Leica, Germany). For unstained preparations, the interactions zones of whole cultures in Petri dishes were viewed by phase contrast microscopy, while stained preparations were viewed using fluorescence microscopy. Images were acquired with a Leica camera and LAZ-EZ software (Leica, Germany).

2.2.3.1 Staining with Nile Red

Staining with Nile red was performed according to the method described by Kimura *et al.* (2003). *S. commune* mycelial samples cut off with scalpel behind the interaction zone,

were flooded with 700 µl of Nile red (0.3 µg/ml) in dimethyl sulfoxide (DMSO) for 5 mins. The samples were rinsed quickly with sterile distilled water and blotted dry with a paper napkin. Cover slips were placed over each sample and slides were viewed as described above.

2.2.3.2 Congo Red staining

S. commune mycelia excised with scalpel behind the contact zone were covered with 500 µl of 0.1% Congo Red solution in distilled water for 2 mins (Sifkin & Cumbie, 1988). Samples were washed with distilled water, blotted dry before covering with cover slip and viewed microscopically as previously described.

2.2.3.3 Evans Blue staining

Mycelia cut off behind the interaction zone were stained as described by Semighini *et al*. (2006). 500 µl of 1% Evans blue dye in phosphate buffered saline (PBS – pH 6) was applied mycelial samples for 2 mins, rinsed three times with PBS and blotted dry before covering with cover slip for microscopy.

2.3 Results

2.3.1 Morphological changes in the mycelia of *S. commune* interacting with *T. viride* on agar

Mycelial reactions characterised by slowed rate of mycelial extension and reduced mycelial density at the spreading front were observed mainly in *S. commune* before contact between both species (figure 2.4A). This was followed by sealing-off of the mycelial front by *S. commune* within 24 hours of contact with *T. viride* (figure 2.4B), 24 hours post contact, *S. commune* developed a barrage in the contact zone, most likely to resist the spreading mycelial front of *T. viride* (figure 2.4C), with concomitant development of a brownish pigment along the line of contact. Pigmentation spread away from the contact zone further into the domain occupied by *S. commune* and increased in intensity with increasing duration of contact (figure 2.4 D, E, F). *S. commune* was overgrown by *T. viride* after 120 hours of mycelial interaction between both species

2.3.1 Effects of Calcium ionophore A23187 on the morphology of *S. commune*

Mycelia of *S. commune* growing on PDA containing 6 μM calcium ionophore A23187 produced brownish pigmentation, similar to pigmentation in mycelia confronted by *T. viride*. Pigmentation began to appear on the underside of the culture plates after 48 hours of mycelial growth and intensified with time. Contrary to the patterns in co-cultures of *S. commune* and *T. viride*, where pigmentation emanated from the contact zone, pigmentation in calcium ionophore-containing cultures began to appear around the points of inoculation, spreading outwards towards the contact area (figure 2.5). In addition, whereas pigmentation on confrontation plates was predominantly visible at the undersides of the plates, it was visible on both sides of the plates in calcium ionophore-containing cultures.
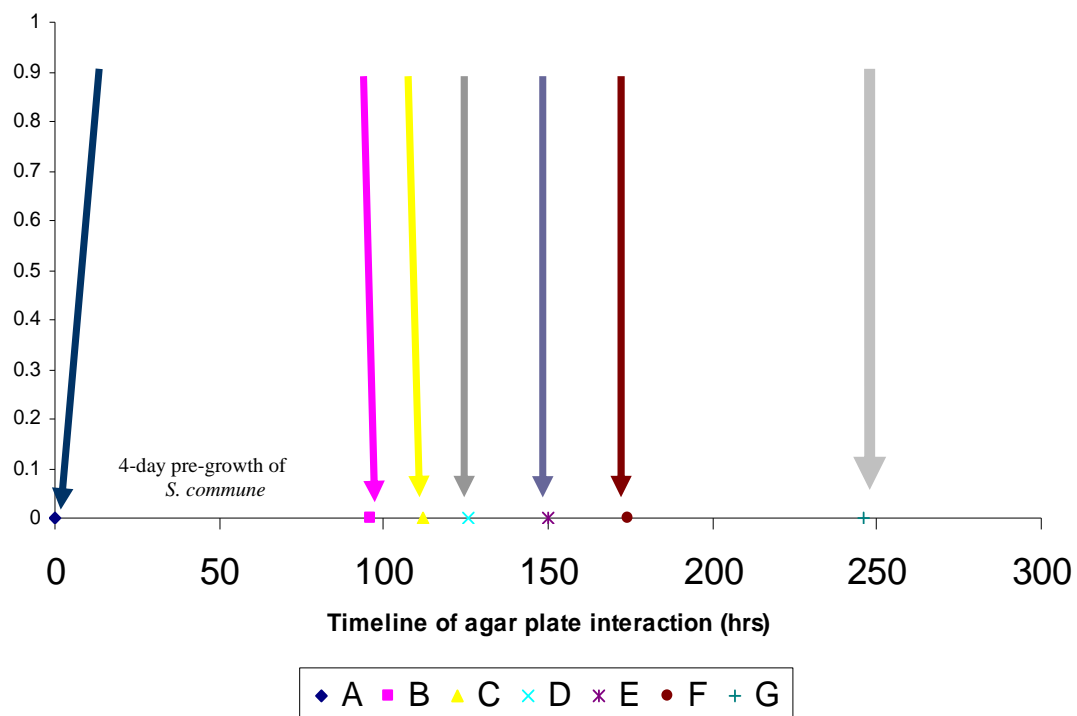
Figure 2.3 Timeline of agar plate interaction assay between *S. commune* and *T. viride* over 120 hours. A: O hours, inoculation of *S. commune*; B: 96 hours (4 days post *S. commune* inoculation), inoculation of *T. viride*; C: 112 hours (16 hours post *T. viride* inoculation), observation of initial mycelial rejection before contact; D: 126 hours (30 hours post *T. viride* inoculation), mycelial contact between both species; E: 150 hours (24 hours post contact); F: 174 hours (48 hours post contact); G: 246 hours (over-growth of *S. commune* by *T. viride*, 5 days (120 hours) after contact.
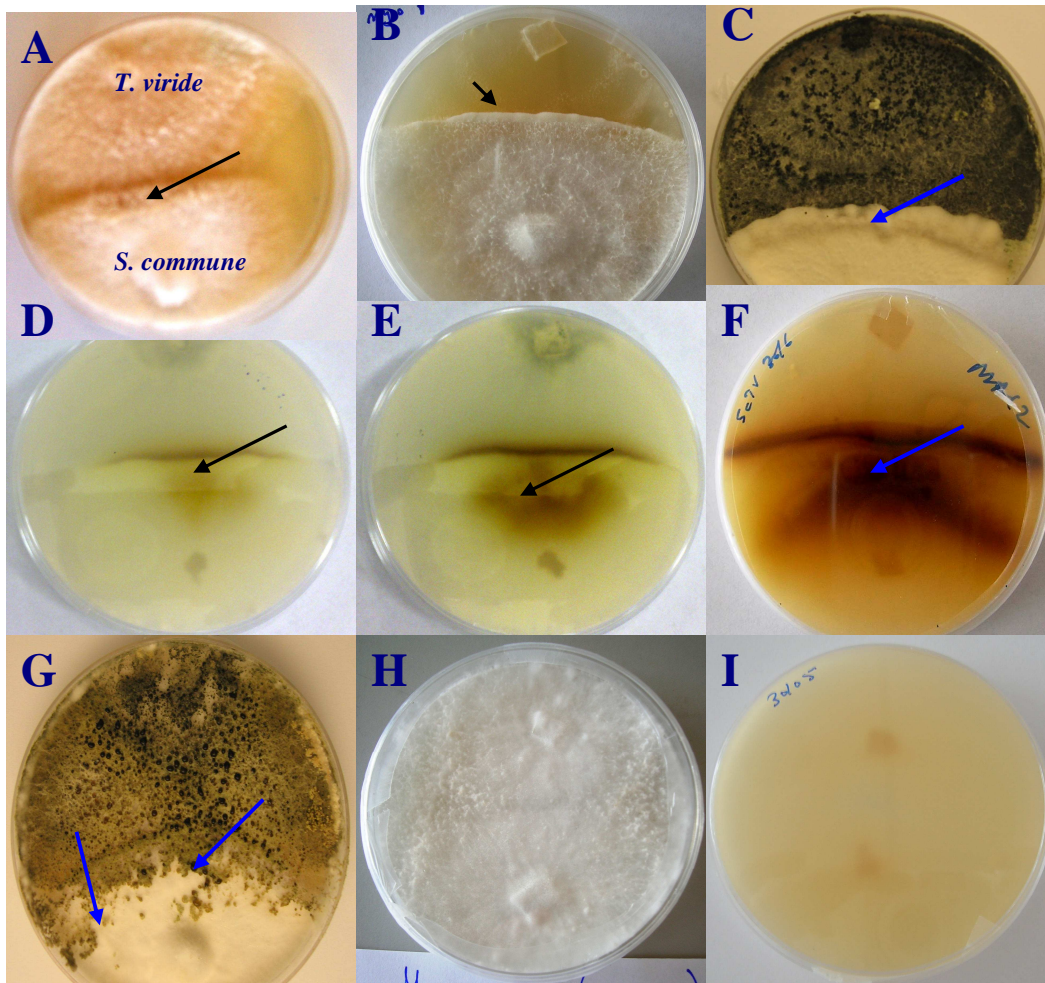
Figure 2.4 Macroscopic morphological changes in the mycelia of *S. commune* interacting with *T. viride* on representative interaction plates. A – Initial mycelial rejection between S. *commune* and *T. viride* before contact (point C in figure 2.3); B – Sealing-off of *S. commune* mycelial front 24 hours after contact with *T. viride* (point E in figure 2.3); C – Barrage formation by *S. commune* after 48 hours of contact with *T. viride* (point F in figure 2.3); D – underside of Petri dish showing the inception of pigmentation in the mycelia of *S. commune* after 16 hours of interaction with *T. viride*; E & F – increase in the intensity of pigmentation after 30 and 96 hours of contact respectively; G – overgrowth of *S. commune* by *T. viride* after 120 hours of contact (point G in figure 2.3); H – Self-paired culture of *S. commune* after 120 hours of mycelial contact; I – underside of self-paired culture of *S. commune* 120 hours post mycelial contact.
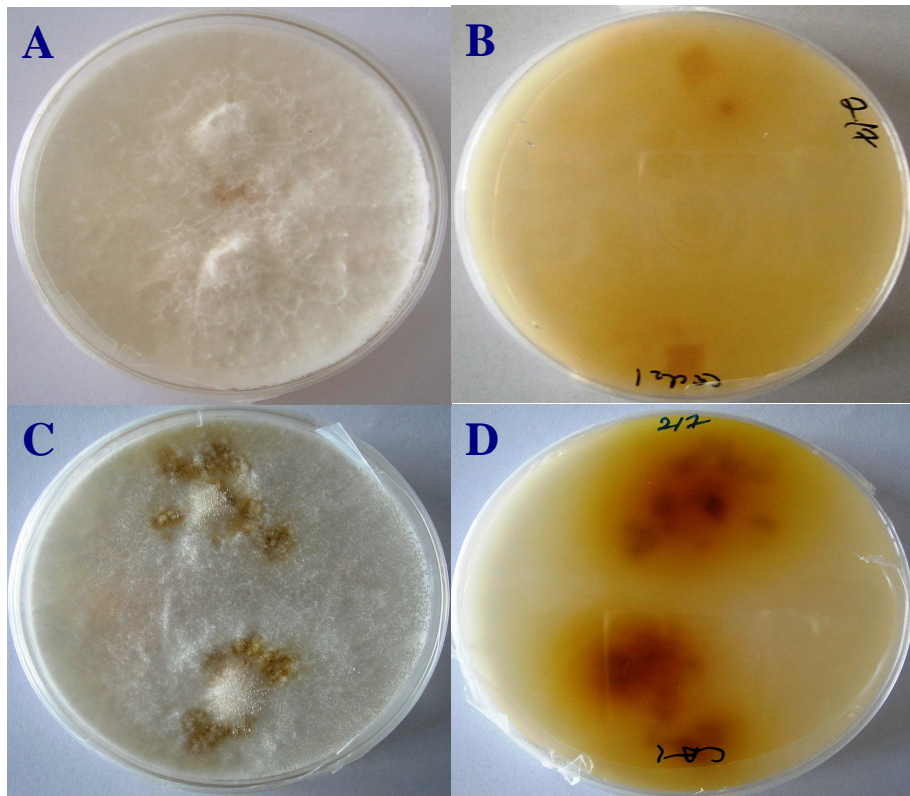
Figure 2.5 Development of pigmentation in cultures of *S. commune* growing on calcium ionophore A23187-containg PDA after 120 hours of inoculation. A & B – top and undersides of control plates containing DMSO. C & D - Brownish pigmentation in plates containing calcium ionophore A23187 dissolved in DMSO before incorporation in PDA. Pigmentation above bears similarity to the pigmentation induced by contact with *T. viride* in fig 2.4 D-F.

2.3.2 Unstained microscopic examination of the mycelia of *S. commune* interacting with *T. viride*

Figure 2.6 (phase contrast microscopic image) reveals the degeneration of the protoplasmic components of *S. commune* at the points of contact with *T. viride*. Hyphal compartments away from the points of contact appeared protected from the protoplasmic aggregation in the affected adjoining compartments. Furthermore, contact between both species elicited profuse growth and extension of the mycelia of *T. viride* towards *S. commune*, followed by systematic coiling around the hyphae of its host. The enveloped

31

hyphae of *S. commune* underwent pigmentation, resulting in brownish discolouration in the affected areas (figure 2.7 A & B).
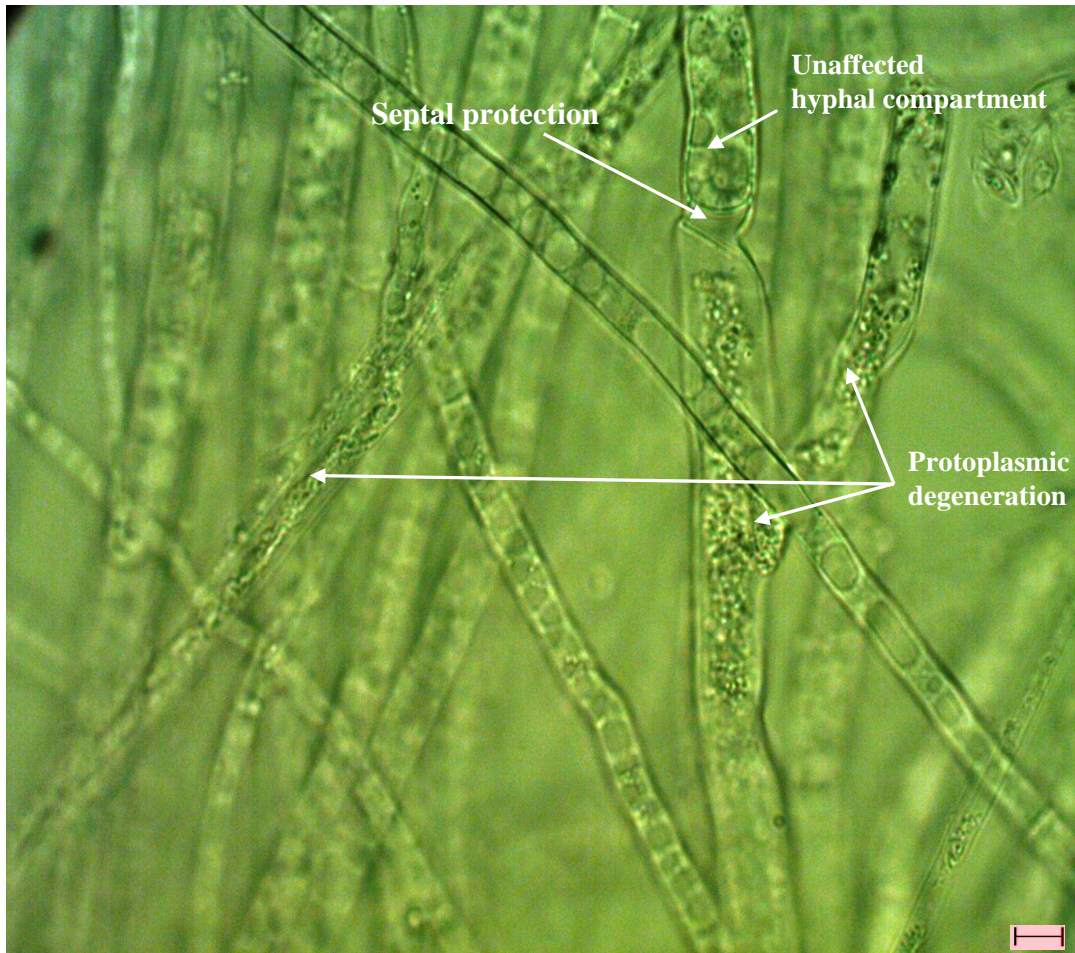
Figure 2.6 **Micrograph depicting the formation of intracellular aggregates (protoplasmic degeneration) within the hyphae of *S. commune* at points of contact, after 48 hours of interaction with *T. viride*. Bar = 10 μm**
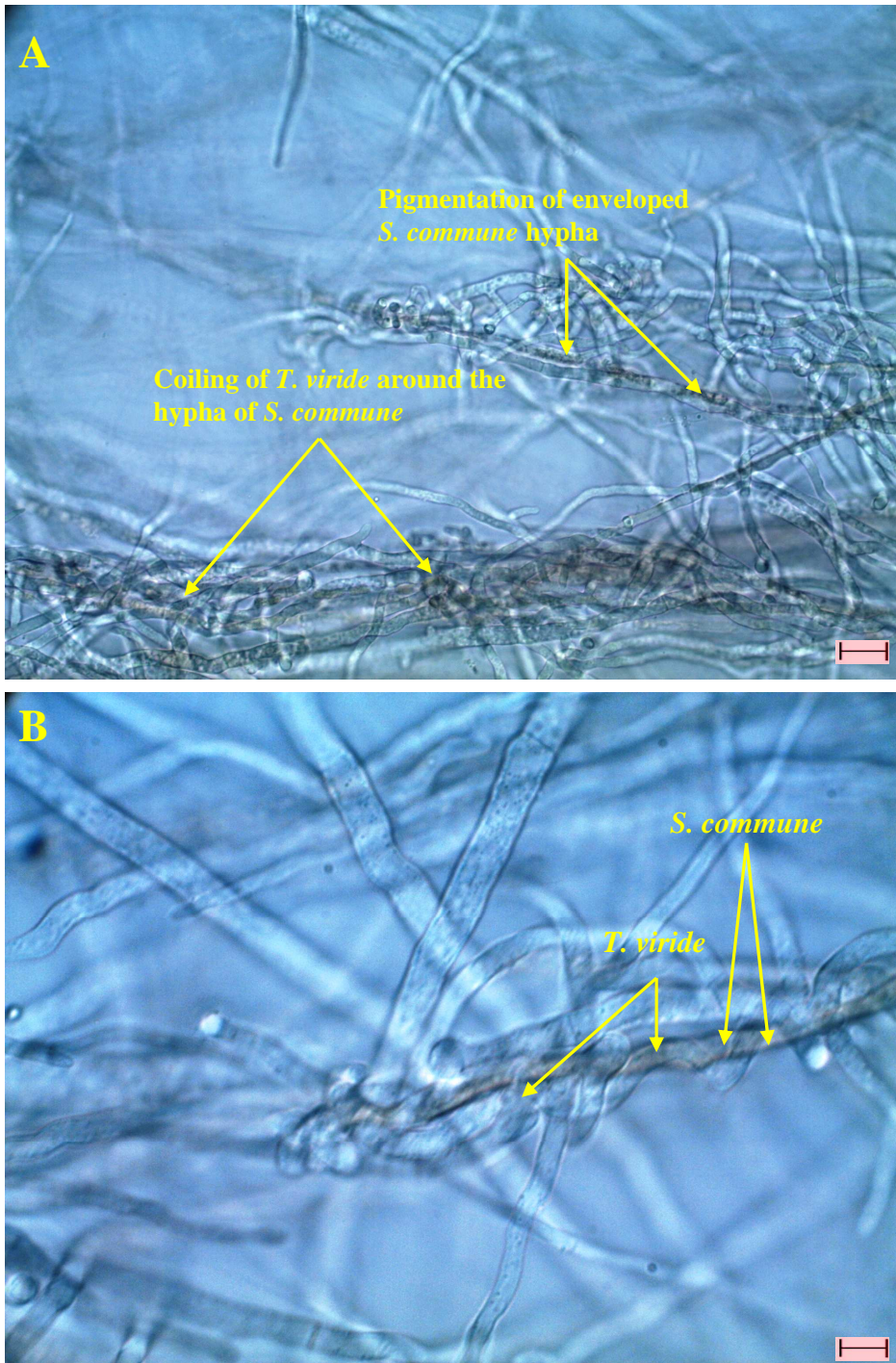
Figure 2.7 Systematic coiling of the mycelia of *T. viride* around the hyphae of *S. commune* and the induction of pigmentation in the enveloped hyphae of the former. Bar = 10 µm

2.3.3 Examination of stained (Nile Red, Congo, Red, Evans Blue) preparations of mycelial samples of *S. commune* confronted by *T. viride*

Contact between *S. commune* and *T. viride* resulted in cell wall lysis, enlargement of hyphal compartments, protoplasmic degeneration, and weakening of septal walls between adjoining compartments in the mycelia of *S. commune*. However, self-interacting cultures of the same fungus retained their cell wall with no observed indications of degeneration or septal damage after 48 hours of contact (figures 2.8 - 2.10).
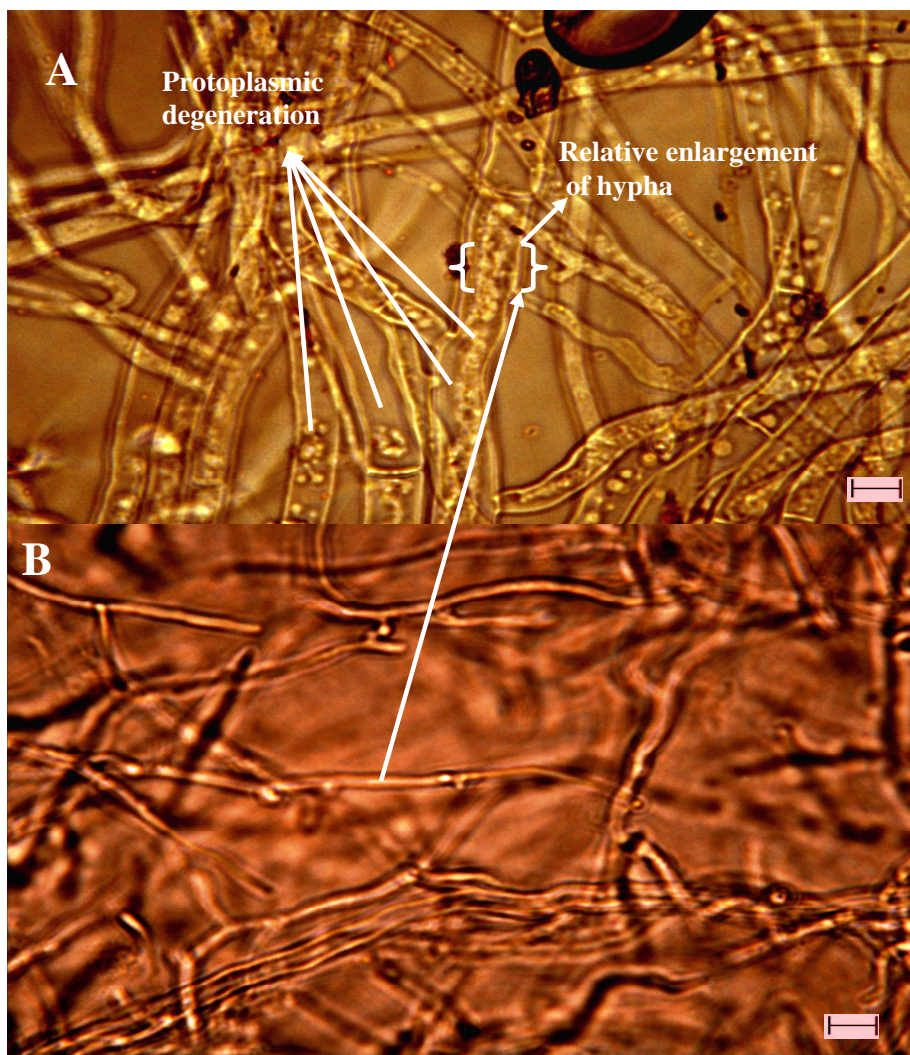
Figure 2.8 Relative enlargement of *S. commune* mycelia, cell wall lysis and degeneration of protoplasmic components following 48 hours contact with *T. viride*, revealed by mycelial staining with Nile Red. A – *S. commune* mycelia paired with *T. viride*; B – Self-paired mycelia of *S. commune*. Bar = 10 μm
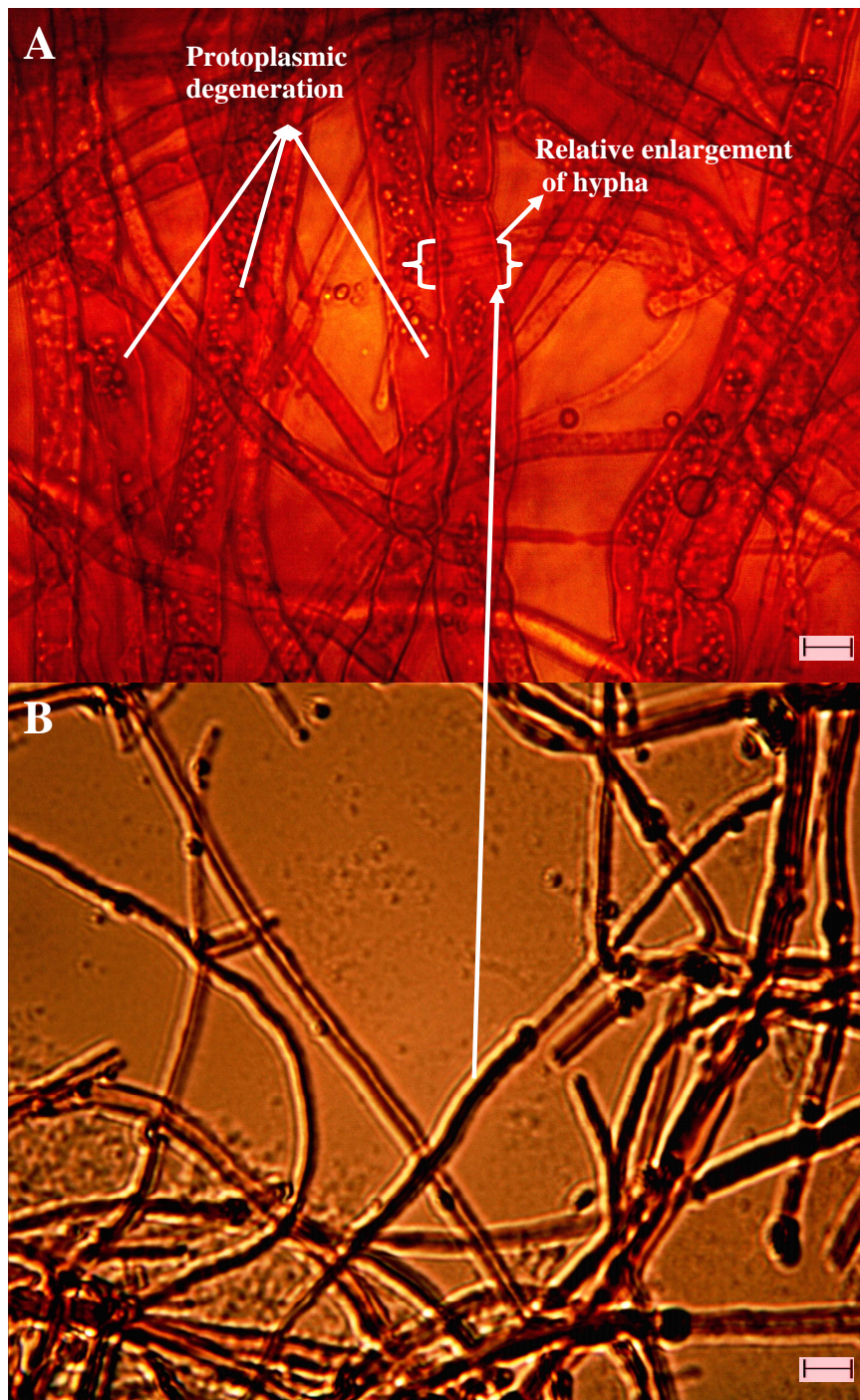
Figure 2.9 Micrograph illustrating comparative enlargement of *S. commune* mycelia, lysis of cell wall and of protoplasmic degeneration following 48 hours contact with *T. viride*, revealed by mycelial staining with Congo Red. A – *S. commune* mycelia paired against *T. viride*. B – Self-paired *S. commune* mycelia. Bar = 10 μm
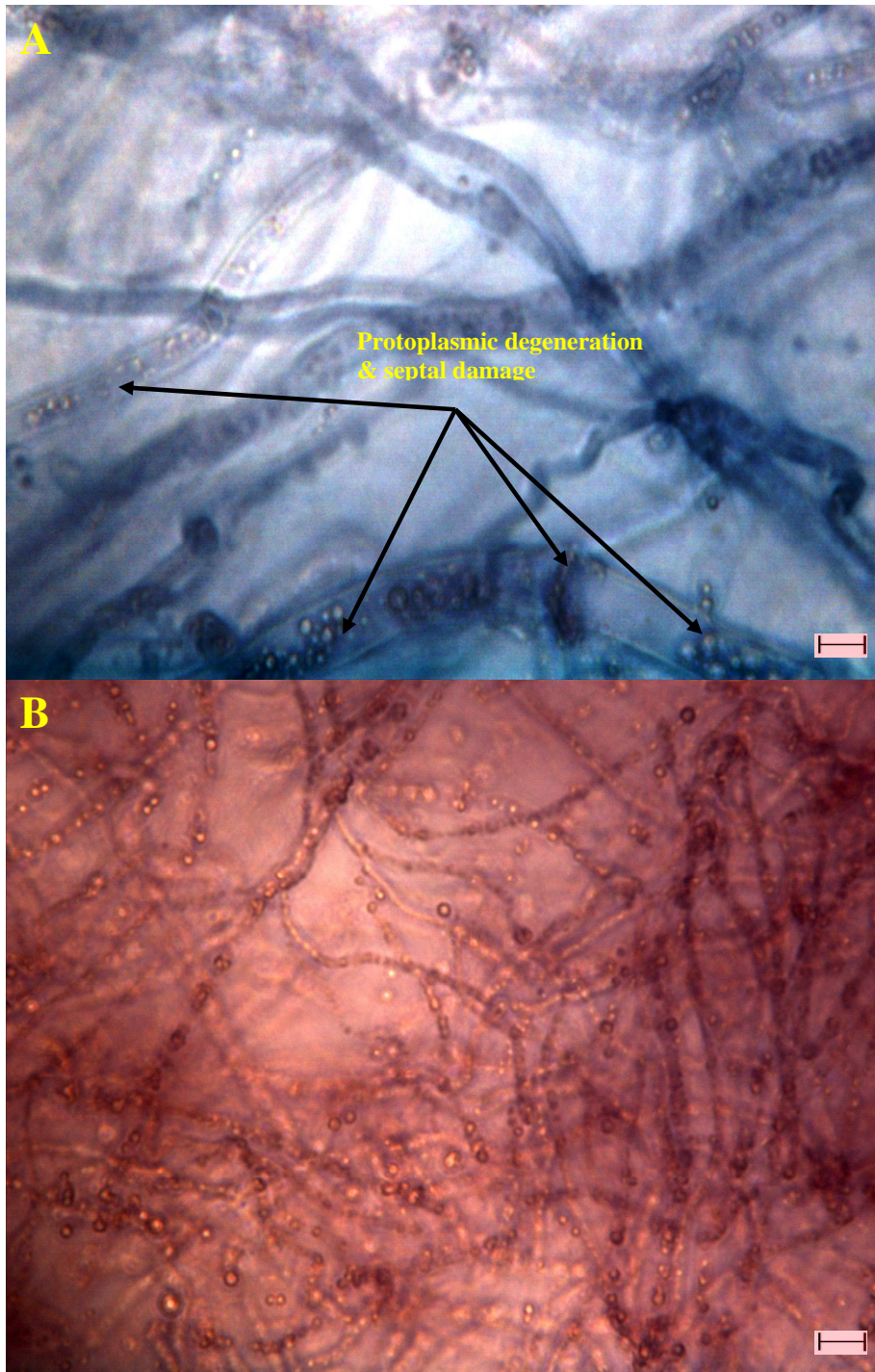
Figure 2.10 Septal damage revealed by Evans Blue stain in addition to mycelial enlargement and cell wall lysis. **A** − Mycelia of *S. commune* interacting with *T. viride*; B – self-interacting mycelia of *S. commune*. Bar = 10 μm

After 168 hours (7 days) of mycelial contact with *T. viride*, complete lysis of the mycelia of *S. commune* was observed. Cellular fragments following lysis were more clearly revealed by staining with Nile Red and Congo Red (figures 2.11 - 2.12).
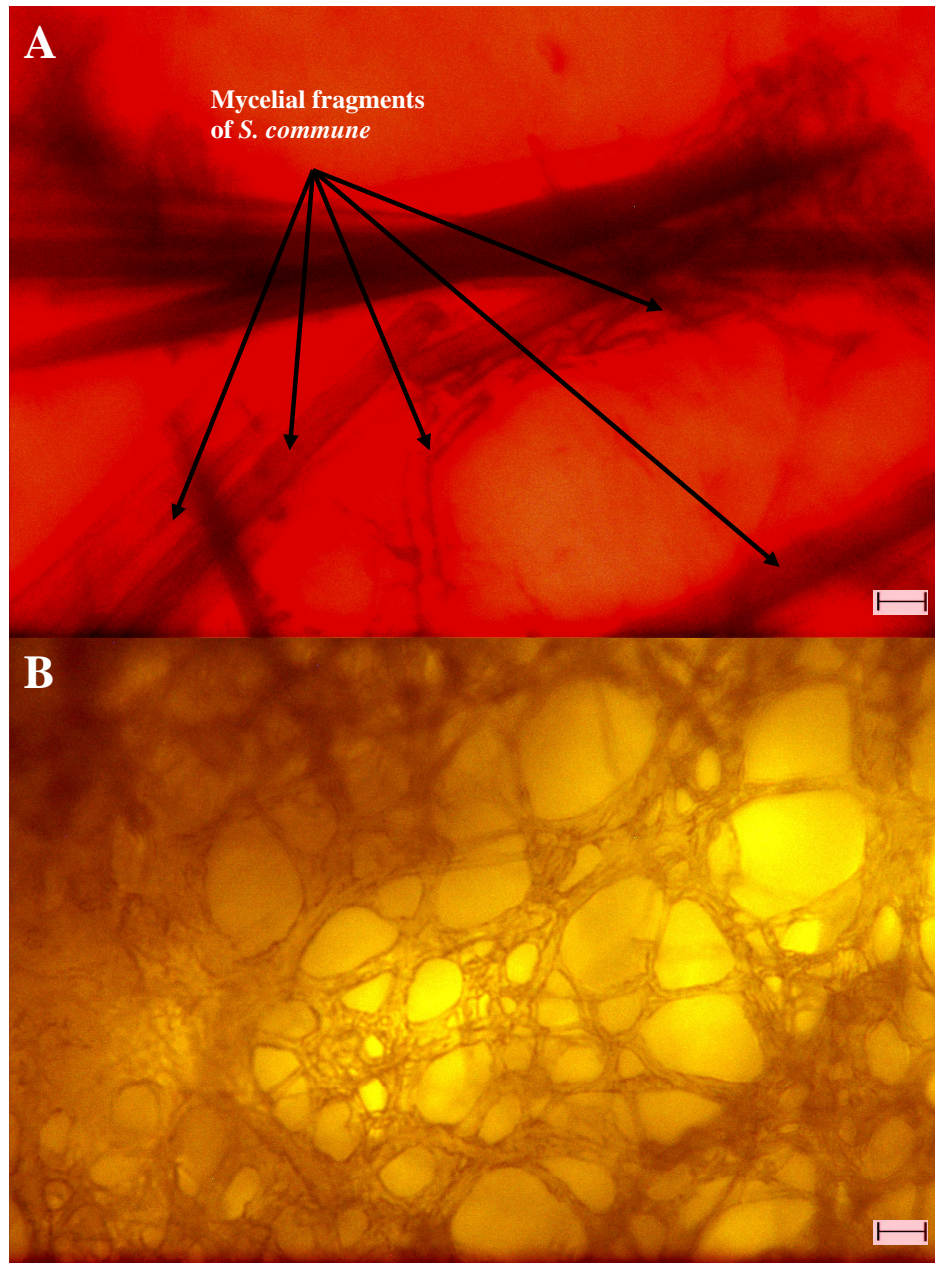
Figure 2.11 Complete lysis and cell death of *S. commune* mycelia in the contact areas with *T. viride* (A) relative to intact mycelial mesh in self-paired cultures of *S. commune* (B). Bar = 10 μm
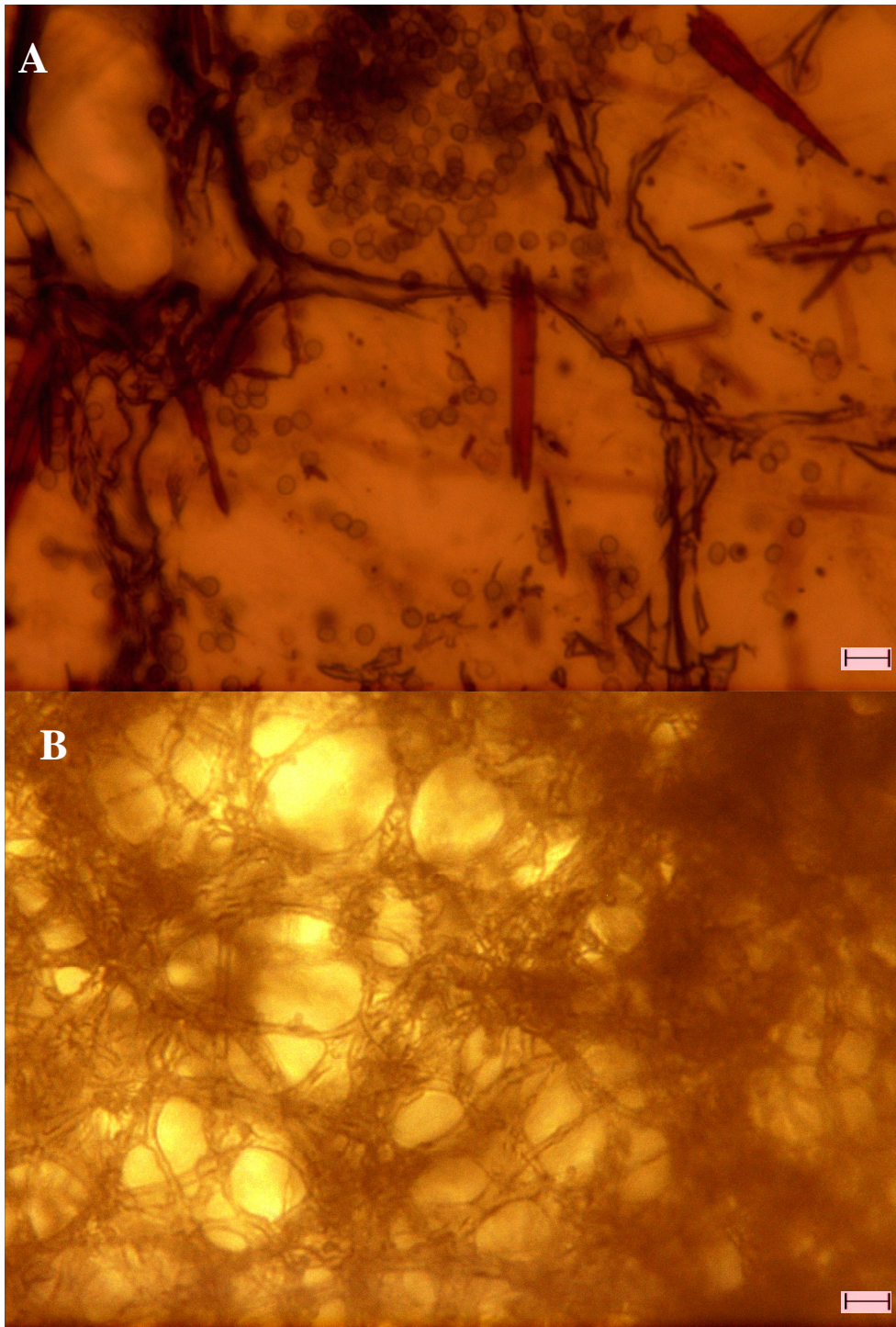
Figure 2.12 Lysis and release of intracellular components of *S. commune* mycelia following 168 hours of contact with *T. viride* (A) revealed by staining with Congo Red in comparison with intact mycelia in self-paired cultures (B). Bar = 10 μm

2.4 Discussion

2.4.1 Macro- and microscopic changes in the morphology of *S. commune* mycelia confronted by *T. viride*.

Changes observed in the mycelia of *S. commune* following contact with *T. viride* were initial mycelial rejection prior to contact, cell wall lysis, pigmentation, barrage formation, formation of protoplasmic aggregates within the affected mycelia and cell death, confirm the high degree of antagonism of the *T. viride* strain used in this study. More importantly, it sheds light on the results presented in subsequent chapters. Interestingly, although hydrolysis of *S. commune* cell wall was observed after 48 hours of contact with *T. viride*, mycelial disintegration was not observed until after 168 hours of contact. This suggests that *S. commune* perhaps possesses some form of resistance, to *T. viride*. It has been reported that white-rot fungi are resistant to biological control with *Trichoderma* species and *Gliocladium virens* on wood (Highley & Ricard 1988). Although these biocontrol fungi were able to kill the white rot fungi tested in the study of Highley and Ricard (1988), cell death was only observed after 5 weeks of incubation. In the present study, cell death was localized in the areas of contact and spread as *T. viride* overgrew *S. commune*. The implication is that *S. commune*, a white-rot fungus possibly undergoes biochemical/physiological modulations that allow it to stave off cell death temporarily during conflict with *T. viride*.

The early rejection observed between both fungi, particularly in *S. commune* agrees with the suggestion that some form of non-self recognition mechanism exists both intra- and interspecifically in fungi (Saupe, 2000; Demethon *et al*., 2003; Pinan-Lucarré *et al*., 2007; Rayner, 1991a&b; Rayner *et al*., 1995; Griffith *et al*., 1994). Heilman-Clausen & Boddy (2005) demonstrated the use of infochemicals by wood-rot fungi for a passive defence strategy, which affects later colonizers, thereby contributing to the structuring of microbial communities. Data in chapters 3 and 4 (sections 3.3.2 and 4.3.2) show the rise in levels of phenol compounds after 48 hours and 24 hours of mycelia interactions respectively. Although the levels of phenol compounds were not measured prior to mycelial contact, it is not impossible that increased production of phenol compounds may

have started before contact, and, if that is the case, phenolic or other classes compounds may form part of the signal exchanges that drive non-self recognition between both fungi.

In another vein, superoxide anion has been implicated in fungus-fungus interactions (Baker and Orlandi, 1995) and results in Chapter 4 (section 4.3.2) show a sharp increase in superoxide anion levels in the mycelial domain of *S. commune* within 24 hours of contact with the antagonist. Chances are that these radicals might contribute to the web of non-self recognition mechanisms during the early stages of contact between both fungi.

Microscopic observation of cell wall disruption in *S. commune* upon 48 hours of mycelial contact with *T. viride*, confirms the widely reported mycoparasitic properties of *Trichoderma* species, mediated by cell wall-degrading enzymes (Highley & Ricard, 1988; Marra *et al.*, 2006; Bruce *et al.*, 1995; Bruce & Highley, 1991; Lu *et al.*, 2004; Howell, 2002; Chet, 1987). Furthermore, figure 2.6 shows systematic coiling of *T. viride* around the mycelia of *S. commune* and profuse mycelial extension of the antagonist towards the latter. This underlines the combative proficiency of *T. viride* and agrees with the work of Lu *et al.* (2004) who reported similar coiling and development of specialized antagonistic structures in *Trichoderma aureoviride* in a three-way *Trichoderma*-pathogen-plant pairing. In chapter three, GC-MS analyses show up-regulation of polyols predominantly in the mycelia of *T. viride* during interaction with *S. commune*. This systematic coiling in part explains the build up of sugar alcohols in *T. viride*. As described in detail in Chapter 3, accumulation of polyols in *T. viride* may have been influenced to some degree by the need to generate inner hydrostatic turgour pressure required for the development of and coiling of specialized structures and mycelia around host mycelia.

There exists a possibility that lysis of the cell wall of *S. commune* mycelia in the contact areas might be connected with pigmentation and up-regulation of phenol compounds. Up-regulation of phenylalanine ammonia lyase (PAL) is discussed in chapter seven (section 2.4.2). PAL is a stationary phase enzyme of plants, fungi and actinomycetes, which catalyzes the entry point into the phenylpropanoid pathway; a major source of

phenol compounds (Kim *et al*., 2001; Kalghatgi & Rao, 1976; Gomez-Vasquez *et al*., 2004; Kurosaki *et al*., 1986). Wounding is one of the stress factors that trigger PAL synthesis (Jones, 1984; Hahlbrock & Scheel, 1989; Kim *et al*., 2001). It is possible that compromised cell integrity in the form of cell wall lysis (wounding), may have elicited PAL synthesis in *S. commune*. This in turn may have contributed to the increased production of phenol compounds, which would promote the synthesis, and secretion of phenoloxidases by substrate induction (Crowe & Olsson, 2001). Pigmentation during fungus-fungus interactions has been strongly attributed to changes in the activity and levels of phenoloxidases (Boddy, 2000; Crowe & Olsson, 2001; Griffith *et al*., 1994, Rayner *et al*., 2005; Iakovlev *et al*., 2004). Data in chapter Five (sections 5.3.1; 5.3.2.4; 5.3.3) show significant increases in the levels and activities of laccase and manganese peroxidase in mycelial samples of *S. commune* in response to confrontation by *T. viride*, which perhaps account for pigmentation in the *S. commune* domain following contact with *T. viride*.

Another possible explanation for pigmentation during this interaction is possible induction of secondary metabolism. Figure 2.3B illustrates the sealing-off of mycelial front by *S. commune* in response to *T. viride*. Griffith *et al*. (1994) proposed that fungus-fungus interaction elicits the sealing-off of the mycelial front, which in turn limits nutrient supply, thus shifting fungal growth to secondary metabolism. This, according to the authors, results in pigmentation as a result of the interplay between phenolic build-up and phenoloxidase activity. Considering the sealing-off of mycelial front by *S. commune* within 24 hours of contact with *T. viride*, mycelial growth in the former may have switched to secondary metabolism, which perhaps accounts in part for the up-regulation of phenolic compounds and phenoloxidases and the resulting pigmentation.

Although the aggregation of the intracellular protoplasmic contents in *S. commune* interacting with *T. viride* after 48 hours of contact bears strong semblance to hyphal interference (Rayner & Boddy, 1988), the considerable delay in hyphal disintegration tends to suggest that other physiological reactions, which differ from hyphal interference, may have occurred. The pattern of degeneration in the affected mycelia of *S. commune*

suggests some form of shock typical of heat damage of macromolecules and membranes. This could be attributed to oxidative damage in form of lipid peroxidation (Chapter 4) of membranes (Halliwell & Aruoma, 1991), stemming in part from likely disruption of mitochondrial functioning leading to electron leakage (Iakovlev *et al*., 2004). The aggregation of intracellular components would most likely alter the mitochondrial membrane potential (Chapter 4) thereby disrupting electron transport. Furthermore, intracellular aggregation of protoplasmic components is synonymous with protein synthetic stress in fungi (Krysan *et al*., 2009), as discussed in Chapter 7. The patterns of protein expression in the mycelia of *S. commune* paired against *T. viride* for 48 hours (Chapter 7) strongly point to a possible induction of protein synthetic stress. Taken together, it could be deduced that both oxidative and protein synthetic stresses may have, to different degrees played a part in the degeneration of protoplasmic contents in the mycelia of *S. commune* confronted by *T. viride*.

A likely consequence of autolysis in *S. commune* is starvation. Given that the cell wall plays a critical role in the vesicular transport of nutrients into the hypha, loss of cell wall function restricts nutrient acquisition in fungi (Casadevall *et al*., 2009). This would result in starvation, thereby warranting the need to harness the recycling of intracellular sources of carbon and nitrogen. Perhaps this may have influenced *S. commune* protein expression patterns presented in chapter 7 depicting the up-regulation of enzymes involved in amino acid hydrolysis, most of which are mainly secondary metabolic enzymes. In addition, possible involvement of the up-regulated malate dehydrogenase isoforms in β-oxidation, an important source of carbon (essential to several metabolic processes) is also discussed in chapter 7. It is also likely that limiting nutrient acquisition owing to cell wall lysis, and the resultant starvation, may have contributed to the high levels of protein carbonyls detected in *S. commune* confronted by *T. viride* (Chapter 4). Protein carbonylation has been reported to be enhanced by starvation (Stratman, 2001; Yan *et al*., 1997; Yan & Sohal, 1998).

2.4.2 Induction of pigmentation in the mycelia of *S. commune* by calcium ionophore A23187

Interestingly, calcium ionophore A23187 to some degree mimicked the induction of pigmentation in the mycelia of *S. commune* as in the case of mycelia confrontation with *T. viride*. Crowe and Olsson (2001) showed that the addition of calcium ionophore A23187 to liquid cultures of *Rhizoctonia solani* caused a significant increase in laccase activity. Calcium ionophore A23187 mobilizes calcium across membranes (Ramos *et al*., 1989; Schmid & Harold, 1988). In addition, Calcium ionophore A23187 has antifungal properties (Bernard *et al*., 1979). However, there was no indication of growth inhibition (antifungal activity) at the concentration tested in this study. In view of this, it is likely that the induction of pigment production in *S. commune* by calcium ionophore A23187 was via the calcium influx pathway. A possible means of calcium mobilization is via the inositol triphosphate pathway (Gadd, 1994; Larson *et al*., 1992). Results from metabolomic studies (Chapter 3) showed a significant increase in the levels of a metabolite potentially identified as inositol phosphate. Whereas it cannot be concluded that the molecule in question was inositol triphosphate, it does suggest that the inositol triphosphate pathway might exist in *S. commune*. Furthermore, this result implicates calcium influx/mobilization from intracellular stores in the induction of pigmentation in *S. commune* confronted by *T. viride*.

2.5 Conclusions

The results presented in this chapter serve as a prelude to subsequent chapters. Furthermore, they provide both macro- and microscopic insight into the metabolomic, genomic and proteomic studies. For instance, protoplasmic degeneration points to the possible involvement of oxidative (Chapter 4) and protein synthetic (Chapter 7) stresses in the sequence of processes, and reactions that accompany the response of *S. commune* to *T. viride*. In addition, the resultant pigmentation following contact with *T. viride* sheds more light on the levels of phenolics (Chapter 3) and phenoloxidase levels and activities (Chapter 5) in the mycelia of *S. commune*. Microscopic observation of *S. commune* cell wall lysis not only explains the sharp increases in the activity of chitinase in mixed cultures of both fungi (Chapter 5), but also underlines the antagonistic proficiency of *T.*

viride. The loss of cell wall function in part accounts to some extent for the marked increase in amino acid degrading enzymes, otherwise expressed during stationary phase of growth, in *S. commune* paired against *T. viride*, as well as the up-regulation of malate dehydrogenase isoforms (Chapter 7). More importantly, these results portray the complex interplay of physiological and biochemical responses, which appear to form the hallmark of *S. commune* response and possible temporary resistance to *T. viride* before, cell death.

CHAPTER 3

Metabolomics of the antagonistic interaction Between *S. commune* and *Trichoderma viride*

3.1 Introduction

Field studies of wood-rotting basidiomycetes have indicated that earlier colonizers of wood exert a marked influence on the development and structure of communities of later colonizers, and laboratory pairings of these two groups have showed that initial inoculation of early colonizers on agar prior to the inoculation of late colonizers affected the hyphal extension and survival of the latter (Niemelä *et al*., 1995; Heilman-Clausen & Christensen, 2003; Renval, 1995). Holmer *et al* (1997) speculated that wood-rotting fungi might use bioactive secondary metabolites to influence the establishment of other fungal species on the same wood material. Similarly, several studies have demonstrated the mediation of antagonism at a distance during interspecific combative interactions by different basidiomycetes paired on agar (Boddy, 2000; Gloer, 1995; Griffith *et al*., 1994; Humphris *et al*., 2001; Strongman *et al*., 1987; Wheatley 2002). There are speculations that antagonism at a distance is mediated by diffusible or volatile compounds, which cause mycelial degeneration of one of the interacting species before replacement (Sonnenbichler *et al*., 1994; Boddy, 2000; Gloer, 1995; Griffith *et al*., 1994; Humphris *et al*., 2001; Heilman-Clausen & Boddy, 2005). However, reactions vary depending on the interacting species and in some cases, mutual inhibition has been observed indicating a mutual exchange of chemical signals and their detection at a distance (Boddy, 2000; Rayner & Webber, 1984).

Heilman-Clausen & Boddy (2005) demonstrated, reduced hyphal extension rate, delayed growth or total inhibition of 46% of the studied fungal species inoculated on agar following initial placement of wood previously inhabited and decayed by *Stereum hirsutum* on the same agar medium. The authors hypothesized that *S. hirsutum* may employ chemicals in a passive defence strategy. In the same study, wood materials pre-decayed by *Fomes fomentarius* stimulated the growth of 45% of the species studied. These results support the earlier suggestion that wood degrading fungi might use 'infochemicals' to influence the establishment of later colonizers, as well as those of competing species on wood. Furthermore, Hynes *et al* (2007) reported the up-regulation of volatile organic compounds, mainly sesquiterpenes such as benzoic acid methyl ester, benzyl alcohol and a quinolium type compound during 25-day mycelial interactions

between *Hypholoma fasciculare* and *Resinicium bicolor* in a sealed mini fermentation vessel. In another study by Svensson *et al*. (2001), *Physisporinus sanguinolentus* up-regulated secondary metabolites inhibitory to *Heterobasidion annosum*.

Profiling of metabolites associated with combative interactions between *Stereum hirsutum* and its competitors, *Coprinus micaceus* and *Coprinus disseminatus* showed the up-regulation of 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid in *S. hirsutum* whereas 1,2-dihydroxyanthrquinone was up-regulated in *C. disseminatus* (Peiris *et al*., 2008). Pyridoxine and 2-methyl-2,3-dihydroxypropanoic acid were found to be down-regulated in *S. hirsutum* in the same study. Gregorio *et al*. (2006) reported that addition of filter-sterilized fermentation broth of *Marasmius pallescens* to fermentation cultures of *Maramiellus troyanus* resulted in the up-regulation of laccase in the latter. They attributed this to the possible presence of phenolic compounds known to induce laccases by substrate recognition in the supernatant of *Marasmius pallescens* culture broth.

Rayner *et al*. (1994) concluded that fungal interactions induce the secretion of hydrophobic metabolites, which are converted to free radicals and polymers by laccases thereby stabilizing the cell wall and generating quinone compounds, which might exert protective functions against competitors. Generally, it appears the secretion of both primary and secondary metabolites is strongly modulated in interacting mycelia depending on the species. Studies of fungal interactions have led to the discovery of scytalidin from *Scytalidium* sp. (Weber *et al*., 1988) and culmorin from *Leptospaeria oraemaris* (Strongman *et al*., 1987). Similarly, a volatile organic compound produced by *Trichoderma harzianum* has been considered as bio-preservatives for timber (Schoeman *et al*., 1996).

Whereas several studies have demonstrated inhibitory or stimulatory effects of metabolites secreted during different interactions, very little is known about the possible roles these compounds may play during fungus-fungus interactions. Investigation of the patterns of metabolite secretion (metabolomics) during inter-specific combative interactions offers promises to understanding the underlying physiological basis of non-

self rejection and combative interactions in fungi. Metabolomics is the broad analysis of intra- and extra-cellular metabolites in a biological system at any point in time (Mashego *et al.*, 2007). Metabolomics plays a complementary yet key role to genomics and proteomics in the understanding of the phenotypes exhibited by a system at any point (Jewett *et al.*, 2006). For instance, identification of the function(s) of a singe metabolite and the patterns of its secretion might shed light on a wide array of genes and proteins involved in the production of the metabolite, thereby aiding broader understanding of the molecular and physiological mechanisms at play within the system.

A metabolomic study entails sample collection, preparation, analysis and data processing (Dunn *et al.*, 2005). However, metabolomic studies might be limited by inability to extract certain metabolites to significant levels, and poor amenability of different chemical species to certain analytical techniques (Goodcare *et al.*, 2004; Jewett *et al.*, 2006). In this chapter, the metabolite profiles of both *S. commune* and *T. viride* paired on PDA relative to their self-paired cultures are presented. Metabolite profiling was carried out by high performance liquid chromatography (HPLC) followed by identification using gas chromatography-mass spectrometry (GC/MS). GC/MS was carried out at the University of Manchester, UK in collaboration with Dr. Warwick Dunn.

3.2 Materials and Methods

3.2.1 Metabolite extraction

Agar plate interaction assays were set up as described in chapter 2 (section 2.2.1). After 2 days of mycelial contact, 3.5g of mycelial strips were cut off from the sampling zone indicated in figure 3.1. Strips were freeze-dried for 48 hours, crushed with a glass rod in 50 ml tubes and extracted in 10 ml of methanol. Extraction was carried out overnight at 4°C. Excess ethanol was removed from the extracts by drying under vacuum in a centrifugal evaporator (Genevac Ltd). Extracts were stored at -20°C in 7 ml vials.
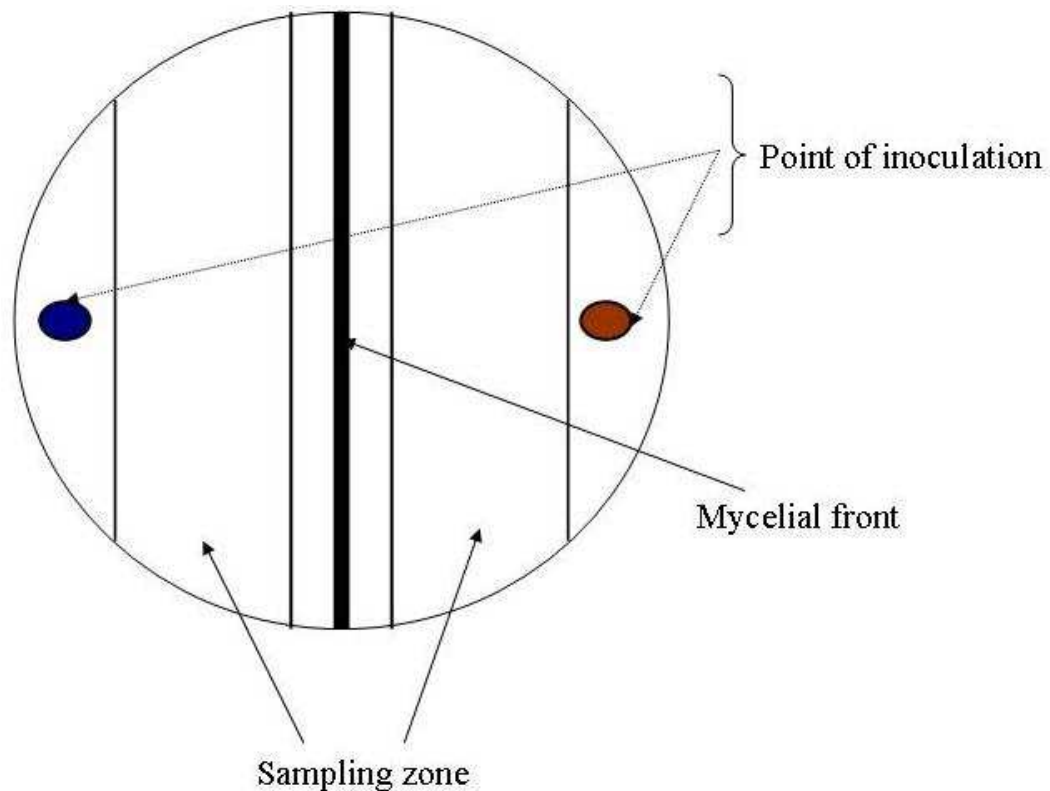
Figure 3.1 **Schematic representation of the sampling zones from interaction cultures.**

3.2.2 High performance liquid chromatography (HPLC)

Methanolic extracts of both *S. commune* and *T. viride* were profiled by HPLC in collaboration with Hypha Discovery limited, using a Waters 600E controller, 717 autosampler, 60 F pump module and a 996 photodiode-array detector. 10 µl of extract reconstituted in methanol was injected into the Waters symmetry® C18 (54 X 4.6 mm i.d., 3.5 µm) with a gradient elution: linear gradient from 90:0:10 to 0:90:10, A:B:C (A: water; B: Acetonitrile (Hipersolve, BDH); C: 0.1% v/v Trifluoroacetic acid (Hipersolve, BDH) in Acetonitrile) over 8 minutes, then held for another 4 minutes at 0:90:10 before re-applying the initial conditions for 1 minute prior to a 6 minute equilibration. A flow

rate of 1.5 ml/min was applied. Absorbance was measured at 205 nm. Metabolite profiles are presented in maxplot (UV against retention time).

3.2.3 Gas Chromatography-Mass Spectrometry (GC/MS)

Extracts for GC/MS were prepared in replicates of 10 for both control and test cultures and dispatched to the School of Chemistry, University of Manchester, UK where they were analyzed (see Appendix I, section 1). Univariate Kruskal Wallis analyses were used to assess the statistical differences between metabolite peaks from self-paired and interspecific cultures. Peaks with $P \leq 0.02$ were considered as statistically up-/down-regulated in the test samples in comparison to the control cultures.

3.2.4 Statistical analysis

Unpaired T-test was used to determine the level of significance of the observed up-/down-regulation of the different metabolites, by using Graphpad T-test calculator software (Graphpad software, San Diego California: http://www.graphpad.com/quickcalcs/ttest1.cfm). P values < 0.05 were considered significant.

3.3 Results

3.3.1 HPLC profile

HPLC profiles showed varying peak patterns in both *S. commune* and *T. viride* after 48 hours of mycelial contact compared to the self-paired cultures (figures 3.2 to 3.5). For instance, after 7.807, 8.52 and 9.14 minutes, peaks not present in the self-paired (SCSC) samples were detected in *T.viride*-confronted mycelia (SCTR) of *S. commune*. Similarly, new peaks were detected in *T. viride* following mycelial contact with *S. commune* at 8.1 and 9.6 minutes.

Figure 3.2 HPLC chromatogram showing the patterns of metabolite peaks in mycelia-agar samples of *S. commune* paired against self for 48 hours. 10 biological replicas (samples) were extracted in methanol for HPLC using a linear gradient from 90:0:10 to 0:90:10 - A:B:C (A: water; B: Acetonitrile; C: 0.1% (v/v) Trifluoroacetic acid in Acetonitrile).

Figure 3.3 HPLC chromatogram showing the patterns of metabolite peaks in mycelia-agar samples of *S. commune* paired against *T. viride* for 48 hours. Arrows represent new peaks or increase in peak area in samples from cultures of *S. commune* confronted by *T. viride*, compared to samples from self-paired cultures.

Figure 3.4 HPLC chromatogram showing the patterns of metabolite peaks in mycelia-agar samples of *T. viride* paired against self for 48 hours.

Figure 3.5 HPLC chromatogram showing the patterns of metabolite peaks in mycelia-agar samples of *T. viride* paired against *S. commune* for 48 hours. Arrows are as in fig 3.3.

3.3.2 GC/MS

Mass/ion fragments of selected peaks are shown in appendix I. Figure 3.6 represents data from Kruskal Wallis test, where the highlighted peaks are significantly different in area between test and control samples. 108 peaks were detected by mass spectrometry and 38 of them (35%) were potentially identified. The identified metabolites were mainly sugar alcohols and cyclitols (cyclic sugar alcohols), fatty acids, phenolic compounds, organic acids, amino acids, aldehydes, sugar phosphate and vitamins.

Patterns of metabolite up- and down-regulation based on variations in peak area detected by mass spectrometry are summarized in tables 3.1 and 3.2. Sugar alcohols were predominantly up-regulated after mycelial contact between both fungi. Although glycerol was down-regulated in both *T. viride* and *S. commune* in co-cultures, xylitol was 57% up in peak area in *T. viride* paired against *S. commune* whereas threitol was up-regulated in *S. commune* paired against *T. viride*. In addition, peaks 44 and 79 identified as sugar alcohols were up-regulated in *T. viride* following mycelial contact with *S. commune* with 80% and 55% increases in peak area respectively. Hexanetetrol, a cyclic sugar alcohol (cyclitol) was barely detected in samples from self-paired *S. commune* cultures; however, its peak area increased 97% after 2 days of mycelial contact with the antagonist. Fatty acids particularly, hexadecanoic acid, octadecanoic acid and octadecenoic acid were down-regulated in *S. commune* following combative interaction with *T. viride*. Tropic acid, a typical phenolic compound was not detected in self-paired cultures of *S. commune*; however, it was significantly present in *S. commune* mycelia after 48 hours of mycelial contact. Tropic acid also showed 60% increase in peak area in mycelia of *T. viride* after 48 hours of mycelial interaction.
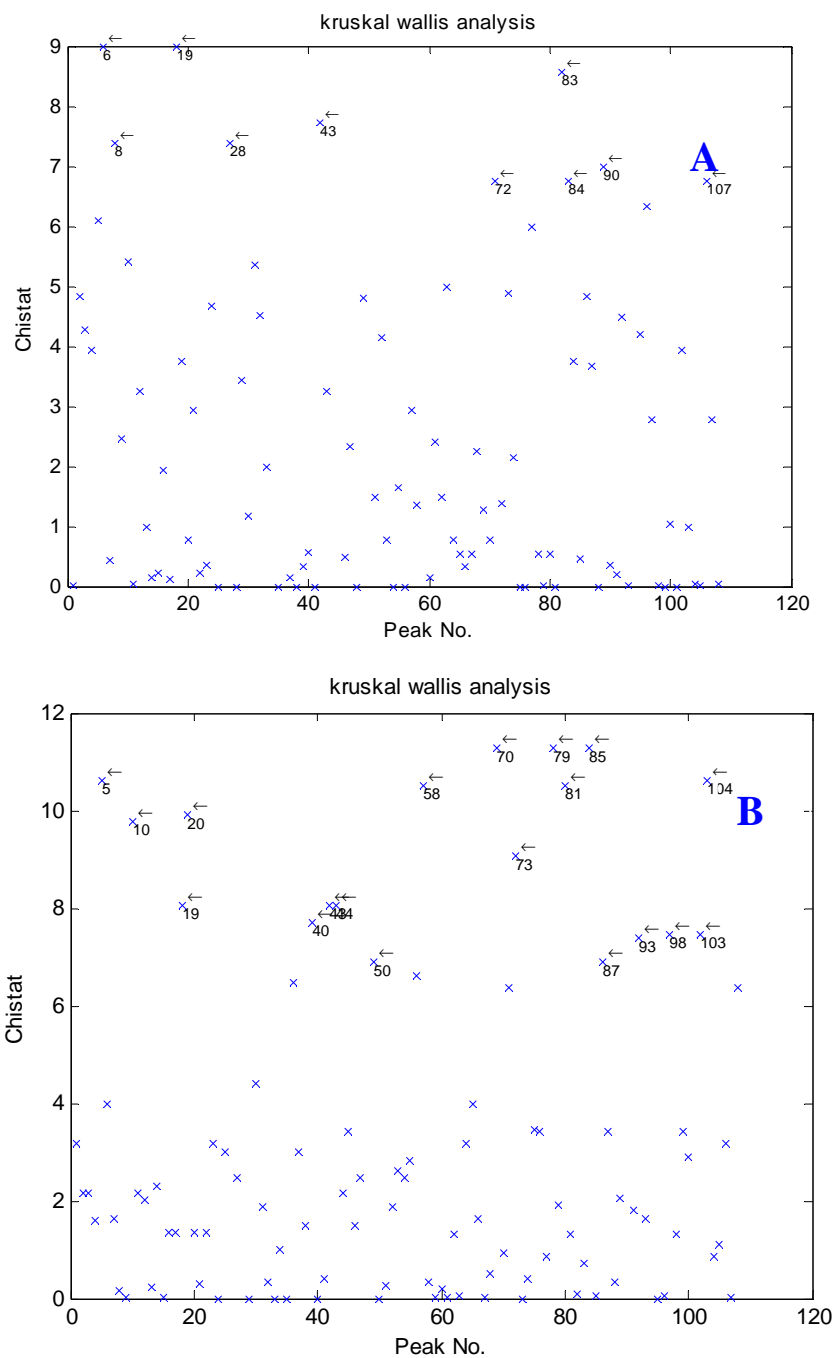
Figure 3.6 Kruskal Wallis analysis indicating peaks (represented by figures) that showed variations in area between interacting mycelia of *S. commune* and *T. viride* relative to their self-paired mycelia after 48 hours of contact. A: Peaks with variations in area between the mycelia-agar samples of *S. commune* paired against *T. viride* in comparison to self-interacting cultures. B: Peaks with variations in area between self-paired *T. viride* mycelia and those interacting with *S. commune*. 10 biological replicas were analysed by GC-MS for both test and control samples.

Mandelic acid, another phenolic compound was up-regulated in both fungi during paired growth on PDA, while 4-hydroxyphenyl ethanol detected only in *T. viride* was up-regulated with a 61% increase in peak area after contact. γ-aminobutyric acid (GABA) was detected at extremely low levels in both fungi. However, after contact, its peak area increased by 99.8% in *T. viride*-confronted *S. commune*. Similarly, pyridoxine was strongly up-regulated in *S. commune* following contact with *T. viride*. Malic, phosphoric and citramalic acids were up-regulated in *S. commune* during interactions, while maleic acid was down-regulated. 3-hydroxypropanoic acid was strongly up-regulated in both fungi in co-cultures. 2 unidentified peaks, 74 and 58 showed variations in area, with peak 74 up-regulated in both fungi while peak 54 was down-regulated in *T. viride*. Phosphoric and acetic acids showed a decrease in peak area in *T. viride* following contact and pyruvic acid and alanine were down-regulated in *S. commune* mycelial samples. Myo-inositol phosphate was not detected in self-paired *S. commune*. However, following contact with *T. viride*, there was strong detection of myo-inositol phosphate in mycelia of *S. commune*.

Table 3.1 Up-regulated metabolites following 48 hours of mycelial contact between *S. commune* and *T. viride*

Peak	Metabolite identity	Source (sample)	% Increase in peak area	*P* value
5	3-Hydroxyporpanoic acid	**SC**TR	60	0.0198
		TRSC	74	0.0001
9	GABA	**SC**TR	99.8	0.0005
10	Phosphoric acid	**SC**TR	55	0.0001
19	Erythritol/isomer	**SC**TR	60	0.0001
28	Malic acid	**SC**TR	41	0.0003
33	Citramalic acid	**SC**TR	72	0.0016
38	Mandelic acid	**SC**TR	77	0.0191
		TRSC	71	0.0001
40	2-hydroxyglutaric acid	**TR**SC	90	0.0047

43	Xylitol	**TR**SC	57	0.0004
46	4-Hydroxyphenyl ethanol	**TR**SC	61	0.0046
53	2-Furancarboxylic acid	**SC**TR	44	0.0002
		TRSC	41	0.0001
57	Tropic acid	**SC**TR	100*	0.0001
		TRSC	60	0.0212
69	Pyridoxine	**SC**TR	81	0.0001
89	Myo-inositol phosphate	**SC**TR	100*	0.0001
76	N-acetylglucosamine	**SC**TR	99.7	0.0002
		TRSC	36	0.0012
81	Galactosylglycerol	**TR**SC	50	0.0013
44	Sugar alcohol	**TR**SC	80	0.0001
79	Sugar alcohol	**TR**SC	55	0.0005
50	2,3,4-trihydroxybutanal	**TR**SC	33	0.0013
48	Hexanetetrol	**SC**TR	97	0.0001
74	Unidentified	**SC**TR	70	0.0001
		TRSC	100*	0.0001

* Metabolites, which were detected only in test but not in control samples. **SC**TR: *S. commune* paired against *T. viride*; **TR**SC: *T. viride* paired against *S. commune*. **SC**SC: Self-paired *S. commune*; **TR**TR: Self-paired *T. viride.* The first two letters of the sample source abbreviation (in bold) represent the section of the interaction assay (the fungus occupying the domain) from which the sample was taken for analysis. In each case, the designated metabolite is up-regulated in the paired culture (SCTR or TRSC- depending on the domain from which sample was taken from paired cultures) compared to the self-paired cultures (SCSC or TRTR).

Table 3.2 **Down-regulated metabolites in paired cultures of** *S. commune* **and** *T. viride*

Peak	Metabolite identity	Source (sample)	% Decrease in peak area	*P* value
3	Acetic acid	**TR**SC	68	0.0005
4	Pyruvic acid	**SC**TR	67	0.0013
6	Glycerol	**SC**TR	60	0.0005
		TRSC	40	0.0131
10	Phosphoric acid	**TR**SC	68	0.0007
14	Alanine	**SC**TR	52	0.0012
16	Maleic acid	**SC**TR	43	0.0073
19	Erythritol	**TR**SC	35	0.0003
43	Xylitol	**SC**TR	56	0.0241
79	Sugar alcohol	**SC**TR	60	0.0018
58	Unidentified	**TR**SC	55	0.0003
50	2,3,4-trihydroxybutanal	**SC**TR	48	0.0328
72	Hexadecanoic acid	**SC**TR	46	0.0007
83	Octadecanoic acid	**SC**TR	64	0.0001
84	Octadecenoic acid	**SC**TR	37	0.0034

Same key as in table 3.1 for samples source/identity applies to table 3.2. The designated metabolite is down-regulated in the paired cultures (SCTR or TRSC) in comparison to the self-paired cultures (SCSC or TRTR).
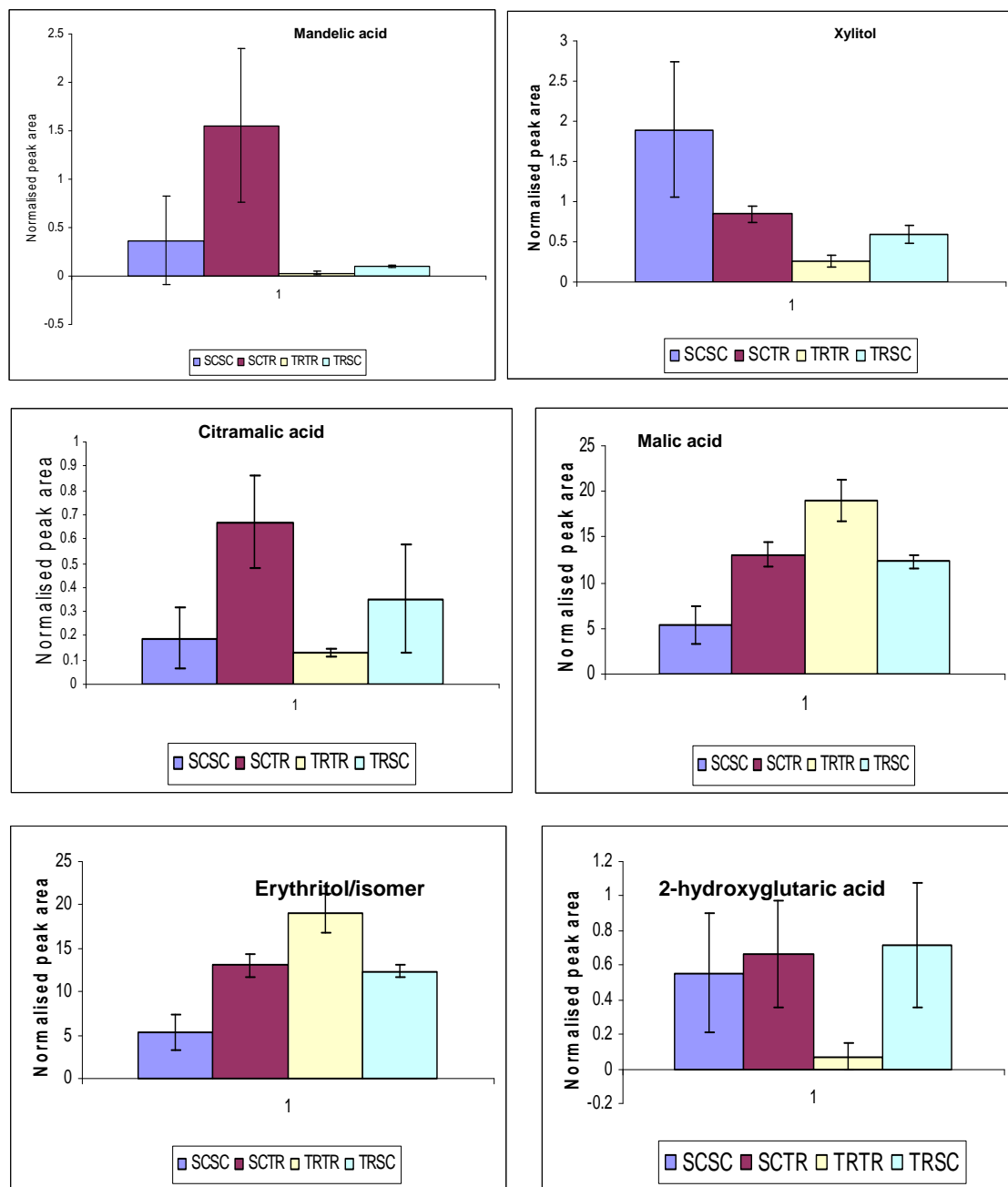
Figure 3.7 Plots of metabolite peak areas indicating down- or up-regulation of designated metabolites in *S. commune* and *T. viride* in dual cultures relative to their self-paired cultures. Up-regulated metabolites in *S. commune*: Mandelic acid, citramalic and malic acids and erythritol; Down-regulated metabolites: Xylitol; Up-regulated metabolites in *T. viride*: Xylitol, citramalic and 2-hydroxyglutaric acids; Down-regulated metabolites: Malic acid and erythritol. 10 biological replicas were analysed and error bars represents standard deviation.
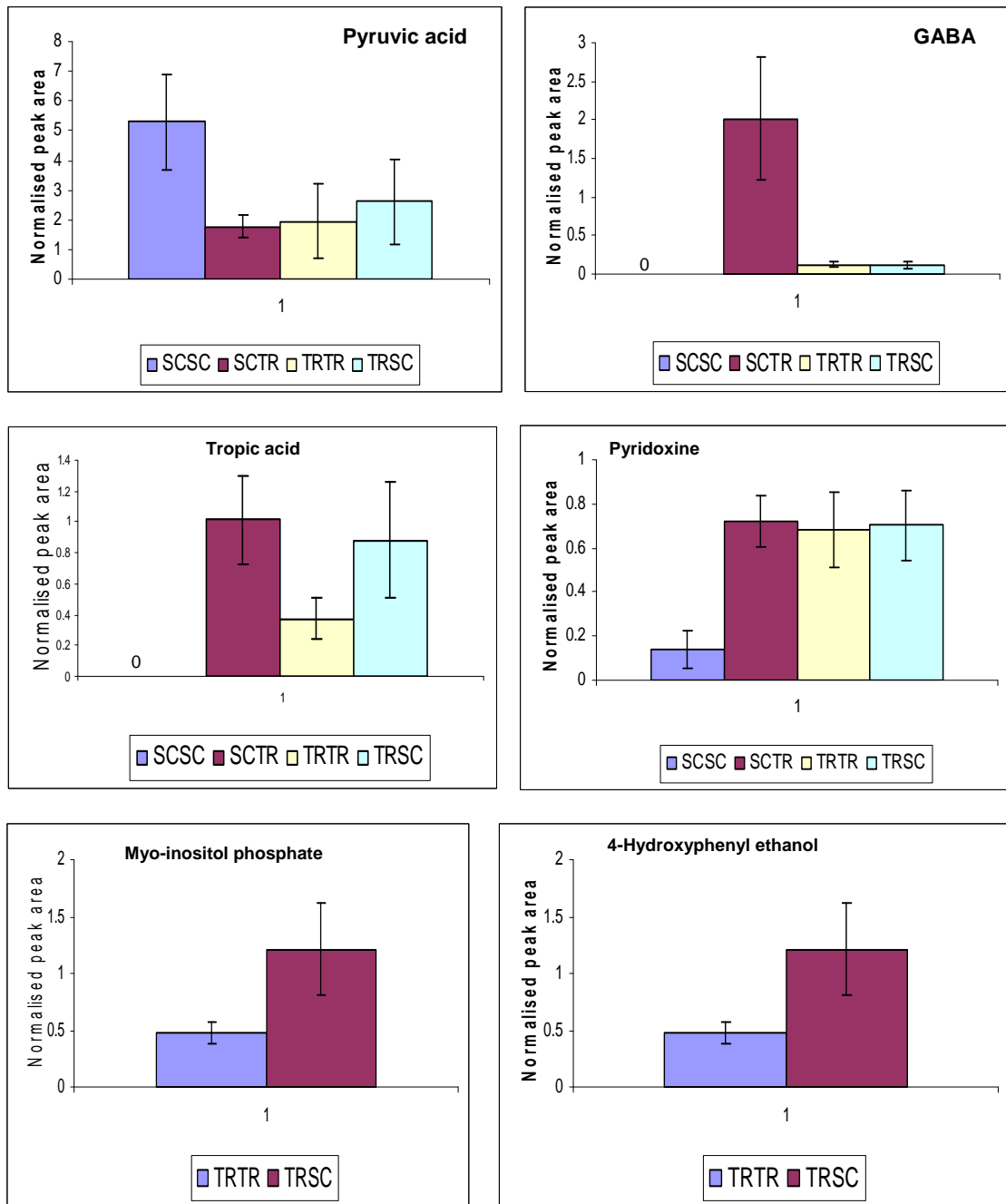
Figure 3.8 Comparative plots of metabolite peak areas (10 biological replicas). *S. commune*: **Up-regulation of GABA, tropic acid, pyridoxine, myo-inositol phosphate and down regulation of pyruvic acid;** *T. viride*: **Up-regulation of tropic acid and 4-hydroxyphenyl ethanol. Error bars represents standard deviation.**
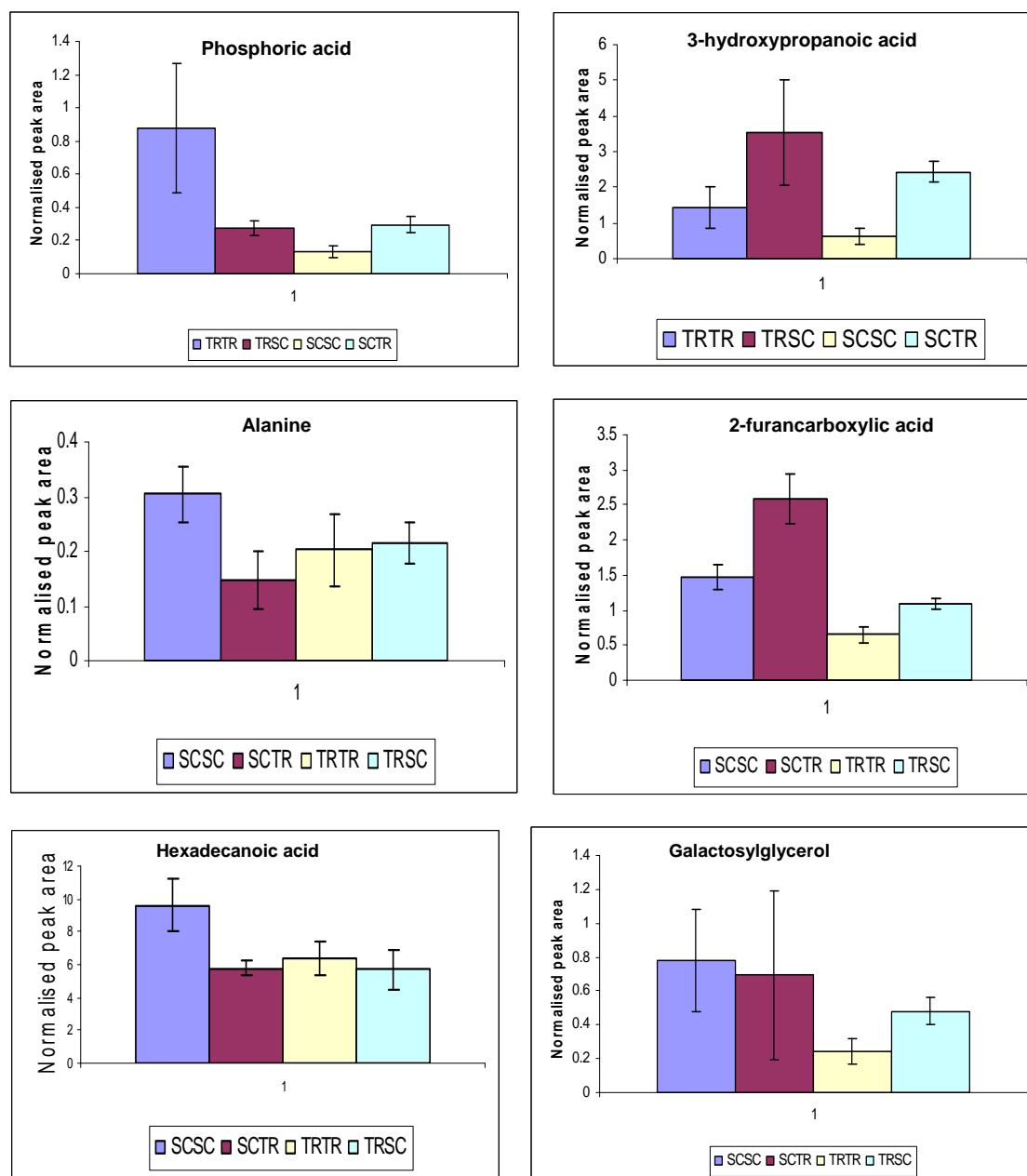
Figure 3.9 Plots of metabolite peak areas indicating down- or up-regulation of designated metabolites in *S. commune* and *T. viride* in dual cultures relative to the self-paired cultures. *S. commune*: Up-regulation of phosphoric, 3-hydroxypropanoic and 2-furancarboxylic acids and down-regulation of alanine and Hexadecanoic acid; *T. viride*: Up-regulation of 3-hydroxypropanoic and 2-furancarboxylic acids and Galactosylglycerol and down-regulation of phosphoric acid (additional metabolite peaks are included in appendix I). Error bars represents standard deviation.

3.4 Discussion

The aim of this aspect of the work was to comparatively analyse the patterns of metabolite expression in paired cultures of both fungi, relative to their self-interacting mycelia. Results from this aspect of the study showed that sugar alcohols were predominantly up-regulated in both species. For instance, xylitol and galactosylglycerol alongside two other peaks (44 and 79; table 3.1) identified as sugar alcohols were strongly up-regulated in *T. viride*. Similarly, erythritol and hexanetetrol were also up-regulated in *S. commune*. Phenolic secondary metabolites namely mandelic acid, tropic acid and 4-hydroxyphenyl ethanol were up-regulated in either or both species. Peak areas indicate that primary metabolites and metabolic intermediates such as fatty acids and pyruvic acid were down-regulated in *S. commune* paired against *T. viride*. GABA, synthesized via a shunt that bypasses steps in the Kreb's cycle was strongly up-regulated in mycelial samples of *S. commune* interacting with *T. viride*, with a corresponding increase in organic acids namely, 3-Hydroxypropanoic acid, malic and citramalic acids. Furthermore, pyridoxine, myo-inositol phosphate and N-acetylglucosamine increased significantly in *S. commune* mycelia confronted by *T. viride*.

3.4.1 Up-regulation of sugar alcohols

Increased solute concentration in any environment relative to the cytoplasmic concentration of living cells elevates osmotic pressure, which can inhibit metabolic functions (Adler *et al*., 1982). Yeasts and filamentous fungi respond to cytoplasmic osmotic pressure via the accumulation of ions and synthesis of well-suited osmolytes such as polyhydric alcohols (polyols/sugar alcohols), proline and trehalose (Davis *et al*., 2000). These compatible solutes (osmolytes) do not interfere with metabolic activities at high intracellular concentrations and allow the cell membrane to remain tightly pressed against the cell wall (Prescott *et al*., 1999; Shen *et al*., 1999; Ramirez *et al*., 2004). Different polyols such as glycerol, sorbitol, mannitol, arabitol, and erythritol have been implicated in osmotic stress response in filamentous fungi (Shen *et al*., 1999; Ramirez *et al*., 2004; Davis *et al*., 2000; Delgado-Jarana *et al*., 2006). In addition, galactosylglycerol (also known as isofloridoside), a low molecular weight carbohydrate has been strongly

implicated in osmotic stress response especially in algae (Kauss 1968, 1969 and 1973; Reed *et al*., 1980; Gerwick *et al*., 1990).

Given the strong up-regulation of these sugar alcohols, it is logical to infer that post-contact activities between both fungi may have resulted in an increase in the local osmotic pressure. This may have warranted an increase in the synthesis of sugar alcohols to counteract the pressure exerted on the cytoplasm by the external solute concentration. Osmotic stress response is mediated by an MAPK cascade referred to as high osmotic glycerol (HOG) pathway (Shen *et al*., 1999; Delgado-Jarana *et al*., 2006). Delgado-Jarana *et al* (2006) using HOG-GFP fusion as a biomarker demonstrated that the HOG pathway influences mycoparasitic behaviour in *Trichoderma harzianum* in addition to osmotic stress response. During mycoparasitism, *Trichoderma* species lyse the host's cell wall leading to total mycelial disintegration in the contact areas. Hence, *Trichoderma* species grow across a variety of metabolites, cell wall polymers and different intracellular environments during inter-specific mycelial interactions (Delgado-Jarana *et al*., 2006). The accumulation of sugar alcohols in the mycelia of *T. viride* is attributable in part to this phenomenon. Furthermore, microscopic images of the mycelia of both fungi within the interaction zone (chapter 2; section 2.3.2) show *T. viride* aggressively coiled around *S. commune*. *In vivo* studies of interactions between a GFP-tagged strain of *T. aureoviride* and two other fungal species, *Phytium ultimum* and *Rhizoctonia solani* showed the development of specialized structures – appressoria, papillae and hooks before and during contact with the host species (Lu *et al*., 2004).

Chet *et al* (1981) reported the development of similar structures when *Trichoderma hamatum* was paired against *R. solani*. Lu and co-workers (2004) hypothesized that since the fungal tip is an actively growing and sensitive area, the development of these specialized structures was most likely a response to osmotic pressure changes. Thines *et al* (2000) demonstrated that *Magnaporthe grisea* penetrates plant hosts with the aid of appressoria following the generation of high inner hydrostatic turgour pressure by the accumulation of molar concentrations of glycerol. Profuse mycelial growth and coiling of *T. viride* around *S. commune* lead us to argue that the accumulation of sugar alcohols in

T. viride may have been influenced to some extent by the need to generate inner hydrostatic turgour pressure required for the development of and coiling of specialized structures and mycelia around the host mycelia. *Metarhizium anisopliae* an entomopathogenic fungus employs a similar osmoregulatory mechanism during its attack on insects. For instance, disruption of a gene, *Mos1* that encodes an osmosensor in *Metarhizium anisopliae* resulted in alterations in the expression of genes involved in hyphal development, cell membrane stiffness, and generation of intracellular turgour pressure with a corresponding increase in mycelial sensitivity to osmotic and oxidative stresses (Wang *et al*., 2007).

Although most sugar alcohols were down-regulated in the mycelia of *S. commune*, erythritol and hexanetetrol were up-regulated. Following cell wall lysis, mycelia of *S. commune* must have been exposed to increased osmotic stress thereby, requiring biochemical osmoprotectants to cushion the increasing pressure on the cell membrane. This most likely accounts for the up-regulation of erythritol and hexanetetrol. Two or more sugar alcohols were up-regulated in each fungus. Fungi often produce a combination of omsolytes instead of a single sugar alcohol, because a mixture of sugar alcohols is more efficient for containing stress related to water activity/osmotic shock (Davis *et al.,* 2000; Ramirez *et al*., 2004). In addition, a mixture of sugar alcohols reduces toxicity, which might emerge from high concentrations of a single osmolyte as well as circumvents feedback inhibition that can arise from high concentrations of a single compound (Ramirez *et al*., 2004). Accumulation of sugar alcohols may contribute to oxidative stress adaptation/redox control by lowering NADH levels, which reduces the production of reactive oxygen radicals in the respiratory chain (Shen *et al*., 1999).

Furthermore, the production of most polyols for instance erythritol generates large amounts of NADPH (figure 3.9), which provides reducing power for antioxidant enzymes and oxidoreductases. Under conditions of oxidative stress, which most possibly was at play in *S. commune* during contact with *T. viride*, generation of NADPH helps to alleviate the deleterious effects of oxidative stress. Similarly, although xylitol may be synthesized directly from xylose, it may also be produced from ribulose-5-phosphate,

synthesized in the pentose phosphate pathway (PPP) (Diano *et al*., 2006). In addition to the NADPH, molecules generated in the PPP, xylitol synthesis further yields 5 molecules of NADPH. Up-regulation of xylitol in *T. viride* does suggest possible need to balance the redox status of the intracellular environment in addition to osmregulation.
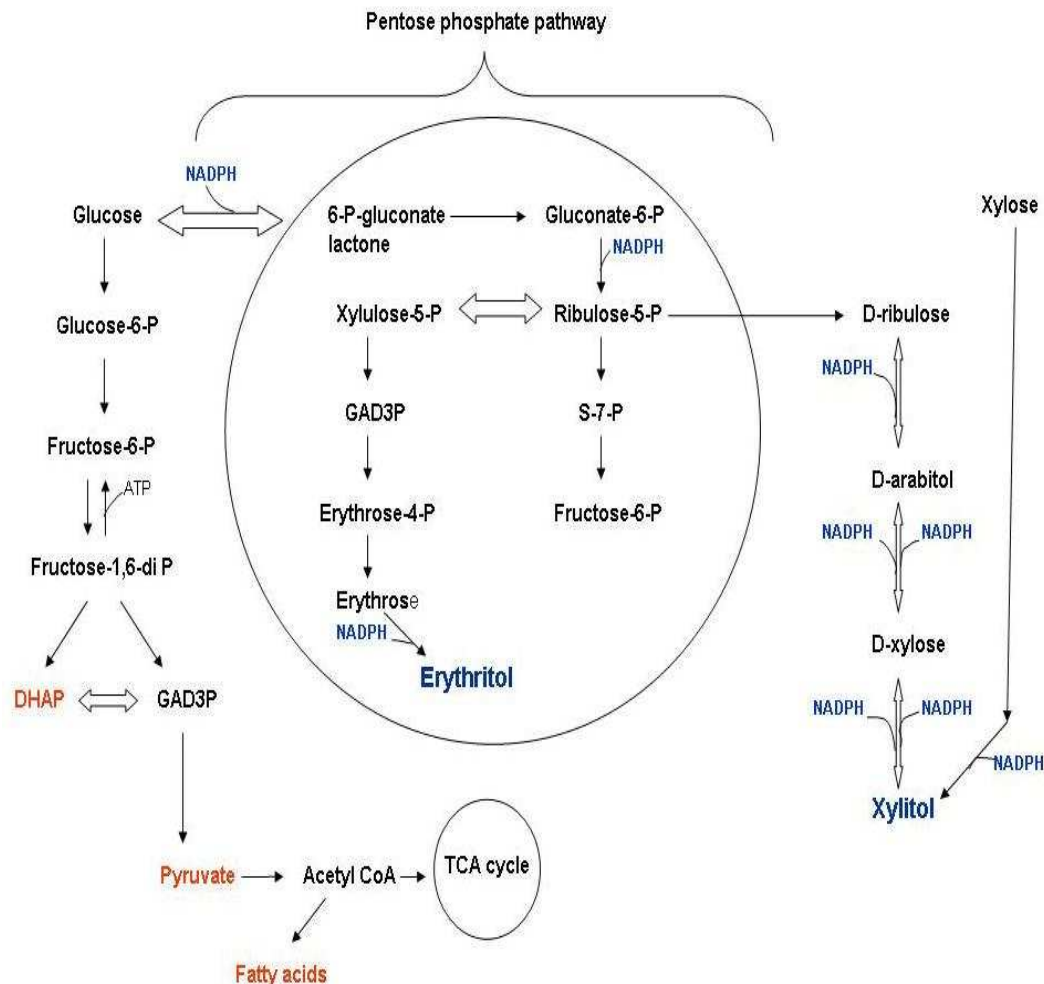
Figure 3.10 Schematic representation of erythritol and xylitol biosynthetic pathways (Modified from Diano *et al*., 2006; Lee *et al*., 2003). Dihydroxyacetone phosphate (leading to glycerol synthesis), pyruvate and fatty acids (all in red) were down-regulated in *S. commune*. Steps leading to erythritol synthesis (up-regulated in *S. commune*) pass through the PPP, generating NADPH, a key source of reducing power for antioxidant enzymes. Xylitol (up-regulated in *T. viride*) biosynthesis also, generates high levels of NADPH molecules (see tables 3.1 and 3.2 and figures 3.6 and 3.8).

3.4.2 Down-regulation of fatty acids and up-regulation of pyridoxine

Hexadecanoic, octadecanoic and octadecenoic acids (fatty acids) were down-regulated in *S. commune* (table 3.2 and figure 3.8). This may be as result of the down-regulation of fatty acid synthetic enzymes or hydrolysis of fatty acids to supplement the cell's energy requirements under conditions of impaired energy generation and nutrient acquisition. Both factors may have contributed to the decrease in the levels of fatty acids detected in *S. commune* paired against *T. viride*. For instance, RT-PCR showed that the gene, which codes for acyl carrier protein (ACP), a key enzyme in the fatty acid synthetic (FAS) pathway, was up-regulated after 24 hours of contact with *T. viride*. However, after 48 hours of mycelial contact, the expression level dropped (Chapter 6; section 6.3). Given that samples for metabolite profiling were taken after 48 hours of contact, down-regulation of this gene is in agreement with the assumption that fatty acid synthesis may have been down-regulated in *S. commune*. Nonetheless, caution ought to be exercised in the interpretation of this result considering that enzymes might be regulated at the protein level. Having noted this, the fact that the same gene was up-regulated on the first day of contact, and down-regulated by the second day, does suggest that ACP might be regulated at the gene level and that its expression was perhaps affected by inter-specific mycelial contact.

FAS enzymes are housekeeping enzymes, which play primary roles in cellular metabolism and cell multiplication (Schweizer & Hoffman, 2004). Their expression occurs mainly at an intermediate constitutive rate; however, they are subject to metabolic regulation given the high metabolic energy requirements of FAS (Schweizer & Hoffman, 2004; Stryer, 1995). Perhaps down-regulation of FAS is a physiological adaptation under conditions of intense stress, which allows the channelling of metabolic resources to the immediate need of stress adaptation and adjustment. Conversely, *S. commune* might have utilized fatty acids as substrates to meet its energy needs by hydrolysis. Under limiting conditions of growth, most organisms mobilise intracellular energy reserves such as lipids. Proteomic studies (Chapter 7) revealed the up-regulation of two proteins that bear conserved sequences of glyoxysomal malate dehydrogenase leading us to argue that the β-oxidation cycle might have been activated in *S. commune*.

The electrons arising from fatty acid oxidation are funnelled into ATP synthesis via the electron transport chain whereas the resulting acetyl-CoA from the same oxidation process is used to keep the TCA cycle running thereby conserving further energy (Kunze *et al*., 2006). It is worthy of mention that β-oxidation is a potent generator of reactive oxygen species and could be a key contributor to the oxidative stress indicators observed in this study. Pyridoxine is an essential co-factor of several enzymes that catalyze amino acid decarboxylation, transamination and racemation reactions (Ristilä *et al*., 2006). Furthermore, pyridoxine has been repeatedly implicated in antioxidation reactions during which it is thought to quench singlet oxygen and hydrogen peroxide (Ristilä *et al*., 2006; Ehrenshaft & Daub, 2001; Gonźalez *et al*., 2007; Shi *et al*., 2002; Denslow *et al*., 2007). Strong up-regulation of pyridoxine in the mycelia of *S. commune* supports the data in chapter 5 showing the induction of oxidative stress in *S. commune* following contact with *T. viride*.

3.4.3 Induction of GABA

It is worth mentioning that the simultaneous up-regulation of pyridoxine and GABA may not be unconnected. GABA, a non-protein amino acid is synthesized in the GABA bypass (GABA shunt) which obviates a key regulatory step in the Kreb's cycle; Conversion of α-ketoglutarate to succinate via Succinyl-CoA (discussed in chapter 6; section 6.4 – Down-regulation of α-ketoglutarate dehydrogenase) (Panagiotou *et al*., 2005; Bauché *et al*., 2003; Solomon *et al*., 2002). The GABA shunt involves amino acid decarboxylation and transamination reactions of which the enzymes that catalyse these reactions require pyridoxine as co-factor. In addition, activation of GABA shunt occurs when the TCA cycle is blocked as a result of down-regulation or repression of α-ketoglutarate dehydrogenase, which is sensitive to redox imbalance. RT-PCR results in chapter 6 show that this enzyme was repressed upon contact with *T. viride* suggesting that redox imbalance/oxidative stress might have been at play within the mycelia. This as in the case of pyridoxine points to possible prevalence of oxidative stress in the mycelial of *S. commune* following contact with the mycelia of *T. viride* (See chapter 5, section 5.4.3 for further discussion on the implication of GABA shunt oxidative stress response).

3.4.4 Up-regulation of Phenolic metabolites

Rayner *et al*. (1994) proposed that fungus-fungus contact elicits secondary metabolism in the interacting species. Secondary metabolism is characterized by increased secretion of phenolic compounds some of which are for defence/attack purposes (Peiris *et al*., 2007; Lardner *et al*., 2006). Increased production of phenolic compounds is considered one of the factors that promote the secretion of phenoloxidases during combative interactions in fungi (Gregorio *et al*., 2005; Peiris *et al*., 2007; Rayner *et al*., 1994). Mandelic acid was markedly up-regulated in *S. commune*, whereas tropic acid was strongly induced in the same fungus and up-regulated in *T. viride* in addition to the up-regulation of 4-hydroxyphenyl ethanol. Induction of phenylalanine ammonia lyase (PAL) a key enzyme of the phenylpropanoid pathway (a major source of phenolic compounds) is extensively discussed in chapter 7 (Section 7.4.1.3). Up-regulation of PAL, an enzyme of secondary metabolism suggests that the up-regulation of phenolic compounds is perhaps a sequel to the induction of secondary metabolism.

Furthermore, considering the sequence of events, leading to the up-regulation of phenylalanine ammonia lyase (PAL) (Chapter 7), phenolic compounds (above and in Chapter 4) and phenoloxidases (Chapter 5) it is possible that the production of phenolic compounds is most likely a response rather than an attack mechanism, especially in *S. commune*. However, considering that PAL and phenoloxidases were not detected in *T. viride*, it is unlikely that both fungi secrete phenolic compounds by the same mechanism. In addition, *T. viride* might employ phenolic compounds for attack purposes given its antagonistic nature. Albeit antibiosis has been implicated in the attack machinery of *Trichoderma* species (Schirmböck *et al*., 1994; El Hajj *et al*., 1989), there is no mention of phenolic compounds as part of their attack strategy. Nonetheless, it is not impossible that they might employ phenolics for antibiosis during combative interactions.

3.4.5 Up-regulation of organic acids

Fungi thrive at an acidic pH hence; they tend to acidify their immediate environment as a means of discouraging the growth of competitors (Magnuson & Lasure, 2004). The levels

of organic acids observed for both fungi tend to suggest that they may have employed acidification as a means of protecting their territory from invasion. Although both fungi up-regulated organic acids, 3-hydroxypropanoic, phosphoric, malic, citramalic and 2-furancarboxylic acids were up-regulated in *S. commune* mycelia while only 3-hydroxypropanoic, 2-hydroxyglutaric and furancarboxylic acids were up-regulated in *T. viride*. Chances are that both fungi possess mechanisms for dealing with acidification, thus it is unlikely that acidification may have played significant role in the outcome of interactions between *S. commune* and *T. viride* particularly on agar.

3.4.6 Down-regulation of pyruvic acid (metabolic intermediate)

The strong down-regulation observed for pyruvic acid in the mycelia of *S. commune* confronted By *T. viride* is attributable to either down-regulation of glucose-hydrolytic pathways such as glycolysis or an increased flux through the Kreb's cycle. Down-regulation of *S. commune* glyceraldehyde-3-phosphate dehydrogenase gene (chapter 6) suggests that glycolysis may have been biochemically scaled down. This notwithstanding, alternative pathways such as pentose phosphate pathway (PPP) might have been up-regulated as suggested by the increase in the activity of glucose-6-phosphate dehydrogenase, a key enzyme of the PPP.

In the light of this, it is unlikely that slower flux through the glucose-hydrolytic pathways is responsible for the drop in the levels of pyruvate detected in *S. commune*. On the other hand, high levels of malic acid and up-regulation of 2 isoforms of malate dehydrogenase (chapter 7; section 7.4.1.5) do suggest that there may have been an increase in flux through the Kreb's or glyoxylate cycle which is most probably responsible for the depletion of pyruvate. Under severe stress, it is likely that either cycle would play a key role in the supply of energy required to maintain cellular activities particularly, those associated with stress response. Down-regulation of alanine might be as a result of reduced production of the amino acids owing to secondary metabolism resulting in the hydrolysis of amino acids as sources of carbon and nitrogen. Proteomic studies (Chapter 7; sections 7.4.1.3 & 7.4.1.5) shows the up-regulation of two enzymes of secondary metabolism involved in amino acid hydrolysis namely PAL and branched-chain α-

ketoacid dehydrogenase E2 subunit (BCKAD). It is likely that both factors are involved in the down-regulation of alanine. Rise in the levels of N-acetylglucosamine, the product of chitin hydrolysis (Lorito *et al*., 2001; De la Cruz *et al*., 1993) confirms the digestion of *S. commune* cell wall by chitinase of *T. viride* after mycelial contact between both organisms.

3.4.7 Induction of myo-inositol phosphate

Although there is no clear specification regarding the actual identity of the myo-inositol phosphate detected in the mycelia of *S. commune* paired against *T. viride*, both inositol biphosphate and inositol triphosphate have been strongly implicated in osmotic stress response in yeast (Perera *et al*., 2003) and *Arabidopsis* cell culture (Takahashi *et al*., 2001). The fact that inositol phosphate was barely detected in the control samples, but showed marked increase in peak area strongly suggests that it plays a vital role during interaction with the antagonist. Although there is not enough data to understand the possible role of this compound during this interaction, in the light of events stemming from mycelial contact with *T. viride*, osmotic shock response is its possible role. Homan *et al* (1998) showed that inositol biphosphate is required for the integrity of the subplasmalemmal cytoskeleton. In view of the fact that *S. commune* cell wall was hydrolysed following contact with *T. viride*, which increases the exposure of *S. commune* to osmotic stress, strengthening of the cytoskeleton, which serves as the main protective envelope under this condition, would be paramount. In addition, inositol phosphates are important second messengers involved in a wide range of molecular and physiological activities (Perera *et al*., 2003). Hence, there is a possibility that they might be involved in the sensing and signalling of stress upon contact with *T. viride*.

3.5 Summary and conclusions

HPLC profiling showed alteration in peak patterns suggesting that mycelial contact between both organisms elicited the up- and down-regulation of metabolites. This was confirmed by GC-MS, where the patterns of metabolite expression suggest that contact between *S. commune* and *T. viride* engendered osmotic stress in both fungi and oxidative stress predominantly in the mycelia *of S. commune*. This is reflected by the increase in

peak areas of sugar alcohols in both organisms, which most likely helped to counterbalance the rising concentration of the local environment because of cell wall hydrolysis and subsequent disintegration of the mycelia of *S. commune*. Generation of NADPH during the biosynthesis of sugar alcohols might contribute to the provision of reducing power required for antioxidation. Rise in the levels of pyridoxine and GABA buttress the assumption that redox imbalance hence; oxidative damage might be at play within the mycelia of *S. commune* following 48 hours of mycelial confrontation with *T. viride*. The levels of phenolics and alanine are pointers to probable onset of secondary metabolism during interaction, especially in *S. commune* whereas *T. viride* might employ phenolic compounds for attack purposes. Both fungi appear to acidify their growth domains as a means of checking invasion by competitors. Myo-inositol phosphate, which was induced in *S. commune*, might mediate osmotic stress detection and response.

CHAPTER 4

Indicators of oxidative stress and induction of Secondary metabolism in the mycelia of *S. commune* interacting with *T. viride*

4.1 Introduction

Generally, the strong reactions observed in mycelia involved in interspecific combative interactions such as sealing-off of the mycelial front and cessation of growth upon contact, pigmentation, barrage formation and protoplasmic degeneration in the interaction zone, suggest the prevalence of stress, particularly oxidative damage, in the combat zone. Baker and Orlandi (1995) reported rises in the levels of reactive oxygen species (ROS) in interacting fungal mycelia, favouring wood degradation when fungal mycelia of different wood-rot species interact on wood. Pigmentation, a consistent feature of combative interactions, has been suggested to arise from the build up of free radicals in the hyphal tip region (Chi *et al*., 2006). Up-regulation of phenoloxidases is also thought, in part, to be influenced by the physiological need to scavenge reactive oxygen species in the mycelial confrontation zone (Hanson *et al*., 1999; Crowe & Olsson, 2000). Furthermore, protoplasmic degeneration, another principal feature of interspecific combative interactions, is thought to occur as a result of the accumulation of phenolic compounds and toxic free radicals in paired mycelia (Iakovlev & Stenlid, 2000).

Using differential a gene expression strategy, Iakovlev *et al*. (2004), generated molecular data implicating oxidative damage in the changes associated with the adaptation of *Heterobasidion annosum* (a phytopathogen) to antagonistic confrontation by a potential biocontrol fungus, *Physisporinus sanguinolentus*, on agar plates. A gene encoding a mitochondrial import protein was repressed in the mycelia of *H. annosum* 24 hours post contact with *P. sanguinolentus*. This is a potential indicator of mitochondrial dysfunction/damage, which ultimately promotes electron leakage, and possible production of ROS, such as hydroxyl radical. Furthermore, a gene homologous to *Coprinus cinereus rah1* (*recA* homologue) was up-regulated in *H. annosum* (Iakovlev *et al*., 2004). *recA* codes for a RAD51 protein that is involved in DNA repair and homologous recombination under conditions of stress (Nathan & Shiloh, 2000; Bishop, 1994; Game, 1993; Stassen *et al*., 1997). γ-irradiation, known to induce the production of hydroxyl radicals that damage DNA also triggered a rise in the expression of RAD51 in *C. cinereus* (Stassen *et al*., 1997) and *S. cerevisiae* (Mikosch *et al*., 2001). Iakovlev and his colleagues (2004) concluded that up-regulation of a *recA* homologue in *H. annosum*

paired against *P. sanguinolentus* was most likely related to the production of free radicals and oxidative damage.

Electron leakage in the mitochondrion and the resulting oxidative damage, has been reported to be associated with the induction of secondary metabolism (Rayner & Griffith, 1994). Hence, it is worth mentioning that the repression of a mitochondrial import protein and up-regulation of *recA* homologue are also indicative of a switch to secondary metabolism. Iakovlev *et al*. (2004) also reported the repression of cystathionine gammalyase gene, and a fimbrin protein-encoding gene (involved in actin polarization), both of which are associated with primary metabolic growth. This is a further indication of the induction of secondary metabolism. Rayner and Griffith (1994) put forward the most widely upheld explanation for the physiological changes that characterise antagonistic interactions in fungi. They suggested that fungus-fungus interactions promote the secretion of hydrophobic metabolites, which are oxidised by phenoloxidases, leading to the generation of free radicals and cell wall-sealing polymers. These biochemical events dislocate nutrient supply, leading to reduction or cessation of growth, thereby triggering secondary metabolism. In view of this, disruption of ATP synthesis and electron transport, a major source of ROS, which have been implicated in fungus-fungus interactions, most likely stem from the sharp inception of a secondary metabolic-dominated phase of growth when mycelia of opposing species make contact (Rayner, 1997).

Gregorio *et al*. (2006) reported that mycelial contact between *Marasmius pallescens* and *Maramiellus troyanus* caused protoplasmic degeneration, an indication of secondary metabolism and oxidative damage in both fungi, but mainly in the former. Copper, paraquat, and alcohol treatments, established elicitors of oxidative damage (although copper is also laccase co-factor, hence transcriptionally elicits laccase gene expression), mimicked laccase secretion on *Rhizoctonia solani* as in combative interaction with other species (Crowe & Olsson, 2000). More importantly, these treatments also triggered lipid peroxidation and dry weight loss in *R. solani*. Despite strong indications that oxidative stress may be at the core of the physiological factors/signals that drive or ensue

combative interactions in fungi, very few studies have directly investigated the incidence of oxidative stress indicators in paired fungal cultures (Baker and Orlandi, 1995; Crowe & Olsson, 2000).

Hence, the aim of this chapter was to investigate the levels of lipid peroxidation, protein carbonylation, and superoxide anion ($\dot{O}_2^-$) in the mycelia of *S. commune* interacting with *T. viride*. Levels of phenolic compounds and ATP in growing cells are strong indicators of the prevailing phase of growth. In the light of this, the levels of ATP were assayed in *S. commune* mycelia whilst the levels of phenolic compounds were assayed in the mycelia of both interacting fungi.

4.2 Materials and Methods

4.2.1 Lipid peroxidation assay

Lipid peroxidation is an established indicator of cellular damage in both plants and animals. Hence, it is used as a pointer of oxidative stress in cells and tissues (Esterbauer *et al.*, 1991; Carbonneau *et al.*, 1991). Lipid peroxides are unstable; they decompose to form a complex chain of compounds such as reactive carbonyl compounds (Esterbauer *et al.*, 1991; Carbonneau *et al.*, 1991). Polyunsaturated fatty acid peroxides produce malondialaldehyde (MDA) and 4-hydroxyalkenals (4-HNE) on decomposition (Esterbauer *et al.*, 1991; Carbonneau *et al.*, 1991). As a result of this, measurement of levels of these two markers; malonaldehyde and 4-hydroxyalkenals, is used as an indicator of lipid peroxidation ((Esterbauer *et al.*, 1991). Lipid peroxidation assay was carried out using Oxis Bioxytech® LPO-586™ colorimetric lipid peroxidation assay kit (Oxis international), following the manufacturer's protocol.

0.2 g of mycelia-agar samples was cut off with scalpel every 24 hours, within the domain occupied by *S. commune*, 10 mm from the interaction line. Samples were homogenised in a Fastprep-24 homogeniser (MP, UK) and homogenates were reconstituted in 1 ml of sterile distilled water. Auto- or photo oxidation was prevented by the addition of

butylated hydroxytoluene to the samples to a final concentration of 5 mM, and assay was carried out in the dark.

200 μL of the supernatant after centrifugation (13,000 rpm for 10 minutes) was used for the assay, which is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-HNE at 45°C. One molecule of either of MDA and 4-HNE reacts with two molecules of N-methyl-2-phenylindole to yield a stable chromatophore with maximal absorbance at 586 nm. 200 μl of sample was mixed with 650 μl of N-methyl-2-phenylindole (pre-formulated in acetonitrile by the manufacturer. Information on the concentration is not provided) in a propylene micro centrifuge tube in triplicate. The mixture was vortexed gently, and then 150 μl of methanesulfonic acid was added. The reaction mixture was vortexed gently, and incubated at 45°C for 1 hour. The resulting turbid samples were centrifuged at 12,000 rpm for 15 minutes to obtain a clear supernatant. The clear supernatant was transferred to a cuvette and absorbance was measured at 586 nm. MDA was used as standard at concentrations ranging from 0.5 to 4.0 μM to plot a standard curve (see Appendix II for MDA standard curve).

4.2.2 Protein carbonylation assay

Protein carbonyl content was measured in *S. commune* mycelia over the same period as lipid peroxidation, according the method described by Reznick *et al*. (1994) with a few modifications. 0.2 g of mycelial samples homogenised as described in section 4.2.1 were reconstituted in 1 ml of 1X phosphate-buffered saline (PBS) with 25 μL of fungal protease inhibitor cocktail (Sigma-Aldrich, UK). 100 μg of protein homogenate was mixed with 500 μL of 10 mM 2,4-dinitrophenyl hydrazine (DNPH), dissolved in 2 M HCL and incubated at 37^0C for 1 hour. Proteins were then precipitated with 20% trichloroacetic acid for 20 mins at 4^0C, centrifuged at 13,000 rpm for 15 mins and the resulting pellet was washed 3 times with ethanol: ethylacetate (1:1) to remove excess DNPH. The pellet was redissolved in 1.5 ml of 6M guanidine hydrochloride (pH 2) and absorbance was read at 380 nm. With a molar absorption coefficient ($\square$) of 22,000 M^{-1} cm^{-1}, protein carbonyl was calculated as nanomoles of DNPH incorporated (protein carbonyls) per milligram of protein. Protein concentration was quantified according to

Bradford method (1976).10 µL of each sample was diluted by making it up to 200 µL (in PBS) and mixed with 800 µL of Bradford reagent (Sigma). Absorbance was read at 595 nm in Novaspec II spectrophotometer (Amersham, UK). Standard curves were prepared using Bovine serum albumin (BSA) as standard over a concentration range of 0.1 to 1.5 mg/ml (see Appendix II for protein standard curve).

4.2.3 Assay for the concentration of phenol compounds

The contents of phenol compounds in mixed and self-paired cultures of *S. commune* and *T. viride* were measured spectrophotometrically by the diazosulphanilamide method described by Malarczyk *et al*. (1989). 0.2 g of freeze-dried mycelia-agar samples were ground to powder, reconstituted in 1 ml of sterile distilled water and centrifuged at 12,000 rpm for 15 minutes at 4^oC. The assay mixture contained 100 µL of supernatant, 100 µL of diazosulphanilamide (1 % diazosulphanilamide in 10 % HCl) and 100 µL of 5 % (w/v) sodium nitrite. The resulting solution was alkalized by the addition of 1 ml of 20 % (w/v) sodium carbonate, and change in absorbance was measured at 400 nm. Phenol content was expressed as a function of absorbance.

4.2.4 Measurement of extracellular superoxide anion content

The levels of Superoxide anion radicals were rapidly assessed spectrophotometrically by measuring the superoxide anion-dependent formation of formazone with nitrotetrazolium blue (NBT) in an alkaline medium (Malarczyk *et al*., 1997). The reaction mixture consisted of 3 ml of sterile distilled water, 50 µL of 1 M sodium hydroxide and 100 µL of 5 mM NBT solution, to which 0.1 g of fresh mycelia-agar samples were added. The mixture was incubated at room temperature for 10 minutes, spun at 13, 000 rpm for 2 minutes, and the absorbance of the supernatant was read at 560 nm. The superoxide anion content of the mycelial samples was expressed as a function of absorbance reading at 560 nm.

4.2.5 ATP assay

ATP was first extracted by boiling 0.2 g of *S. commune* mycelial samples in TE buffer (0.1 M Tris, 2mM EDTA pH 7.75) for 5 mins. ATP content of the samples was measured by the use of a bioluminescence-based ATP assay kit (Sigma-Aldrich), according to the

manufacturer's protocol. The assay is based on the utilization of ATP by firefly luciferase to produce light from luciferin. The amount of light emitted (in equation 2 below) is proportional to the amount of ATP present in a sample. 100 μL of ATP assay mix solution was allowed to stand for 3 mins at room temperature. This allows endogenous ATP to be hydrolyzed. Reaction was started by rapidly adding 100 μL of sample/standard to the assay solution, mixed quickly by swirling and measuring the amount of light produced in a luminometer (see Appendix II for standard curve).

$$\text{ATP} \quad + \quad \text{Luciferin} \quad \xrightleftharpoons[\text{Mg}^{++}]{\text{Firefly luciferase}} \quad \text{Adenyl-luciferin} \quad + \text{PPi} \quad (1)$$

$$\text{Adenyl-luciferin} \ + \ O_2 \ \longrightarrow \ \text{Oxyluciferin} \quad + \text{AMP} + CO_2 + \text{Light} \ (2)$$
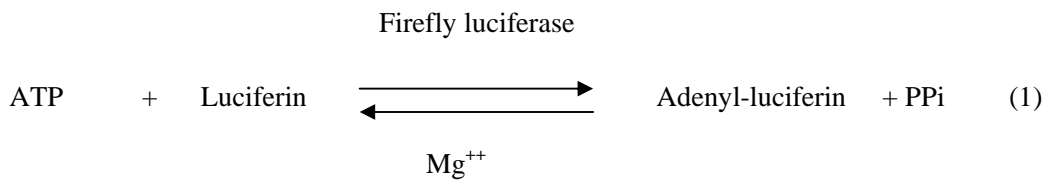
4.3 Results

4.3.1 Levels of lipid peroxides and intracellular carbonylated proteins in the mycelia of *S. commune* following contact with *T. viride*

Levels of lipid peroxides (LPO) and protein carbonyls in *S. commune* mycelia are presented in figures 4.1 and 4.2 respectively. Differences in levels detected in test (SCTR) and control samples (SCSC) for each assay were analysed using unpaired *t*-test to determine their levels of significance. LPO increased sharply after 24 hours of contact with *T. viride* 2.6 fold higher than levels detected in self-paired mycelia of *S. commune* ($P = 0.0026$). Afterwards, LPO decreased with increasing duration of mycelial contact. However, LPO levels remained significantly higher in the mycelia of *S. commune* interacting with *T. viride* after 48 and 72 hours of contact, 3.7 fold and 2.0 fold ($P = 0.0022$; $P = 0.0331$) respectively than in the self-interacting cultures. Measurements of protein carbonyl content showed higher levels of protein carbonylation in mycelia of *S. commune* paired against *T. viride* during the first 3 days of contact with *T. viride*, 1.3

folds, 2.7 fold and 2.2 fold ($P = 0.0002$; $P < 0.0001$; $P < 0.0001$) respectively than in self-paired cultures. This was followed by a decline in protein carbonyl levels in the interacting cultures of *S. commune* and *T. viride*, and a modest rise in self-paired cultures.
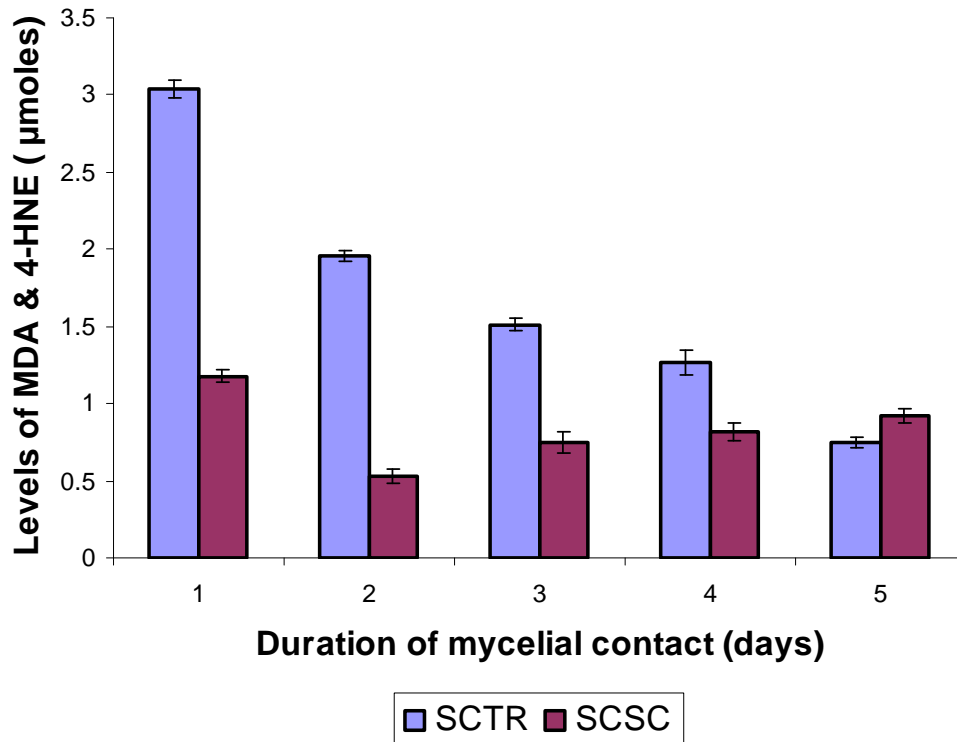
Figure 4.1 Levels of malonaldehyde and 4-HNE in the mycelia of *S. commune* paired against *T. viride* in comparison to levels in self-paired cultures over an interaction period of five days. All experiments were carried out in triplicates, using 3 independent biological samples (cultures) in each for both test and control samples. Error bars represent standard deviation. SCTR: *S. commune* vs. *T. viride*; SCSC: *S. commune* vs. *S. commune*.
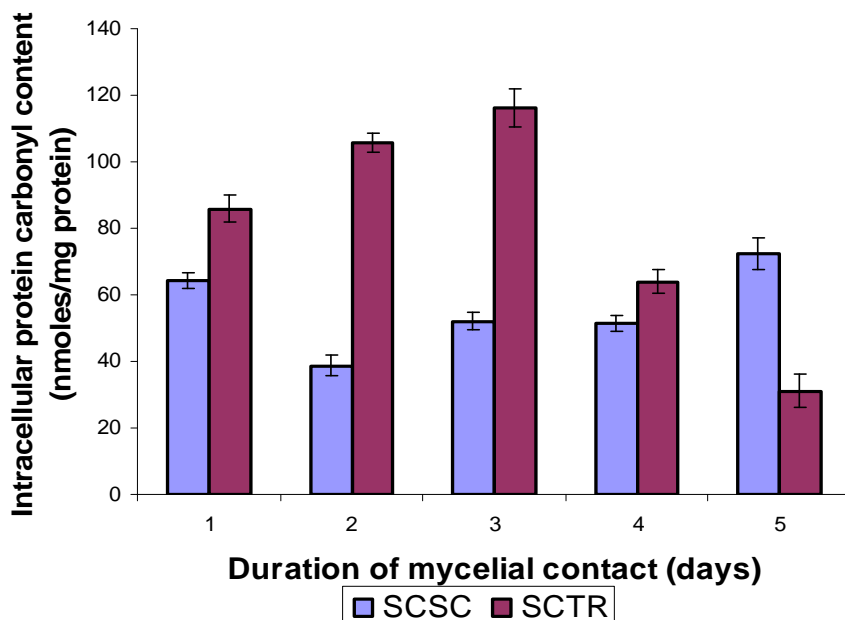
Figure 4.2 Comparative levels of intracellular protein carbonyl content in the mycelia of *S. commune* paired against self and against *T. viride* (SCSC: self-paired *S. commune*. SCTR: *S. commune* paired with *T. viride*).

4.3.2 Levels of superoxide anion and phenol compounds in interacting cultures of *S. commune* and *T. viride*

Levels of superoxide anion radicals and phenolic compounds were measured as a function of absorbance readings at 560 nm and 400 nm respectively (figures 4.3 and 4.4) according to the methods of Malarczyk *et al*. (1997 and 1989 respectively). Extracellular superoxide anion radicals increased rapidly in the mycelial domains of both *S. commune* and *T. viride* in interacting cultures compared to the controls. Absorbance readings for superoxide anion radicals increased 12 fold, 9.2 fold and 4.4 fold after 24, 48, and 72 hours ($P < 0.0001$; $P < 0.0001$; $P = 0.0024$) of contact respectively, in the mycelia of *S. commune* confronted by *T. viride*, relative to the self-paired cultures. Absorbance readings for *T. viride* mycelia paired against *S. commune* were 3 fold and 2.3 fold ($P = 0.0009$; $P = 0.0075$) higher than in the self-interacting cultures after 24 and 48 hours of contact respectively. Levels of superoxide anion in the mycelia of *S. commune* paired against *T. viride* dropped after 72 hours of contact, and increased modestly after 48 hours

in the mycelia of *T. viride* interacting with *S. commune*. However, levels of superoxide anion radicals in self-paired mycelia of *T. viride* increased progressively, with increased duration of contact, until they reached relatively similar levels as in *T. viride* mycelia interacting with *S. commune*.

Based on the absorbance readings, phenol compounds were 2.3 fold ($P = 0.0002$) and 1.5 fold ($P = 0.0004$) higher in the mycelia of *S. commune* paired against *T. viride*, 24 and 48 hours post contact respectively. The amounts of phenol compounds however decreased after 48 hours to similar levels as in self-paired cultures where they stabilized for the rest of the experiment. Increases in the amounts of phenol compounds were also detected in the mycelia of *T. viride* paired against *S. commune*. After 24 hours of interspecific mycelial contact they reached an absorbance reading of 1.29; 1.4 fold ($P = 0.0016$) higher than levels detected in self-paired *T. viride* (figure 4.4).
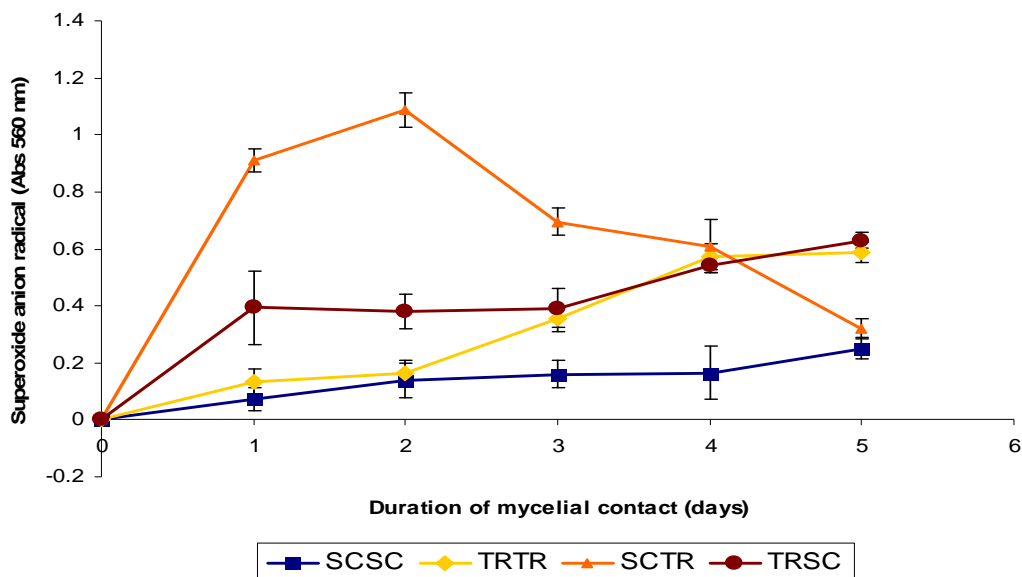
Figure 4.3 Levels of superoxide anion radicals in interacting cultures of *S. commune* and *T. viride* relative to their self-paired controls (SCSC: *S. commune* paired against self; SCTR: *S. commune* paired against *T. viride*; TRTR: *T. viride* paired against self; TRSC: *T. viride* paired against *S. commune*). Experiments were carried out in triplicates and error bars represent standard deviation.
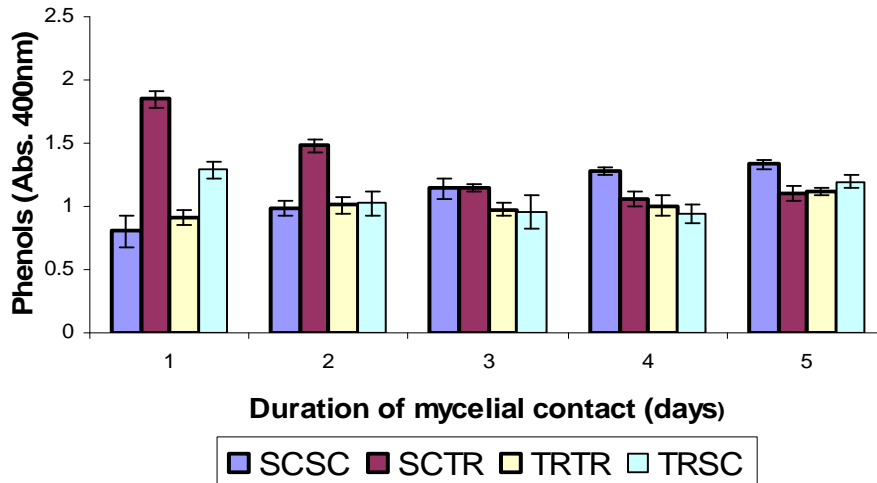
Figure 4.4 Levels of phenol compounds in co-cultures of *S. commune* and *T. viride* relative to their self-paired cultures (SCSC: self-paired *S. commune*. SCTR: *S. commune* paired with *T. viride*). Experiments were carried out in triplicates. Error bars represent standard deviation.

4.3.3 Amounts of ATP in the mycelia of *S. commune* paired against *T. viride* in comparison to self-paired mycelia

There were no significant differences between the amounts of ATP detected in the mycelia of *S. commune* paired against *T. viride* and the self-paired mycelia of *S. commune* until after 5 days of mycelial contact. After 5 days of interspecific mycelial contact between both organisms, the amounts of ATP in the mycelia of *S. commune* interacting with *T. viride* decreased 1.2 fold ($P = 0.0002$) relative to the self-paired mycelia.
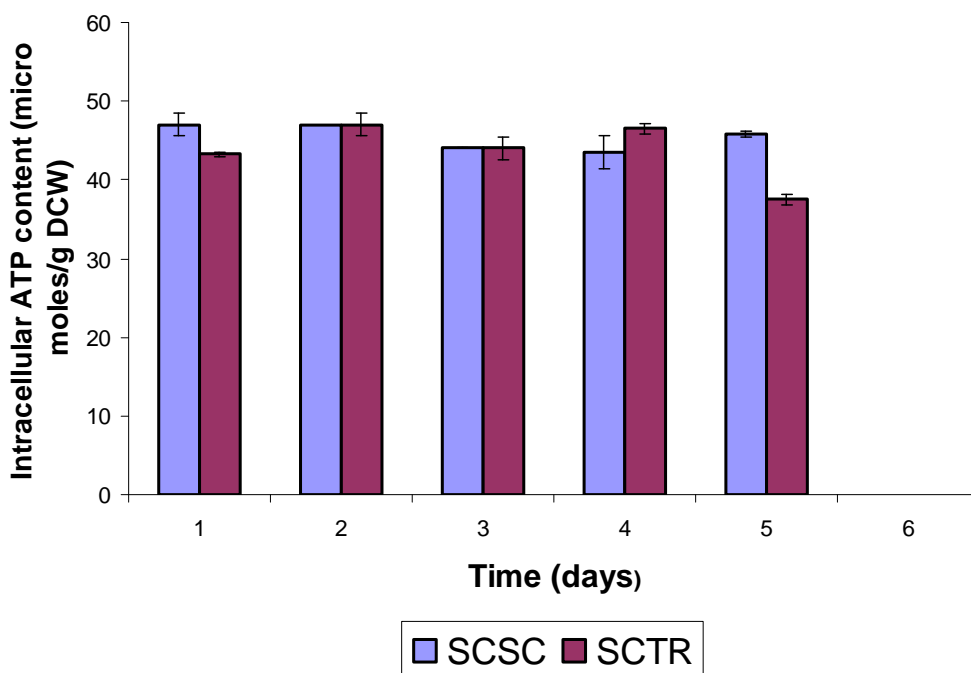
Figure 4.5 The amounts of ATP in the mycelia of *S. commune* paired against *T. viride*, relative to self-paired *S. commune*. Assay was performed in triplicates, and error bars represent standard deviation. SCSC: self-paired *S. commune*. SCTR: *S. commune* paired with *T. viride*.

4.4 Discussion

The levels of lipid peroxides, intracellular protein carbonyl content and superoxide anion radicals detected in the mycelial samples of *S. commune* interacting with *T. viride* strongly implicate the induction of both extracellular and intracellular oxidative stress/damage in the mycelia of *S. commune*, compared to its self-interacting mycelia. In addition, together with the increased levels of phenol compounds in the early stages of interspecific mycelial contact, these data are also indicative of a possible switch of mycelial growth to secondary metabolism during contact with the antagonist. The amounts of ATP in the mycelia of *S. commune* confronted by *T. viride*, suggest that the former may have activated some mechanism(s) for sustaining ATP generation and perhaps key metabolic processes necessary for survival prior to the later stages of

combat. Furthermore, whereas lipid peroxidation and protein carbonylation were not assayed in *T. viride*, the levels of superoxide anion radicals detected in its mycelia also suggest an early induction of oxidative burst upon contact with *S. commune*, although significantly milder than levels detected in the mycelia of *S. commune*. However, given the robust biochemical and physiological abilities of *T. viride* to attack and utilize *S. commune* mycelial components as nutrients, it is very unlikely that the slight increase in phenol compounds in the mycelia of *T. viride* after 24 hours of interspecific contact is related to the induction of secondary metabolism in the latter.

4.4.1 Increase in the levels of lipid peroxidation and superoxide anion in the mycelia of *S. commune* paired against *T. viride*.

A common feature of reactions between free radicals and non-radicals is the initiation of chain reactions (Halliwell & Chirico, 1993). For instance, extremely reactive radicals such as hydroxyl radical ($OH^•$) attack biological molecules such as lipids by removing a hydrogen atom from the lipid molecule (Halliwell & Chirico, 1993). The resulting lipid radical according to the equation below ensures the elongation of a lipid peroxidation chain reaction by functioning as a radical itself (Halliwell & Chirico, 1993). Polyunsaturated fatty acids, because of their possession of multiple double bonds are more susceptible to attack by radicals.

$$L\text{-}H \quad + \quad OH^• \quad \longrightarrow \quad H_2O \quad + \quad L^•$$
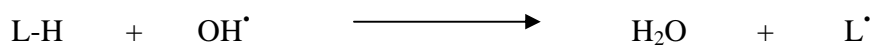
Given the role of free radicals in the initiation of lipid peroxidation, lipid peroxidation assay over the last three decades has become one of the vital tools for the detection of oxidative damage in living cells (Esterbauer *et al*., 1991; Aruoma *et al*., 1989; Comporti, 1985; Halliwell & Chirico, 1993). The concomitant increases in the levels of superoxide anion and lipid peroxides in the mycelium of *S. commune* paired against *T. viride* implicate oxidative damage in the complex cascade of physiological changes in *S. commune* mycelia interacting with *T. viride*. Although superoxide anion is not as reactive as hydroxyl radical hence, it is not as reactively deleterious to DNA and lipid membranes

as hydroxyl radical, it promotes the generation of hydroxyl radical, which directly damages lipid membranes (Halliwell & Aruoma, 1991). Although lipid peroxidation has not been previously studied in direct relation to fungal interactions, Crowe & Olsson (2000) demonstrated that compounds such as paraquat, copper sulphate, isopropanol and caffeine caused significant increases in laccase activity in liquid cultures of *Rhizoctonia* solani with a corresponding rise in levels of lipid peroxides. The laccase activity levels in their study were similar to levels detected when *R. solani* was confronted by an antagonistic strain of *Pseudomonas fluorescens*. This led them to conclude that oxidative stress was perhaps associated with interspecific interactions involving fungi and might be one of the factors promoting laccase secretion in most interacting fungal mycelia. It is likely that a correlation exists between the increase in the levels of laccase and manganese peroxidase (Chapter 5; sections 5.3.2.4 and 5.3.3) and superoxide anion and phenolic compounds detected in the mycelia of *S. commune* confronted by *T. viride*.

Increased levels of superoxide anion in the both interacting species soon after contact are in agreement with the work of Baker and Orlandi (1995), who reported that fungus-fungus contact causes an oxidative burst in the contact zone. From an extracellular point of view, increased levels of ROS in the interaction zone with concomitant loss of cell wall in mycelia of *S. commune* confronted by *T. viride* would make the lipid components of the cell membrane more susceptible to oxidative damage by lipid peroxidation. Furthermore, the work of Bao *et al.* (1994) provided evidence pointing to manganese peroxidases as key players in lignin depolymerization. More importantly, their work showed that manganese peroxidase degrades phenolic and non-phenolic lignin by lipid peroxidation.

 Activity patterns for manganese peroxidase (Chapter 5; sections 5.3.2.4 and 5.3.3) suggest that *S. commune* appeared to secrete more manganese peroxidase than laccase, and for longer, during mycelial conflict with *T. viride*. In the light of this, the activities of manganese peroxidase, coupled with the levels of ROS in the interaction zone may have promoted lipid peroxidation in *S. commune* paired against *T. viride*. On the other hand, chances are high that intracellular aggregation of cytoplasmic components in mycelial

filaments of *S. commune* in contact with *T. viride* (Chapter 2; sections 2.3.2 and 2.3.3) may have disrupted mitochondrial membrane potential necessary for electron transport and ATP synthesis (Iakovlev *et al*., 2004). This is very likely to cause electron leakage and consequently oxidative stress. In essence, this would promote lipid peroxidation. There are possibilities that oxidative damage in form of lipid peroxidation increased in the mycelia of *S. commune*, both intra- and extracellularly.

4.4.2 Increase in the levels of protein carbonyls in the mycelia of *S. commune* and phenol compounds in both fungi during mycelial confrontation between both species.

Detection of high levels of carbonylated proteins in the mycelia of *S. commune* paired against *T. viride* further supports the assumption that a high degree of oxidative damage may have occurred within the mycelia of *S. commune* during contact. Protein carbonylation occurs by the introduction of carbonyl groups into the side chains of specific amino acids (lysine, threonine, proline, arginine etc), an irreversible reaction, which renders proteins non-functional, thereby subject to degradation (Levine, 1983 & 2002). In yeast, increased carbonylation has been found to be associated with pronounced tendency of ageing mitochondria to produce ROS (Aguilaniu *et al*., 2001; Jazwinski, 2004). Protein carbonylation is also enhanced by starvation and advanced cell age (Stratman, 2001; Yan *et al*., 1997; Yan & Sohal., 1998).

Studies on ageing animals identified oxidative carbonylation as a major contributor to protein removal by degradation (Oliver *et al*., 1987; Levine, 2002). Interestingly, mycelial contact between both fungi caused a rise in the levels of phenol compounds in both species. However, this was more pronounced in *S. commune*. Increase in the levels of phenol compounds points to a possible switch of mycelial growth to secondary phase of metabolism (ageing), a potent cause of mitochondrial dysfunction (Iakovlev *et al*., 2004) in addition to cytoplasmic degeneration. Such dysfunction is likely to promote electron leakage and consequently oxidative damage, which may have contributed to increase in the levels of protein oxidation in the mycelia of *S. commune* interacting with *T. viride*.

It is very unlikely that that the higher levels of phenol compounds detected in the mycelia of *T. viride* soon after contact with *S. commune* was as a result of switch of mycelial metabolism to secondary phase. This is because, contact with *S. commune* promoted a wide range of aggressive mechanisms in the mycelia of *T. viride* (chapter 2; sections 2.3.2 and 2.3.3) leading to lysis of *S. commune* mycelia. Thus, contact with *S. commune* may have enhanced nutrient supply to *T.* viride. This was most unlikely to favour secondary metabolism to the latter. Perhaps, *T. viride* up-regulates some phenol compounds for combat purposes. For instance, a 61% increase in peak area was detected by GC-MS for 4-hydroxyphenyl ethanol in the mycelia of *T. viride* following 48 hours of contact with *S. commune* (chapter 3), and this compound was not detected at all in the mycelial of the former.

However, in addition to ROS-driven oxidation of proteins, it has been demonstrated that the increased oxidation of proteins in ageing cells might be a result of impaired transcriptional/translational fidelity thereby, implicating carbonylation in protein quality control (figure 4.6) (Dukan *et al*., 2000; Bota & Davies, 2002; Grune *et al*., 2004). Dukan *et al*. (2000) showed that treatment of *E. coli* with streptomycin, an established inducer of mistranslation (Edelmann & Gallant, 1977), and concomitant induction of heat shock chaperones enhanced protein carbonylation. Streptomycin was added to growing cells at a concentration (1 μg/ml) which was shown to be incapable of increasing the production of superoxide anion or limiting cell growth (Dukan *et al*., 2000). This treatment increased protein carbonylation 9 fold despite a corresponding rise in the levels of heat shock chaperones, hence establishing a link between protein misfolding and carbonylation (Dukan *et al*., 2000).

In the same study (Dukan *et al*., 2000); *E. coli* was subjected to additional treatments capable of inducing protein mistranslation or termination of translation. Such treatments included, exposure to puromycin, an inducer of premature termination of translation, introduction of multicopy plasmid Pkk724G, which promotes mistranslation, introduction of a mutation that decreases transcriptional fidelity thereby causing mistranslation as a result of mistranscription (Dukan *et al*., 2000). All of these treatments aimed at producing

aberrant proteins generated marked increases in the levels of carbonylated proteins as well as heat shock chaperones (Dukan *et al*., 2000). These data implied that cellular carbonylation of proteins during cell growth of *E. coli* is not exclusively influenced by levels of ROS, but mainly by the levels of aberrant proteins (Dukan *et al*., 2000). Based on this observation, Dukan *et al*. (2000) concluded that rapid oxidation of misfolded proteins might ensure that they are directed to the proteolytic machinery rather than being incorporated into the protein maturation machinery (figure 4.6).

The patterns of protein expression in the mycelia of *S. commune* confronted by *T. viride* presented in chapter 7 suggest possible initiation of endoplasmic reticulum stress (stemming from increased flux through the protein synthetic machinery), a major elicitor of protein misfolding. The unfolded protein response (UPR), a restorative machinery that streamlines protein synthesis, and directs misfolded proteins towards refolding (cyclophilins) or degradation (polyubiquitins), also appeared to be up-regulated. Based on these, coupled with the evidence highlighted above, it could be argued that generation of aberrant/misfolded proteins, in part played a key role in the increased generation of carbonylated proteins in mycelia of *S. commune* paired against *T. viride*.

Polyubiquitination is a major adaptation of eukaryotic cells to stress and is highly selective compared to the lysosomal proteolytic pathway (Staszczak, 2008). Polyubiquitins selectively degrade aberrant proteins under various conditions of stress. In addition to the up-regulation of a Probable E3 ubiquitin-protein ligase (Chapter 7; section 7.3.1), the polyubiquitin gene was also strongly up-regulated in the mycelia of *S. commune* paired against *T. viride* (chapter 6; section 6.3). The selectivity of the polyubiquitin pathway allows for the specific labelling and degradation of aberrant proteins only. Up-regulation of components of this pathway in the mycelia of *S. commune* paired against *T. viride* most likely points to a marked rise in the levels of aberrant proteins in the former, which might include both misfolded proteins (as a result of endoplasmic reticulum stress) and carbonylated proteins stemming from both oxidative stress.
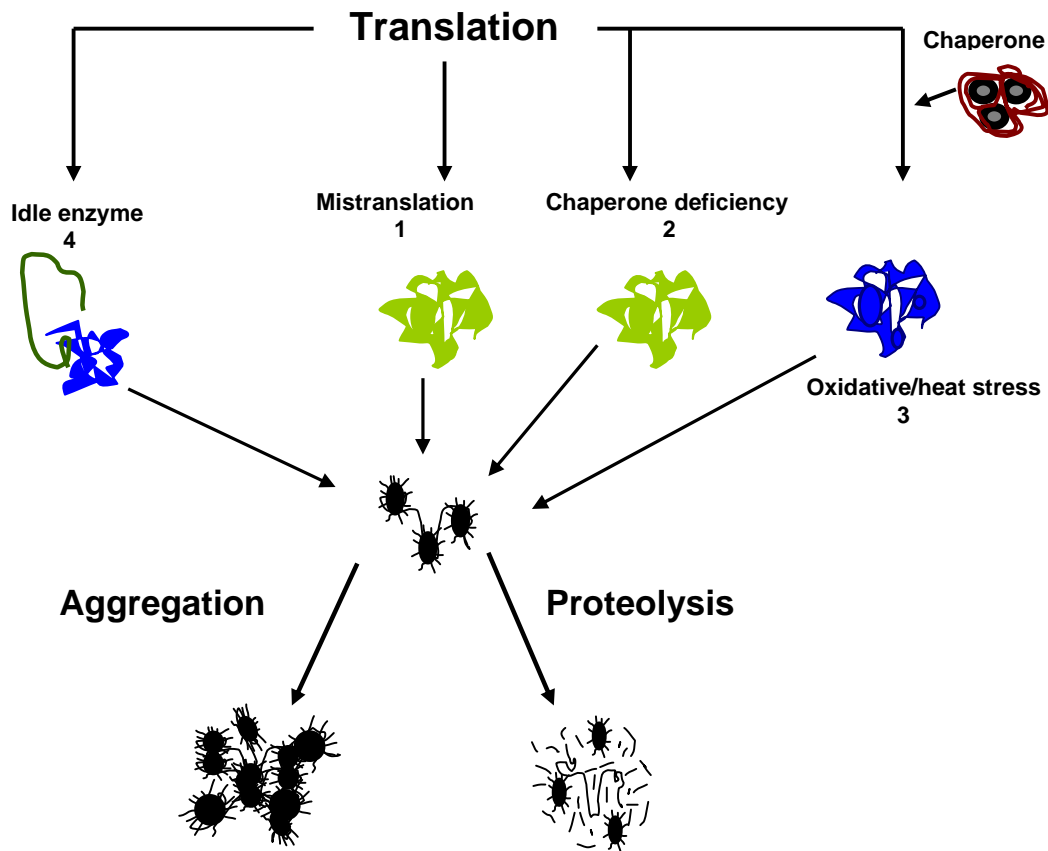
Figure 4.6 Schematic representation of processes that generate proteins predisposed to carbonylation and the fate of such proteins (Adapted from Nystrom, 2005). Process 1 depicts the generation of aberrant proteins by mistranslation. Process 2 represents the formation of misfolded proteins during chaperone deficiency. In process 3, functional proteins are produced by the protein synthetic mechanisms; however, aberrant protein structures are generated by stress conditions such as oxidative/heat stress. Process 4 illustrates the hypothetical oxidation of an idle enzyme most likely under conditions of substrate deficiency. Soluble carbonylated proteins are usually destined for proteolysis while highly carbonylated proteins from high-molecular-weight aggregates tend to be resistant to proteolysis.

4.4.3 Levels of ATP in the mycelia of *S. commune* paired against *T. viride*.

Despite the strong indications of stress (cell wall lyses, pigmentation, cessation of growth and subsequent hyphal disintegration) when *S. commune* made mycelial contact with *T. viride*, ATP levels detected in the mycelia of the former do not suggest any decrease in

the energy generation machinery within the first 4 days of contact. The genes which code for cytochrome C_1 and ATP synthase (β-chain), key components of the electron transport and ATP synthetic machinery of the mitochondrion were down-regulated and suppressed respectively (Chapter 6; section 6.3), in *S. commune* mycelial following contact with *T. viride*. The sources of ATP in *S. commune* mycelia paired against *T. viride* remain unclear. In addition, the levels of ATP detected in the mycelia of *S. commune* confronted by *T. viride* is further complicated by the fact that nucleoside diphosphate kinase, another source of ATP, was down-regulated in the former (Chapter 7; section 7.3.1). However, it is worthy of mention that the δ-chain of ATP synthase was up-regulated in *S. commune* after 48 hours of contact with *T. viride* (Chapter 7; section 7.3.1).

One explanation for this is that *S. commune* might possess multiple copies of some of the genes that encode proteins involved in ATP synthesis. Another possible explanation is that ATP synthetic enzymes might be regulated at the protein level. Although ATP can be generated by substrate level phosphorylation in the Kreb's cycle and in the pentose phosphate pathway, which appeared to be up-regulated in preference to the glycolytic pathway (Chapters 3 & 5), ATP yields from substrate level phosphorylation are extremely low (Prescott *et al.*, 1999). Based on this, the arguments that ATP synthetic enzymes are regulated at the protein level and/or presence of multiple genes for some of these enzymes appear more plausible. Whatever the mechanism employed by *S. commune* to sustain ATP synthesis, it is important to note that there must have been high physiological requirements for ATP during combat with *T. viride*, prior to mycelial death.

Patterns of gene and protein expression as well as enzyme activity levels suggest that polyubiquitination (Chapters 6 & 7), protein folding and transport by cyclophilins (Chapter 7), and β-oxidation of fatty acids (Chapters 3 & 7) were up-regulated in the mycelia of *S. commune* following contact with *T. viride* (figure, 4.7). These, among other physiological processes, may account for the need to maintain ATP levels despite the prevailing stress conditions. For instance, the polyubiquitin pathway requires 26S proteasome, an ATP-dependent multi-subunit protease (Staszczak, 2008). Similarly, protein folding and transport by cyclophilins proceeds in an ATP-dependent manner

(Matouschek, *et al*., 1995). β-oxidation of fatty acid species is a high ATP-requiring process (Singh *et al*., 1987). Helicases were also up-regulated (chapter 7; section 7.4.1.5), and helicase activity is an ATP-requiring process.

Figure 4.7 Schematic representation of the hypothetical causes (red arrows) and effects (blue arrows) of physiological/biochemical changes (green arrows) in the mycelia of *S. commune* interacting with *T. viride*.

4.5 Summary and Conclusions

The results presented in this chapter most of all reflect the stress to which the mycelia of *S. commune* were subjected as a result of contact with the highly antagonistic strain of *T. viride* used in this study. Increase in the levels of lipid peroxidation, superoxide anion and protein carbonylation, all point to severe physiological stress both extra- and intracellularly. In addition, early increase in the amounts of phenol compounds detected particularly in the *S. commune* domain, against a backdrop of morphological changes (sealing-off of mycelial front, cessation of growth, loss of cell wall and subsequent cell

(Matouschek, *et al*., 1995). β-oxidation of fatty acid species is a high ATP-requiring process (Singh *et al*., 1987). Helicases were also up-regulated (chapter 7; section 7.4.1.5), and helicase activity is an ATP-requiring process.

Figure 4.7 Schematic representation of the hypothetical causes (red arrows) and effects (blue arrows) of physiological/biochemical changes (green arrows) in the mycelia of *S. commune* interacting with *T. viride*.

4.5 Summary and Conclusions

The results presented in this chapter most of all reflect the stress to which the mycelia of *S. commune* were subjected as a result of contact with the highly antagonistic strain of *T. viride* used in this study. Increase in the levels of lipid peroxidation, superoxide anion and protein carbonylation, all point to severe physiological stress both extra- and intracellularly. In addition, early increase in the amounts of phenol compounds detected particularly in the *S. commune* domain, against a backdrop of morphological changes (sealing-off of mycelial front, cessation of growth, loss of cell wall and subsequent cell

death) reported in chapter 2 support the assumption that contact between both species resulted in a switch of *S. commune* growth to secondary metabolic phase. Equally, worthy of note is that oxidative stress may not be the sole inducer of protein oxidation in *S. commune* during contact. This is because the data in chapter 7 implicate protein synthetic stress, which has been demonstrated to favour protein carbonylation in the sequence of physiological responses by *S. commune* to *T. viride*. It is likely that the stress responsive mechanisms recruited by *S. commune* such as ubiquitination and β-oxidation, may account for the inexplicably high levels of ATP detected in the mycelia of *S. commune,* despite unfavourable conditions of growth following contact with the antagonist.

CHAPTER 5

The activities of selected enzymes during Mycelial confrontation between *S. commune* And *T. viride*

5.1 Introduction

By means of their elaborate extracellular enzyme secretory mechanisms, fungi colonise and metabolise a wide range of resources in nature. They respond to environmental triggers via regulated shifts in patterns of enzyme secretion (Archer & Wood, 1995). Since they rarely form mono-specific populations in nature, invasion and colonisation of nutrients results in intra- and inter-specific mycelial contacts (Heilmann-Clausen & Boddy, 2005). Competition for nutrients and space might lead to antagonism, which elicits morphological, physiological and biochemical changes in the interacting mycelia. Enzymes play vital roles in the acquisition of nutrients, defence/attack, neutralization of toxic metabolites (often associated with antagonism in fungi) and exchange of chemical signals between interacting mycelia. There is a vast body of literature demonstrating the strong involvement of phenol-oxidases (laccase, manganese peroxidase, and tyrosinase) in the biochemical sequence of events that ensue fungus-fungus contact especially amongst wood-rot fungi (Boddy, 2000; Rayner & Webber, 1984; Griffith *et al*., 1994; Gregorio *et al*., 2006; Chi *et al*., 2006; Score *et al*., 1996; Freitag *et al*., 1991).

Aggressive saprotrophic fungi such as *Trichoderma* species parasitize a wide range of fungi by means of their ability to secrete a battery of lytic enzymes namely; chitinases, laminarase, proteases and cellulases in addition to other antagonistic mechanisms (Highly, 1991; Chet *et al*., 1998; Lorito *et al*., 2001; Markovich & Kononova, 2003). Macroarray study of the patterns of gene expression during competitive interaction between the biocontrol fungus, *Phlebiopsis gigantea* and the conifer pathogen, *Heterobasidion parviporum* showed a strong induction of genes that encode enzymes involved in the processing of nutrients (fructose biphosphate aldolase, glutamine synthethase, arginase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglucomutase, etc) in the biocontrol fungus which out-grew the pathogen (Adomas *et al*., 2006).

These suggest that both intra- and extracellular enzymes mediate key functions that have significant bearings on the outcome of antagonistic interactions (White & Boddy, 1992; Score *et al*., 1997; Baldrian, 2004). The aim of this section of the study was to investigate the involvement of phenoloxidases (laccase and manganese peroxidase -MnP), glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD), catalase, chitinase,

cellulase, and succinic semialdehyde dehydrogenase (SSADH) in mycelial confrontations between *S. commune* and *T. viride*.

5.2 Materials and Methods

5.2.1 *In situ* detection of phenoloxidase activity

Agar plate interaction assays for the detection of phenoloxidases were set up as described in chapter two (section 2.2.1). However, for *in situ* detection of phenoloxidases, potato dextrose agar (PDA) was saturated with 0.01% (w/v) Remazol brilliant blue (RBB) dye. Phenoloxidases decolourise RBB from blue to yellow; hence, this allows visualisation of enzyme activity *in situ* during mycelial confrontations.

5.2.2 Culture conditions and sample preparations

Samples for SOD, catalase, G6PDH, SSADH, laccase and manganese peroxidase assays were taken from solid agar cultures (PDA), while chitinase activity was assayed using samples from liquid cultures. Agar plate interaction assays were set up as previously described and samples for SOD, catalase, G6PDH, SSADH, laccase and manganese peroxidase assays were taken from the *S. commune* domain every 24 hours post mycelial contact with *T. viride*. 200 mg of mycelial/agar samples were cut off 10 mm behind the barrage zone, stored overnight at -80°C and freeze-dried afterwards. Freeze-dried samples were ground to powder using a mortar and pestle, with the addition of small amounts of acid-washed sand (Simga). The resulting powder was suspended in 2 ml of 1X phosphate buffered saline –pH 6.5 (PBS – see Appendix III for recipe) for 1 hour at 4°C.

Sample mixtures were spun at 15,000 rpm for 20 mins at 4°C and the supernatant was used for the respective assays. Samples for in-gel laccase and manganese peroxidase activity staining were also prepared as described above. To assay for chitinase activity, cultures were grown in potato dextrose broth. Inocula were prepared by agitating 10 mycelial plugs in sterile distilled water with the addition of sterile glass beads. 1 ml of the resulting colloidal suspension was used to inoculate 60 ml of broth in 250 Erlenmeyer flasks. Both organisms were pre-inoculated and grown in separate flasks, with *T. viride*

inoculated three days after *S. commune*. This was because of the former's faster growth rate. Four days after the inoculation of *S. commune* (1-day post *T. viride* inoculation), both cultures were mixed for chitinase activity determination as well as for the monitoring of pH profile. Cultures were grown in rotary shakers at 28°C and 150 rpm. 1 ml of culture broth was taken every 24 hours for assay and centrifuged as above. 25 μL of protease inhibitor cocktail (Sigma-Aldrich, UK) was added to each sample (from both solid and liquid cultures, except for SSADH assay where samples were processed slightly differently) during sample preparation and total protein content was determined according to Bradford method (described in chapter 4).

5.2.3 Enzyme activity assays

a) SOD assay

The reaction mixture consisting of 1 ml of 50 mM Tris-HCl (pH 8.5) with 1 mM EDTA and 200 μl of sample homogenate (sample) containing 40 μg of protein, was incubated at 30°C for 5 min. 100 μl of 5mM pyrogallol in 10 mM HCL was added to initiate the reaction. Change in absorbance was recorded at 320 nm for 1 min in a Perkin Elmer UV spectrophotometer. Data was recorded and analysed using 'Win Lab' software. SOD activity was defined based on the percentage inhibition of auto-oxidation of pyrogalol, where one unit equals 50% inhibition according to Marklund & Marklund (1974). Activity was expressed as U/mg of protein.

b) Catalase assay

40 μg of protein sample made up to 200 μl in 1X PBS (pH 6.5) was mixed with 1.9 ml of reagent grade water and 1 ml of 0.059 M hydrogen peroxide according to the method described by Beers and Sizer (1952). Rate of breakdown of hydrogen peroxide was monitored by measuring the decrease in absorbance at 240 nm. One unit of catalase was defined as the amount required to decompose 1 μmole of hydrogen peroxide per minute at pH 6.5 and 25°C. Activity was expressed as U per mg of protein.

c) G6PDH assay

G6PDH was assayed according to the method of Noltman *et al*. (1961). The reaction mixture consisted of 21 ml of deionised water, 5 ml of 250 mM glycine (pH 7.4), 1 ml of 60 mM glucose-6-phosphate, 1 ml of 20 mM β-nicotinamide adenine dinucleotide phosphate (β-NADP) and 1 ml of magnesium chloride. 2.9 ml of the reaction mixture above was mixed with 200 μl of sample (containing 40 μg of protein) and increase in absorbance was measured at 340 nm for 5 mins. One unit was defined as the amount of enzyme that oxidised 1 μmole of D-glucose-6-phosphate to 6-phospho-D-gluconate per min at pH 7.4 and at 25^0C in the presence of β –NADP and expressed as U per mg of protein.

d) Laccase assay

Laccase activity assay was carried out spectrophotometrically according to the method of Teerapatsakul *et al*. (2007). 200 μL of 2.5 mM ABTS in 0.1 M sodium tartrate buffer (pH 3) was mixed with 50 μl of sample supernatant and 950 μL of 0.1 M sodium tartrate buffer (pH 3). Oxidation of ABTS was measured at 414 nm for 2 minutes with an extinction coefficient $\varepsilon = 3.6 \times 10^4$ M^{-1} cm^{-1}. One unit of laccase was defined as the amount that produced 1 μM of product per minute and expressed as U per mg of protein.

e) Manganese peroxidase assay

Manganese peroxidase assay mixture contained 950 μL of assay buffer   (0.1 M sodium tartrate pH 4.5, 0.1 mM hydrogen peroxide, and 0.1 mM manganese sulphate) and 50 μl of sample supernatant. Change in absorbance was measured for 4 minutes at 470 nm with an extinction coefficient $\varepsilon = 2.75 \times 10^4$ M^{-1} cm^{-1}. One unit of enzyme was expressed as the amount that produces 1 μM of product per minute and expressed as U per mg of protein.

f) Chitinase assay

Colloidal chitin was prepared according to the method described by Sandya *et al* (2004) with slight modifications. 200 gm of commercial chitin (Sigma-Aldrich) were mixed in 1500 ml of concentrated hydrochloric acid (HCl) and kept overnight at 4°C. The resulting

mixture was then filtered through a sieve with the addition of pre-chilled water with constant stirring. The filtrate was left to settle for 2 hours at 4°C, allowing the formation of gelatinous white precipitate. The white precipitate was collected by centrifugation at 13,000 rpm for 15 mins at 4°C. The gelatinous material was washed with cold distilled water until a pH of 6 was attained. The resulting colloidal chitin was stored at refrigeration temperature.

Chitinase activity was assayed according to the method of Yanai *et al.* (1992). 250 µl of colloidal chitin was mixed with 250 µl of 0.2 M sodium acetate buffer (pH 4) and 500 µl pre-centrifuged fermentation broth. The reaction mixture was incubated for 2 hours at 37°C. After incubation, the reaction mixture was spun at 13,000 rpm for 5 mins, 500 µl of the supernatant was mixed with 100 µl of 0.8 M boric acid to terminate the reaction, and the pH was adjusted to 10.2 with KOH. The solution was then heated for 3 mins in boiling water and then allowed to cool. 3 ml of p-dimethyl aminobenzaldehyde [DMAB – 1 g of DMAB dissolved in 100 ml of glacial acetic acid with the addition of 1% (v/v)] HCl) solution was added and the resulting mixture was incubated at 37°C for 20 mins. Absorbance was measured at 585 nm against water as blank. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of N-acetylglucosamine per min under assay conditions.

g) SSADH assay

SSADH was assayed according to the method of Solomon & Oliver (2002). Ground *S. commune* mycelial material was suspended in 4 ml of extraction buffer [50 mM PBS (pH 7), 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 20 mins and then centrifuged at 15,000 rpm for 20 mins. The resulting supernatant was used for the activity assay. 1 ml of the reaction mixture contained 100 mM sodium pyrophosphate buffer (pH 9), 14 mM β-mercaptoethanol, 0.5 mM NAD^+, made up with 200 µl (containing 40 µg of protein) of supernatant sample. Following equilibration at 30°C for 5 mins, the reaction was started by the addition of 100 µM succinic semialdehyde. Absorbance was measured at 340 nm. One unit of SSADH activity was defined as the amount of enzyme required to produce 1 µmol of NADH per min at 30°C.

5.2.4 In-gel activity staining for laccase and manganese peroxidase

The activities and levels of laccase and manganese peroxidase were assayed in 12% polyacrylamide gel under native conditions according to the method of Laemmli (1970).

The resolving gel consisted of;

Water	5 ml
0.5 M Tris-HCl buffer (pH 8.8)	2.6 ml
40% Bis acrylamide	3 ml
N, N, N', N'-tetramethylethylenediamine (TEMED)	10 μl
10% (w/v) ammonium persulfate	100 μl

Stacking gel

Water	3.65 ml
0.5 M Tris-HCl buffer (pH 6.8)	625 μl
40% Bis acrylamide	500 μl
N, N', N'-tetramethylethylenediamine (TEMED)	10 μl
10% (w/v) ammonium persulfate	50 μl

30 μg of protein was run in the gel at 120 V for 3 hours at 4°C (see Appendix II for gel running buffer recipe). Laccase staining buffer contained 2.5 mM ABTS [2, 2'-azinobis (3-ethylbenzathiazoline-6-sulfonic acid); (Sigma-Aldrich), in 0.1 M sodium tartrate buffer (pH 3). Manganese peroxidase staining buffer consisted of 1 mM 2,6-dimethoxy phenol, 0.4mM hydrogen peroxide, and 1 mM manganese sulphate in 0.1 mM sodium tartrate (pH 4.5). For laccase activity, gels were stained at room temperature, while the activity of manganese peroxidase was stained at 30°C for 10-15 minutes. Gel images were acquired and densitometric analyses were performed using Bio-Rad Quantity 1 software.

5.2.5 Measurement of pH profile in liquid co-cultures

1 ml of sample was taken from liquid co-cultures of *S. commune* and *T. viride* every 24 hours and the pH was measured against the pH of self-paired liquid cultures of each fungus. Cultures were prepared as described in section 5.2.2.

5.2.6 Statistical analysis

Unpaired *T*-test was used to determine the level of significance of the observed increase or decrease in enzyme activity and protein levels for the assays described in this chapter, using Graphpad *T*-test calculator software (Graphpad software, San Diego California: http://www.graphpad.com/quickcalcs/ttest1.cfm). P values < 0.05 were considered significant.

5.3 Results

5.3.1 *In situ* detection of phenoloxidase activity

Contact between both fungi on PDA containing RBB resulted in dye degradation shown by the decolourisation in the contact zone, 48 hours post contact (figure 5.1). Decolourisation appeared to have started after 24 hours of contact and intensified after 48 hours compared to self-paired cultures. Given that phenoloxidases decolourise RBB by oxidation, decolourisation in the contact area is indicative of localized secretion of laccase and manganese peroxidase in the mycelial mat around the area of contact between *S. commune* and *T. viride*.

5.3.2 Enzyme activities

5.3.2.1 SOD activity in *S. commune* mycelia paired against *T. viride* on agar

In comparison to the self-paired cultures, SOD activity increased 2.4, 2.5 and 2.0 fold in mycelia of *S. commune* paired against *T. viride* on days 1-3 post contact respectively ($P =$ 0.0009, 0.0003, 0.0003 respectively). Activity in the *T. viride*-confronted however, decreased after 3 days of contact (Figure 5.2).
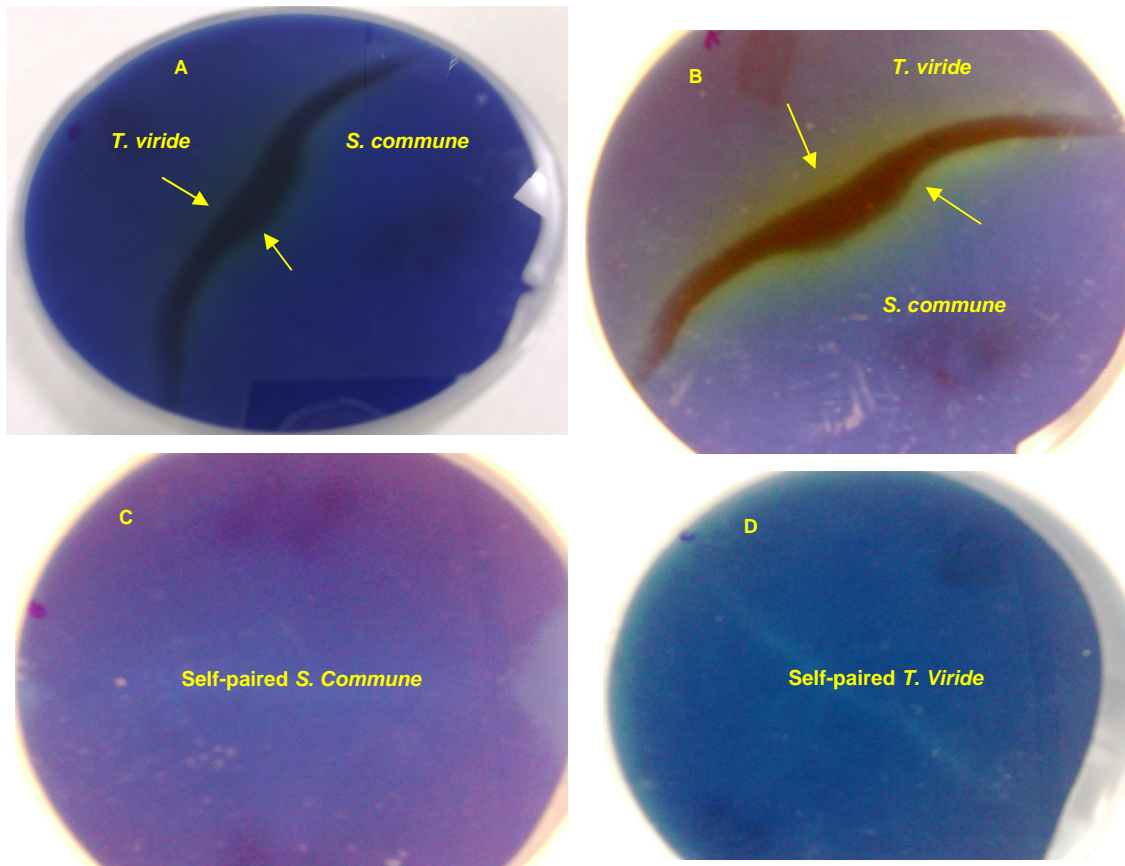
Figure 5.1 Macroscopic images depicting the decolourisation of remazol brilliant blue (RBB) following contact between *S. commune* and *T. viride*. A: Interacting culture of both fungi 24 hours after contact showing minimal signs of dye decolourisation; B: Intensified dye decolourisation after 48 hours of interspecific mycelial contact; C & D: Self-paired *S. commune* and *T. viride* respectively without dye any degradation following contact.
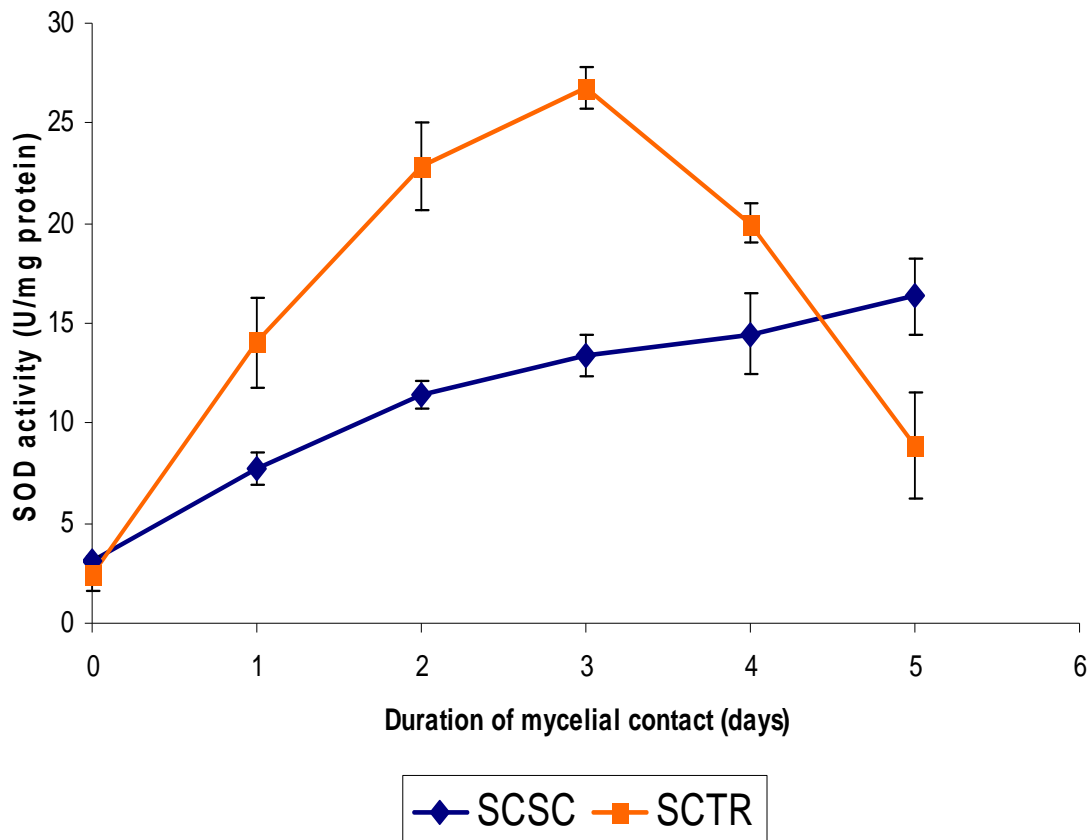
Figure 5.2 Superoxide dismutase (SOD) activities of *S. commune* mycelia paired against *T. viride* (SCTR), compared to enzyme activity in self-paired cultures of the former (SCSC). Experiments were carried out with 3 separate biological samples. Error bars represent standard deviation.

5.3.2.2 Catalase activity in *S. commune* mycelia paired against *T. viride* on agar

Catalase activity was up in *T. viride*-confronted mycelia after days 2-3 of mycelial contact 1.5 and 3.5 fold respectively (P = 0.0106 and 0.0034 respectively). Similar to SOD activity, enzyme activity peaked on day 3 of mycelial contact and dropped after 4 days of interaction (Figure 5.3).
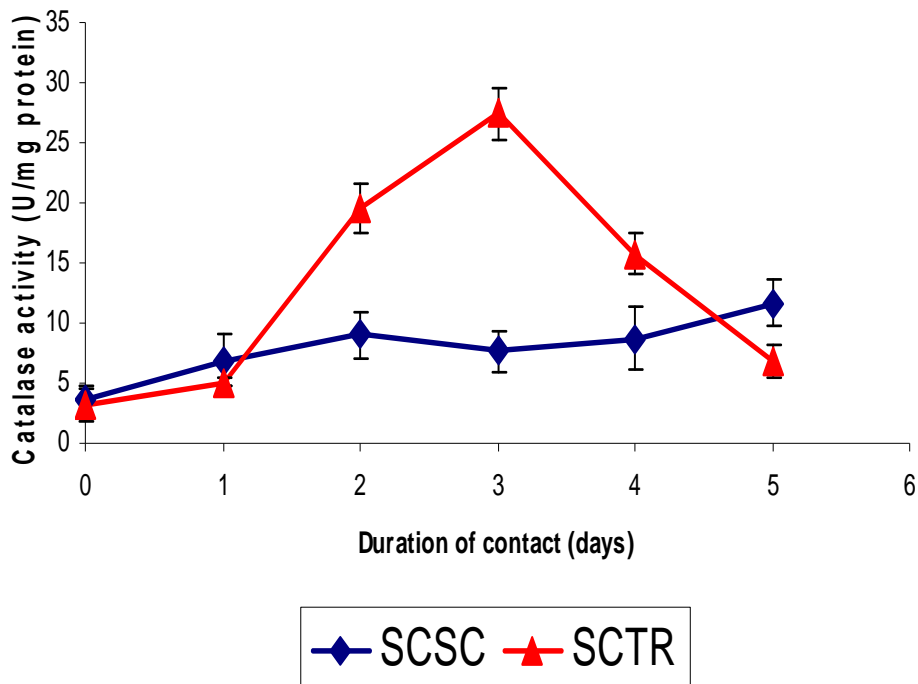
Figure 5.3 Patterns of catalase activity in the mycelia of *S. commune* following self-and non-self interactions with *T. viride* SCSC: self-paired *S. commune*. SCTR: *S. commune* interacting with *T. viride*). 3 biological samples (agar plate replicas) were used for assay. Error bars represent standard deviation.

5.3.2.3 **Patterns of G6PDH activity in mycelia of *S. commune* paired against *T. viride***
There was an increase in G6PDH activity following early contact (24 hours) with *T. viride*. Enzyme activity increased progressively through 48 hours of contact and peaked after 72 hours, at which time an activity of 13.075 U/mg proteins was detected. Enzyme activity however decreased afterwards (Figure 4.4). Overall, G6PDH activity increased 1.6, 1.9, and 3.2 fold within the first 3 days of interspecific interaction respectively ($P = 0.0025, 0.0018, 0.0001$ respectively).
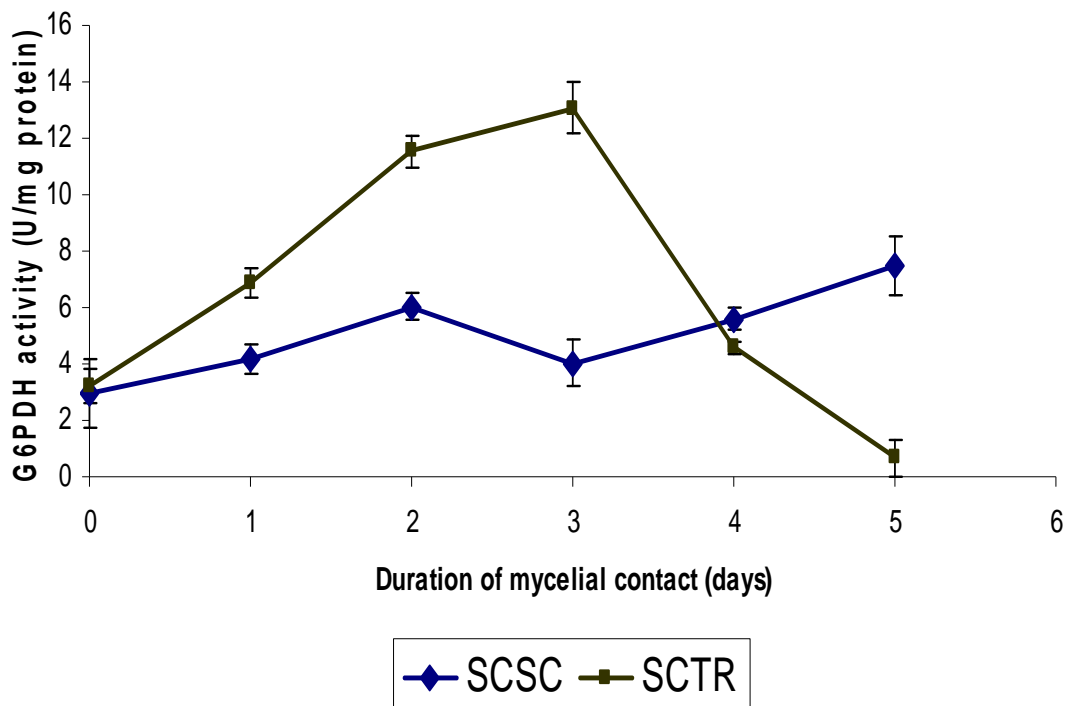
Figure 5.4 G6PDH activity in the mycelia of *S. commune* **paired against** *T. viride* **compared to self-paired cultures of the former on agar (SCSC: self-paired** *S. commune*. **SCTR:** *S. commune* **paired with** *T. viride*)**. Experiments were carried out in triplicates. Error bars represent standard deviation.**

5.3.2.4 Patterns of laccase and manganese peroxidase activities *S. commune* **domain following confrontation by** *T. viride*.

Contact with *T. viride* elicited a burst of laccase activity within 24 hours in the mycelial domain of *S. commune* surrounding the contact zone as shown in figure 5.5. However, laccase activity dropped progressively in the *T. viride*-confronted cultures, but increased moderately in the self-paired cultures with increasing duration of contact. A burst of laccase activity after contact with *T. viride* resulted in 11 (P = 0.0001) fold increase in activity compared to self-paired cultures. Manganese peroxidase activity increased strongly from the first to the third day of mycelial contact with *T. viride*, reaching a peak specific activity of 12.78 U/mg proteins on the second day of contact (Figure 4.6). Manganese peroxidase activity of *S. commune* increased 7.6 fold (P = 0.0001), 13.6 fold

103

(P = 0.0001) and 3.8 fold (P = 0.0001) in the first 3 days following interspecific mycelial contact compared to self-interacting mycelia.
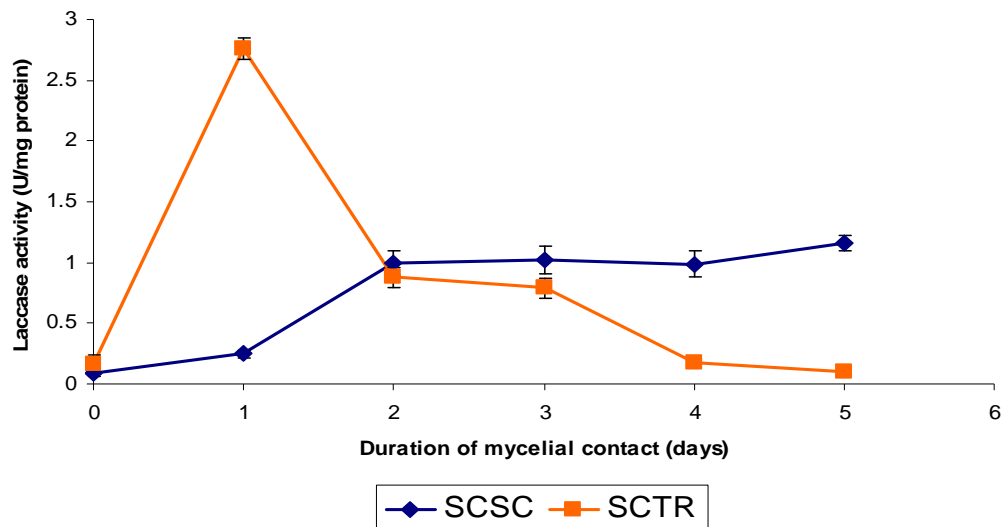
Figure 5.5 Laccase activity within the domain of *S. commune* paired against *T. viride* on PDA (SCSC: self-paired *S. commune*. SCTR: *S. commune* paired with *T. viride*). Assay was performed in triplicates, using samples from 3 separate cultures. Error bars represent standard deviation.
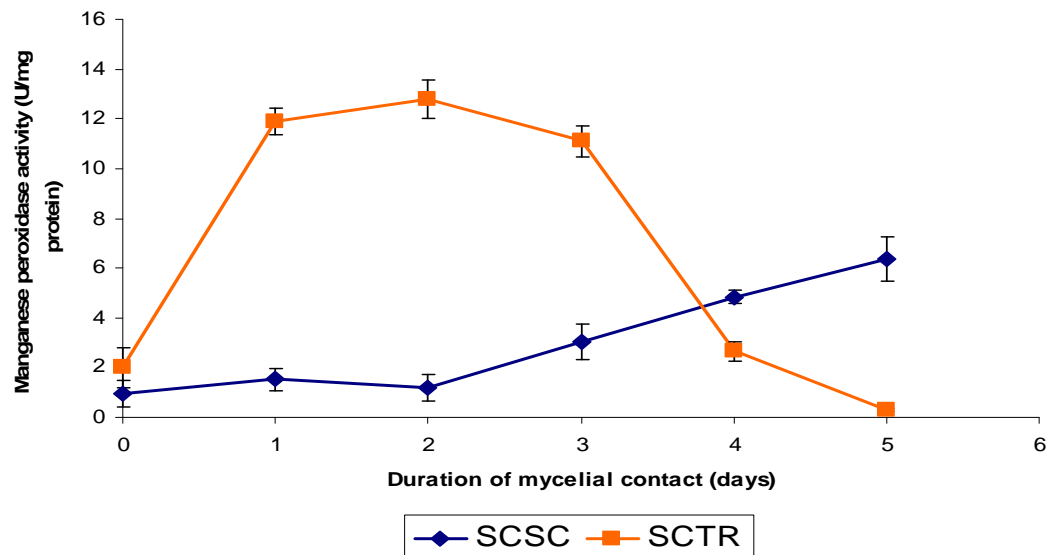
Figure 5.6 Manganese peroxidase activity within the domain of *S. commune* paired against *T. viride* on PDA (SCSC: self-paired *S. commune*. SCTR: *S. commune* paired with *T. viride*). Assay was carried out in triplicates. Assay was run in triplicates, using samples from 3 different plate cultures. Error bars represent standard deviation.

5.3.2.5 Chitinase activity in liquid co-cultures of *S. commune* and *T. viride*

Both organisms formed morphologically distinct pellets in potato dextrose broth (Figure 4.8). Within 48 hours of mixing established cultures of both fungi, chitinase activity increased markedly, reaching a peak activity of 8.9 U/ml on day 9 of fermentation (5 days post mixing). Compared to the mono-cultures, chitinase activity remained significantly higher in the dual cultures from 3 days after mixing through to the 9th day post mixing ($P = 0.0001$), when fermentation was terminated. Whereas chitinase activity was barely detected in mono-cultures of *S. commune*, activity increased moderately in self-mixed cultures of *T. viride* (figure 4.7). Furthermore, the numbers of *S. commune* pellets reduced gradually during fermentation (after mixing), and disappeared completely 7 days post mixing (day 11 of fermentation).
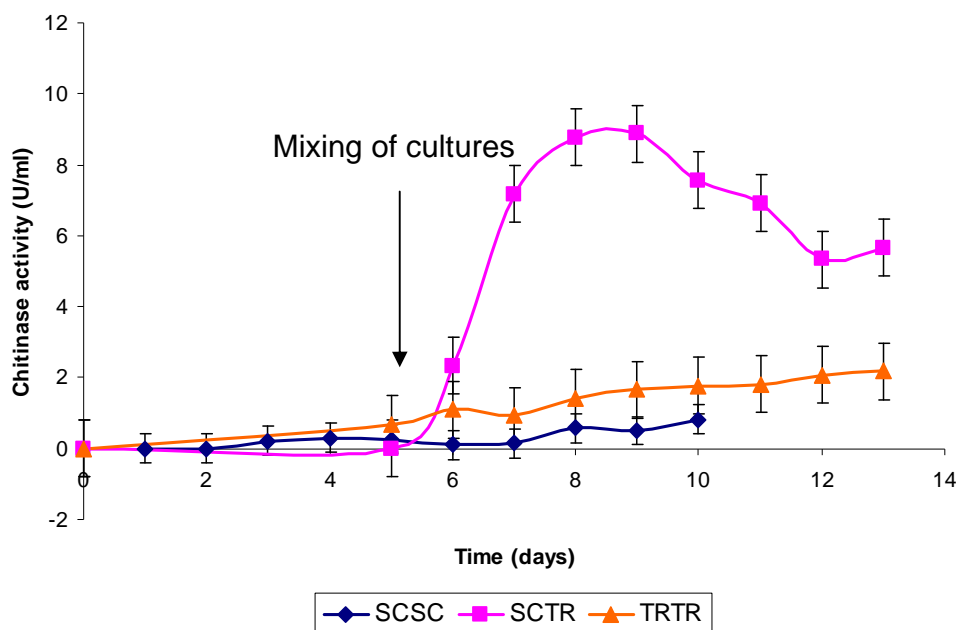
Figure 5.7 Chitinase activity in mixed liquid cultures of *S. commune* and *T. viride*, compared to the self-paired of both fungi (SCSC: self-paired *S. commune*. SCTR: *S. commune* paired with *T. viride*. TRTR: self-paired *T. viride*). Experiments were carried out in triplicates. Error bars represent standard deviation.

T. viride: Smaller pellets

S. commune: Bigger pellets
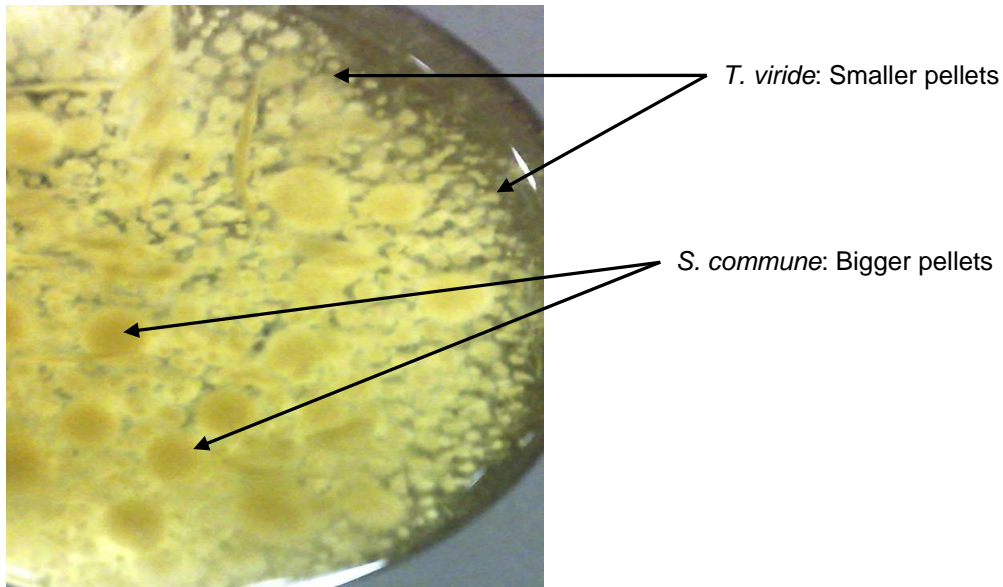
Figure 5.8 Morphologically distinct pellets (macroscopic) produced *S. commune* and *T. viride* in potato dextrose broth. *S. commune* forms larger pellets than *T. viride* as shown immediately after mixing (4 days post *S. commune* inoculation (pre-mixing) and 2 days after the inoculation of *T. viride*).

5.3.2.6 SSADH activity in liquid co-cultures of *S. commune* and *T. viride*

SSADH activity increased 2.6 fold ($P = 0.0029$) in *S. commune* mycelia after 48 hours of contact with *T. viride* (figure 5.10). However, activity fell sharply afterwards in the interacting mycelia with no significant change in the self-paired cultures.
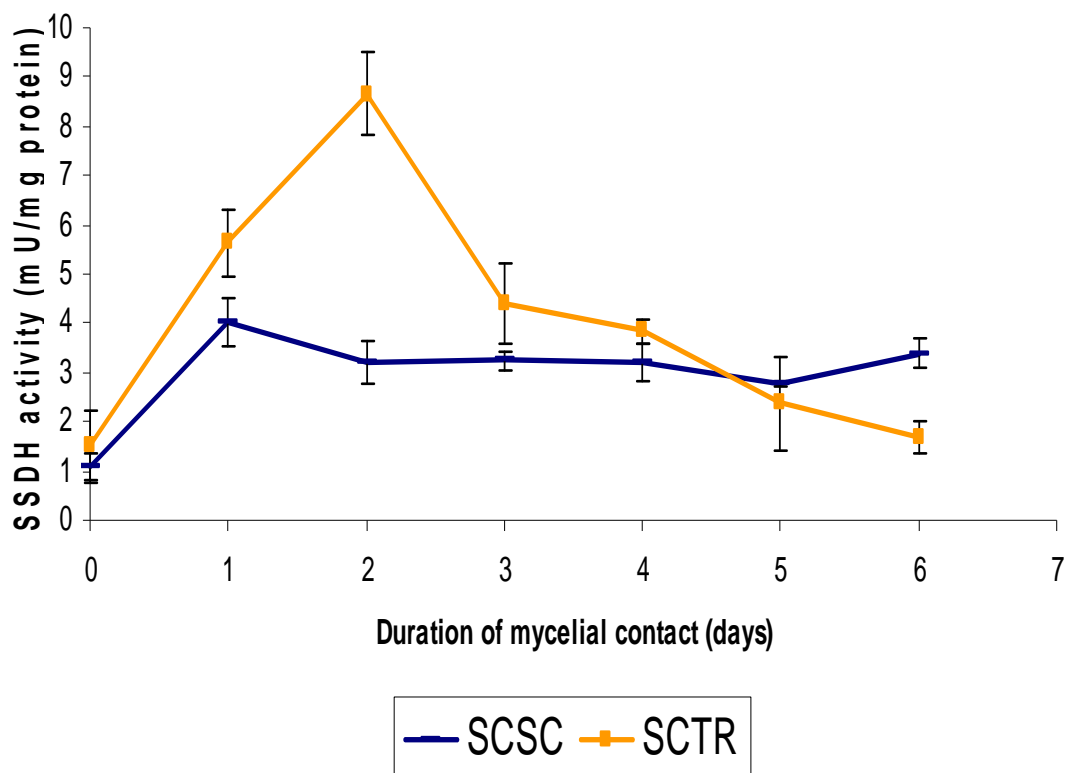
Figure 5.10 Specific activity of SSADH in mycelia of *S. commune* paired against *T. viride*, relative to co-cultures of *S. commune* (See figure 5.2 for key to SCSC and SCTR).

5.3.3 In-gel determination of the levels of laccase and manganese peroxidase in *S. commune* domain during contact with *T. viride*

Results of in-gel activity staining for both laccase and manganese peroxidase were in agreement with the activity levels detected by spectrophotometric assays. Protein levels were expressed as a function of band volumes resulting from enzyme/substrate binding in the gel. Laccase levels in agar/mycelial samples taken from *S. commune* domain around the contact zone increased 2.9 fold ($P < 0.0001$) compared to samples from the same area of self-paired cultures. However, similar to the activity pattern, the band volume of laccase dropped 1.6 fold ($P = 0.0115$) compared to self-interacting cultures. Manganese peroxidase was up-regulated 7.6 and 13.6 fold ($P < 0.0001$) respectively after 24 and 48 hours of contact (figure 5.11).
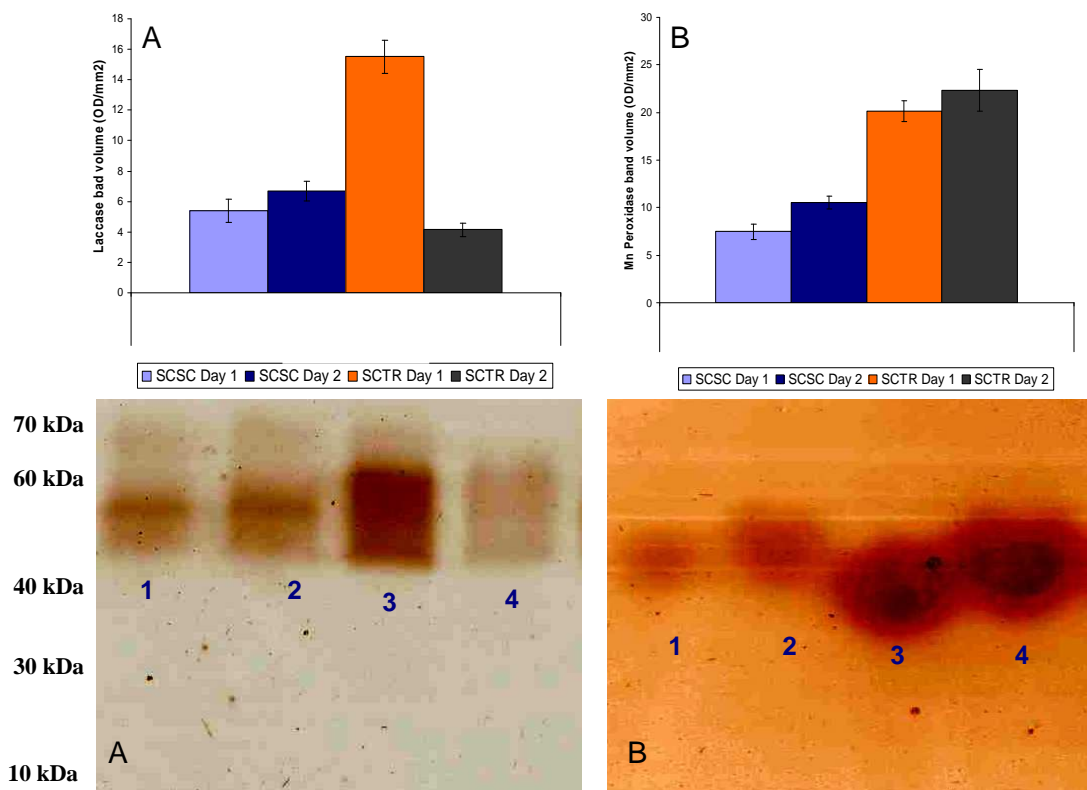
Figure 5.11 Protein gel electrophoresis run under native conditions showing levels of laccase and manganese peroxidase within the domain of *S. commune* paired against *T. viride* for 48 hours, in comparison to its self-paired cultures. A1 and B1 represent plots of band volumes detected by densitometric analyses for laccase and manganese peroxidase respectively. A2 and B2 show changes in band volumes, hence protein levels during interactions. 1 & 2: Self-paired *S. commune* after 1 and 2 days of contact respectively; 3 & 4: *S. commune* paired against *T. viride* after 1 and 2 days of interaction. Wells were loaded with 30 μg of protein. Error bars in the band volume plot represent standard deviation.

5.3.4 pH variations during mycelial interactions in liquid cultures

Broth was adjusted to pH 7 before inoculation. *S. commune* reduced medium pH to an average of 5.2 while the pH of *T. viride* cultures remained relatively stable before mixing

of cultures. The pH of self-paired and co-cultures of both fungi increased post mixing before stabilizing at 6.1 and 7.8 for self-paired cultures of *S. commune* and *T. viride* respectively, while the pH of their co-cultures showed the same pattern as *T. viride* cultures from 7 days post mixing (figure 5.12).
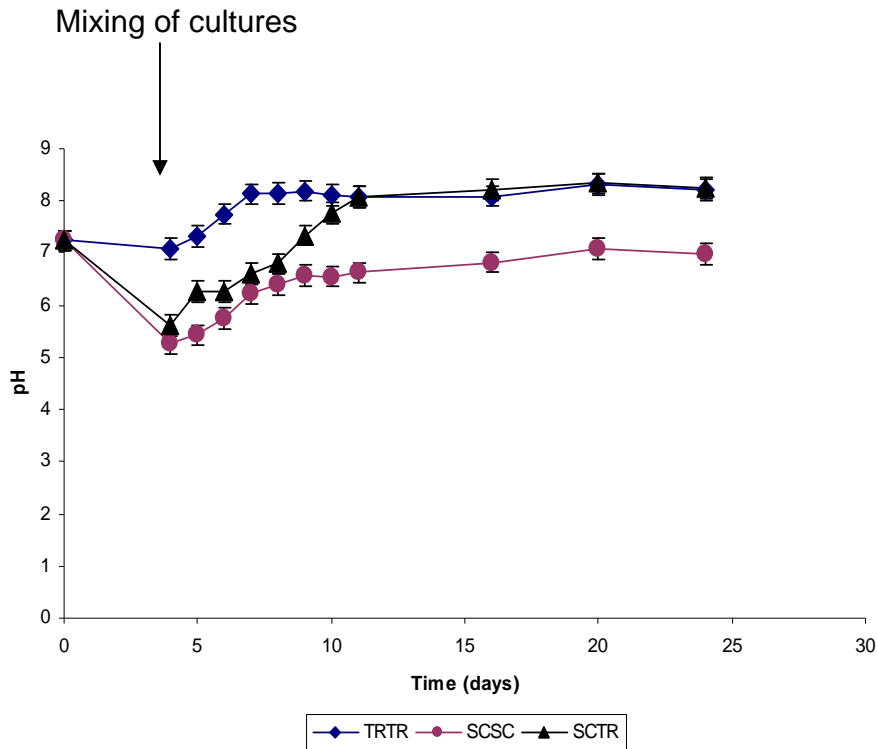
Figure 5.12 Changes in medium pH of mixed cultures of *S. commune* and *T. viride*, in comparison to self-interacting cultures of both fungi ((See figure 5.7 for key to SCSC, SCTR and TRTR). 6 replica cultures were set up for experiment, out of which 3 uniform flasks were used for experiment. Error bars represent standard deviation.

5.4 Discussion

Fungal interactions trigger a complex cascade of physiological reactions, most of which are mediated by a wide range of intra- and extracellular enzymes. SOD, catalase, G6PDH, SSADH, laccase, and manganese peroxidase activities were assayed following

the detection of increased levels of protein carbonyls, markers for lipid peroxidation, superoxide anion, phenolic compounds and GABA (chapters 3and 4), in addition to earlier studies implicating phenoloxidases, phenolic compounds and oxidative stress in interspecific combative interactions in fungi (Rayner *et al*., 1995; Griffith *et al*., 1994; Boddy, 2000; Gregorio *et al*., 2006; Peiris *et al*., 2008; Peiris, 2009 (PhD thesis); Score *et al*., 1997; Crowe & Olsson, 2001; Iakovlev & Stenlid, 2000; Chi *et al*., 2006).The body of evidence on the roles of chitinases as part of the mycoparasitic machinery of *Trichoderma* species (Howell, 1987; Lorito *et al*., 1998; 2001; Bruce *et al*., 1995; Bruce & Highley, 1991) prompted the need to assay for chitinase activity. Sharp up-regulation followed by down-regulation was consistently detected for all the enzymes assayed in mycelial samples of *S. commune* from 24 to 96 hours post contact. While laccase activity was higher after 24 hours of contact with *T. viride*, manganese peroxidase activity was significantly higher in the *S. commune* domain confronted by *T. viride* for 72 hours after contact with the antagonist. In-gel activity staining confirmed a similar pattern of protein levels for both enzymes.

5.4.1 Up-regulation of laccase and manganese peroxidase in *S. commune* mycelia following contact with *T. viride*

With regards to combative interactions in filamentous fungi, phenoloxidases namely laccase, manganese peroxidase and tyrosinase have been extensively studied because of their up-regulation during interspecific mycelial contact. However, laccase is the most strongly implicated phenoloxidase in fungus-fungus interactions (Rayner *et al*., 1995; Griffith *et al*., 1994; Boddy, 2000; Gregorio *et al*., 2006; Peiris *et al*., 2008; Score *et al*., 1997; Crowe & Olsson, 2001; Iakovlev & Stenlid, 2000; Chi *et al*., 2006; White & Boddy, 1992). Laccase and manganese peroxidase are part of the lignin-degrading repertoire of white-rot fungi (Tsujiyama & Minami, 2005; Xiaobin *et al*., 2007) and they have been shown to participate in the detoxification of xenobiotic compounds and fungicides (White & Boddy, 1992).

Production of free radicals and polymers by the oxidation of hydrophobic metabolites (stemming from the induction of secondary metabolism) which leads to the strengthening

of cell wall and synthesis of protective quinones and melanin (Rayner *et al*., 1994) is the most widely accepted mechanism underlying the up-regulation of phenoloxidases during fungus-fungus interactions. The results from different aspects of this work are in agreement with this mechanism, although they implicate additional mechanisms. Firstly, increased secretion and localization of phenoloxidases within the interaction zone (figure 5.1) confirm earlier reports suggesting that phenoloxidases perform key functions specifically around the contact area (Tsujiyama & Minami, 2005; Iakovlev & Stenlid, 2000). Detoxification of toxic compounds secreted by one or both of the interacting species which according to Rayner and co-workers (1994) leads to the strengthening the cell wall is possibly one of these functions. According to Gregorio *et al*. (2006), addition of filter-sterilized culture supernatant of *Maramius pallescens* to cultures of *Maramiellus troyanus* resulted in rapid induction of laccase and manganese peroxidase, possibly due to phenolic or other classes of compounds present in the former.

GC/MS profiling of the metabolites associated with interactions between *Stereum hirsutum* and its competitors *Coprinus micaceus* and *Coprinus disseminatus* showed increased production of aromatic compounds such as 1-methyl-3,5-dihydroxybenzene and 1,2-dihydroxyanthraquinone in *S. hirsutum* (Peiris *et al*., 2007) which possess the typical phenoloxidase substrate structure. Furthermore, both laccase and manganese peroxidase were up-regulated in the same study (Peiris, 2009). Crowe and Olsson (2001) also demonstrated the implication of substrate recognition in the induction of phenol-oxidising enzymes, particularly laccase in cultures of *Rhizoctonia solani* paired against *Pseudomonas fluorescens*.

Up-regulation of phenylalanine ammonia lyase (PAL - which catalyses the committed step in the phenolpropanoid pathway; a major source of phenolic compounds), in the mycelia of *S. commune* after 48 hours of contact with *T. viride* (chapter 7; section 7.3.1) points to the "phenolics-phenoloxidases" interplay during interactions between *S. commune* and *T. viride*. Furthermore, GC/MS detection of increased levels of mandelic and tropic acids, both of which are phenolic compounds in the mycelia of *S. commune* after contact with *T. viride* and 4-hydroxyphenyl ethanol in the latter (chapter 3; section

3.3.2) coupled with the increased levels of phenolic compounds, as detected by spectrophotometric method (chapter 4; section 4.3.2) lend further weight to the substrate recognition hypothesis as one of the factors that may have elicited the induction of laccase and manganese peroxidase in the present study. In figure 4.4 (chapter 4), the initial build up of phenolic compounds following mycelial contact reduced to similar levels in self-paired cultures of *S. commune* and *T. viride* by 48 hours of contact. This suggests that contact between both fungi may have caused an increase in the secretion of phenolics with corresponding rise in phenoloxidase secretion, which may have oxidised phenolic compounds to non-toxic levels in the interaction zone. Fungi with more robust phenoloxidase production capacities have been observed to be more predominant during interactions, perhaps due to their ability to detoxify toxic compounds, which may accumulate during fungus-fungus interactions (Tsujiyama & Minami, 2005; Chi *et al*., 2006).

Secondly, Crowe and Olsson (2001) inferred that the up-regulation of laccase in *R. solani* paired against an antagonistic strain of *P. fluorescens* was also a possible response to cellular damage (compromised cell integrity) via the activation of calcium influx and heat shock pathway. Lysis of *S. commune* mycelia after contact with *T. viride* (chapter 2) is potentially fatal and consequently compromises cellular integrity. Under such condition, phenoloxidase-derived free radicals are likely to restore homeostasis via the polymerization of phenolic compounds described above, thereby rendering the cell wall less porous. Thirdly, phenoloxidases can scavenge reactive oxygen species (ROS) during substrate oxidation, thereby generating transient free radicals, which undergo further reactions leading to the production of protective melanin (pigmentation) (Gregorio *et al*., 2005; Thurston, 1994; Rayner, 1997; Reinhammer and Malstrom, 1981; Misall *et al*., 2005). ROS destabilize membranes and macromolecules in a manner similar to lipophilic damage of microbial structures by alcohols (Piper, 1995; Panaretou & Piper, 1992) which have been demonstrated as potent inducers of laccase (Crowe & Olsson, 2001). The work of Iakovlev *et al*. (2004) also implicated oxidative damage in the sequence of reactions that ensued mycelial contact between *Heterobasidion annosum* and *Physisporinus sanguinolentus*. Taken together, it could be suggested that the levels of superoxide anion

soon after contact between both fungi (chapter 4; section 4.3.2), may be part of a range of factors that triggered increased laccase and manganese peroxidase production in *S. commune*.

It has been reported that stimulation of the release of calcium from intracellular stores through inositol 1,4,5-triphosphate-sensitive channels promotes laccase synthesis (Crowe & Olsson, 2001). Inositol phosphate was strongly up-regulated in *S. commune* mycelia after 48 hours of contact with *T. viride* (chapter 3; section 3.3.2). Although it could not be specifically confirmed whether this was inositol triphosphate, inositol phosphates have been generally implicated in cellular signalling (Brighton *et al*., 1992; Liu *et al*., 2009). Similarly, a heat shock protein (HSP 70)-encoding gene (path of the heat shock pathway) was up-regulated in the mycelia of *S. commune* after 24 hours of contact with *T. viride* (chapter 6; section 6.3). Taken together, it could be deduced that substrate recognition, oxidative stress and cellular damage were among the inducers of laccase and manganese peroxidase in the mycelia of *S. commune* paired against *T. viride*.

It is worth mentioning that increase in the activity of laccase and manganese peroxidase was very likely as a result of *de novo* protein synthesis given the rise in the levels of protein detected by in-gel activity staining (figure 5.11). This is buttressed by RT-PCR results in chapter six, which show that the *S. commune* laccase gene was up-regulated in response to *T. viride*. To this end, it could be concluded that laccase and manganese peroxidase were transcriptionally activated during mycelial confrontation with *T. viride*. However, contrary to extensive reports, *S. commune* appeared to have employed more of manganese peroxidase than laccase during interaction with *T. viride*, considering the patterns of activity and protein levels detected for both enzymes (figures 5.5, 5.6 and 5.11). *Schizophyllum* sp. F17, found to be efficient at the degradation of structurally diverse azo dyes, predominantly produced manganese peroxidase, whereas lignin peroxidase and laccase were hardly detected during dye degradation (Xiaobin *et al*., 2007). Although there is not enough evidence to confirm this, it does appear that *Schizophyllum* species might preferentially synthesize more of manganese peroxidase for detoxification or homeostatic functions.
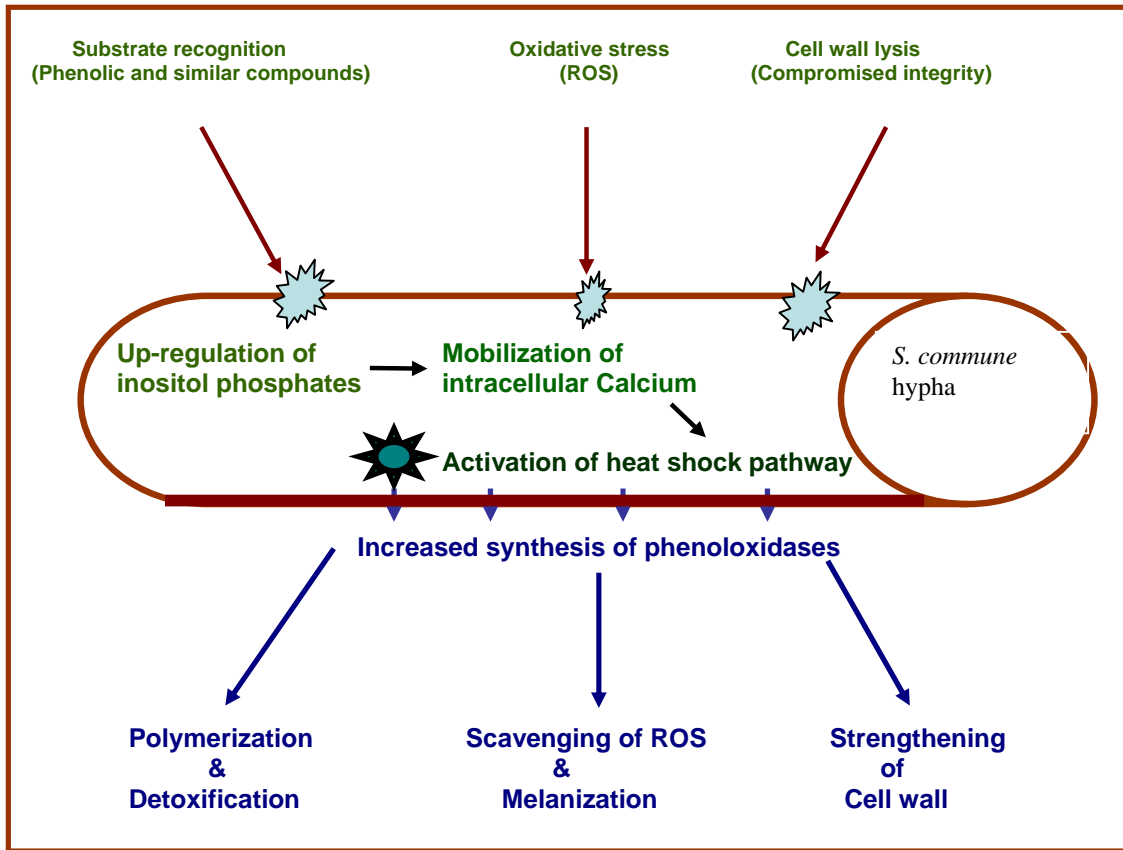
Figure 5.13 Schematic representation of the factors that most likely triggered the secretion of laccase and manganese peroxidase in *S. commune* confronted by *T. viride* and the possible underlying biochemical reactions promoting their synthesis and secretion.

5.4.2 Increase in SOD and catalase activity in the mycelia of *S. commune* paired against *T. viride*.

Although the present study has not made any distinctions between extracellular and intracellular SOD and catalase, activities for both ROS scavenging enzymes increased within the *S. commune* mycelial domain after contact with *T. viride* before dropping to or below control levels. These rises in activities are indicative of increased levels of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which are scavenged by SOD and

114

catalase respectively (Kang *et al*., 1998; Fink-Boots *et al*., 1999; Dolashka-Angelova *et al*., 1999; Garre *et al*., 1998; Kurakov *et al*., 2001).

Extracellularly, fungus-fungus interactions lead to the production of ROS, which harness degradation on wood (Hammel *et al*., 2002). On the other hand, protoplasmic degeneration, which very often arises from combative interactions in fungi, is an indication of switch to secondary metabolism and response to the corresponding rise in ROS (Iakovlev & Stenlid, 2000). Repression of a gene encoding a mitochondrial import protein in *Heterobasidion annosum* paired against *Physisporinus sanguinolentus* suggested that the structure and functioning of mitochondria were disrupted in the former; an indication of secondary metabolism (Iakovlev *et al*., 2004). Such disruption results in electron leakage hence, oxidative damage. Protoplasmic degeneration, rise in the levels of phenolic compounds and the levels of superoxide anion were detected in the mycelia of *S. commune* paired against *T. viride* (Chapters 2, 3 and 4 respectively), which suggest possible prevalence of oxidative damage and secondary metabolism in *S. commune* during contact.

Implication of the up-regulation of malate dehydrogenase isoforms in the β-oxidation pathway (a major source of ROS), is discussed in chapter 7. Against this backdrop, it could be inferred that *S. commune* is subjected to both extra- and intracellular oxidative stress during confrontation with *T. viride*, accounting for the rise in the activities of SOD and catalase both of which function to alleviate oxidative stress. Fink-Boots *et al*. (1999) demonstrated simultaneous increase in the activities of laccase and SOD as well as phenolic compounds and superoxide anion in selected fungi. Although heat was the stressor applied in their study, the results of this study (Chapters 4 and 5) suggest that oxidative stress might mimic heat damage in its effect to macromolecules and membranes resulting in the degeneration of protoplasmic components. Down-regulation of genes that code for cytochrome C_1 and a component of the mitochondrial ATP synthase complex, both of which are components of the mitochondrial electron transport chain (Chapter 6; section 6.3) further implicate oxidative stress in the response patterns observed for *S. commune* following contact with *T. viride*.

5.4.3 Elevated G6PDH and SSADH activities in *S. commune* confronted by *T. viride*

Detection of elevated SSADH activity in the mycelia of *S. commune* 48 hours post contact with the confronting mycelia of *T. viride* supports the detection of increased levels of GABA in *S. commune* mycelia (Chapter 3). In plants, GABA shunt has been shown to function in the regulation of cytosolic pH, balance of nitrogen and carbon metabolism, and adaptation to stress such as wounding, starvation, drought, cold and heat stresses (Bown & Shelp, 1997; 2003; Snedden & Fromm, 1999). Activation of GABA shunt is strongly influenced by the redox status of the cell as discussed in chapters 6 and 3. Catabolism of GABA leads to the terminal intermediate in the shunt, succinic semialdehyde, which is converted to succinate by SSADH and fed back into the TCA cycle with the generation of NADH (figure 5.14).

The study of Bouché *et al.* (2002) demonstrated that a functional GABA shunt is essential for suppressing the accumulation of hydrogen peroxide in plants. The authors hypothesized that this was perhaps owing to the ability of GABA shunt to produce NADH and succinate under conditions that hamper the TCA cycle, limit respiration and promote the production of ROS. Suppression of α-ketoglutarate dehydrogenase (via which GABA shunt is activated) critically restricts NADH production under conditions of oxidative stress (NADH/NAD$^+$ balance) (Tretter & Adam-Vizi, 2000). To this end, GABA shunt ensures the continuity of the respiratory and energy-generating machinery of the mitochondrion with corresponding production a vital co-factor, NADH. In addition, given that GABA shunt bypasses NADH-producing steps in the TCA cycle, the shunt perhaps helps to maintain NADH concentration to levels optimum for cellular functioning relative to NAD$^+$ levels.

Knockout of GABA shunt genes in yeast resulted in increased sensitivity to hydrogen peroxide, while over-expression of these genes led to increased tolerance of oxidative stress (Coleman *et al.*, 2001). In the study of Bouché *et al.* (2002), *Arabidopsis ssadh* mutants were hypersensitive to white light and heat with rapid accumulation of hydrogen peroxide when exposed to these stress conditions. Rapid increase in the activity of SSADH and increase in the levels of GABA (chapter 3) following contact between *S.*

commune and *T. viride* strongly suggest that the GABA shunt is activated in the former. More importantly, as in plants, it is very likely that GABA shunt plays similar roles in alleviating stress stemming from damage, and oxidative stress especially with the possible block of the TCA cycle as a result of the suppression of α-ketoglutarate dehydrogenase (figure 5.14).

G6PDH, the first enzyme in the pentose phosphate pathway (PPP) is one of the NADPH-producing enzymes, which are important to all living organisms because of their roles in biosynthesis and regulation of cellular redox state (Bériault *et al*., 2005). NADPH-producing enzymes make key contributions to the intracellular NADPH pool and are critical in the maintenance of a reducing environment under aerobic conditions (Bériault *et al*., 2005). There is a growing body of evidence suggesting that G6PDH might play a pivotal role in oxidative stress response (Shenton and Grant, 2003; Izawa *et al*., 1998; Liu *et al*., 2007). Shenton and Grant (2003) reported decrease in the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a corresponding increase in G6PDH in *Aspergillus niger* B1-D under conditions of oxidative stress.

Shenton and Grant (2003) argued that this might have been as a result of the oxidative damage of glycolytic enzymes such as GAPDH, thereby causing a shift in glucose metabolism to the PPP with greater potential to produce NADPH a vital source of reducing power for antioxidant enzymes. Similarly, Liu *et al*. (2007) showed that exposure of kidney beans to sodium chloride-induced oxidative stress resulted to a rapid increase in G6PDH activity and protein levels. Although GAPDH was not assayed in this study, suppression of GAPDH gene is reported for *S. commune* in chapter 6 after 48 hours of mycelial contact with *T. viride*. Although this does not completely rule out the possibility that the enzyme might still be active, it is a strong indication that its activity and protein levels may have dropped following suppression of the gene. Down-regulation of other glycolytic proteins (chapter 7; section 7.3.1) further buttresses the assumption that the glycolytic pathway may have been shelved under the described conditions of growth. More importantly, rapid increase in the activity of G6PDH suggests that the PPP may have functioned as an alternative glucose-metabolizing pathway in the mycelia of *S.*

commune during confrontation. The PPP is a major source of NADPH, which provides reducing power for antioxidant enzymes and oxidoreductases.
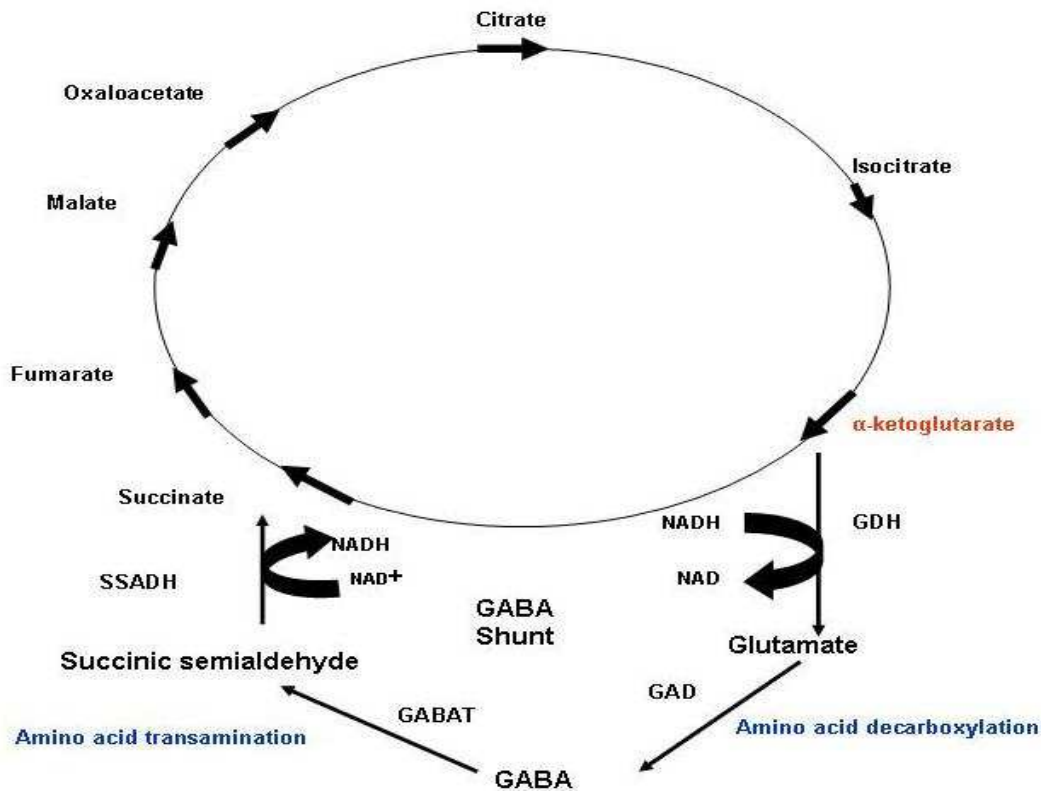
Figure 5.14 Schematic representation of GABA shunt, which bypasses 2 steps in the TCA cycle as a result of block in the conversion of α-ketoglutarate (highlighted in red as the point of TCA block) to Succinyl-CoA by α-ketoglutarate dehydrogenase (adapted from Panagiotou *et al*., 2005; Bouché *et al*., 2002). Pyridoxine-requiring enzymes (highlighted in blue), which catalyse decarboxylation and transamination reactions of amino acids, are involved within the GABA shunt (implication of pyridoxine in oxidative stress is discussed in chapter 3).

Erythritol was one of the sugar alcohols up-regulated in the mycelia of *S. commune* paired against *T. viride* (*chapter 3*). Interestingly, erythritol is synthesized via the PPP (Chapter 3; figure 3.9). Based on this, it could be argued that *S. commune* may have up-regulated the PPP with corresponding drop in glycolytic activity as a means of containing oxidative stress as well as osmotic shock. This would be less energy expensive under

conditions of stress considering that it allows for simultaneous accumulation of both NADPH and erythritol through a single pathway.

5.4.4 Increase in the activity of chitinase and pH variations in liquid cultures of *S. commune* and *T. viride*

The pH profile of liquid co-cultures of *S. commune* and *T. viride* (figure 5.12) confirms the physiological dominance of *T. viride* over the former. More importantly, it demonstrates that *T. viride* kills *S. commune* during mycelial interactions. This is indicated by the complete disappearance of *S. commune* pellets (which were morphologically distinct from those of the antagonist) 7 days post mixing. The mycoparasitic properties of *Trichoderma* species are well documented (Chapter 1; section 1.2.1) (Bruce *et al*., 1995; Highley & Ricard, 1988; Bruce & Highley 1991; Mendoza-Mendoza *et al*., 2003; Howell *et al*., 1993; Lorito *et al*., 1998). To this end, increase in the activity of chitinase is in agreement with several studies, which demonstrated the employment of this enzyme mainly in the attack response of *Trichoderma* species against host fungi (Bruce *et al*., 1995; Highley & Ricard, 1988; Bruce & Highley 1991; Mendoza-Mendoza *et al*., 2003).

The fungal mycelium is chiefly composed of polysaccharides such as chitin, glucan and chitosan, which determine the shape of the hypha (Gooday, 1995). These polymers are embedded in a protein matrix (Gooday, 1995). The molecular structure of chitin confers mechanical rigidity to the fungal cell wall (Gooday, 1995). Mycoparasites such as *Trichoderma* species digest host cell wall leading to total lysis of the mycelium, thereby allowing them to utilize host constituents as nutrients (mycoparasitism is discussed in details in chapter 1) (Aluko & Hering, 1970; Baek *et al*., 1999; Bliss, 1951). Increase in chitinase activity, pH profile of liquid co-cultures of both fungi and the disappearance (lysis) of *S. commune* pellets confirm the induction of the mycoparasitic machinery of *T. viride* paired against *S. commune*.

5.4.5 Summary and conclusions

The activity patterns of the studied enzymes presented in this chapter suggest that *S. commune* was dominated and subsequently killed by *T. viride* in the contact areas on solid cultures and in liquid cultures. This is supported by the pH profile of both fungi in liquid co-cultures as well as the lysis of *S. commune* pellets 7 days post contact with *T. viride*. Considering the dynamics of interactions in liquid cultures in comparison to agar plate interactions, lyses and death are more likely to occur quicker in liquid cultures. A combination of increasing levels of phenolics in the contact area on agar, response to cell wall damage and build up of ROS appear to be among the cascade of factors that may have triggered increased synthesis of laccase and manganese peroxidase in *S. commune* paired against *T. viride*. The physiological requirement to contain intra- and extracellular oxidative stress, to generate reducing power for antioxidant enzymes by sustaining the production of NADPH and to regulate NADH/NAD$^+$ (redox) status in the cell most possibly account for the rise in the activities of SOD, catalase, SSADH and G6PDH in the mycelia of *S. commune*. Enzyme- driven mycoparasitic attack of *S. commune* by *T. viride* is confirmed by the sustained rise in chitinase activity in liquid co-cultures of both fungi.

CHAPTER 6

Studies of expression patterns of selected Genes in *S. commune* paired against *T. viride* using RT-PCR

6.1 Introduction

The complex morphological and physiological changes that occur during interspecific mycelial interactions strongly reflect the possible involvement of diverse molecular modulations, which elicit a myriad of defence and/or attack mechanisms. Such molecular modulations might involve over-expression, down-regulation or repression of different genes based on the functions of the proteins they encode and their modes of regulation. This makes gene expression analyses a vital tool in the effort to broaden understanding of the underlying molecular bases of antagonistic mycelial interactions. Genomic tools such as cDNA macroarrays (Adomas *et al.*, 2006) and mRNA differential display (Ikovlev *et al.*, 2004) have been successfully used to probe for differentially expressed genes in paired cultures of *Phlebiopsis gigantea* versus *Heterobasidion parviporum* and *Heterobasidion annosum* versus *Physisporinus sanguinolentus* respectively. In addition, Peiris (2009) used quantitative RT-PCR to investigate the expression patterns of laccase genes in interacting mycelia of *Stereum hirsutum*, *Coprinus micaceus* and *Coprinus disseminatus*.

Application of genomic tools to such a study depends greatly on the availability of sequenced fungal genomes on the database, although homologous matching with similar or other fungal genes/genomes might suffice. Albeit *S. commune* has not been fully sequenced, expressed sequence tags (ESTs) of some *S. commune* genes are available on the NCBI database (Guettler *et al.*, 2003). This makes it possible to design primers for the available genes, thereby allowing the application of a specific gene expression analytical tool such as reverse transcriptase-polymerase chain reaction (RT-PCR) for the specific investigation of the mRNA abundance for selected genes of *S. commune*. Expression profiles of genes encoding proteins involved in varying physiological functions in *S. commune* paired against *T. viride* are presented in this chapter. Although this does not by any means provide a global picture of the molecular response of *S. commune* to *T. viride*, it offers specific insight into the expression of selected genes of *S. commune* in response to the antagonists at 24 and 48 hours of mycelial interactions, relative to unpaired and self-paired mycelia of *S. commune*. Hence, assemblage of the mRNA abundance levels of the selected genes and the results of other tools (metabolomics and proteomics) employed

in the whole study enables further understanding of the complex phenomenon of non-self recognition and consequent rejection between the white-rot basidiomycete, *S. commune* and the antagonist, *T. viride*. Genes encoding proteins involved in functions such as glycolysis, electron transport and ATP generation, trans-membrane transport, protein synthesis, stress response, cell wall biogenesis, amino acid synthesis and cellular replication were among the studied genes. The aim of this section of the study was to isolate, quantify and compare the mRNA abundance of selected genes of *S. commune* in mycelia of *S. commune* paired against *T. viride* relative to levels in unpaired and self-paired mycelia.

6.2 Materials and methods

6.2.1 Isolation and quantification of total RNA

RNA was isolated from *S. commune* mycelia interacting with self, with *T. viride* and from unpaired mycelia, according to the protocol presented in figure 6.1. 0.2 g of mycelial samples were cut off from the agar with scalpel, frozen quickly in liquid nitrogen, stored at -80°C overnight and freeze-dried. Freeze-dried mycelial samples were stored at -80°C for a maximum period of 2 months. Tri reagent^{TM} (Sigma-Aldrich, UK) was used for RNA isolation with slight modifications of the manufacturer's protocol aimed at reducing the levels of contaminating phenolics and polysaccharides, which strongly interfere with RNA extraction. Freeze-dried samples were ground to powder in mortar with the addition of small amounts of acid-washed sand (Sigma-Aldrich, UK) and suspended in 3 ml of Tri reagent^{TM}. Tri reagent^{TM}-mycelial powder mixtures were vortexed vigorously for 1 min and incubated at room temperature for 20 mins. 0.2 volume of chloroform was used for phase separation per 1 ml of Tri reagent^{TM} used. The mixture was inverted gently for 15 seconds and incubated at room temperature for 3 mins before spinning at 13,000 rpm for 15 mins at 4°C. The top aqueous layer was collected afterwards.

Triple extractions rather one, were carried out by reconstituting the resulting aqueous solution after phase separation in 500 μL of Tri reagent^{TM} for second and third extraction steps respectively, without vigorous mixing. Phase separations with

chloroform were carried out as described above (figure 6.1). RNA was precipitated from the resulting final aqueous phase after the 3rd extraction step by mixing with 200 μL of isopropanol, and incubation at -20°C after a gentle inversion for 4 hours. RNA pellets after precipitation were suspended in 50-100 μL of formamide and stored at -80°C. All RNA samples were run on 1.2% agarose gel for the detection of 5s, 18s and 28s RNA and quantified spectrophotometrically (Uvette spectrophotometer). Samples with 260/280 (nm) absorbance ratio of 1.8 and 3 clear bands (5.8s, 18s and 28s rRNAs) on 1.2% agarose gel were used for RT-PCR.

6.2.2 Primer design

Primers were designed online with the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) based on expressed sequence tags (Guettler *et al*., 2003) and in some cases, full-length coding sequences of the selected genes available on the NCBI database (http://www.ncbi.nlm.nih.gov/). The primer sets used for each of the selected genes are shown in table 6.1.

6.2.3 RT-PCR

RT-PCR was carried out in triplicates with the Qiagen® OneStep RT-PCR kit by following the manufacturer's protocol. The reaction mixture composed of;

dNTP mix (containing 10 mM of each dNTP) 2 μL (400 μM of each dNTP)

5X Qiagen® OnStep RT-PCR Buffer 10 μL (1X)

Qiagen® OnStep RT-PCR enzyme mix 2 μL

RNase inhibitor 5 units/reaction

5X Q-solution 10 μL

Primers (forward and reverse) 0.6 μM

Template RNA 2 μg/reaction

The final volume was made up to 50 μL with RNase free water.

Homogenisation → Ground mycelia + 3 ml of Tri reagent™
(vigorous vortexing)

Phase separation → Add 0.2 vol of Chloroform per ml of Tri reagent™
Invert gently (15 seconds)
Incubate at room temp (3 min)
Spin (13,000 rpm; 15 min; 4ºC)
Collect aqueous phase

Second extraction → Homogenisation: Reconstitute in 500 µL of Tri reagent™
Incubate for 45 mins at room temp
Phase separation: Add 0.2 vol of Chloroform
Incubate (15 mins; room temp)
Spin as above
Collect aqueous phase

Third extraction → Homogenisation and phase separation: As in second extraction
Incubate at room temp (5 mins)
Spin (8 mins; 4ºC)
Collect aqueous phase

Precipitation → Mix with 200 µL of isopropanol
Incubate at 120ºC (4 hours)
Spin (13,000 rpm (30 mins)
Collect pellet

RNA wash → Wash twice with 70% ethanol

RNA solubilization → Air-dry pellet for 3 mins
Reconstitute in 50 – 100 µL
Leave on ice for 2-3 hours to dissolve

Figure 6.1 Schematic representation of the stages of RNA isolation from ground mycelia of *S. commune*.

Table 6.1 Genes studied by RT-PCR, their primer sequences and product sizes.

Gene name/ putative function	NCBI Accession number	Primers (Forward and reverse)	Predicted size (base pairs)
GAPDH	BG673933	AATAATGGCCGTGAAGGTTG TGGACGGTGTCGTACTTGAA	428
18SrRNA *	EU520239	CAACGGATCTCTTGGCTCTC CTCCAGCAGACCTCCACTTC	640
Polyubiquitin **	U74318	AGGTCGAATCCTCTGACACG TCAGAGACCACCACGGAGAC	1079
Mitochondrial ATP Synthase (beta chain)	BG713751	CCCCATCCAGATTCCTGTC CCTCCGAAGAGACCGATCTTG	540
Vacuolar ATP Synthase (C subunit)	BQ102562	CACCAAGAACCTCATCAGCA GTGATGCCAACACAGACACC	486
Serine/threonine protein kinase kinase (MAPKK)	BQ134634	ACGCCTACGAATCTGAGCAT GAGGATATTGTCCGCCTTCA	541
Serine/threonine protein kinase (MAPK)**	AB218429	ATCTACGCGCTCAAGACGAT GAAGTTCCGCGCTGTAGAAG	2054
Glutamine synthethase	BQ173853	ATGCCAAAAAGACGATGGAC GGTAGTGGGCCTCAATCAGA	498
Alpha tubulin	BF942492	CGACCTGCTCTACTCCAAGC GCGACAGAGAAAGCGTAACC	425
Heat shock cognate protein (sks2)	BG673919	CAACGGCTTGTTGAAGGTCT GTGGATGTACGCCTCAAGGT	404

Laccase **	AB015758	GCAAGACCAGCTCAATGACA	1822
		GCCGAACTCGTAGACGAAAG	
Pyruvate decarboxylase	BG713762	ACGTCGTACACCGTCAACAA	449
		TCCCGATTATCATGCACAAA	
2-Oxoglutarate dehydrogenase (E1 component)	BI135399	GACTCCGTCGACCAGATGAT	474
		GCAGCACCACAAGGGTAAGT	
Acyl carrier protein	BG739705	TCGGTATCGAAATCCCTGAC	503
		AACTACTGTCGGCGGGTATG	
Plasma membrane H $^+$ATPase	BI324797	TACCACCAACAAGCTCACCA	494
		AGTCGAGGAGCTTGATACCG	
Chitin synthase *	M82956	GTGTGCATAGTGTCGGATGG	509
		CGTGACAGGCTTCTTGTTGA	
Neutral trehalase	BQ102603	CTGCAGAACAGCGAAGAGTG	550
		AACGAGTTCGACACCAGCTT	
Cytochrome C_1	BQ102564	AAATGTTCTCGAGGCTGTCG	489
		GATAGACCCGACCGAGACAA	
Ribosomal protein S8	BI324810	CCGCAAAAAGAGAAAGTTCG	499
		TTGTACGGACAAGCTCGTTG	
Actin ** (control)	AF156157	CACGGTATCGTCACCAACTG	2475
		TGATCTGCGTCATCTTCTCG	

Most primers were designed based on *S. commune* ESTs available on the NCBI database with exception of asterisked genes above. * Partial sequence, ** complete coding sequence. MAPKK – mitogen activated protein kinase kinase; MAPK – mitogen activated protein kinase; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase. Actin gene was used as control.

RT-PCR amplification conditions were as follows;

Reverse transcription:	55°C	30 mins
Initial PCR activation:	95°C	15 mins

3-step cycling

Denaturation:	94°C	1 min
Annealing:	50°C	1 min
Extension:	72°C	1 min
Cycles: 35		
Final extension:	72°C	1 min

6.2.4 Visualisation and quantification of products

Products were run on 1.2% agarose gel in TAE (Tris-Acetate-EDTA) buffer stained with ethidium bromide for product visualisation and quantification of band volumes. The volumes of the resulting bands were determined and compared using the Uvitec gel analysis software (Cambridge Scientific). mRNA levels for the genes under investigation were expressed as a function of band volume after RT-PCR amplification.

6.2.5 Statistical analysis

Unpaired T-test was used to determine the level of significance of the observed up-/down-regulation of the different genes, by using Graphpad T-test calculator software (Graphpad software, San Diego California: http://www.graphpad.com/quickcalcs/ttest1.cfm). P values < 0.05 were considered significant.

6.3 Results

Gel electrophoresis after RNA isolation showed bands corresponding to 5.8s, 18s and 28s rRNAs in 1.2% agarose gel (figure 6.2). Concentrations of total RNA ranged from 200-9000 µg/ml with absorbance ratios at 260 nm and 280 nm (260/280) ranging from 1.8 – 2.5. 260/230 absorbance ratios were between 1.8 and 2.2. Triple extraction appeared to reduce the phenolic and sugar (contaminants) contents of the total RNA samples.

Figure 6.3 and table 6.2 show a sharp increase in the expression of genes coding for chitin synthase, an MAPKK, an MAPK, laccase, Polyubiquitin, HSP 70, ribosomal protein S8 and acyl carrier protein after 24 hours of mycelial contact with *T. viride*. Although neutral trehalase was down-regulated after 24 hours of contact with *T. viride*, it was up-regulated 3 and 3.2 fold ($P < 0.0008$) compared to unpaired and self-paired cultures respectively. 18SrRNA gene was slightly up-regulated relative to the un-paired samples after 24 hours of contact. However, after 48 hours of contact, the band volume increased 4 and 2 fold ($P = 0.004$) relative to the unpaired and self-paired cultures respectively.

2-oxoketoglutarate dehydrogenase E1 component, α-tubulin and H^+-transporting ATPase genes were repressed after 24 hours of interaction, whereas pyruvate decarboxylase gene was only repressed after 48 hours of contact. Genes encoding GAPDH and Vacuolar ATP synthase were down-regulated after 24 hours of contact, while Cytochrome C_1, mitochondrial ATP synthase and glutamine synthethase genes were down after 24 hours of mycelial contact. Although cytochrome C_1 gene was down-regulated in mycelia paired against *T. viride* relative to unpaired mycelia, comparison of band volumes for self-paired mycelia (where this gene was repressed) and mycelia confronted by *T. viride* show stronger band volumes in the former (figure 6.3). Similarly, there was no significant difference in the expression patterns observed for glutamine synthethase between self-paired mycelia and mycelia paired against *T. viride*.

However, it was down-regulated in the self-paired mycelia and mycelia paired against *T. viride,* compared to the unpaired cultures. Genes for acyl carrier protein, chitin synthase, ribosomal protein S8 and Cytochrome C_1 were strongly down-regulated in self-paired cultures following 24 hours of mycelial contact. Interestingly, in comparison to unpaired and self-paired cultures, all the genes, which were up-regulated in the mycelia confronted by *T. viride* after 24 hours of mycelial conflict (laccase, acyl carrier protein, Polyubiquitin, HSP 70, chitin synthase, ribosomal protein S8, MAPK and MAPKK),

showed clear drops in band volume by the end of 48 hours of mycelia contact. For all the genes presented in this chapter, products of the predicted size were detected in agarose gel after staining with ethidium bromide.
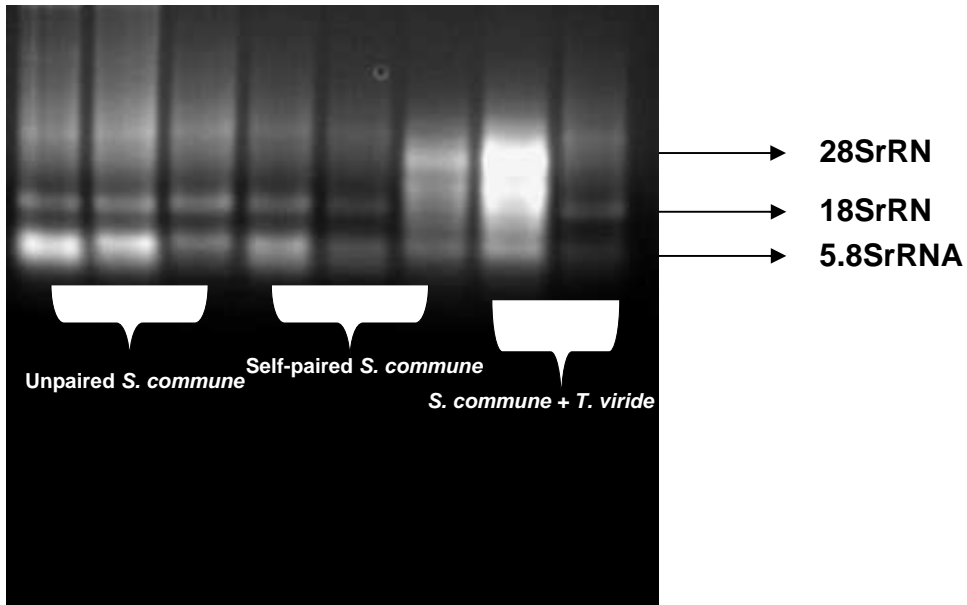
Figure 6.2 RNA bands on representative 1.2% agarose gel showing 28SrRNA, 18SrRNA and 5.8SrRNA from unpaired, self-paired mycelia of *S. commune* and mycelia of confronted by *T. viride*.

Glutamine synthethase

GAPDH

Polyubiquitin

Heat Shock protein cognate protein (sks2)

MAPK

MAPKK

Vacuolar ATP synthase C subunit

Laccase

Pyruvate decarboxylase

α –Oxoketoglutarate dehydrogenase E1

Acyl carrier protein

Plasma membrane H^+ ATPase

α-Tubulin

Chitin synthase

Neutral Trehalase

Cytochrome C_1

Mitochondrial ATP synthase beta chain

18SrRNA

Ribosomal protein S8

Actin

1   2   3   4   5   6

Figure 6.3 Representative DNA bands on ethidium bromide-stained 1.2% agarose gel showing the expression patterns of the investigated genes of *S. commune* paired against *T. viride*. 1 & 2: mRNA from unpaired mycelia of *S. commune* corresponding to 24- and 48-hour contact periods respectively in paired cultures; 3 & 4: mRNA from self-paired mycelia of *S. commune* after 24 and 48 hours of contact respectively; 5 & 6: mRNA from *S. commune* mycelia paired against *T. viride*, after 24 and 48 hours of contact respectively. PCR reactions were set up in triplicates and 3 band replicas of test samples were compared against triplicate bands of control samples.

Table 6.2 Up-regulated genes in mycelia of *S. commune* following mycelial conflict with *T. viride*.

Gene function/name	*P* value	Fold increase in band volume			
		Relative to SC		Relative to SCSC	
		24 hours of contact	48 hours of contact	24 hours of contact	48 hours of contact
Polyubiquitin	< 0.0001	10.7		11.2	
Laccase	< 0.0001	4.2		2.0	
Acyl carrier protein	< 0.0001	2.0		6.0	
Chitin synthase	0.0006	2.0		5.0	
Neutral trehalase	0.0008		3.0		3.2
Ribosomal protein S8	< 0.0001		2.0		9.0
18SrRNA	0.004		4.0		2.0
MAPKK	0.0019	1.5		1.6	
MAPK	0.0053	10.8		8.7	
HSP 70	0.0106	2.3		2.5	

SC- unpaired mycelia of *S. commune*; SCSC, self-paired mycelia of *S. commune*. Experiments were carried out in triplicates.

Table 6.3 Genes down-regulated/repressed in *S. commune* following mycelial conflict with *T. viride*.

Gene function/name	*P* value	Fold decrease in band volume			
		Relative to SC		Relative to SCSC	
		24 hours of contact	**48 hours of contact**	**24 hours of contact**	**48 hours of contact**
GAPDH	< 0.0001		4.3		5.0
Pyruvate decarboxylase			repressed		repressed
Glutamine synthethase*	<0.0001	2.0			
Vacuolar ATP synthase	0.0007		3.5		2.7
2-Oxoketoglutarate dehydrogenase		repressed		repressed	
H^+-transporting ATPase		repressed		repressed	
Alpha tubulin		repressed		repressed	
Neutral trehalase	0.0008	5.4		8.9	
Cytochrome C1*		2.2			
Mitochondrial ATP Synthase (β-chain)	< 0.0001	6.3		2.4	

SC- unpaired mycelia of *S. commune*; SCSC, self-paired mycelia of *S. commune*. Experiments were carried out in triplicates (see Appendix IV for a plot of gene band volumes).

6.4 Discussion

Although the results presented in this chapter are only a snap shot of the transcriptional changes in *S. commune* paired against *T. viride*, they indicate strong modulation of the molecular machinery of *S. commune*. Expression patterns of laccase, chitin synthase, neutral trehalase, polyubiquitin, HSP 70, an MAPK and MAPKK-encoding genes are strong indicators of a rapid physiological and molecular response by *S. commune* to the mycoparasitic attack of *T. viride* within 24 hours of mycelial contact. The proteins encoded by these genes are key players in stress detection and response in most eukaryotic systems. Up-regulation of chitin synthase gene is an indication of direct response to cell wall damage, while increase in polyubiquitin gene points to the implication of the protein product to protein quality control, a most likely response to cell wall, protein synthetic and oxidative stresses, as well as starvation. This is supported by the expression pattern of neutral trehalase gene, which regulates intracellular levels of trehalose that participates in the maintenance of protein conformations during various conditions of stress, a function also performed by HSP 70 of which the encoding gene was up-regulated after 24 hours of mycelial interaction with *T. viride*.

The up-regulation of genes that code for 18SrRNA and ribosomal protein S8 suggest possible increase in the activities of the protein synthetic machinery of the endoplasmic reticulum. The expression patterns of the beta chain of ATP synthase and cytochrome C_1 genes suggest possible disruption of mitochondrial function. Down-regulation of the E1 component of α -Oxoketoglutarate dehydrogenase is a strong indication that the Kreb's (tricarboxylic acid) cycle may have been blocked at the α -Oxoketoglutarate stage of the cycle, an indication of redox imbalance. Arrest of cell replication/growth could be the reason for the repression of α-tubulin gene. Perhaps the prevailing stress conditions during contact with the antagonist limits primary metabolic functions such as amino acid synthesis, accounting for the down-regulation of glutamine synthethase especially after 48 hours of mycelial interaction. Up-regulation of genes encoding two proteins putatively involved in signalling (an MAPKK and an MAPK) implicate mitogen activated protein kinases in the web of signalling activities responsible for the detection and activation of physiological response to the mycelia of *T. viride*. Down-regulation of GAPDH gene

suggests that glycolysis may have been down-regulated in the mycelia of *S. commune* interacting with *T. viride*.

It is noteworthy that most genes, which were up-regulated after 24 hours of mycelial contact, were down-regulated following 48 hours of interaction. Perhaps this is reflective of the severity and continuity of the stress to which *S. commune* mycelia were subjected following contact with *T. viride*. Progressive cell wall lyses and subsequent disintegration of *S. commune* mycelia suggest a gradual loss of viability in the confronted mycelial of *S.commune* especially in the contact zone.

6.4.1 Up-regulation of laccase gene

Pronounced laccase secretion is a predominant response by most basidiomycetes during inter-specific combative interactions (Griffith *et al*., 1994a, b & c; Rayner *et al*., 1994; Score *et al*., 1997; Boddy, 2000; Crowe & Olsson, 2001; Baldrian, 2004; Gregorio *et al*.,, 2006; Peiris *et al*., 2007; Peiris, 2009). Up-regulation of laccase gene confirms that increased levels of laccase protein and activity in cultures of *S. commune* confronted by *T. viride* (Chapter 5; sections 5.3.2.4 & 5.3.3) was a result of *de novo* protein synthesis. Rayner *et al* (1997) concluded that elevated levels of laccases during combative interactions in fungi allows the interacting species to convert free radicals and hydrophobic metabolites released into the contact zone to polymers, thereby sealing hyphal boundaries. On the other hand, Crowe and Olsson (2001) ascribed this phenomenon to the activation of the heat shock and calcium influx pathways in response to compromised cell integrity as laccase-derived products may play homeostatic roles in polymerizing and rendering the cell wall less permeable or by the detoxification of antifungal compounds released by either or both species.

 It is unlikely that that a single factor was responsible for the up-regulation of laccase in the mycelia of *S. commune* confronted by *T. viride*. Compromised cell integrity is most likely one of the factors that elicited laccase up-regulation in this study, considering that *T. viride* lysed *S. commune* cell wall within 48 hours of hyphal contact on agar. In addition, GC/MS and biochemical assays showed rise in levels of phenolic compounds in

the interaction zone. Phenolic compounds can be potentially toxic to fungi at elevated concentrations and are substrates for laccases and other phenol oxidases. Furthermore, possible switch to secondary metabolic phase of growth could be part of the web of events that triggered laccase up-regulations in *S. commune*. Following the sealing-off of the mycelia front, particularly by *S. commune* soon after contact with *T. viride*, nutrient supply would be disrupted which most likely arrests mycelial biogenesis, hence initiating secondary metabolism (Rayner *et al*., 1994). Secondary metabolism is associated with increased secretion of phenolic compounds, some of which are for defence/attack purposes or a response to diminishing mycelial growth (Peiris *et al*., 2007; Lardner *et al*., 2006). Oxidative burst, as in plant-fungus interactions, is a major characteristic of combative interactions between fungi (Iakovlev *et al*., 2004; Galhaup *et al*., 2002; Rayner *et al*., 1994; Rayner, 1997; Chi *et al*., 2006) and reactive oxygen species (ROS) harness laccase-driven oxidation of phenolic compounds (Thurston, 1994; Mayer and Staples, 2002). Thus, laccase oxidations might contribute to the scavenging of toxic ROS that accompany mycelial confrontations. Addition of isopropanol and ethanol were shown to significantly increase laccase secretion in *Rhizoctonia solani* (Crowe & Olsson, 2001). These alcohols are lipophilic and therefore destabilize membranes and proteins in a manner similar to heat shock (Crowe & Olsson, 2001; Piper, 1995; Panaretou & Piper, 1992). Alcohol-based lipophilic damage could be likened to oxidative damage such as lipid peroxidation (chapter 5; section 4.3.1) which may also account for laccase up-regulation in *S. commune*. The links between the induction of secondary metabolism (increased phenolic synthesis), cell wall damage (compromised cellular integrity), oxidative stress, up-regulation of laccase and possible up-regulation heat shock pathway (below) are presented in figure 6.4.

6.4.2 Up-regulation of HSP 70 gene

Although aggregation of cellular components has been shown to occur during cell wall damage (Krysan, 2009), it has also been demonstrated during combative interactions not involving a mycoparasitic fungus with the capability to secrete a battery of lytic enzymes. For instance, Ikediegwu (1976) reported the formation of dense streak-like cytoplasmic materials in paired hyphae of *Peniophora gigantea* and *Heterobasidion annosum* neither

of which have demonstrated cell wall lytic properties. These dense streak-like cytoplasmic materials are similar to the cellular aggregates shown in chapter 2 (section 2.3.2). While cell wall damage may have played a role in the formation of these aggregates in the areas of inter-specific hyphal contact, it is very possible that these aggregates mimic response to heat shock, which permanently damages intracellular and cell wall-bound macromolecules (Prescott *et al*., 1999). It is very likely that oxidative stress (lipid peroxidation) is implicated in the formation of these aggregates in *S. commune* paired against *T. viride* considering that ROS have a similar damaging effect on macromolecules (Takemoto *et al*., 2007). This perhaps accounts for the rapid up-regulation of HSP 70 within the first day of mycelial contact. Under conditions of heat shock or other stress conditions that mimic heat damage, HSPs are up-regulated because of their chaperone function, which assists in the maintenance of protein conformation under stress thereby preventing the formation of toxic protein aggregates (Prescott *et al*., 1999). HSP 70 proteins also contribute the sequestration of damaged, denatured or improperly folded proteins during conditions of stress (Panaretou & Piper, 1992; Craig & Gross, 1991; Linquist & Craig, 1988). HSP 70 appears to be among the first line of molecular defences recruited by *S. commune* in response to the oxidative onslaught arising from hyphal contact with *T. viride*.

6.4.3 Down-regulation of cytochrome C_1 and repression of mitochondrial ATP synthase (beta chain) genes

Although both Cytochrome C_1 and the beta chain of mitochondrial ATP synthase involved in electron transport and ATP synthesis in the mitochondrion, were down-regulated in self- and *T. viride*-paired cultures of *S. commune*, cell wall lysis and cellular aggregation were observed only in the latter. It is likely that damage to the mitochondrial membrane, which would affect proton gradient hence, electron transport in the mitochondrial matrix following cellular aggregation may have occurred in the mycelia confronted by *T. viride*, thereby leading to electron leakage, redox imbalance and oxidative stress. This may have contributed to intracellular oxidative insult leading to the activation of heat shock response and laccase up-regulation. It is possible that multiple electron transport and ATP synthetic mechanisms exist in *S. commune*, since ATP levels

remained comparatively high in the stressed mycelia as in the self-paired cultures (Chapter 4; section 4.3.3), despite down-regulation of cytochrome C_1, and the beta chain mitochondrial ATP synthase genes in both sets of cultures. Similarly, cellular aggregation may have affected the vacuoles as well, thereby limiting vacuolar functions. This perhaps contributed to the down-regulation of vacuolar ATP synthase gene.
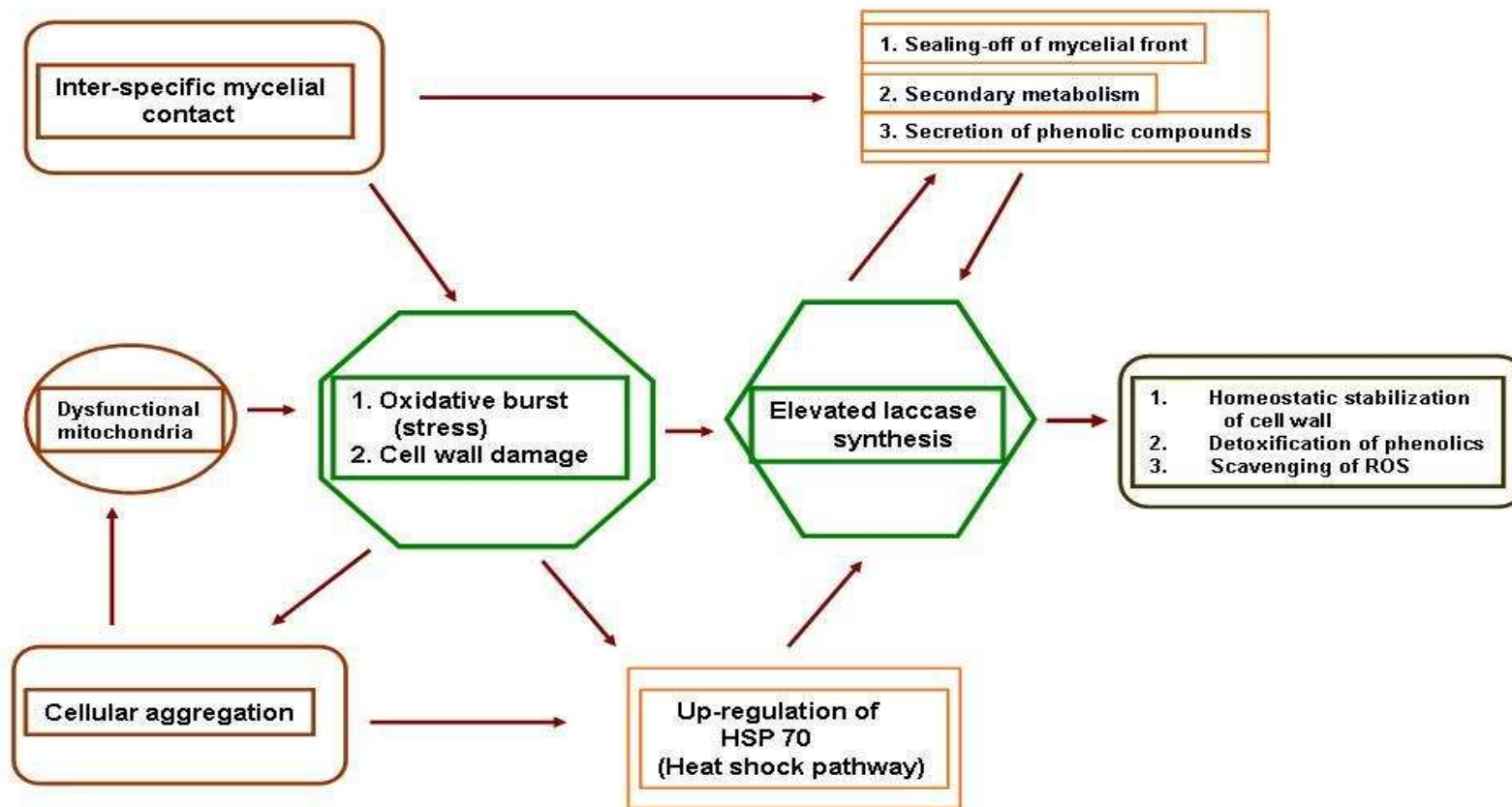
Figure 6.4 Hypothetical scheme of events leading to laccase up-regulation and the physiological roles of laccase in *S. commune* following mycelial confrontation with *T. viride*.

6.4.4 Up-regulation of genes that code for an MAPKK, an MAPK chitin synthase, and acyl carrier protein and down-regulation of α-tubulin

Serine/threonine protein kinases (mitogen-activated protein kinases-MAPKs) form the core of fungal cell wall integrity pathway. This cascade detects and responds to cell wall perturbation by physical or chemical agents in the environment (Nobel *et al*., 2002; Jung and Levin, 1999; Duran and Nombela, 2004; Cid *et al*., 1995) as well as to oxidative stress in fungi (Fernandez *et al*., 1998; Arana *et al*., 2005). Both stresses may have elicited the up-regulation of both MAPKK and MAPK genes. The main function of protein kinases during cell wall stress is to activate/promote the expression of key proteins, which directly participate in the biosynthesis of cell wall components (Duran & Nombela, 2004; Nobel *et al*., 2000; Ketela *et al*., 1999). An example is chitin synthase, responsible for the synthesis of chitin, which constitutes the foundation of the fungal cell wall architecture (Duran & Nombela, 2004). This perhaps explains the up-regulation of chitin synthase gene in *S. commune* after 24 hours of mycelial conflict.

Up-regulation of acyl carrier protein (ACP) gene might as well be one of mechanisms targeted towards synthesis and incorporation of polymers into the cell wall in response to the activities of cell wall lytic enzymes. ACPs are key components of the fatty acid synthesis pathway (Prescott *et al*., 1999). It has been demonstrated that they are involved in the synthesis of phospholipids (Magnuson *et al*., 1993) and cell wall polymers in bacteria (Heaton and Neuhaus, 1994). Perhaps ACP is involved in the construction of cell wall polymers or in the strengthening of cell membrane structural integrity, considering that after cell wall lysis, the cell membrane is the only protective envelope around the cell. Similarly, the up-regulation of chitin synthase could be interpreted as a direct response to cell wall lysis, given that chitin is the major source of rigidity to the fungal cell wall (Gooday, 1995). α-tubulin is a part of the cytoskeleton involved mainly in cytokinesis (Heath, 1995). α-tubulin gene was repressed 24 hours post interspecific contact. This could mean a complete termination of nuclear division and arrest of cell growth in *S. commune* mycelia interacting with *T. viride*. Considering the stress imposed on *S. commune* following interspecific contact, cell replication is an unlikely process to be retained during mycelial conflict, accounting for the repression of this gene compared

to self-paired and unpaired cultures. Iakovlev *et al.* (2004) also reported the repression of a gene, which encodes a fimbrin protein, involved in the polarization of actin cytoskeleton during inter-specific combat between *Heterobasidion annosum* and *Physisporinus sanguinolentus*.
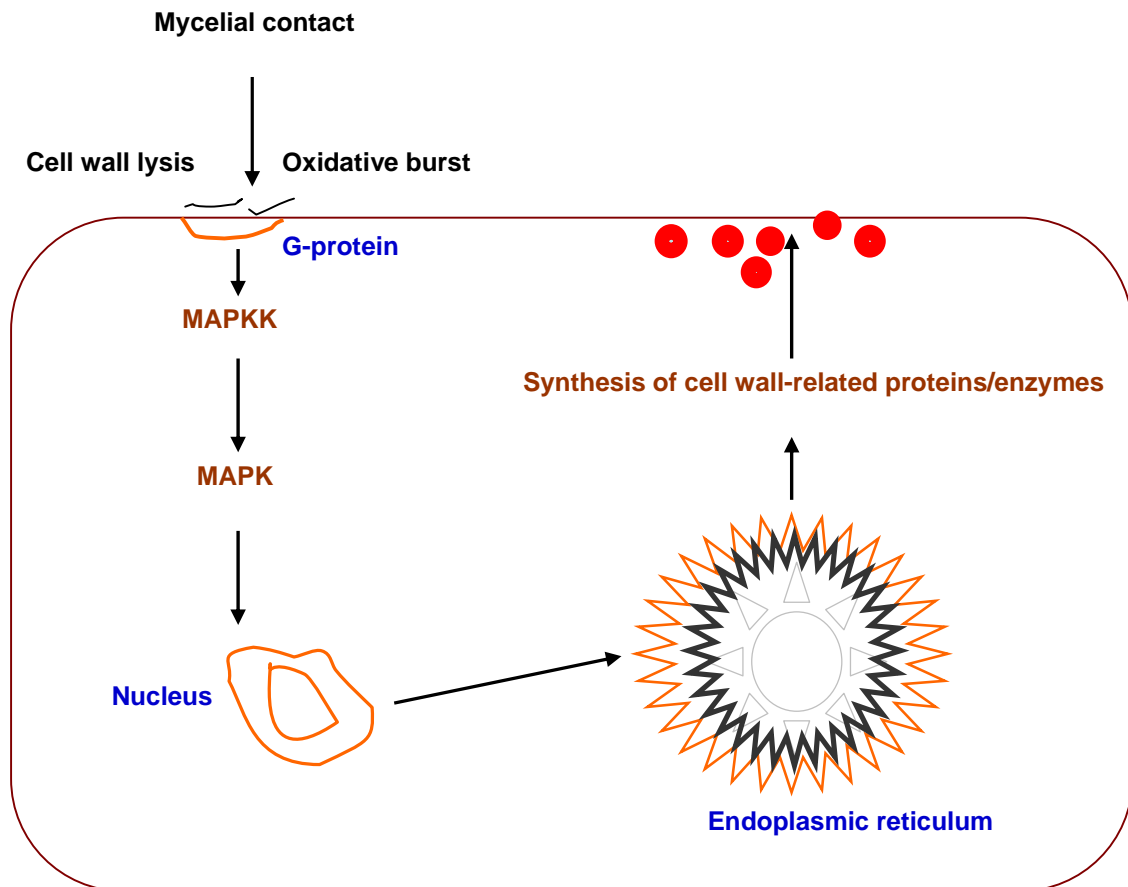
Figure 6.5 Hypothetical sketch depicting possible implication of the MAPK cascade in the activation of cell wall integrity pathway leading to the synthesis of cell wall proteins and enzymes involved in the construction of cell wall polymers.

6.4.5 Repression of the gene encoding the E1 component of α –ketoglutarate dehydrogenase, and down-regulation of GAPDH and pyruvate decarboxylase genes

The gene for the E1 component of α –ketoglutarate dehydrogenase (AKDH) was repressed after 24 hours of contact, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate decarboxylase (PD) genes were down-regulated and repressed

respectively after 48 hours of mycelial contact with *T. viride*. AKDH is a vital metabolic enzyme, which generates NADH in the TCA cycle and a precursor for amino acid synthesis (Singh *et al.*, 2007). However, it functions as a regulatory point in the tricarboxylic acid (TCA) cycle, being sensitive to NADH/NAD$^+$ ratio (redox state) (Van Laere, 1995). Its sharp repression is indicative of an unfavourable redox state and unbalanced AMP/ATP ratio (Van Laere, 1995) which very often leads to the activation the γ-aminobutyric acid (GABA) bypass under oxidative insult (Coleman *et al.*, 2001; Panagiotou *et al.*, 2005). GABA synthesis generates succinate, which is fed back into the TCA cycle, bypassing key NADH-producing steps of the TCA cycle (Juhnke *et al.*, 1996; Singh *et al.*, 2007). GC-MS analyses and enzyme assay showed increased amounts of GABA and Succinic semialdehyde dehydrogenase (SSADH) activity respectively (Chapters 3; section 3.3.2 and Chapter 5; section 5.3.2.6). Taken together, it can be argued that the cascade of events following mycelial contact between *S. commune* and *T. viride* perhaps creates, an unbalanced redox state, altered sequence of electron transport and ATP synthesis, all which can induce oxidative stress consequently leading to the transcriptional down-regulation of AKDH.

GABA shunt is perhaps one of the pathways employed by *S. commune* to balance NADH/NAD ratio, in response to physiological stress. Increased activity of G6PDH, the committed enzyme of the pentose phosphate pathway (PPP), a major source of NADPH described in chapter 3 further supports this assumption. PD is associated with fermentation, catalyzing the conversion of pyruvate to acetaldehyde and carbon dioxide (Prescott *et al.*, 1999). This process competes with the TCA cycle especially under conditions of limiting oxygen supply (Panagiotou *et al.*, 2005). Repression of PD gene 48 hours after mycelial contact means that most of the pyruvate generated from glucose metabolism is perhaps channelled towards the TCA cycle most likely to boost ATP generation. The TCA cycle has an ATP turn over of 15 ATP molecules per pyruvate (Prescott *et al.*, 1999).

Indication that the PPP might be active in *S. commune* during mycelial confrontation suggests that the glycolytic pathway might have been down-regulated in favour of a less

energy expensive pathway (PPP) which favours the generation of antioxidant NADPH. Oxidative stress has been shown to reduce glucose and nitrogen uptake in yeast by possibly oxidizing enzymes involved in glucose and nitrogen metabolism (Shenton & Grant, 2003). Li *et al* (2008) demonstrated that oxidative stress has a similar effect on *Aspergillus niger* B1-D glyceraldehyde-3-phosphate dehydrogenase (GAPDH) thereby reducing glucose uptake. Although they observed decreased uptake of nitrogen, activities of enzymes involved in ammonia assimilation remained unchanged. This possibly accounts for the down-regulation of GAPDH and glutamine synthethase suggesting that this response might be transcriptionally regulated in the mycelia of *S. commune* interacting with *T. viride*. Proteomic studies (chapter 7; sections 7.3.1 & 7.3.3-tables 7.3 & 7.4) strongly support this assumption, given that all the identified glycolytic and amino acid synthetic proteins were down-regulated.

6.4.6 Up-regulation of genes that encode ribosomal proteins, neutral trehalase and polyubiquitin

The relationship between cell wall damage and increased protein synthesis is discussed in details in chapter 7 (section 7.4.1). Up-regulation of ribosomal protein S8 and 18SrRNA genes after mycelial contact especially 18SrRNA gene strongly support the conclusion that cell wall lysis may have promoted protein synthesis in *S. commune* particularly proteins associated with cell wall repair and remodelling (Krysan. 2009; Richie *et al*., 2009). Furthermore, up-regulation of polyubiquitin gene makes case for the activation of restorative mechanisms such as degradation of carbonylated proteins (caused by oxidative stress) and toxic aggregates of misfolded proteins stemming from increased synthetic flux through the protein synthetic machinery (endoplasmic reticulum stress) (Panaretou & Piper, 1992; Krysan, 2009; Dukan *et al*., 2000). Hence, both oxidative and protein synthetic stresses (Chapter 7; section 7.4.1) may have elicited the transcriptional activation of polyubiquitin. In addition, in the light of the described stress conditions to which the mycelia of *S. commune* were subjected after contact with *T. viride*, selective degradation of expression factors for genes encoding housekeeping proteins not required under severe stress and idle enzymes (Nystrom, 2005) is a likely additional role of polyubiquitination proteins during this interaction in *S. commune*.

Accumulation of trehalose in stressed mycelia is an established stress-alleviating mechanism in fungi (Fernández *et al*., 1998; Oćon *et al*., 2007). Trehalose build up has been demonstrated in fungi subjected to oxidative (Crowe *et al*., 1987; Fernández *et al*., 1998), chemical (Chang *et al*., 1989), heat (Enjalbert *et al*., 2003) and osmotic (Döhlemann *et al*., 2006) stresses. Trehalose has been shown to stabilize proteins under conditions of stress allowing them to maintain their native conformation (Singer and Linquist, 1998; Eroglu *et al*., 2000). However, trehalose interferes with protein refolding explaining the paradoxical up-regulation of trehalose synthesizing enzymes during stress and trehalose hydrolysing enzymes upon stress recovery (Fernández *et al*., 1998; Oćon *et al*., 2007). This scenario fits almost perfectly with the expression pattern observed for neutral trehalase (the key enzyme associated with trehalose hydrolysis during stress conditions or stress recovery) in this study. *S. commune* neutral trehalase gene was strongly down-regulated within 24 hours of mycelial contact, most likely to allow trehalose build up following antagonism from *T. viride*. However, it was up-regulated after 48 hours of mycelial interaction between both species, possibly to allow the refolding of key proteins required under the unfavourable conditions of growth at this stage of mycelial confrontation.

6.4.7 Down-regulation of plasma membrane proton-transporting ATPase (H$^+$ ATPase)

As mentioned earlier, heat and ethanol stresses permeabilise membranes thereby perturbing transmembrane ion gradients required for pH regulation, potassium balance and nutrient uptake involving proton-transporting ATPases (Panaretou & Piper, 1992; Kobayashi *et al*., 1986; Serrano *et al*., 1986). Transmembrane gradient is susceptible to dissipation by different stresses potentially causing a toxic drop in intracellular pH, thereby stimulating proton-transporting ATPases, which promote proton efflux (Panaretou & Piper, 1992; Coote *et al*., 1991). This helps to counteract internal acidification (Panaretou & Piper, 1992; Coote *et al*., 1991). Despite its important role in stress response, Panaretou & Piper (1992) observed reductions in the levels of proton-transporting ATPase and an increase in HSP 30 in yeast subjected to heat stress, as well

as in cells in stationary phase. However, the authors had earlier reported that despite reductions in the levels of proton-transporting ATPase, the gene encoding the ATPase in *Saccharomyces cerevisiae* was still being transcribed. They suggested that perhaps cells under severe stress were incapable of synthesizing ATPase. In the present study, proton-transporting ATPase was repressed in *S. commune* soon after contact with *T. viride*. Inexplicably, it appears ATPase is transcriptionally shut down in *S. commune* confronted by *T. viride*. It is worth pointing out that oxidative shock following hyphal contact might mimic heat or ethanol stress, which would potentially wipe out transmembrane ion gradient thereby affecting the activity of proton-transporting ATPase. The reason why the gene that encodes this protein was repressed remains unclear, perhaps the protein is regulated at the translational/protein level.

6.5 Summary and conclusions

The results presented in this chapter point to the possible prevalence of cell wall, oxidative and protein synthetic stresses in the mycelia of *S. commune* paired against *T. viride*. For instance, genes that encode GAPDH, 18SrRNA, and ribosomal protein S8 often used as controls in RT-PCR assays were either strongly down- or up-regulated. Whereas actin remained relatively same across the test and control samples, there is a strong possibility that this was mainly due to its role in the maintenance of membrane integrity especially following cell wall damage. The patterns of gene expression suggest induction of metabolic shifts in *S. commune* leading to the down-regulation of NADH-producing and energy expensive processes such as glycolytic reactions and the reaction catalysed by AKDH. This, most likely promotes higher production of antioxidant NADPH via alternative pathways. Up-regulation of genes that encode proteins involved in protein synthesis, HSP 70 and polyubiquitin suggest an increase in the protein synthetic flux, with possible corresponding increase in the activities of counteracting mechanisms that check protein misfolding or recycling of aberrant proteins. Laccase up-regulation implicates compromised cell integrity, oxidative burst and increased synthesis of phenolic compounds as part of the mesh of biochemical and molecular events arising from mycelial contact with *T. viride*. Part of the restorative measure recruited by *S.*

commune most likely includes promoting the synthesis of cell wall polymers and regulation of trehalose metabolism to meet intracellular requirements under stress.

CHAPTER 7

Protein profiles of interacting cultures of *S. commune* and *T. viride*

7.1 Introduction

Enhanced secretion of extracellular enzymes such as phenol-oxidases, changes in morphological forms and pigmentation, are some of the core characteristics of inter-specific interactions in filamentous fungi (Boddy, 2000; Rayner *et al*., 1994; Gregorio *et al*., 2006). The processes that drive these changes, during mycelial confrontation are mediated predominantly by proteins, which are far more directly related to functions/phenotypes than genes, making proteomics a more reliable analytical tool for studying the physiological and biochemical processes underlying various responses and cellular states in living systems (Bhadauria *et al*., 2007; Horie *et al*., 2008). Proteomics is the qualitative and quantitative functional analyses of the total protein expressed by a genome, cell, tissue or organism (Rohrbough *et al*., 2007; Bhadauria *et al*., 2007). Recent advances in protein sequencing coupled with completion of the sequencing of over 25 fungal genomes make proteomics more amenable to studies of complex fungal systems (Bhadauria *et al*., 2007).

However, difficulties such as the inherent complexity of extracting membrane proteins, poor detection of low-abundance proteins and lack of an amplification tool such as PCR (in the case of DNA and RNA) limit the application of proteomics (Rohrbough *et al*., 2007). Despite these limitations early indications from the application of proteomics to fungi have led to suggestions that proteomics is a powerful tool for studying changes of protein expression profiles in response to various stresses (Teixeira *et al*., 2005; Qin *et al*., 2006). A key step in fungal proteomic studies is the protein extraction, given the very robust nature of fungal cell wall (Bhadauria *et al*., 2007). Several methods have been developed and the method adopted may vary from species to species. However, it should reproducibly capture all the different proteins present in the proteome with little or no contamination (Bhadauria *et al*., 2007).

Following protein extraction, protein samples are profiled by two-dimensional (2D) gel electrophoresis. 2D gel electrophoresis generates a global picture of the expression patterns of a proteome under varying conditions (Bhadauria *et al*., 2007). 2D gel electrophoresis separates proteins firstly, by their isoelectric point (pI) during isoelectric

focusing (IEF: first dimension) and secondly based on molecular mass during sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE: second dimension) (O'Farrell *et al.*, 1997). The resulting profile allows the detection of proteins of interest, which are identified by mass spectrometry. The low number of annotated or sequenced fungal genomes limits the possibility of identifying proteins from un-sequenced organisms. However, search tools such as basic local alignment search tool (BLAST) can be useful for the elucidation of possible functions, location and modification of proteins of interest by determining their homology with available sequences on the database.

In a three-way interaction study, Marra *et al.* (2006) used 2D gel electrophoresis to investigate the proteomes of an antagonistic strain of *Trichoderderma atroviride* (mycoparasite), interacting with bean plants and two phytopathogens; *Botrytis cinerea* and *Rhizoctonia solani* in an effort to understand the induction of systemic resistance in bean plants by *T. atroviride* against these two fungal pathogens. Horie *et al.* (2008) used a combination of 1D and 2D gel electrophoresis to study the proteomes of the fruiting bodies of two mushrooms; *Sparassis crispa* and *Hericium erinaceum*, while Qin *et al.* (2006) demonstrated the crucial role of antioxidant proteins and hydrolytic enzymes in the pathogenicity of *Penicillium expansum* using 2D protein gel electrophoresis. Extacellular proteins involved in cellulose, lignin and hemicellulose degradation were found to increase in abundance in *Phanerochaete chrysosporium* grown on different substrates using 2D gel electrophoresis (Sato *et al.*, 2007).

Whereas genomic tools have been used (Iakovlev *et al.*, 2004; Adomas *et al.*, 2006) in the study of inter-specific fungal interactions, 2D gel electrophoresis has been sparingly employed to this area of research. Earlier work in our laboratory (Peiris, 2009) showed up-regulation of proteins involved in homeostatic response to oxidative stress and heat shock proteins in paired cultures of *Stereum hirsutum* and its competitors, *Coprinus micaceus* and *Coprinus disseminatus*. However, several questions remain to be answered as to whether all fungi recruit the same or similar mechanisms to inter-specific confrontation. Furthermore, the up-regulation of antioxidant proteins requires further investigation, if their roles in mycelial interactions are to be better understood. Data

presented in this chapter represent an attempt to use the 2D gel electrophoresis to further our knowledge of fungal interactions especially the response of a wood degrader (*S. commune*) to a highly antagonistic strain of a biocontrol fungus (*T. viride*).

7.2 MATERIALS AND METHODS

A schematic summary of the stages involved in this aspect of the work, from interaction assays, to protein extraction, SDS-PAGE, and protein identification are in figure 7.1.
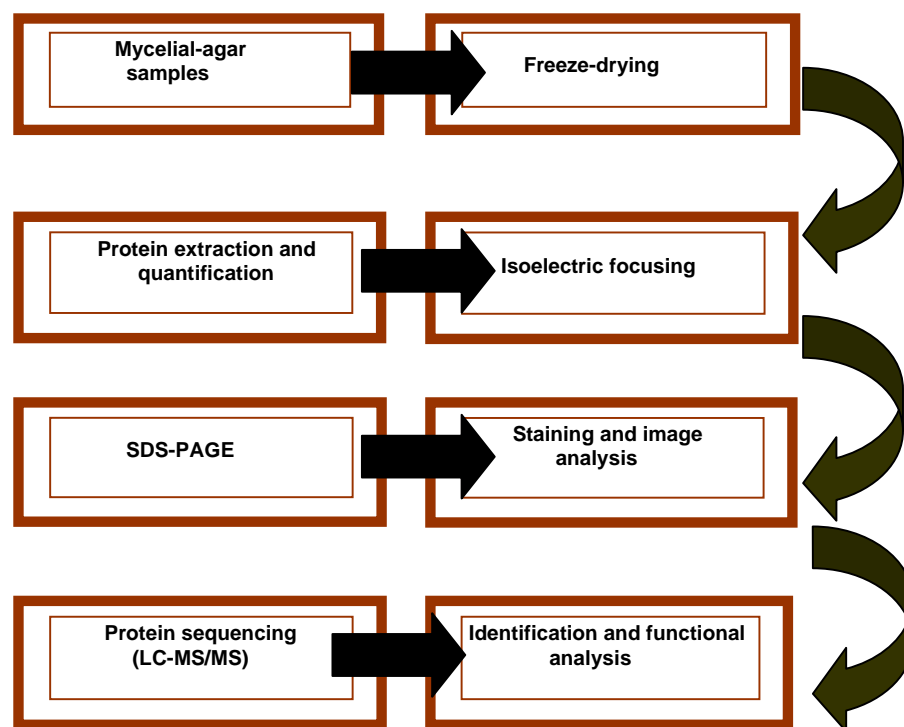
Figure 7.1 **Schematic representation of the experimental procedures employed for profiling the proteomes of *S. commune* and *T. viride* from their self-paired and interacting cultures.**

7.2.1 Agar plate interaction assay and sample preparation

Mycelial confrontation assays were set up as described previously (Chapter 2; section 2.2.1). Cultures were grown in replicates of 15 for each batch of protein extraction from which five uniform plates, based on patterns of pigmentation and barrage formation were selected for protein extraction. Samples for protein extraction were taken after 48 hour of

mycelial contact as described in Chapter 3 (section 3.2.1). However, for protein extraction, mycelial samples were cut off together with agar to capture of both intra- and extracellular proteins. Samples were frozen at -80°C overnight before freeze-drying.

7.2.2 Protein extraction

Samples for protein extraction were taken away from the interaction line, in the domain occupied by each fungus as described in Chapter 2 (section 2.2.1). Protein extraction was carried out according to the method described by Horie *et al*. (2008). 250 mg of freeze-dried samples for each extraction batch were ground to powder with a mortar and pestle with the addition of small amounts of acid-washed sand (Sigma-Aldrich). The powder was dissolved in 8 ml of Tris-buffered (0.1 M; pH 8.8) phenol (80 g/l) and 8 ml 0f extraction buffer (0.9 M sucrose, 0.1 M Tris (pH 8.8), 10 mM EDTA and 0.4 % (v/v) 2-mercaptoethanol in sterile distilled water). The resulting mixture was allowed to vortex for 30 mins and then centrifuged at 15,000 rpm for 30 mins, after which the top phenol layer was removed and spun again for 15 mins at 15000 rpm. 2 ml of Tris-buffered phenol and 2 ml of extraction buffer were added to the lower aqueous residue after the first centrifugation.

The mixture was vortexed for 1 minute and spun at 15, 000 rpm for 15 mins. The resulting top phenol layer was collected and the two phenol layers were pooled after which 5 volumes (5X) of the precipitating solution was added (0.1 ammonium acetate in 100 % methanol), mixed by vortexing for 15 seconds and incubated at -20°C overnight. The suspension was centrifuged at 15000 rpm for 20 mins at 4°C to pellet proteins. The pellet was washed twice with 1 ml of 0.1 M ammonium acetate in 100 % methanol, once with 80 % ice-cold acetone and once with 70 % ethanol. The pellet was dried for 10 mins at 37°C and dissolved in 200 µL rehydration buffer (6 M urea, 2 M thiourea, 0.5 % CHAPS (w/v) and 0.5 % (v/v) Pharmalyte (pH 3-10; Amersham) excluding dithiothreitol (DTT). Samples were stored at -80°C until use.

7.2.3 Protein quantification

The protein content of each extract was performed according to the method described by Bradford (1976). 10 µL of each sample was diluted by making it up to 200 µL (in the

same buffer) and mixed with 800 µL of Bradford reagent (Sigma). Absorbance was read at 595 nm in Novaspec II spectrophotometer (Amersham, UK). Standard curves were prepared using Bovine serum albumin (BSA) as standard over a concentration range of 0.1 to 1.5 mg/ml. Each sample was also run on 1-D gels after quantification.

7.2.4 Rehydration of IPG strip

Following the optimisation of the amounts of protein required for loading the strips for each fungus, volumes containing 200 µg and 150 µg of protein for *S. commune* and *T. viride* respectively, were made up to 130 µL with rehydration buffer (6 M urea, 2 M thiourea, o.5 % (w/v) CHAPS and 0.5 % (v/v) Pharmalyte (pH 3-10; Amersham) and 0.4 % (w/v) DTT). The mixture was loaded onto a swelling tray (Amersham, UK) and allowed to absorb onto an 18 cm Immobiline gel strip, pH 3-10 non-linear (GE healthcare) overnight. The strip was covered with mineral oil (Sigma-Aldrich, UK) during overnight rehydration. All experiments were carried out in replicates of 5, using proteins samples from different cultures/extraction batches.

7.2.5 Isoelectric focusing

Following rehydration and protein absorption on gel strip, strips were gently blotted dry on tissue paper. Isoelectric focusing was carried out on a Multiphor II (Pharmacia BIOTECH) electrophoresis unit. Temperatures during isoelectric focusing were maintained at 17.2°C using Amersham Multiptemp III thermostatic circulator cooling unit. Strips were placed gel-side-up onto the dry strip aligner tray and electrode strips were placed on the anode and cathode ends of the IPG strip after soaking them in distilled water. A small amount of mineral oil was poured onto the cooling ceramic cooling block and the aligner tray was placed over the block. Electrodes were connected to the IEF electrophoresis unit and the IPG strips were focused for 5 hours for *S. commune* samples, and 4 hours 10 mins for *T. viride* samples, (table 7.1) using an Amersham EPS 3501 power pack. Preliminary runs showed that proteins from both fungi required different durations to be focused along the strip.

Table 7.1 IEF programmes for *S. commune* and *T. viride* protein samples

IEF steps	Voltage	Duration
Step 1	150 V (for proteins from both fungi)	30 mins (SCSC/SCTR)
		5 mins (TRTR/TRSC)
Step 2	300 V (for proteins from both fungi)	30 mins (SCSC/SCTR)
		25 mins (TRTR/TRSC)
Step 3	600 V (SCSC/SCTR)	30 mins (for proteins from both fungi)
	800 V (TRTR/TRSC)	
Step 4	3500 V (for proteins from both fungi)	3 hrs 30 mins (SCSC/SCTR)
		3 hrs 10 mins (TRTR/TRSC)

SCSC/SCTR: Time or voltage applied to *S. commune* protein samples from self-paired cultures and mycelia interacting with *T. viride*; TRTR/TRSC: Time or voltage applied to *T. viride* protein samples from self-paired cultures or mycelia paired against *S. commune*.

7.2.6 Equilibration of IPG strips

After focussing, strips were equilibrated to reduce disulfide bonds and unfolding of proteins with a view to promoting the transfer of proteins from the IPG strips onto SDS-PAGE. Equilibration was performed for 15 mins by rocking the strips gently in 5 ml of 50 mM Tris-HCl, pH 8.8; 6 M urea; 30 % (v/v) glycerol; 2 % (w/v) sodium dodecyl sulphate (SDS); and 1 % (w/v) DTT and for another 15 mins in 5 ml of 50 mM Tris-HCl, pH 8.8; 30 % (v/v) glycerol; 2 % (w/v) SDS; and 2.5 % (w/v) iodoacetamide.

7.2.7 SDS-PAGE

The second dimension was run by placing the focussed and equilibrated strips onto a 1 mm thick 8 x 7 cm 12 % (w/v) SDS-PAGE resolving gel. SDS-PAGE resolving gels were prepared by mixing water (4.9 ml), 1.5 M Tris base (pH 8.8; 2.6 ml), 40 % Bis acrylamide (3 ml), 10 % (w/v) ammonium persulfate (100 µL), N,N,N',N'-tetramethylethylenediamine (TEMED) (10 µL), and 10 % (w/v) SDS (100 µL) and

allowed to set within the gel plates. Molecular weight markers were loaded onto a small piece of filter paper placed on the low pH end of the strip. IPG strips were sealed onto the SDS-PAGE resolving gel by pouring sealing buffer (0.5 % (w/v) agarose, 25 mM Tris, 192 mM glycine, 0.1 % SDS and trace amounts of bromophenol blue) over the strips. This ensured that the strip was intact with the resolving gel during electrophoresis (protein separation). Electrophoresis was carried out using a Biorad protein electrophoresis unit at 80 V for 30 mins and at 110 V until the dye (bromophenol blue) front reached the base of the gel (see Appendix III for the composition of gel running buffer). Altogether, 20 gels were run with different biological samples for each of test and control cultures for both fungi respectively, out of which 5 gels (most uniform) were used subsequently for image analysis.

7.2.8 Protein visualization

After separation, proteins were fixed and stained for visualization by microwave-heating gels (with intermittent cooling) in 10 % (v/v) acetic acid containing 0.1 % coomassie brilliant blue (Sigma-Aldrich, UK) until protein spots became visible. Afterwards, gels were destained with 10 % (w/v) acetic acid allowing removal of excess coomassie blue. Gels were preserved in 10 % (w/v) acetic acid.

7.2.9 Image analysis

Stained gels were scanned with GS800 densitometer (Biorad, UK) and the resulting images were acquired with Quantity II software. Each image was converted to a TIFF file and analysed using Progenesis PG240 Samespot software (Nonlinear Dynamics, UK). Five gels from each of test and control batches were uploaded onto the Progenesis programme for anlysis. The amount of protein in each spot was determined as a function of spot intensity, and images (spots) from self-paired samples were compared against those from interacting cultures. Fold changes in protein spots between samples from self-paired and co-cultures were calculated and ranked based on p-value from one way ANOVA.

7.2.10 Sequencing (LC-MS/MS) and identification

Spots, which differed significantly in volume between self-paired and interacting cultures, were excised and dispatched to the University of York Mass Spectrometry Facility (UK) where they were sequenced by LC-MS/MS and identified by sequence matching on the NCBI database. Both fungi are not sequenced; hence, protein identification was achieved by peptide fragmentation and mass fingerprinting followed by sequence alignment with available proteins on the database. Where matches resulted in hypothetical proteins, further confirmation of protein function was achieved by matching the sequence obtained from York University against proteins on the NCBI database to identify the possible presence of conserved domains with other known proteins. This was carried out with the NCBI conserved domain software (www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi).

7.3 Results

Results of protein analyses are presented in two sections;

7.3.1 – Comparative patterns of expressed proteins from the mycelia of *S. commune* paired against self and against *T. viride*.

7.3.2 – Comparative patterns of expressed proteins from the mycelia of *T. viride* paired against self and against *S. commune*.

7.3.1 Comparative patterns of expressed proteins from the mycelia of *S. commune* paired against self and against *T. viride*.

Figures 7.2 and 7.3 show remarkable differences in the patterns of protein spots between self-paired mycelia of *S. commune*, in comparison to its mycelia interacting with *T. viride*. About 682 spots were resolved between pH 3-10, and 284 spots were significantly different between self-paired cultures of *S. commune* and cultures of interacting with *T. viride* (fold difference ≥ 2; $p < 0.05$). Out of these 284 spots, 121 were up-regulated while 163 were down-regulated. 38 spots (21 up-regulated and 17 down-regulated) were selected for sequencing by LC-MS/MS based on their level of up-/down-regulation (fold

increase/decrease), and the position of the protein spot relative to other spots. Only spots that could be excised without the risk of cross mixing between spots were selected for sequencing. A plot of normalised spot volumes of selected differentially expressed protein spots are shown in figures 7.4 and 7.5. Since the *S. commune* sequence is not yet available on the database, MS/MS spectra were submitted for search on the database in the Mascot search engine.

The best matches for up- and down-regulated *S. commune* protein spots are presented in tables 7.2 and 7.3 respectively. Identified up-regulated proteins are involved in a wide range of physiological and biochemical functions including, transcriptional/translational regulation (SC6, SC39 and SC93), amino acid metabolism (SC100), cellular metabolism and energy generation (SC28, SC114 and SC150), and protein synthesis and assembly and signalling (SC2, SC40, SC55, SC60, SC87, SC195 and SC394). Other up-regulated spots, SC8, SC20, SC41 and SC75 were found to be involved in homeostatic responses to cell wall damage and oxidative stresses. Proteins associated with glycolysis/gluconeogenesis (SC83, SC345, SC438, and SC465) and trehalose metabolism (SC313) were found to be down-regulated. Other down-regulated proteins include spots SC302 and SC444 (heat shock proteins); SC34, SC350, and SC355 (phosphate metabolism and signalling); SC62 and SC3369 (transcriptional regulation and protein synthesis); and SC69 (amino acid metabolism).
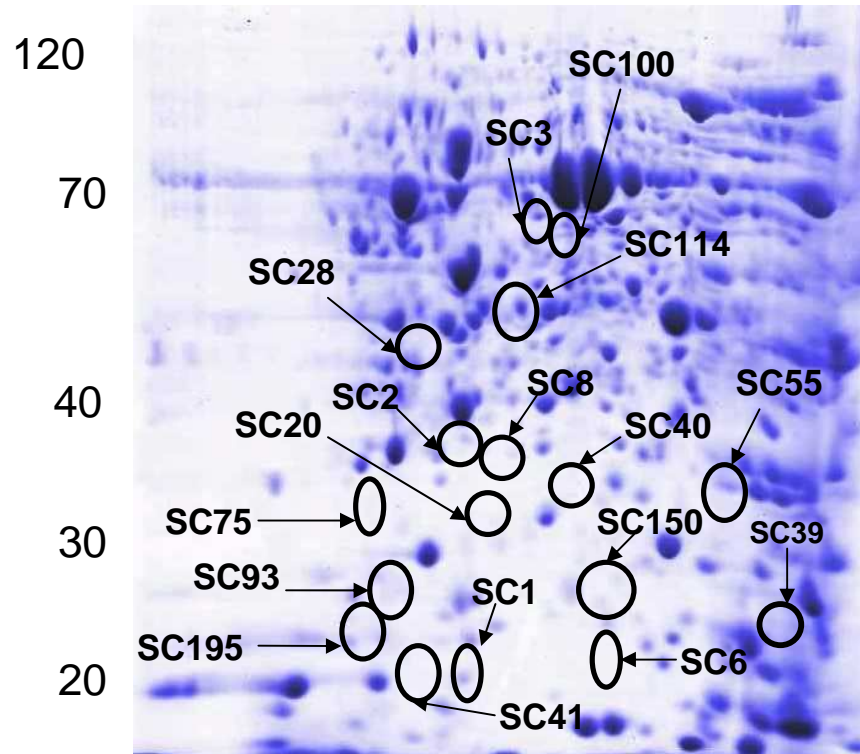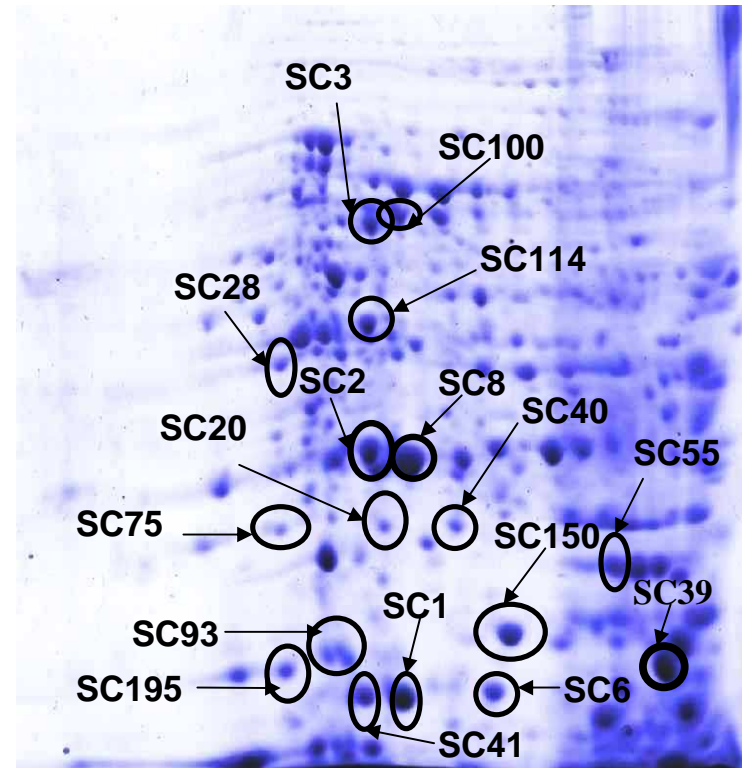
Figure 7.2 Representative gels of protein extracts from the mycelia of *S. commune* **paired with self, and mycelia paired with** *T. viride* **after 48 hours of contact. 200 μg of protein was separated by 2D gel electrophoresis in 12% sds-polyacrilamide gel after IEF. Circled spots represent some of the protein spots (only the sequenced spots out of the up-regulated proteins) up-regulated in mycelia of** *S. commune* **confronted by** *T. viride***, compared to self-interacting cultures. 5 gels were selected for image and statistical analyses out of 20 gels, for each of test and control batches.**
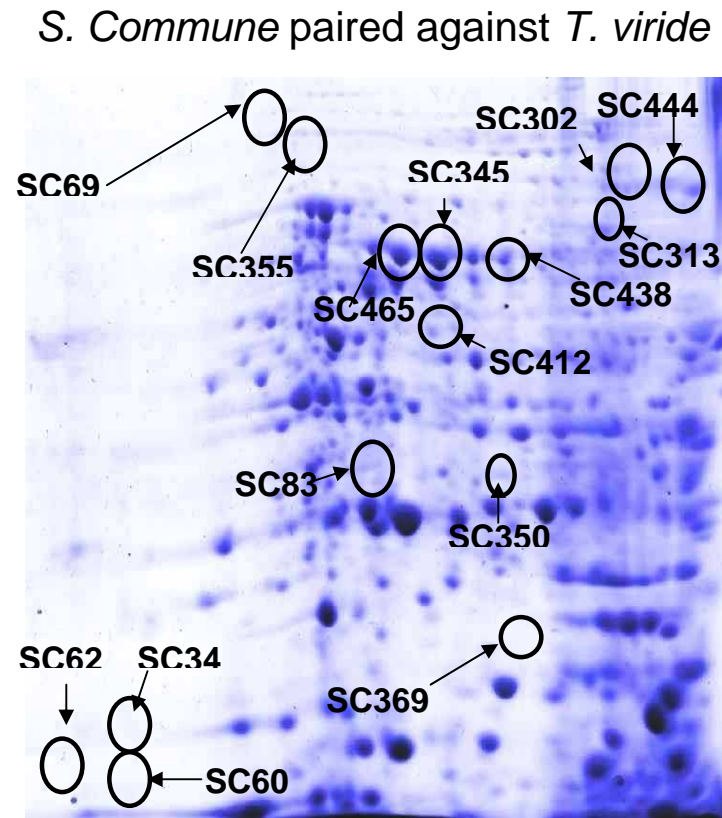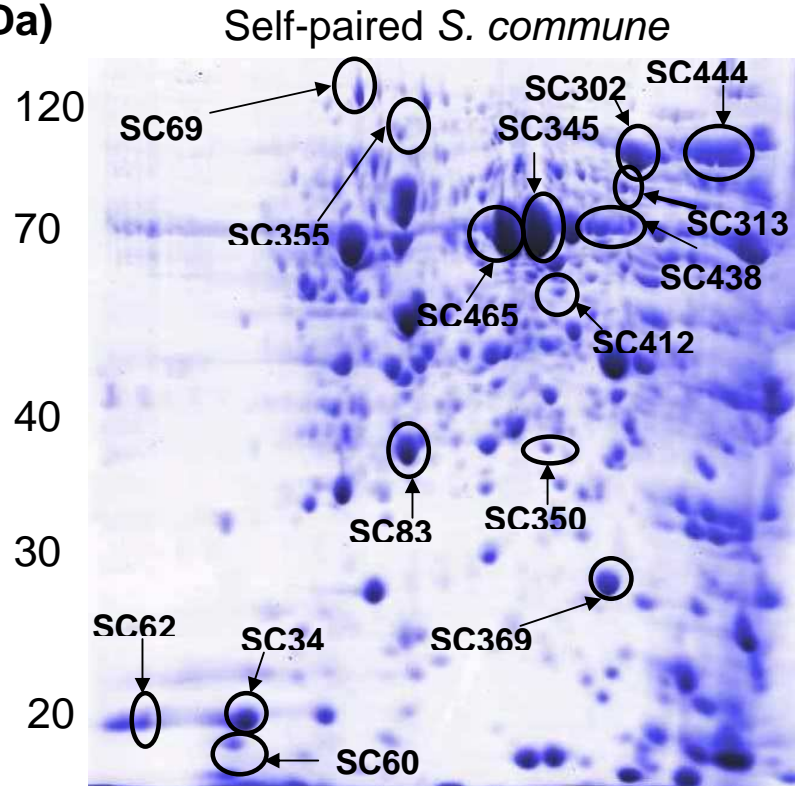
Figure 7.3 Representative gels of protein extracts (200 μg) from *S. commune* mycelia paired with self, and mycelia paired with *T. viride* for 48 hours, separated by 2D gel electrophoresis in 12% gels following IEF. Circled spots represent some of the protein spots (only the sequenced spots) down-regulated in the mycelia of *S. commune* confronted by *T. viride*, compared to self-interacting cultures.
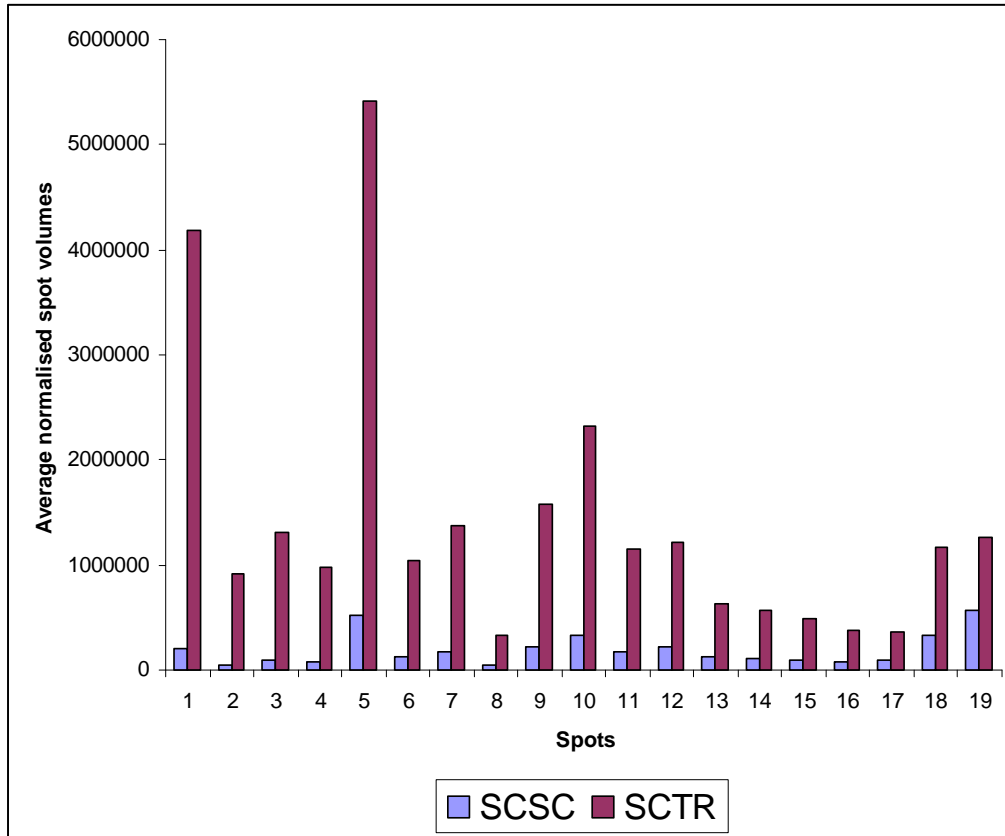
Figure 7.4 Comparative average normalised spot volumes of up-regulated spots: 1-19 on the x-axis represent protein spots as follows; SC1, SC2, SC3, SC6, SC8, SC20, SC28, SC39, SC40, SC41, SC55, SC75, SC87, SC93, SC100, SC114, SC150, SC195, SC394 respectively. SCSC: self-paired *S. commune***; SCTR:** *S. commune* **confronted by** *T. viride***.** (Progenesis software generates the average of the spot volumes of the test and control gels as a single value respectively).
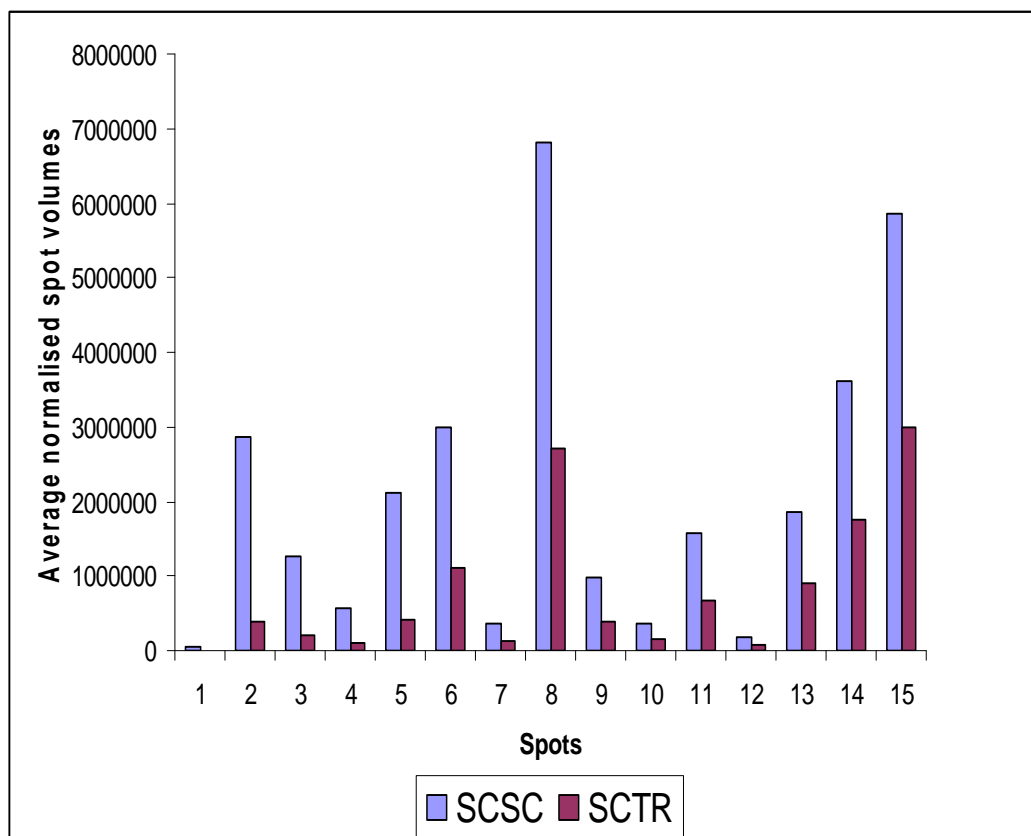
Figure 7.5 Comparative average normalised spot volumes of down-regulated proteins: 1-15 on the x-axis represent the following protein spots; SC33, SC34, SC62, SC69, SC302, SC313, SC345, SC350, SC355, SC369, SC412, SC438, SC444, SC465 respectively. Key to SCSC & SCTR, as in figure 7.4. (Progenesis software generates the average of the spot volumes of the test and control gels as a single value respectively).

Table 7.2 Identities of proteins from the mycelia of *S. commune* up-regulated in response to *T. viride* after 48 hours of contact, compared to self-paired cultures of *S. commune*.

Protein spot	NCBI Ac. No.	P-value (ANOVA)	Fold Increase	Protein identity	Mascot Score	Sequence Coverage (%)	Predicted mol. wt. (kDa)	Predicted pI	No of matched peptides*
SC1	gi|189191156	1.679E-10	19.8	Hypothetical protein	36	1	121	5.7	1
SC2	gi|169779443	4.242E-6	17.5	Hypothetical protein	84	15	18	5.9	2
SC6	gi|38567258	1.938E-5	11.5	transcription factor BTF3a	79	15	16	7.6	2
SC8	gi|209446855	2.294E-11	10.3	phenylalanine ammonia-lyase	61	1	80	5.7	1
SC20	gi|34014958	6.405E-7	8.2	HEX1-Woronin body component	66	8	25	6.6	2
SC28	gi|169865690	2.739E-5	7.8	Hypothetical protein	202	9	35	9.6	3
SC39	gi|68563739	2.211E-4	7.2	RNA polymerase II, 2nd largest subunit	39	4	26	5.5	1
SC40	gi|241889506	1.816E-9	7.6	Hypothetical protein	36	1	85	9.6	1
SC41	gi|187932846	7.598E-13	7.1	glycosyl transferase	29	2	49	5.4	1
SC55	gi|409567	0.006	6.4	Translational initiation factor 5a	104	7	18	5.4	1
SC75	gi|225708148	1.011E-10	5.6	Probable E3 ubiquitin-protein ligase	42	4	34	5.9	1
SC87	gi|409567	1.689E-7	5.2	Translational initiation factor 5a	108	7	18	5.4	1
SC93	gi|190346518	2.986E-10	5.2	Hypothetical protein	39	1	13	7	1
SC100	gi|51247011	3.418E-6	5.0	Branched alpha-ketoacid dehydrogenase	42	3	43	9.1	1
SC114	gi|169865690	1.083E-10	4.7	Hypothetical protein	262	15	35	9.6	4
SC150	gi|145610189	0.003	4.0	Mitochondrial ATP synthase-D chain	75	5	21	10.1	1
SC195	gi|13925731	5.017E-10	3.6	Cyclophilin A-1	84	8	19	9.6	1
SC394	gi|409567	0.002	2.2	Translational initiation factor 5a	104	7	18	5.4	1

*Matched peptide sequences are shown in appendix V. Analyses were carried out in replicates of 5.

Table 7.3 Identities of proteins from the mycelia of *S. commune* down-regulated in response to *T. viride* following 48 hours of mycelial contact, compared to self-paired cultures of *S. commune*.

Protein spot	NCBI Ac. No.	*P*-value (ANOVA)	Fold Increase	Protein identity	Mascot Score	Sequence Coverage (%)	Predicted mol. wt. (kDa)	Predicted pI	No of matched peptides*
SC33	gi|28317	5.166E-8	7.6	Hypothetical protein	72	3	60	5.0	2
SC34	gi|15614217	2.250E-8	7.6	nucleoside diphosphate kinase	54	8	17	5.0	1
SC62	gi|169856964	8.196E-10	6.1	40S ribosomal protein S14	136	34	16	11.2	4
SC69	gi|170084893	1.966E-9	5.9	Hypothetical protein	228	7	85	6.2	2
SC83	gi|46201420	4.284E-10	5.3	Triosephosphate isomerase	79	5	26	6.6	1
SC302	gi|170094730	4.957E-5	2.7	Hypothetical protein	241	8	67	5.4	4
SC313	gi|85816030	0.001	2.6	Trehalose phosphorylase	295	10	82	5.9	6
SC345	gi|169845435	1.494E-9	2.5	Hypothetical protein	237	8	47	5.7	3
SC350	gi|159113158	1.166E-7	2.5	Serine/Threonine protein kinase	24	5	21	4.4	1
SC355	gi|115375356	6.383E-7	2.5	Methyl accepting protein	27	1	64	5.4	1
SC369	gi|166031245	0.012	2.4	Hypothetical protein	33	2	49	5.6	1
SC412	gi|187939647	0.013	2.2	Hypothetical protein	26	5	31	5.4	1
SC438	gi|238599828	5.231E-4	2.1	Hypothetical protein	232	13	34	5.0	3
SC444	gi|50288201	6.816E-8	2.0	Hypothetical protein	301	7	70	4.8	4
SC465	gi|169845435	1.707E-7	2.0	Hypothetical protein	243	8	47	5.7	3

*Matched peptide sequences are shown in appendix V. Analyses were carried out in replicates of 5.

Matches for 6 up-regulated proteins were hypothetical proteins, while 9 of the down-regulated proteins produced best homologies with hypothetical proteins. All hypothetical proteins were searched for conserved domains on the NCBI database to determine possible functions of the spots matching these proteins. Table 7.4 shows the possible functions of the spots that produced the best homologies with hypothetical proteins. The match for SC2, one of the up-regulated proteins contains a conserved domain for cyclophilins, which are mainly involved in protein synthesis and assembly, stress response and cellular signalling. SC28 and SC114 carry conserved domains for glyoxysomal/mitochondrial malate dehydrogenase, one of the enzymes of the tricarboxylic acid cycle. SC40 carries a domain for proteins involved in protein export during protein synthesis while SC93 bears a helicase domain.

Matches for the down-regulated proteins carried domains for functions including; protein folding (SC302 and SC444), Glycolysis/gluconeogenesis (SC345 and SC465), transcriptional regulation (SC369) and amino acid metabolism (SC69).

Table 7.4 Conserved domains and possible functions of *S. commune* proteins up-/down-regulated during interactions with *T. viride*.

Spot	Organism	Conserved domain(s)	Possible function(s)
Up-regulated proteins			
SC1	*Pyrenophora tritici-repentis*	None	Unknown function
SC2	*Aspergillus oryzae*	cd01926, PRK10791, PRK10791,	cyclophilin type peptidyl-prolyl cis-trans isomerase : signalling, stress response ; protein synthesis and assembly
SC28	*Coprinopsis cinerea*	cd01337, PTZ00325, PTZ00325	Glyoxysomal and mitochondrial malate dehydrogenase
SC40	*Gemella haemolysans*	cl11439, SecD_SecF, PRK13024, PRK13024	bifunctional preprotein translocase subunit; protein export membrane proteins
SC93	*Pichia guilliermondii*	cd00046, cd00079, cd00162, pfam00176	DEAD-box helicases superfamily; signal transduction; transcription regulation
SC114	*Coprinopsis cinerea*	cd01337, PTZ00325, PTZ00325	Glyoxysomal and mitochondrial malate dehydrogenase
Down-regulated proteins			
SC33	*Homo sapiens*	pfam00038	Intermediate filament protein
SC69	*Laccaria bicolor*	PRK05222, PRK05222, cd03311,	5-methyltetrahydropteroyltriglutamate—homocysteine S-methyltransferase
SC302	*Laccaria bicolor*	PTZ00009, PTZ00009	heat shock 70 kDa protein
SC345	*Coprinopsis cinerea*	cd03313, PTZ00081, PTZ00081	Enolase: Glycolysis/gluconeogenesis

7.3.2 Comparative patterns of expressed proteins from the mycelia of *T. viride* paired against self and against *S. commune*.

Following 2D analyses of proteins from *T. viride*, 522 spots were resolved between pH 3-10, out of which 30 were differentially expressed (fold difference $\geq$ 2; $p < 0.05$), (figure 7.6). 23 spots were up-regulated while 7 were down-regulated (figure 7.6). Out of the differentially expressed proteins spots, 16 were selected for sequencing and identification (12 up-regulated spots and 4 down-regulated spots). Two spots, TR9 and TR87, were not identified. The best homologies for spots TR22, TR29, TR44, TR59, TR80, and TR115 were hypothetical proteins. Further search on the NCBI database showed that they carry conserved domains for translational initiation factor, transcriptional factor, porin protein, translation elongation factor (GTPase) DNA binding protein and F-actin capping protein respectively (table 7.7). The up-regulated proteins were mainly involved in cellular metabolism such as glycolysis, transcriptional and translational regulation, proteolysis (antagonism) and posttranscriptional processing (table 7.5). Spots TR47, TR59, TR103 and TR115 were down-regulated. TR47, TR59 and TR103 are involved in transcriptional regulation and protein synthesis while TR115 functions in the organisation of actin cytoskeleton (table 7.6).
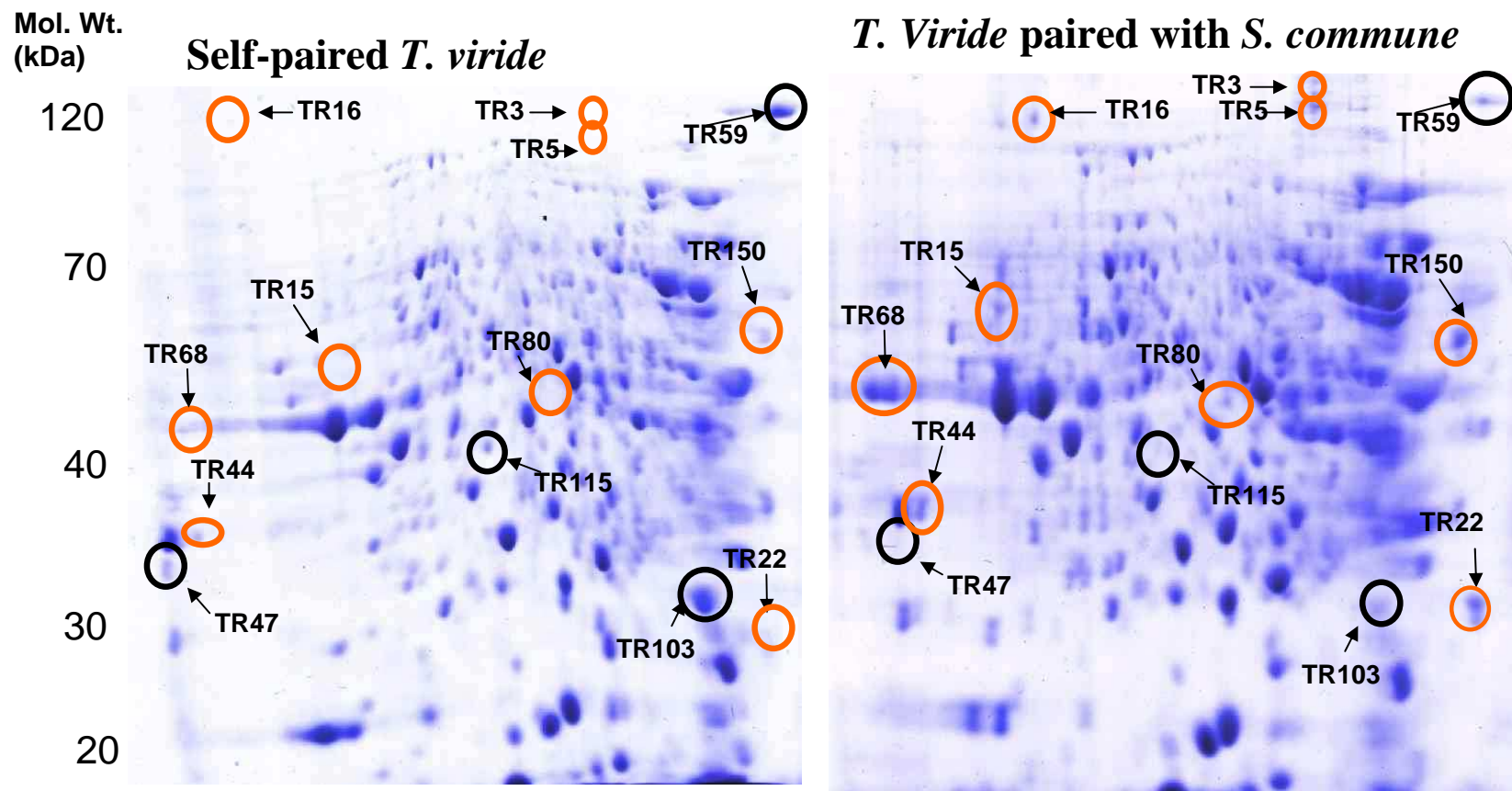
Figure 7.6 Representative gels of protein extracts from the mycelia of *T. viride* **paired with self, and mycelia paired with** *S. commune* **for 48 hours of mycelial contact. 150 μg of protein sample was separated by 2D gel electrophoresis in 12% gels after IEF. Spots circled in red represent some of the proteins, which were up-regulated, while spots circled in black represent some of the down-regulated proteins in the mycelia of** *T. viride* **paired against** *S. commune***, in comparison to self-interacting cultures. 5 gels were selected from test and control batches for image and statistical analyses out of 20 gels for each batch**.
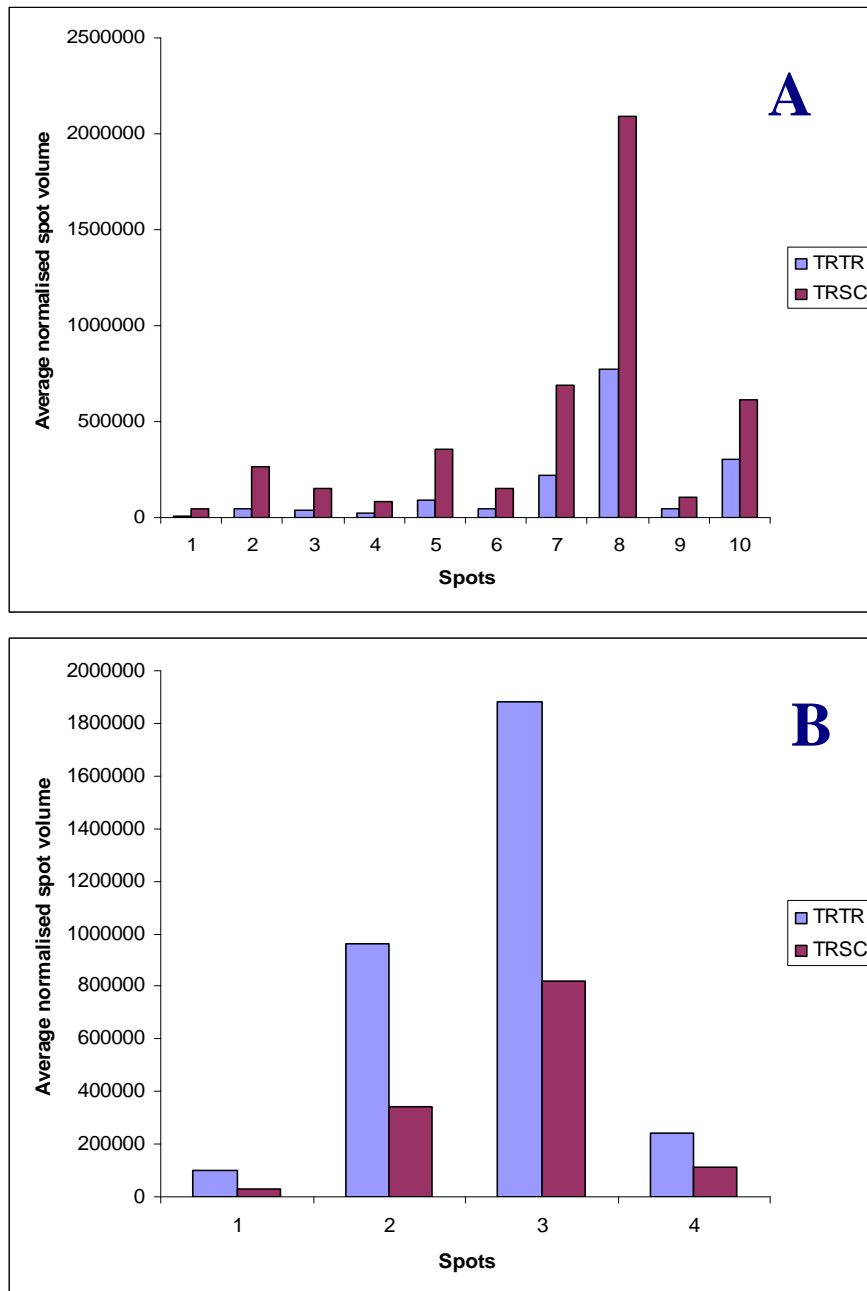
Figure 7.7 Comparative average normalised spot volumes of up-regulated (A) and down-regulated (B) proteins in *T. viride* paired against *S. commune*: A – 1-10 represent spots; TR3, TR5, TR15, TR16, TR22, TR29, TR44, TR68, TR80 and TR150 respectively. B – 1-4 represent spots; TR47, TR59, TR103, TR115 respectively. TRTR: Self-paired *T. viride*; TRSC: *T. viride* paired against *S. commune*. (Progenesis software generates the average of the spot volumes of the test and control gels as a single value respectively).

Table 7.5 Identities of proteins from the mycelia of *T. viride*, up-regulated after 48 hours in response to *S. commune*, compared to self-paired cultures of *T. viride*.

Protein spot	NCBI Ac. No.	*P*-value (ANOVA)	Fold Increase	Protein identity	Mascot Score	Sequence Coverage (%)	Predicted mol. wt. (kDa)	Predicted pI	No of matched peptides*
TR3	gi|108800163	1.887E-6	6.0	short-chain dehydrogenase/ reductase	17	8	30	6.1	1
TR5	gi|115401284	4.097E-14	5.8	Mitochondrial ATP synthase, beta chain	91	7	54	5.3	1
TR15	gi|172052427	1.916E-9	4.2	glyceraldehyde-3-phosphate dehydrogenase	83	10	36	7.7	3
TR16	gi|172052427	0.002	4.2	glyceraldehyde-3-phosphate dehydrogenase	130	6	36	7.7	2
TR22	gi|164658544	1.366E-10	3.9	Hypothetical protein	106	16	13	4.8	2
TR29	gi|27803030	4.548E-10	3.7	Hypothetical protein	92	5	55	10.1	2
TR44	gi|46136835	2.104E-4	3.2	Hypothetical protein	204	15	37	9.9	3
TR68	gi|172052427	2.213E-10	2.7	glyceraldehyde-3-phosphate dehydrogenase	356	17	36	7.7	5
TR80	gi|256727588	3.041E-11	2.6	Hypothetical protein	158	3	93	6.2	2
TR150	gi|47027997	0.003	2.0	Aspartyl protease	134	9	43	5.2	2

Table 7.6 Identities of proteins from the mycelia of *T. viride*, down-regulated in response to *S. commune* after 48 hours of contact, compared to self-paired cultures of *T. viride*.

| Protein spot	NCBI Ac. No.	*P*-value (ANOVA)	Fold Increase	Protein identity	Mascot Score	Sequence Coverage (%)	Predicted mol. wt. (kDa)	Predicted pI	No of matched peptides*
TR47	gi|84702107	9.263E-6	3.0	signal recognition particle-docking protein	37	2	48	4.4	1
TR59	gi|73960395	1.740E-12	2.8	Hypothetical protein	29	9	18	10.3	1
TR103	gi|409567	9.024E-12	2.3	Initiation factor 5a	96	7	18	5.4	1
TR115	gi|145608614	2.203E-9	2.2	Hypothetical protein	84	7	32	9.4	1

Table 7.7 Conserved domains and possible functions of *T. viride* proteins up-/down-regulated during interactions with *S. commune*.

Spot	Organism	Conserved domain(s)	Possible function(s)
Up-regulated proteins			
TR22	*Malassezia globosa*	COG0231, Efp, cd04468	Translation initiation/elongation factor 5A (eIF-5A)**:** Translation, ribosomal structure and biogenesis
TR29	*Podospora anserina*	cd00590	RNA binding protein**:** post-transcriptional gene expression processes - mRNA and rRNA processing, RNA export, and RNA stability
TR44	*Gibberella zeae*	cl03224, cd07306	Eukaryotic porin**:** forms channels in mitochondrial outer membrane; voltage-dependent anion channel (VDAC)
TR80	*Nectria haematococca*	COG0480,cd03700, cd01681,	Translation elongation factor(GTPase)**:** Translation, ribosomal structure and biogenesis, translocation of the peptidyl-tRNA
Down-regulated proteins			
TR59	*Canis familiaris*	cd00083	DNA-binding transcription factor
TR115	*Magnaporthe grisea*	pfam01267	F-actin capping protein alpha subunit

7.3.3 Summary of protein profile results

7.3.3.1 *S. commune* protein patterns

Figure 7.8 shows a summary of the functional categories of up-regulated and down-regulated proteins of *S. commune* paired against *T. viride*. 51% of the up-regulated proteins were proteins involved in transcription, translation, protein folding, protein transport and assemble. Examples include translational initiation factors, cyclophilin A-1, helicases and an ubiquitination-related protein. Proteins associated with cellular metabolism such as the Kreb's cycle and ATP generation made up 17% of the up-regulated proteins. Proteins that function in response to cellular injury and those associated with amino acid metabolism contributed 11% respectively to protein up-regulation, while 6% were involved in proteolysis.

Three down-regulated proteins, (spots SC345, SC438 and SC465) produced good matches with an enolase protein which catalyses the penultimate step in glycolysis as well as catalysing the reverse reaction during gluconeogenesis (Entelis *et al*., 2006). In total, 26% of the down-regulated proteins in *S. commune* function in the glycolytic pathway, making up the major group of down-regulated proteins. Heat shock proteins, particularly HSP 70 and proteins involved in signalling made up 13% of the down-regulated proteins, respectively. Proteins associated with transcription and translation constituted 13% of the down-regulated proteins, while proteins that function during amino acid synthesis and trehalose metabolism contributed 7% each to down-regulated proteins.
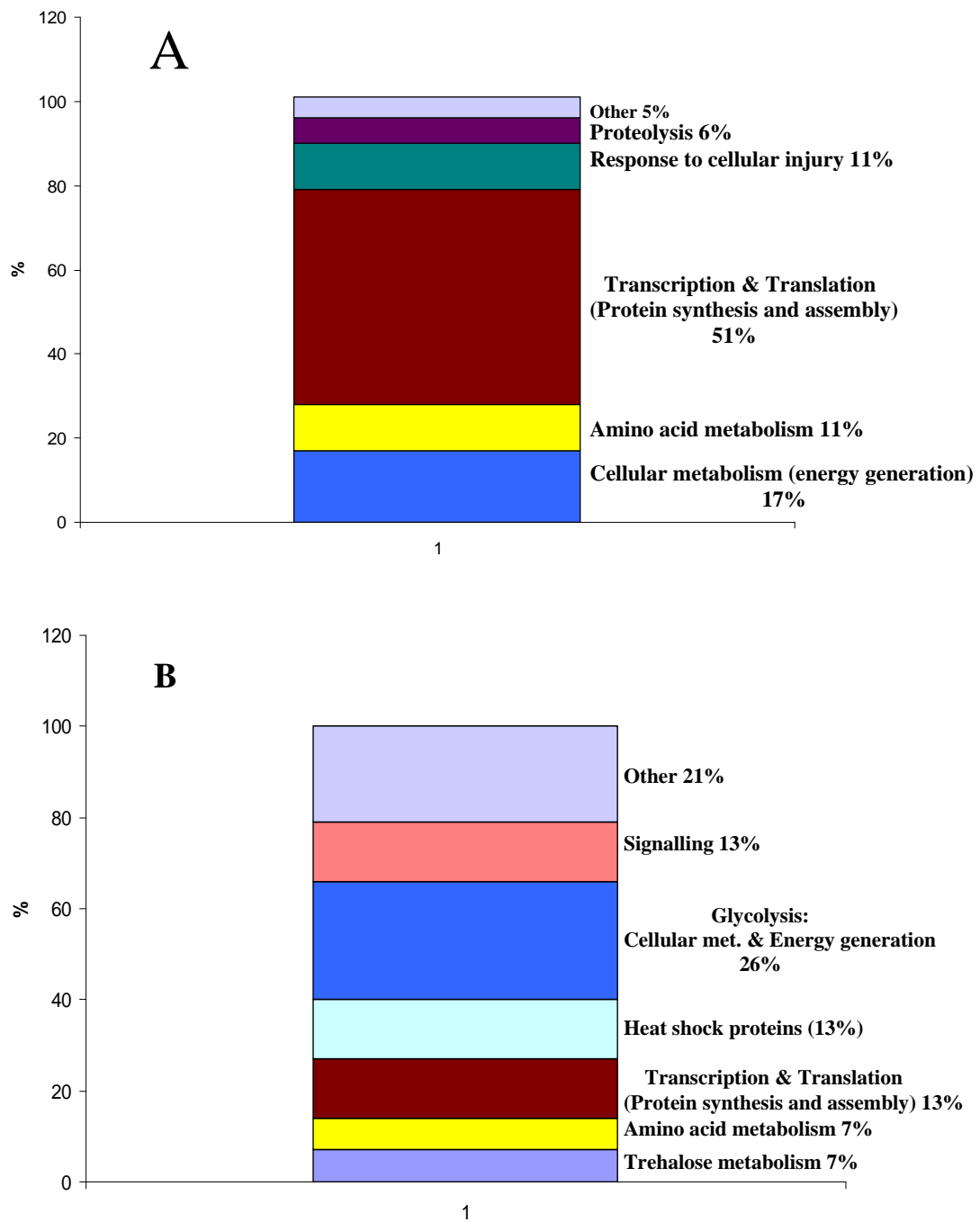
Figure 7.8 Functional grouping of *S. commune* proteins, which were up-regulated (A) or down-regulated (B) in agar plate interaction with *T. viride*.

7.3.3.2 *T. viride* protein patterns

Functional grouping of up-regulated and down-regulated proteins of *T. viride* paired against *S. commune* showed a less complex pattern compared to the patterns observed for *S. commune* (Fig 7.9). Mitochondrial voltage-dependent ion channel proteins, glycolytic and ATP synthesizing enzymes as well as, a short chain dehydrogenase/reductase (all of which are involved in cellular metabolism) were the most predominantly up-regulated proteins, constituting 60% of the spots that increased in volume. Translation initiation factors and RNA-binding proteins made up 30% of the up-regulated proteins, while the remaining 10% was made up by hydrolytic enzyme, an aspartyl protease. Four of the sequenced proteins were down-regulated, and two of them were translation-associated proteins. An F-actin capping protein involved in the organization of actin cytoskeleton and a signal recognition particle-docking protein which participates in protein transport during protein synthesis were down-regulated, each contributing 25% to the pool of down-regulated proteins in *T. viride*.
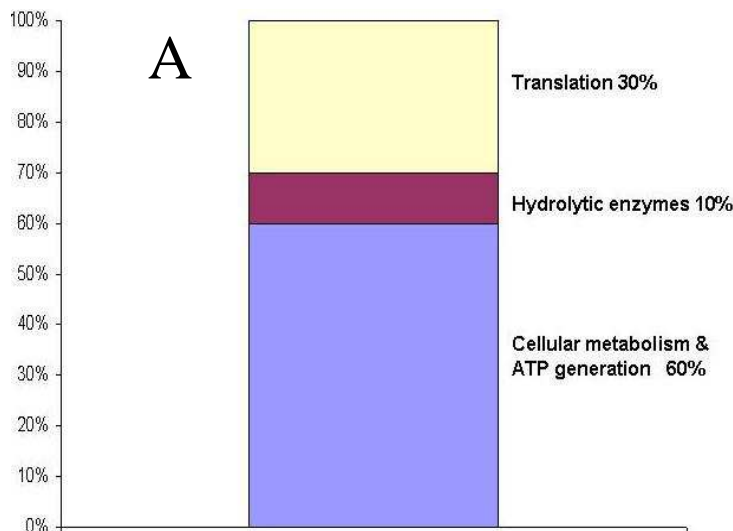
Figure 7.9 Functional grouping of *T. viride* proteins, which were up-regulated during agar plate interaction with *S. commune*.

7.4 DISCUSSION

7.4.1 Comparative analysis of patterns of protein expression in *S. commune* paired against self and against *T. viride*.

Advances in transcript identification and quantification have led to the development of a wide range of molecular tools vis-à-vis, serial analyses of gene expression (SAGE), total gene expression analyses (TOGA), reverse transcriptase-polymerase chain reaction (RT-PCR) etc, for gaining insight into gene expression levels and possibly protein abundance (Rohrbough *et al.*, 2007). However, with the discovery of post-transcriptional/translational modifications, which regulate the rate of protein synthesis and the half-life of proteins, it is now known that mRNA levels for a particular gene do not always correlate with its protein levels (Gygi *et al.*, 2000). Against this backdrop, 2-D protein gel electrophoresis despite some of its limitations is fast becoming a powerful tool for studying the patterns of protein expression and abundance. The rapidly increasing number of fungal genomes available on the NCBI and other databases makes this tool more amenable to analyses of fungal proteomes.

The results presented in this chapter are by no means a reflection of the global (complete) changes in the proteomes of both fungi studied during antagonistic interaction. This is because, the experiment was designed to selectively capture proteins between pH 3 and 10 (based on the isoelectric points of proteins). This therefore, excluded proteins with pI values outside this range. Furthermore, not all differentially expressed proteins were sequenced, because of the cost of protein sequencing. For instance, although laccase and manganese peroxidase had been shown to be up-regulated (Chapter 5) in the mycelia of *S. commune* confronted by *T. viride*, neither protein were identified from the 2D gel-based investigations. It could be that both proteins do not have pI values that allow them to be captured within the studied pH, or they were left out when spots were selected for sequencing.

Despite these limitations, the results in this chapter indicate major shifts in the proteomes of both *S. commune* and *T. viride* following 48 hours of mycelial combat. Most of the changes were observed in *S. commune*, with the up-regulation of proteins involved

mainly in transcriptional and translational regulation, protein synthesis, transport and assembly, response to cellular injury, oxidative stress and cellular metabolism. Proteins involved in glycolysis where mainly down-regulated in *S. commune* as well as those associated with heat shock response, and trehalose metabolism, in addition to some translation-related and amino acid synthetic proteins. Conversely, proteins that function in different cellular and energy-generating metabolic pathways such as glycolysis as well as in translation were up-regulated in *T. viride*. Other translation and transcription-associated proteins made up majority of the down-regulated proteins in *T. viride*.

7.4.1.2 Up-regulation of proteins involved in the protein synthetic machinery of the endoplasmic reticulum, and the unfolded protein response (UPR).

Mycelial confrontation between *S. commune* and *T. viride* induced increased expression of proteins associated with protein synthesis, transport and assembly, despite the immediate stress of cell wall lysis inflicted by the hydrolytic enzymes of *T. viride* (Chapter 2; figures 2.6-2.11) which may have compromised cell integrity. Spot SC195, which increased in volume 3.6 fold, produced strong homology with the cytosolic cyclophilin A. Similarly, spot SC2, up-regulated 17.5 fold, produced a strong match with a hypothetical protein that carries the cyclophilin A domain. Cyclophilins, a conserved group of proteins found in all organisms catalyze the ordinarily slow *cis↔trans* isomerisation of peptide bonds preceding proline residues; hence, they are also referred to as peptidyl-prolyl *cis-trans* isomerases (PPIases) (Viaud *et al.*, 2003; Schonbrunner *et al.*, 1991; Wang & Heitman, 2005). The peptidyl-prolyl *cis-trans* isomerase activity of cyclophilins is not only crucial for protein folding but also plays a key role in the assembly of multidoman proteins (Gothel *et al.*, 1999; Wang & Heitman, 2005). In addition, cyclophilins are multifunctional proteins strongly implicated in cellular processes such as response to cell wall damage, oxidative, disruption of ER homeostasis and temperature stresses, cell cycle regulation, calcium signalling, virulence and regulation of transcriptional repression (Viaud *et al.*, 2003: Richie *et al.*, 2009; Lu *et al.*, 1996; Sykes *et al.*, 1993; Arévalo-Rodriguez *et al.*, 2000)

A possible role of the up-regulated cyclophilin A proteins in the mycelia of *S. commune* would be to facilitate protein folding in the endoplasmic reticulum (ER) (molecular chaperone) via the unfolded protein response (UPR) pathway following cell wall damage. Krysan (2009) reported the activation of the UPR in yeast via the cell wall integrity (CWI) mitogen activated protein (MAP) kinase cascade in response to cell wall perturbation. Richie *et al* (2009) demonstrated a similar response in *Aspergillus fumigatus*. The UPR is an intracellular signalling conduit primarily activated by the accumulation of misfolded proteins in the eukaryotic ER (Krysan, 2009; Scrimale, *et al*., 2009). During protein synthesis, combination of ER-local chaperones and folding enzymes as well as post-translational modifications and disulfide bridge formation carry out the folding of nascent proteins (Helenius & Aebi, 2004; Richie *et al*., 2009). Under normal growth conditions, the ER folding capacity copes favourably with secretory demands (Richie *et al*., 2009).

However, under conditions of stress that elicit increased protein synthesis, the influx of unfolded nascent proteins outstrips the folding capacity of the ER. As a result, a rapid flux builds through the protein synthetic system leading to the accumulation of misfolded proteins in the ER; hence, the ER fails to cope with the secretory load (figure 7.10) (Schubert *et al*., 2000; Richie *et al*., 2009; Krysan, 2009; Scrimale, *et al*., 2009). Accumulation of misfolded proteins is highly deleterious to cell survival, causing the build up of toxic aggregates (Richie *et al*., 2009; Gasser *et al*., 2007). The following ER stress activates a series of homeostatic responses that constitute the UPR (Malhotra & Kaufman, 2007; Richie *et al*., 2009). The UPR is conserved in eukaryotes and upon activation stabilises the functioning of the ER by reducing the flow of proteins into the ER, accelerating protein transport out of the ER, promoting the expression of chaperones and foldases and by degrading recalcitrant improperly folded proteins (Malhotra & Kaufman, 2007; Richie *et al*., 2009; Ron & Walter, 2007) as depicted in figure 7.10.

Accumulation of misfolded proteins is detected by the ER upstream sensor, Ire1p which undergoes autophosphorylation prior to inducing the translation of Hac1p, the transcriptional regulator of the UPR (Krysan, 2009; Richie *et al*., 2009). Null mutation of

Ire1p in *Saccharomyces cerevisiae* resulted in phenotypes similar to mutations in genes involved in cell wall biosynthesis (hypersensitivity to cell wall-damaging drugs and cell wall lysing enzymes, decreased cell wall thickness and pronounced cellular aggregation) (Krysan, 2009). Disruption of Hac1p in *Aspergillus fumigatus* resulted in a mutant incapable of activating the UPR in response to ER stress, loss of cell viability at 45°C, impaired secretary protein capacity and increased sensitivity to cell wall and membrane-active drugs (Richie *et al.*, 2009). Taken together, it could be inferred that there is a strong relationship between cell wall and ER stresses, which triggers the adaptive UPR pathway (Figure 7.10). This is logical, in the sense that during cell wall stress, the delivery of improperly folded or defective proteins to the cell wall would be lethal; as a result, the cell increases its capability to correct the misfolding of proteins thereby decreasing the possibility of integrating defective proteins in the cell wall (Scrimale *et al.*, 2009; Krysan, 2009).

In addition, cell wall damage most likely leads to the up-regulation of a range of cell wall proteins, which might consequently increase the total synthetic flux through the protein synthetic machinery thereby placing extra stress on the ER (Krysan, 2009). In the light of these findings, the pronounced up-regulation of cyclophilins chiefly involved in the acceleration of protein folding, was very likely an adaptive response to cell wall lysis: the major damage inflicted on *S. commune* by *T. viride* during mycelial contact. Cyclophilins have been repeatedly implicated in environmental stress responses, and the fact that they are found in all organisms and in various cell compartments (cytosol, mitochondria, ER, and, in *Escherichia. coli*, periplasmic space) further underscores their importance to cell survival and adaptation (Viaud *et al.*, 2003; Schonbrunner *et al.*, 1991). Adomas *et al.* (2006) also reported strong up-regulation of a cyclophilins in the barrage zone during interspecific combat between *Phlebiopsis gigantea* and *Heterobasidion parviporum*. In addition, *S. commune* mycelia confronted by *T. viride* for 48 hours mimicked *S. cerevisiae* Ire1p null mutant (Krysan, 2009), as shown by micrsocopic observation to have undergone pronounced cellular aggregation within the contact zone (see Chapter 2; figures 2.5; 2.7-2.10). This similarity lends more credence to the correlation between *S. commune* cell wall lysis and possible induction of ER stress. Up-regulation of three

protein spots (SC55, SC87 & SC394) in *S. commune*, which bear very strong resemblance to the eukaryotic initiation factor (eIF-5A), further support the suggestion that *S. commune* may have activated increased protein synthesis in response to cell wall damage.

eIF-5A is highly conserved in eukaryotes and archaea, and has been shown to be involved in the initiation of protein synthesis (Merick, 1979; Chamot & Kuhlemeier, 1992; Saini *et al*., 2009). However, its precise role in the initiation of protein synthesis is not clearly understood. elF-5A is the only protein thus far known to contain hypusine, a distinctive modified amino acid (Park *et al*., 1984; Saini *et al*., 2009; Chamot & Kuhlemeier, 1992). Schnier *et al* (1991) demonstrated that hipusinated elF-5A is important for cell viability in yeast. Furthermore, eIF-5A genes in yeast have been shown to be differentially expressed in response to environmental stress (Schnier *et al*., 1991; Mehta *et al*., 1990). Exhaustion or inactivation of elF-5A in *S. cerevisiae* resulted in increased ribosomal transit time; however, addition of recombinant elF-5A from yeast expedited the rate of tripeptide synthesis *in vitro* (Saini *et al*., 2009).

The same authors also observed that inactivation of elF-5A mimicked eEF2 inhibitor, sordarin. eEF2 transports aminoacyltRNAs to the ribosome and enhances translocation (Preeti *et al*., 2009). Based on these findings, Saini *et al*. (2009) inferred that elF-5A might function jointly with eEF2 to support ribosomal translocation. Given the strong up-regulation observed for 3 proteins in *S. commune* identified as elF-5A, it is logical to deduce that *S. commune* was most likely under increased protein synthetic flux in response to its cell wall damage. This in part explains the levels observed for elF-5A. Nonetheless, this does not exclude the possibility that elF-5A proteins might perform other stress responsive functions during mycelial interactions, considering that they have been implicated in environmental stress response.

Part of the UPR restorative mechanisms involves degradation of irreparably misfolded proteins in the ER (Richie *et al*., 2009; Ron & Walter, 2007). This involves selective hydrolysis of aberrant proteins (Richie *et al*., 2009; Li & Ye, 2008; Staszczak, 2008).

Compared to the conventional lysosomal proteolytic pathway, the polyubiquitin pathway is highly selective and has been strongly implicated in stress response, recycling of oxidatively damaged or irreparably misfolded proteins and in metabolic adaptation where it selectively degrades transcriptional regulators (Staszczak, 2008). Polyubiquitination has also been shown to perform non-degradative functions during DNA damage repair, cellular signalling, intracellular trafficking and ribosomal biogenesis (Li & Ye, 2008). In addition to up-regulation of an *S. commune* polyubiquitin gene (see Chapter 6; section 6.3), a protein homologous to an ubiquitin E3 ligase, a key component of the polyubiquitin degradative machinery showed 5.6 folds increase in abundance in 2-D gels. This could be part of the UPR adaptive mechanisms to clean up toxic misfolded protein aggregates in addition to the recycling of carbonylated proteins due to oxidative damage.

RNA polymerase II and BTF3a were both significantly up-regulated. The former is a key transcriptional initiator in eukaryotes leading to the synthesis of RNAs: precursors for protein synthesis (Prescott *et al*., 1999). BTF3a on the other hand is required for the transcriptional initiation of RNA polymerase II (Zheng *et al*., 1987). BTF3a has also been shown to function as a weak substrate for protein kinase CK2, a highly conserved eukaryotic serine threonine/threonine protein kinase required for cell survival in yeast (Padmanabha *et al*., 1990; Grein & Pyerin, 1999). It is not unlikely that BTF3a might have played multifarious roles in *S. commune* mycelia confronted by *T. viride*. However, synchronized up-regulation of both BTF3a and RNA polymerase II strongly implicates both proteins as participants in a possibly elevated protein synthetic flux in cell wall-stressed mycelia of *S. commune*.
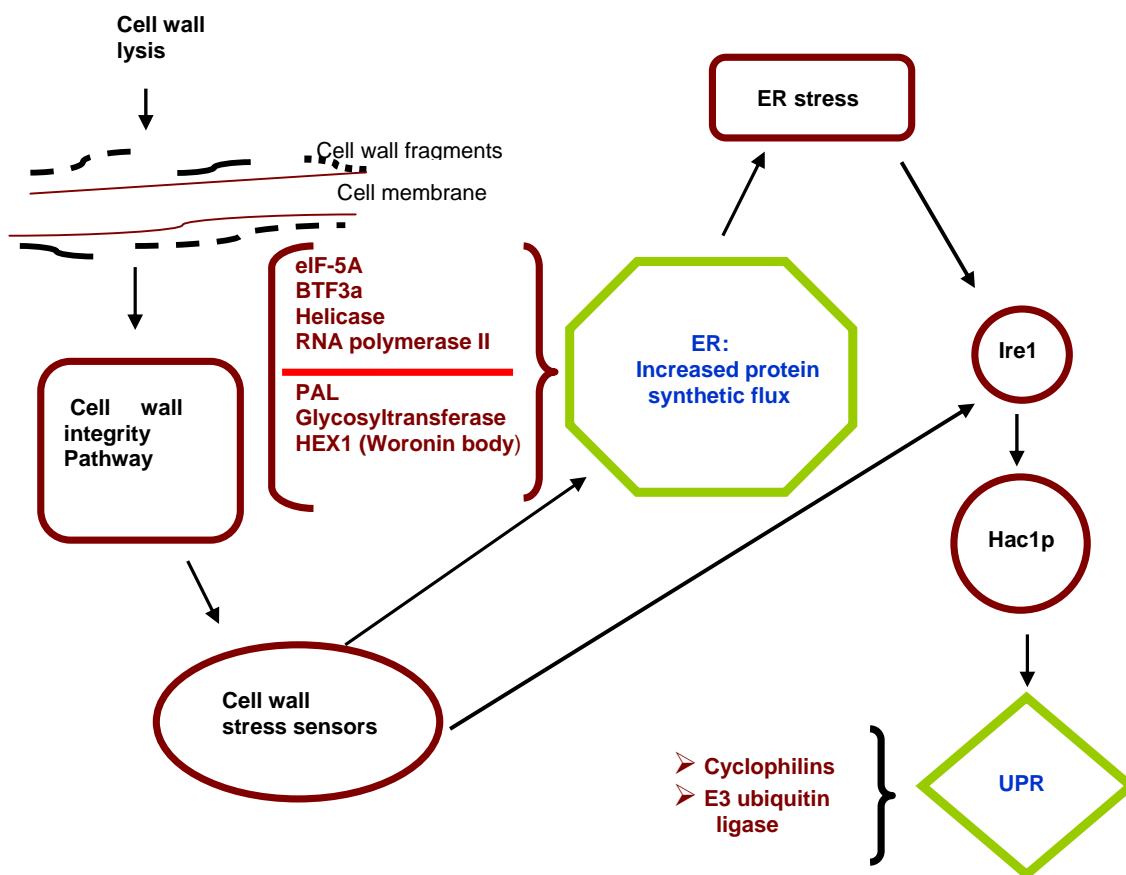
Figure 7.10 Schematic representation of events leading to the induction of the UPR following cell wall lysis in *S. commune* after 48 hours of mycelial contact with *T. viride*. Cyclophilins and the E3 ubiquitin ligase are key components of the UPR, while elF-5A and BTF3a are key parts of the protein synthetic and the transcriptional stream. Helicase also contributes to translational initiation in addition to nucleic acid repair and transcriptional regulation. RNA polymerase II is a key transcriptional initiator in eukaryotes sequel to translation. Phenylalanine ammonia lyase (PAL) is a key phenolic synthetic protein, which could promote the sealing of, and strengthening of cell wall during interactions. Glycosyl transferase is implicated in glycosylation (protein assembly) and synthesis of cell wall polymers, whereas HEX1 is the major component of woronin bodies that plug septal pores during cell wall damage. The highlighted proteins in the diagram were up-regulated in *S. commune* confronted by *T. viride* (Modified from Krysan, 2009).

Up-regulation of a protein that produced a match with a hypothetical protein that bears conserved domains for DEAD-, DEAH- and DEXDc-box helicase (spot SC93), further buttresses the assumption that lysis of *S. commune* cell wall may have elicited increased protein synthesis, resulting in ER stress among other stress responsive functions of helicases. Helicases are ATP-requiring motor proteins that unwind nucleic acids (Vashisht & Tuteja, 2006; Gong *et al*., 2005). Generally, they are involved in DNA repair, nucleo-cytoplasmic transport, translation, RNA metabolism, ribosome biogenesis and organellar expression (Vashisht & Tuteja, 2006; Gong *et al*., 2005; de la Cruz *et al*., 1999; Tanner & Linder, 2001). Different studies have implicated helicases in oxidative stress response (Briolat & Reysset, 2002), response to low and high temperatures (Morlang *et al*., 1999; Seki *et al*., 2001), and salt tress (Montero-Lomeli *et al*., 2002; Pham *et al*., 2000; Sanan-Mishra *et al*., 2005).

More importantly, RNA molecules are susceptible to the formation of stable misfolded structures especially under conditions of stress (Lorsch, 2002; Vashisht & Tuteja, 2006; Gong *et al*., 2005; Jones *et al*., 1996). Data from different workers have led to the suggestion that DEAD-box RNA helicases function as RNA chaperones, which disrupt RNA secondary structures, thereby restoring homeostatic balance, which allows translation of RNA (protein synthesis) to proceed normally (Lorsch, 2002; Vashisht & Tuteja, 2006; Gong *et al*., 2005; Jones *et al*., 1996; Tanner & Linder, 2001). Induction of GABA synthesis, lipid peroxidation and protein carbonylation, and aggregation of intracellular organelles, which might have affected electron transport in the mitochondria, coupled with the activity patterns of catalase, SOD and G6PDH, as shown in earlier Chapters (2-5), are strong indicators of oxidative stress. Oxidative and cell wall stresses would pose a formidable challenge to the survival of *S. commune* paired against *T. viride*. The chances are that the helicase protein may be part of the adaptive mechanisms recruited early on for survival by *S. commune* during the interaction. The role of the helicase may be to stabilize RNA structures thereby promoting translation, in an effort to promote the synthesis and incorporation of proteins and polymers into the damaged cell wall and to contain oxidative insult.

The closest match for spot SC41 for which a 7.1 fold increase in volume was observed is a glycosyltransferase (GT) putatively involved in cell wall biogenesis. GTs are found in both prokaryotes and eukaryotes. They catalyze the synthesis of oligosaccharides, polysaccharides and glyconoconjugates by donating the sugar moiety from an activated nucleotide-sugar molecule to an acceptor, usually, a growing oligosaccharide, a lipid or a protein (Lim & Bowles, 2004; Hashimoto *et al*., 2009; Breton *et al*., 2005). GTs recognise multiple substrates, which accounts for their involvement in a wide range of developmental and metabolic homeostasis (Lim & Bowles, 2004). For instance, plant GTS are involved in biotransformation of secondary metabolites, xenobiotics (pesticides and herbicides), and hormones (Lim & Bowles, 2004; Jones & Vogt, 2001; Ross *et al*., 2001). They also play key roles in the synthesis of cell wall polymers, cell-cell interaction and in cell invasion (Mayer *et al*., 2006; Hashimoto *et al*., 2009). GTs comprise a large group of enzymes of which chitin, cellulose and glycogen synthases are simple examples (Hashimoto *et al*., 2009).

Considering the immediate stress (cell wall lysis) to which *S. commune* mycelia are subjected to upon confrontation with *T. viride*, an up-regulated GT will be most likely involved in the construction of cell wall polymers to annul the effects of cell wall hydrolytic enzymes from *T. viride*. In Chapter 6, it was shown that the genes encoding a chitin synthase and an acyl carrier protein respectively, both of which are involved in the synthesis of cell wall components were both up-regulated soon after contact with *T. viride*. However, *Trichoderma* species are known to employ multiple antagonistic mechanisms, which include antibiosis, (some *Trichoderma* species synthesize peptaibol antibiotics that aid the lysis of host cell wall polymers and cause major damage to membranes) (Schirmböck *et al*., 1994; El Hajj *et al*., 1989). Peptaibols also known as trichorzianines are short peptide antibiotics, which form voltage-gated ion channels in lipids, thereby modifying membrane permeability (El Hajj *et al*., 1989: Le Doan *et al*., 1986).

Although production of peptaibols has not been investigated in the present study, it has been previously reported in *Trichoderma viride* (Jaworski *et al*., 1999). GT-based

detoxification of harmful substrates by glycosylation has been reported in plants, where incorporation of sugar residues onto an aglycone alters its bioactivity (Lim & Bowles, 2004). To this end, possible detoxification of peptaibols or other toxic compounds from *T. viride* might be one of the roles of the up-regulated GT. Furthermore, GTs play key roles in protein assembly during post-translational modifications (glycosylation). This is a major quality control function of GTs during protein synthesis (Hashimoto *et al.*, 2009). In the light of the heightened need for protein synthesis as described above, promotion of glycosylation required for the proper folding and functioning of certain proteins in the ER cannot be ruled out as a possible reason for the up-regulation of a GT in *S. commune*.

7.4.1.3 Up-regulation of Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL) is a stationary phase enzyme of plants, fungi and actinomycetes which catalyzes the non-oxidative deamination of L-phenylalanine to *cis*-cinnamic and free ammonium ion; the entry point into the phenylpropanoid pathway (Kim *et al.*, 2001; Kalghatgi & Rao, 1976; Gomez-Vasquez *et al.*, 2004; Kurosaki *et al.*, 1986). Different studies have shown that injury, light, inception of secondary metabolism, addition of L-phenylalanine and nutrient starvation trigger the synthesis of PAL in plants as well as promote its activity (Jones, 1984; Hahlbrock & Scheel, 1989; Kim *et al.*, 2001). The Key function of PAL in plants challenged by injury of some kind or by a phytopathogen is cell wall strengthening and repair by lignification, which entails the synthesis of phenolic compounds (via the phenylpropanoid pathway), subsequently incorporated into plant lignin cell wall by phenoloxidases (Gomez-Vasquez *et al.*, 2004: Kalghatgi & Rao, 1976; Kurosaki *et al.*, 1986). For instance, working independently, Gomez-Vasquez *et al* (2004) and Kurosaki *et al* (1986) showed that cassava and carrot cell cultures challenged by fungal-derived elicitors and fungal cell wall materials respectively, up-regulated PAL, with corresponding increases in the levels of a wide array of phenylpropanoid pathway-associated phenolic compounds such as *p*-hydroxybenzoic acid, ferulic acid, esculin etc.

Interestingly, Gomez-Vasquez *et al.* (2004) observed visible browning of the challenged cassava cells indicative of elevated phenylpropanoid metabolism. This browning

colouration is also typical of most fungus-fungus interactions, and was observed in the present study, particularly in the mycelia of *S. commune* paired against *T. viride*. These authors also reported corresponding increase in the levels phenol oxidases and ROS in their study. They concluded that in challenged or wounded plants, PAL activates the phenylpropanoid pathway, promoting the accumulation of phenolics, which are utilized by phenol oxidases in the presence of ROS to produce lignin deposited onto the cell wall. Furthermore, this generates quinones (responsible for the brown colouration) which exert antimicrobial activity against infecting pathogens. Similarly, induction of secondary metabolism, carbon limitation, nitrogen starvation, addition of aromatic amino acids (L-phenylalanine and L-tryptophan) particularly during secondary metabolism were shown to increase PAL activity in *Ustilago maydis* and *Rhizoctonia solani* (Kim *et al*., 2001; Kalghatgi & Rao, 1976). According to Kim *et al* (2001), growth temperature, pH and light had no effect on PAL activity.

There is no literature thus far to the best of our knowledge, linking PAL synthesis/activity to fungal cellular injury or to interspecific interactions in fungi, although many authors have reported profound accumulations of phenolic compounds during fungus-fungus interactions. Griffith *et al*. (1994a, b,c) proposed that fungus-fungus interaction shifts fungal growth to secondary metabolism resulting in the sealing-off of hyphal front and pigmentation. In a follow-up, Rayner (1997) and Rayner *et al*. (1994) inferred that non-self recognition in fungi promotes the synthesis and secretion of hydrophobic metabolites from hyphae, which are acted upon by phenoloxidases to generate free radicals and polymers responsible for hyphal sealing. The activity of phenoloxidases also promotes quinone production responsible for pigmentation during interspecific contact. Furthermore, treatment of cultures of single unpaired species with the uncoupling agent, 2,4-dinitrophenol mimicked the morphologies of paired fungal cultures (Griffith *et al*., 1994). Crowe and Olsson (2001) attributed the substantial induction of laccase in *Rhizoctonia solani* paired against antagonistic *Pseudomonas fluorescens* strains to compromised cell integrity.

In the present study, all the morphological and physiological elements of fungus-fungus interaction were observed. Contact between both species resulted in the production of brownish pigmentation and sealing-off of *S. commune* hyphal front (Chapter 2; section 2.3.1). Laccase and manganese peroxidase levels increased in *S. commune*-occupied domains soon after contact with *T. viride*, with corresponding rise in the levels of phenolics and ROS (Chapters 4 & 5). GC-MS analysis showed a rise in the levels of typical phenolic compounds, mandelic acid and tropic acid (Chapter 3; section 3.3.2). Taken together, our results buttress and even more importantly, shed more light on the works of Rayner and his co-workers (1994) and the work of Crowe and Olsson (2001). Cell wall lysis compromises cell integrity hence, is potentially fatal. This, as in plants is the very likely cause of PAL up-regulation in *S. commune*, thereby activating the secretion of phenolic compounds, utilized by the up-regulated phenoloxidases to synthesize hyphae-sealing polymers. To this end, compromised cell integrity indirectly triggers up-regulation of phenoloxidases. The resulting quinones might perform protective functions during contact. 2,4-dinitrophenol that was incorporated in growth medium by Griffith *et al.* (1994), is a classic phenoloxidase substrate. Perhaps this accounts for the similarities in morphology between 2,4-dinitrophenol-containing cultures and interspecifically interacting fungal cultures. This is likely, considering that substrate-induced phenoloxidases would most possibly elicit a similar flow of biochemical events as in injured fungal mycelia.

7.4.1.4 Up-regulation of the Woronin body protein component, HEX-1

Woronin bodies (WBs) are peroxisome-associated organelles unique to filamentous fungi where they plug the septal pore in response to cellular injury (Liu *et al.*, 2008; Soundarajan *et al.*, 2004; Alymore *et al.*, 1984). Although earlier workers described WBs as ascomycete-specific organelles (Woronin, 1864; Trinci & Collinge, 1974), they have been identified repeatedly in basidiomycetes and other higher fungi (Alymore *et al.*, 1984). WBs are made of protein matrix, which consists of a hexagonal (HEX) protein (Liu *et al.*, 2008; Soundarajan *et al.*, 2004; Tey *et al.*, 2005). Environmental conditions such as nutrient starvation have also been demonstrated to induce WBs in *Magnaporthe grisea* (Soundarajan *et al.*, 2004). In addition, Soundarajan *et al* (2004) reported that

WBs are essential for *M. grisea* pathogenesis. We ascribe the up-regulation of WBs in confronted *S. commune* mycelia to the sharp need for the maintenance of cellular integrity upon cell wall lysis. Septal plugging functions to localize the effects of cellular injury to the affected compartments thereby preventing loss of cytoplasm from the intact compartments (Alymore *et al.*, 1984).

7.4.1.5 Stress-induced recycling of intracellular sources of carbon and nitrogen

Cell wall damage is fatal to fungi thus; most metabolic machineries post cell wall lysis would be channelled towards survival. Apart from the immediate stress of cell wall damage, loss of cell wall function limits nutrient acquisition in fungi. For instance, the cell wall plays a key role in the vesicular transport of nutrients into the hypha (Casadevall *et al.*, 2009). This makes limitation of nutrient absorption part of the network of effects arising from fungal cell wall damage. To this end, recruitment of enzymes involved in nitrogen and carbon recycling would make a crucial contribution to cell survival during cellular injury. Perhaps this explains the up-regulation of branched-chain α-ketoacid dehydrogenase E2 subunit (BCKAD) and 2 isoforms of Glyoxysomal/mitochondrial malate dehydrogenase. BCKAD is a complex of enzymes that degrade branched chain amino acids such as valine, isoleucine and leucine (Costeas & Chinsky, 2000). By the oxidative decarboxylation of branched chain amino acids, BCKAD channels metabolic flow towards the synthesis of carbon substrates required to meet changing energy requirements (Costeas & Chinsky, 2000). This might contribute towards the sustenance of metabolic activities under the described conditions of stress.

Malate dehydrogenase (MDH) is a key enzyme of the TCA and the glyoxylate cycles. Although it has been implicated in some other functions in fungi, its core roles lie in these two cycles. In the TCA cycle, MDH converts malate to oxaloacetate, which ensures continuation of the cycle, but more importantly serves as a source of aspartate, a precursor for threonine, isoleucine, asaparagine, methione, lysine and pyrimidines (Prescott *et al.*, 1999). Whereas we cannot rule out the roles of MDH in the TCA cycle as the possible factor that elicited its up-regulation, biosynthetic functions during the prevailing conditions of growth 48 hours post contact with *T. viride* are very unlikely.

Up-regulation of BCKAD and PAL, both of which are amino acid degradative enzymes, coupled with the likely induction of secondary metabolism, suggest alternative functions rather than amino acid biosynthesis for MDH under the conditions of the present study. The two up-regulated MDH proteins bear conserved sequences indicating that they might function in glyoxysomes hence, the glyoxylate cycle. The glyoxylate cycle is an abridged TCA cycle bypassing isocytrate dehydrogenase and α-ketoglutarate dehydrogenase (Prescott *et al*., 1999).

Results in chapters 3 and 5, demonstrate the induction of GABA and rise in the activity of succinic semialdehyde dehydrogenase respectively, both of which indicate possible block of the α-ketoglutarate dehydrogenase. RT-PCR showed suppression of a subunit of the *S. commune* α-ketoglutarate dehydrogenase gene upon contact with *T. viride* (Chapter 6). These data strongly suggest that the strongly up-regulated MDH isoforms might be involved in the glyoxylate cycle. This is more likely considering that the glyoxylate cycle harnesses energy from lipids to drive other energy-requiring pathways and functions (Kunze *et al*., 2006). This implicates MDH in β-oxidation, a key source of Acetyl-coA from fatty acid breakdown (Kunze *et al*., 2006). β-oxidation supplies the carbon fuel required to drive key metabolic functions under stress. For instance, starved rats have been shown to up-regulate MDH isoforms for the mobilization of nutrient reserves (Popov *et al*., 2001).

Increased catalase activity is triggered by β-oxidation, which is a major producer of hydrogen peroxide (Kunze *et al*., 2006). In chapter 5, assays showed a rise in catalase activity, which might be a function of fatty acid breakdown in the glyoxysomes. Over-expression of peroxisomal MDH genes involved in NADH-reoxidation during β-oxidation by Kurita (2003) led to increased intracellular hydrogen peroxide levels, with a corresponding rise in catalase activity. Perhaps the levels of MDH detected in *S. commune* protein samples following contact with *T. viride* are part of a web of biochemical reactions geared towards generation of energy from intracellular stores, which consequently alters the redox (NAD/NADH) balance of the cell, thus promoting oxidative stress.

7.4.1.6 Up-regulation of the δ-chain of ATP synthase and down-regulation of Nucleoside diphosphate kinase

Although the gene encoding the β-chain of *S. commune* ATP synthase was suppressed during interaction with *T. viride* (chapter 6), a protein spot identified as the δ-chain of the same enzyme was up-regulated. Considering that ATP levels in both self-paired and *T. viride*-confronted cultures of *S. commune* were similar (chapter 4), it could be concluded that despite the suppression of ATP synthase β-chain gene, ATP synthesis had progressed unabated in *S. commune* during the earlier stages of mycelial interactions. To this end, it is very likely that ATP synthase is regulated at the protein level. Nucleoside diphosphate kinase (NDK) maintains the cellular pool of nucleoside triphosphates (such as ATP) by catalysing the transfer of phosphoryl group to nucleoside disphosphates (Lee *et al.*, 2009). NDK was down-regulated in *S. commune* mycelia, further supporting the indication that ATP synthase must have played the key role in maintaining the levels of ATP detected in *T. viride*-confronted *S. commune* relative to the self-paired cultures.

7.4.1.7 Down-regulation of glycolysis

Three protein spots (which might be subunits of a polymeric protein) carrying the enolase conserved domain and a triphosphate isomerase, both of which are involved in glycolysis were down-regulated in *S. commune*. Enolase converts 2-phospho-D-glycerate to phosphoenolpyruvate (Pandey *et al.*, 2009) whereas triphosphate isomerase (TIM) catalyses the reversible inter-conversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phopshate (Walden *et al.*, 2004). Down-regulation of a gene that encodes GAPDH, a key glycolytic enzyme is discussed in chapter 6. It does appear glycolytic enzymes were down-regulated following contact with the antagonist. Oxidative stress has been shown to reduce glucose and nitrogen uptake in yeast by possibly oxidizing enzymes involved in the metabolism of both nutrients (Godon *et al.*, 1998). Li *et al.* (2008) demonstrated that oxidative stress has a similar effect on *Aspergillus niger* B1-D glyceraldehyde-3-phosphate dehydrogenase thereby reducing glucose uptake. There is a growing body of evidence suggesting that glucose-6-phosphate dehydrogenase (G6PDH); the first enzyme in the pentose phosphate pathway (PPP)

might be involved in oxidative stress response in yeast (Shenton and Grant, 2003; Izawa *et al*., 1998).

Although, the role of G6PDH in oxidative stress response is has yet to be clearly understood, there are indications that a rise in G6PDH activity during oxidative stress is a pointer to possible up-regulation of PPP, a major source of the antioxidative cofactor, NADPH (Li *et al*., 2008; Shenton and Grant, 2003). Although PPP-related proteins were not found to be up-regulated (among the sequenced proteins), down-regulation of glycolytic proteins does suggest that glycolysis may have been blocked in *S. commune* in favour of a pathway that generates reducing power for antioxidant enzymes. This is in agreement with the rise in G6PDH activity reported in chapter 4. A combination of elevated MDH activity (in the β-oxidation cycle) and cellular aggregation (which is very likely to affect the lipid-bilayer of the mitochondrial matrix hence, electron transport and mitochondrial-cytosolic transport) will potentially herald redox imbalance and intracellular oxidative damage, which might bring about shutting-down of glycolysis and up-regulating PPP to restore redox balance. A similar scenario was observed *in Stereum hirsutum* paired against *Coprinus micacues* and *Coprinus disseminatus*, where glycolytic proteins where down-regulated in favour of PPP proteins (Peiris, 2009).

7.4.1.8 Down-regulation of heat shock proteins (HSP70), 5-methyltetrahydropteroyltriglutamate—homocysteine S-methyltransferase, a transcriptional attenuator and trehalose phosphorylase

Despite their established roles in stress response, two heat shock proteins (HSP70) were down-regulated. A similar pattern was observed by RT-PCR (chapter 6), where a HSP70 gene studied was up-regulated after 24 hours of contact, but was down-regulated by the second day of mycelial interactions. Heat shock proteins function as chaperones aiding the maintenance of protein folding (Prescott *et al*., 1999). It appears *S. commune* employs cyclophilins, two of which were up-regulated (discussed above) for promoting protein folding under stress. Transcriptional attenuators are regulatory proteins that terminate gene expression by causing mRNA to fold into alternative secondary structures (Elf *et al*., 2001). Although the exact gene(s) regulated by this attenuator are not known, its

down-regulation will most probably promote transcription and translation. Perhaps this is part of the response to cell wall lyses promoting the synthesis of stress related proteins (which would be repressed otherwise) required to contain cell wall stress and its consequent effects.
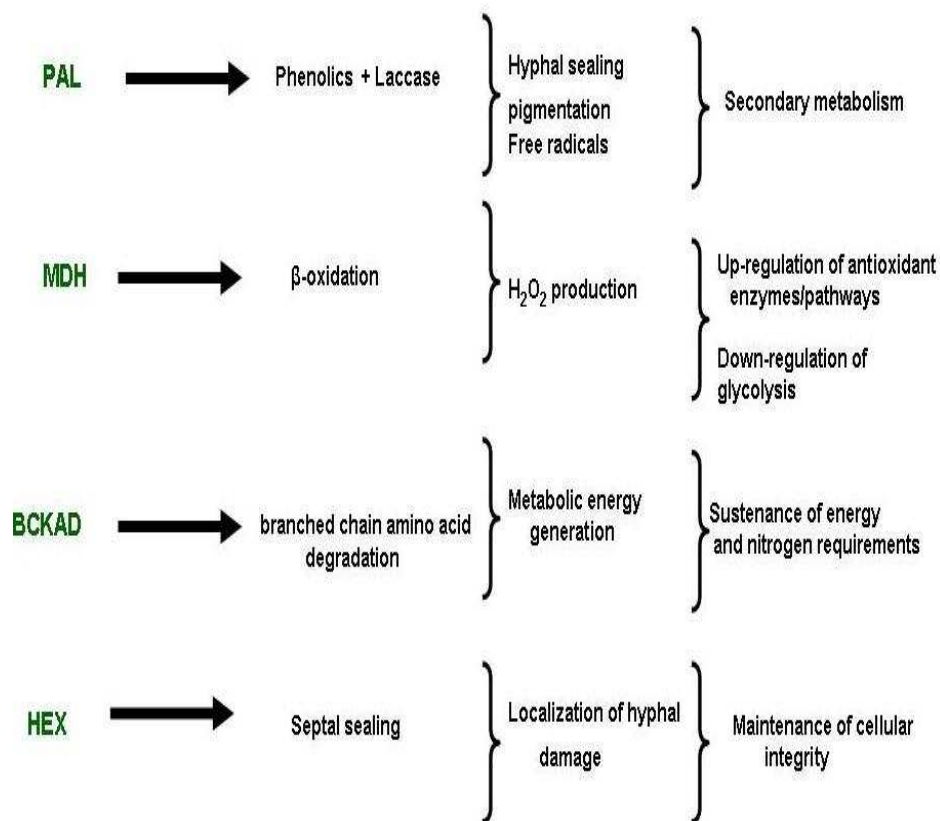
Figure 7.11 **Hypothetical schemes of biochemical events following the up-regulation of PAL, MDH, BCKAD and HEX.**

Down-regulation of 5-methyltetrahydropteroyltriglutamate—homocysteine S-methyltransferase involved in methionine synthesis (Burton *et al.*, 1969) is another indication of the down-regulation of housekeeping functions, which might be energy expensive or are involved in processes not required under the described prevailing conditions of stress. As mentioned in chapter 6, trehalose metabolism plays a key role in stress response in fungi (Fernandez *et al.*, 1998; Oćon *et al.*, 2007). Both trehalose synthetic and hydrolytic enzymes have been implicated in such responses. However,

there is much evidence showing that among the trehalose hydrolytic enzymes, neutral trehalase is the key player in stress response hydrolysing trehalose during or just after the exertion osmotic, oxidative and chemical stresses (Change *et al*., 1989; Dӧhlemann *et al*., 2006; Crowe *et al*., 1987; Fernandez *et al*., 1998). Whereas trehalose phosphorylase which normally catalyses trehalose hydrolysis (Eis & Nidetzky, 1999) was down-regulated, up-regulation of a neutral trehalase gene was reported in chapter 6. We infer that trehalose phosphorylase is not required during stress response, hence was down-regulated following mycelial confrontation.

7.4.2 Comparative analysis of patterns of proteins from *T. viride* paired against self and against *S. commune*.

Most of the up-regulated proteins in *T. viride* 48 hours after mycelial contact with *S. commune* depict a typical mycoparasitic antagonism, and increased metabolism possibly as a result of the utilization of host components as nutrients. Although chitinases and glucanases were not captured within the pH range and other conditions of this study, an aspartyl protease was up-regulated. Proteases form part of the complex web of enzymes employed by *Trichoderma* species for the hydrolysis of host cell walls (Dana *et al*., 2001; Schirmböck *et al*., 1994). Mycoparasitic cell wall hydrolysis releases a rich array of carbohydrates from the host cell wall, which are subsequently metabolised and assimilated by the mycoparasite (Boddy, 2000; Kubicek *et al*., 2001). This may account for the up-regulation of the multiple copies of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), suggesting a possible increase in glycolytic and perhaps overall metabolic activities in *T. viride*. Porins are voltage-dependent anion channels, which are thought to link mitochondrial and cellular energy metabolism by regulating the metabolite flux across the outer mitochondrial membrane (Wu *et al*., 1999). Up-regulation of porin supports the concept that the products of host cell wall lyses may have elicited pronounced metabolic activities in *T. viride*. This is further bolstered by the up-regulation of the ATP synthase β-chain, a strong indicator of vigorous respiration and metabolism.

Also up-regulated was a short-chain dehydrogenase/reductase (SDR). SDRs are highly multifunctional enzymes that catalyse mainly redox reactions utilising a broad spectrum of substrates including alcohols, sugars, steroids, aromatics and xenobiotics (Kellberg *et al.*, 2002; Shafqat *et al.*, 2006). In addition, they also exhibit lyase and isomerase activities (Shafqat *et al.*, 2006). A strong possibility exists that their multifunctional nature may have been the reason for their up-regulation in *T viride*. For instance, whereas *S. commune* up-regulated phenoloxidases to deal with the rising phenolic levels in the interaction zone, as expected phenoloxidases were not detected in *T. viride*. *T. viride* may have employed SDRs for the detoxification of phenolic compounds within the interaction zone. Furthermore, SDRs are involved in carbohydrate metabolism (Kellberg *et al.*, 2002) and may have aided the hydrolysis and utilization of the sugar sources released from *S. commune* cell wall upon hydrolysis. Antibiosis has been shown to augment the antagonistic machinery of mycoparasites (Kubicek *et al.*, 2001; Broglie *et al.*, 1991; Haran *et al.*, 1995) and the work of Butchko *et al.* (2003) demonstrated the implication of SDRs in the synthesis of the toxic polyketide-derived fuminosins (secondary metabolites) in *Gibberella moniliforms*. In view of this, a similar role cannot be ruled out for the SDR up-regulated in *T. viride* interacting with *S. commune*.

Although an elongation factor elf-5a was up-regulated, another was down-regulated. It is worthy of mention that most eukaryotes carry multiples copies of elf-5a (Eyler *et al.*, 2009; Chamot & Kuhlemeier, 1991) and in *S. cerevisiae*, the two copies of elf-5a have been reported to exhibit functional redundancy, hence down-regulation or deletion of one of the proteins does not affect the other (Chamot & Kuhlemeier, 1991). Perhaps up-regulation of one elongation factor and down-regulation of another enables *T. viride* to maintain some form balance during protein synthesis since one of the two is likely to perform independently. F-actin capping protein (CP) has been implicated in the assembly of actin filament and organisation of its architecture, although its precise function has remained elusive (Cooper & Sept, 2008; Nakano & Mabuchi. 2006). Down-regulation of CP might have contributed to the maintenance of cell shape against possible osmotic stress arising form the soluble products of host cell wall hydrolysis. Activation of high osmotic glycerol pathway and accumulation of sugar alcohols not only enable

Trichoderma species to thrive in diverse environments but also allow them to entwine around their hosts during mycoparasitic growth (Delgado-Jarana *et al.*, 2006). Organisation of the actin cytoskeleton might contribute to the resistance against external stresses during mycoparasitic growth.

7.4.3 Summary and conclusions

The data reported here give an insight into some of the physiological and biochemical events that ensue mycelial confrontation between *S. commune* and *T. viride*, 48 hours post contact. The protein patterns especially for *S. commune* reflect a complex response pattern involving pronounced protein synthesis possibly aimed at repairing and containing cell wall damage with consequent ER stress. This may have elicited the UPR to streamline protein synthesis and perhaps momentarily nullify the accumulation of toxic unfolded protein aggregates. There are indications of septal plugging, hyphal sealing, mobilisation of intracellular energy stores, phenolic detoxification, and shift in cellular metabolism in *S. commune* under attack from *T. viride*. On the other hand, the proteome of *T. viride* paired against *S. commune* relatively depicts the recruitment of the mycoparasitic machinery upon contact with *S. commune*. Whereas metabolic activities especially glycolysis may have been pronounced in *T. viride*, the proteome of *S. commune* suggests recruitment of a combination of secondary and primary metabolic activities crucial for the survival of cell wall lysis and perhaps oxidative injury with a greater leaning towards secondary phase of growth. There is little or no indication of an attack response by *S. commune*, leading to the conclusion that the physiological changes in *S. commune* paired against *T. viride* are most likely a combination of defence responses.

CHAPTER 8

Conclusions and future prospects

8.1 Introduction

The aim of the present study was to use *S. commune* as a model to investigate the underlying cellular and molecular mechanisms that drive the response/adaptation of white-rot basidiomycetes to antagonistic mycelial confrontation. A highly antagonistic strain of *Trichoderma viride* was used for confrontation against *S. commune*, in an attempt to replicate strong mycelial combat on agar.

To do this, RT-PCR was used to probe the expression patterns of selected genes in the mycelia of *S. commune* confronted by *T. viride*. 2-D protein gel electrophoresis was employed in the investigation of changes in the proteomes of both fungi paired against each other. The activities of selected enzymes involved in anti-oxidation, detoxification and metabolic shifts were assayed mainly in the mycelial domains of *S. commune* paired against *T. viride*. Microscopy was used to study changes in morphological forms in both fungi interacting on PDA, while GC-MS analysis allowed the investigation of their metabolite patterns. Biochemical assays were also used to investigate the induction of oxidative stress in the mycelia of *S. commune* following mycelial contact with the more antagonistic *T. viride*.

8.2 General Conclusions

Lysis of the cell wall of *S. commune* in the contact areas with *T. viride*, and the subsequent disintegration of mycelia of the former, clearly underlined the pronounced antagonistic abilities of *T. viride*. The initial rejection observed predominantly in *S. commune* prior to contact with the antagonist points to the probable exchange of chemical signals between both species before contact (Chapter 2; section 2.3.1). Perhaps the chemistry of the local environment external to the mycelium of a particular species is characterised by specific chemical signals, which are detected by confronting species. The work of Heilman-Clausen and Boddy (2005) demonstrated the existence of a metabolite-based passive defence/attack system in wood-rot fungi, which influences the establishment of later or same stage colonisers in the same environment. The implication is that some fungi secrete chemical compounds in the course of their colonisation of a substrate, thereby allowing them to stave off invasion from competitors. Although

pigmentation in the mycelia of *S. commune* could be ascribed to a number of factors, oxidation of phenol compounds by phenol-oxidases leading to the production of protective melanin (pigment) is considered central to this phenotypic response to interspecific mycelial contact. Additional explanation for this phenotype is possible switch of mycelial growth to a stationary phase of growth following the sealing-off of the mycelial front (cessation of growth). This would promote the production of secondary metabolites among which are phenol compounds (substrates for phenol-oxidases).

Although protoplasmic degeneration also depicted in Chapter 2 (sections 2.3.2 and 2.3.3) could be described as hyphal interference, it could also be connected to cell wall lysis. The dominance of the mycelium of *T. viride* over that of *S. commune*, illustrated by the morphological changes in the mycelia of both fungi (Chapter 2; sections 2.3.1 – 2.3.3), is further confirmed by the patterns of metabolite expression presented in chapter 3. The products of *S. commune* cell wall lysis and total mycelial disintegration would most likely, alter the water activity/concentration of the surrounding medium relative to cytoplasmic composition. This would result in osmotic stress, warranting homeostatic changes to counterbalance the effect of osmotic shock. This may account for the accumulation of high levels of sugar alcohols in the mycelia of *T. viride* in response to *S. commune*. Sugar alcohols were also up-regulated in *S. commune* although to a lesser extent. Hexanetetrol, which is a cyclic sugar alcohol similar to myo-inositol, was up-regulated significantly only in the mycelium of *S. commune* (Chapter 3; section 3.3.2). Although there is no further evidence to confirm this, hexanetetrol might play signalling roles in *S. commune* similar to myo-inositol. Given that protoplasmic degeneration could possibly alter electron transport in the mitochondrion, it may have led to oxidative stress in the mycelia of *S. commune*. Sugar alcohols might be involved in the production of NADPH, which supplies reducing power for antioxidant enzymes. The high levels of GABA (Chapter 3; section 3.3.2) in *S. commune* point to the fact that the TCA cycle might have been blocked at α-ketoglutarate, a response that arises mainly from NADH/NAD imbalance (redox imbalance), which is indicative of oxidative stress.

The up-regulation of pyridoxine (Chapter 3: section 3.3.2), a key antioxidant and a quencher of singlet oxygen (Ristilä *et al*., 2006; Ehrenshaft & Daub, 2001) corroborates the likelihood of oxidative stress induction in *S. commune*. The induction of pigmentation in *S. commune* by calcium ionophore A23187 (Chapter 2; section 2.3.1) implicates the calcium influx pathway in the production of pigments in the mycelia of *S. commune*, in response to *T. viride*. Inositol phosphates are important secondary messengers involved in a wide range of molecular and physiological activities most of which involve the calcium influx pathway (Perera *et al*., 2003). Based on this link, it could be concluded that the increase in the levels of inositol phosphate in the mycelia of *S. commune* challenged by *T. viride* (Chapter 3: section 3.3.2) might be associated with cell wall lysis and more importantly, pigment production. Perhaps inositol phosphate is involved in a wider network of biochemical/physiological responses to non-self mycelia.

The results presented in Chapter 4 implicate oxidative stress as one of the outcomes of contact with the antagonist. They also suggest that contact with *T. viride* promoted cessation of growth (secondary metabolism) and starvation in *S. commune*. The levels of lipid peroxidation detected in the mycelia of *S. commune* following combative interaction with *T. viride* suggest that severe oxidative damage may have occurred as a result of this interaction. One possible cause of this could be leakage of electrons in the mitochondrion, arising from disruption of mitochondrial membrane potential. In addition, attack on the lipid components of the cytoplasmic membrane by the increased levels of superoxide anion and other radicals, which may have increased in response to non-self mycelial contact, could have contributed to the levels of lipid peroxidation detected in *S. commune*. A major effect of oxidative stress is the irreversible insertion of carbonyl groups into the side chains of specific amino acids in polypeptides (Levine, 1983 & 2002). The high levels of carbonylated proteins in the mycelia of *S. commune* paired against *T. viride* could be linked to oxidative stress. However, carbonylation could be part of the protein quality control machinery, a necessary mechanism following increased protein synthesis (geared towards synthesising the components of the damaged cell wall-Chapter 7; sections 7.3 & 7.4.1.2), starvation caused by cell wall damage (limiting nutrient acquisition ability- Chapter 7; section 7.1.4.5) and induction of secondary

metabolism, confirmed by the concomitant increase in the levels of phenol compounds (Chapter 3: section 3.3.2).

The activity patterns of superoxide dismutase, catalase and glucose-6-phosphate dehydrogenase (G6PDH) reported in Chapter 5 (section 5.3.2) imply that *S. commune* may have activated its antioxidant machinery, to contain oxidative damage arising from contact with *T. viride*. In addition, increase in the activity of G6PDH, a key enzyme in the pentose phosphate pathway (PPP) suggests that that this pathway may have been favoured for glucose metabolism. This is supported by the fact that erythritol, up-regulated in *S. commune* (Chapter 3: section 3.3.2) is synthesized via the PPP, a major source of NADPH. The activity and protein levels of laccase and manganese peroxidase in the mycelial domain of *S. commune* (Chapter 5; sections 5.3.1 & 5.3.2.4) strongly implicates them in detoxification, cell wall polymerization and possibly, synthesis of protective melanin-related compounds hence, accounting for the progressive induction of pigments after contact with the antagonist (Chapter 5; sections 5.3.2 - 5.3.3). Furthermore, the sharp and short-lived increase in the activity of succinic semialdehyde dehydrogenase (SSADH) confirms the activation of GABA shunt, to bypass a limiting step in the TCA cycle, a biochemical mechanism most likely directed towards restoring redox balance (Chapter 5; section 5.3.2.6).

The gene expression patterns presented in Chapter 6 are not by any means a global picture of the transcriptome of *S. commune* in response *T. viride*. Nonetheless, the expression of specific genes studied, provide insight into the physiological response of *S. commune* to *T. viride*, based on the functions of the selected genes. Interestingly, differences in gene expression patterns were observed between self-paired and unpaired samples, indicating that self-interaction may have caused a certain degree of physiological changes in the mycelia of *S. commune*. Given the down-regulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-encoding gene in response to contact with *T. viride* (Chapter 6; sections 6.3 & 6.4.5), it could be inferred that perhaps glycolysis was down-regulated in *S. commune*. This supports the assumption that a rise in the levels of G6PDH activity (Chapter 5; section 5.3.2.3) and the levels of erythritol

(Chapter 3; section 3.3.2) is an indication that glucose metabolism in the confronted mycelia of *S. commune* may have taken place via the PPP. Down-regulation of cytochrome C_1 and ATP synthase genes (Chapter 6; sections 6.3 & 6.4.3) are in support of the suggestion made earlier that protoplasmic degeneration arising from interspecific mycelial contact, may have disrupted the functioning of the mitochondria in *S. commune*. However, this does not explain the high levels of ATP detected in the mycelia of *S. commune* paired against *T. viride*, in comparison to the levels in self-paired mycelia (chapter 4; section 4.3.3). The patterns of chitin synthase and neutral trehalase genes suggest that the protein products of these genes may be involved in cell wall repair, and stress alleviation.

Up-regulation of ubiquitin gene (Chapter 6; sections 6.3 & 6.4.6) could be linked to the physiological requirement to recycle oxidised and improperly folded proteins as a result of oxidative and protein synthetic stresses (chapter 7; sections 7.3.1 & 7.4.1.2), secondary metabolism and starvation. Ubiquitin may also be involved in the degradation of expression factors for non-required proteins and degradation of dormant enzymes, a measure required under stress to regulate the "biochemical economics" of the cell. Cessation of growth following contact with *T. viride*, is further confirmed by the repression of the alpha-tubulin gene in *S. commune* after 24 hours (Chapter 6; sections 6.3 & 6.4.4). The repression of the α-ketoglutarate dehydrogenase gene after 24 hours of interaction with *T. viride* (Chapter 6; sections 6.3 & 6.4.5) is in agreement with the assumption that GABA shunt may have been activated in *S. commune*, in response to redox imbalance in the mycelia of the latter. It is interesting to note that the supposition that lysis of *S. commune* cell wall triggered increased protein synthesis (chapter 7; sections 7.3.1 & 7.4.1.2) is supported by the expression patterns of genes encoding ribosomal protein S8 and 18SrRNA (Chapter 6; sections 6.3 & 6.4.6), particularly the latter after 48 hours of contact with *T. viride*.

As with the gene expression studies (Chapter 6), the proteomics study (Chapter 7) represents only a snapshot of the changes in the proteomes of both fungi, during interactions. This is because, the studies only covered a pH range (3-10) out of which

only a fraction of the significantly down- or up-regulated proteins were sequenced owing to the cost of protein sequencing. In view of this, the results in Chapter 7 are not a representation of the global protein expression in both fungi, during interaction relative to their self-paired mycelia. However, the results from this chapter provide powerful insights into the response of both species to each other. For instance, the up-regulation of multiple copies of the expression factor, BTF3a, RNA polymerase, glycosyltransferase, initiation factor, elF5a, and helicase is a strong indication of increased transcriptional and translational activities, synonymous with cell wall damage. Such a flux through the protein synthetic machinery (endoplasmic reticulum; ER) may have triggered ER stress, thereby eliciting the up-regulation of cyclophilins and components of the ubiquitin pathway (members of the unfolded protein response –UPR– pathway) to streamline protein synthesis and detoxify misfolded protein aggregates. The degeneration of protoplasmic components in *S. commune* in the areas of contact with *T. viride* is characteristic of cell wall damage and ER stress. Furthermore, helicases are key stress alleviators as well as being involved in the restoration of homeostatic balance, which allows translation of RNA (protein synthesis) to proceed normally (Lorsch, 2002; Vashisht & Tuteja, 2006; Gong *et al*., 2005; Jones *et al*., 1996; Tanner & Linder, 2001).

Up-regulation of Phenylalanine ammonia lyase (PAL) (Chapter 7; sections 7.3.1 & 7.4.1.3) confirms the proposition that contact between both fungi resulted in secondary metabolism and pronounced synthesis of phenol compounds, which indirectly triggers synthesis and secretion of phenol-oxidases. The up-regulation of HEX1 (Chapter 7; sections 7.3.1 & 7.4.1.4), the protein component of the Woronin body suggests that *S. commune* may have employed this organelle to localise damage to the affected hyphal compartments. The patterns of malate dehydrogenase (MDH and branched-chain α-ketoacid dehydrogenase E2 subunit (BCKAD) (Chapter 7; sections 7.3.1 & 7.4.1.5) suggest that they may be involved in the recycling of lipids and amino acids respectively, in response to starvation resulting from cell wall lysis.

On the other hand, increase in the levels of ATP synthase, mitochondrial porin and GAPDH (Chapter 7; sections 7.3.2 & 7.4.2), point to increased metabolic activities in *T.*

viride paired against *S. commune*. This is very likely considering the release of carbohydrates from *S. commune* cell wall, following lysis. Hence, contact with the wood-rot fungus most likely favours carbon metabolism in *T. viride*. Furthermore, the up-regulated aspartyl protease in *T. viride* is a potential candidate in the battery of enzymes involved in host cell wall hydrolysis.

Efforts in this study were aimed mainly at understanding the mechanisms employed by *S. commune* to resist attack against a highly combative strain of *T. viride*. Although *S. commune* was subsequently killed in the contact areas, the ability of the former to survive cell wall lysis for a considerable period (120 hours), suggests that it possesses resistance mechanisms, which may be amplified in natural environments. Highley and Ricard (1988) have previously reported such resistance, particularly in wood and other niches, by white-rot fungi. Some of the most significant findings of this work highlight some of such mechanisms. For instance, the indication that ER stress may have been induced in *S. commune* paired against *T. viride* resulting to the up-regulation of UPR based on protein expression patterns (Chapter 7) is supported by protoplasmic degeneration and up-regulation of polyubiquitin, 18SrRNA, ribosomal protein S8 and neutral trehalase genes (Chapters 2 and 6 respectively). In addition, up-regulation of PAL (Chapter 7) agrees with the up-regulation of phenolic compounds (mandelic and tropic acids) (Chapter 2), rise in levels of phenol compounds as detected by phenol assay (Chapter 4), increase in the activities and protein levels of phenol-oxidases in the confronted mycelia of *S. commune* (Chapter 5) as well as the observed pigmentation (Chapter 2).

Increase in the levels of GABA (Chapter 3) is in conformity with the down-regulation of α-ketoglutarate dehydrogenase gene (Chapter 6) and increase in the activity of SSADH (Chapter 5). This can also be linked to the up-regulation of PPP - increase in the activity of G6PDH (Chapter 5) and increased levels of erythritol (Chapter 3), which may have enhanced the synthesis of NADPH, thereby helping to contain oxidative stress. In addition, down-regulation of GAPDH gene (Chapter 6) coincides with the down-regulation of triosephosphate isomerase and enolase (Chapter 7) both of which are glycolytic enzymes. This lends weight to the assumption that *S. commune*, under the

conditions of study, may have preferred PPP to glycolysis for glucose metabolism. A similar pattern was observed in the pairing of *Stereum hirsutum* against *Coprinus micaceus* and *Coprinus disseminatus* (Peiris, 2009). The repression of α-tubulin gene (Chapter 6) may be connected with the sealing-off of mycelial front and cessation of growth in *S. commune* after contact with *T. viride* (Chapter 2), both of which are indicative of secondary metabolism. In addition, related to secondary metabolism is the up-regulation of MDH and BCKAD (carbon and nitrogen recycling, respectively) (Chapter 7).

One of the most significant findings of this work is the up-regulation of key components of the UPR with concomitant increase in the expression of proteins involved in protein synthesis, assembly and transport (Chapter 7; sections 7.3.1 & 7.4.1.2). This strongly suggests that ER stress may have been triggered, as a physiological response to cell wall damage. Although protoplasmic degeneration has long been reported as one of the responses of fungi to non-self (Ikediugwu *et al*., 1970; Ikediugwu, 1976; Boddy, 2000), there is no evidence in literature connecting this phenomenon, to ER stress and the UPR.

The rise in the levels of lipid peroxidation and protein carbonylation in the mycelia of *S. commune*, provide new insight into reports that fungus-fungus interactions lead to secondary metabolism and disruption of mitochondrial functioning, which enhance oxidative stress. In addition, the up-regulation of MDH, PAL and BCKAD, point to the recycling of intracellular carbon and nitrogen sources, a central characteristic of secondary metabolic phase of growth. These findings, therefore lend more credence to the existing assumption that fungus-fungus interactions lead to the switch of mycelial growth to the secondary metabolic phase. Furthermore, the up-regulation of PAL, a stationery phase enzyme, provides fresh evidence in support of the assumption that interspecific mycelial combat promotes the secretion of phenolic compounds, another characteristic of secondary metabolism. In addition, increases in the levels of GABA and pyridoxine, and increased succinic semialdehyde dehydrogenase activity shed more light on the same assumption that interspecific mycelial contact leads to secondary metabolism and consequently, oxidative stress (Iakovlev *et al*., 2004; Rayner *at al*., 1994; Griffith *et*

al., 1994 a, b & c; Rayner *et al*., 1994). This is further confirmed by the repression of the gene that codes for α-tubulin, which is involved in cellular replication/growth.

A recent study by Peiris (2009), demonstrated possible switch in energy metabolism following interspecific combat between *S. hirsutum* and its competitors, *C. micacues* and *C. disseminatus*. This finding is supported by strong indications from this study that *S. commune* may have down-regulated glycolysis with a corresponding increase in the expression of proteins involved in the PPP. Such switch in metabolism could alleviate oxidative stress by the generation of NADPH.

The up-regulation of HEX-1, a strong indication of physiological attempt to localize cellular damage by septal plugging, is also a new finding in the study of antagonistic interactions in fungi.
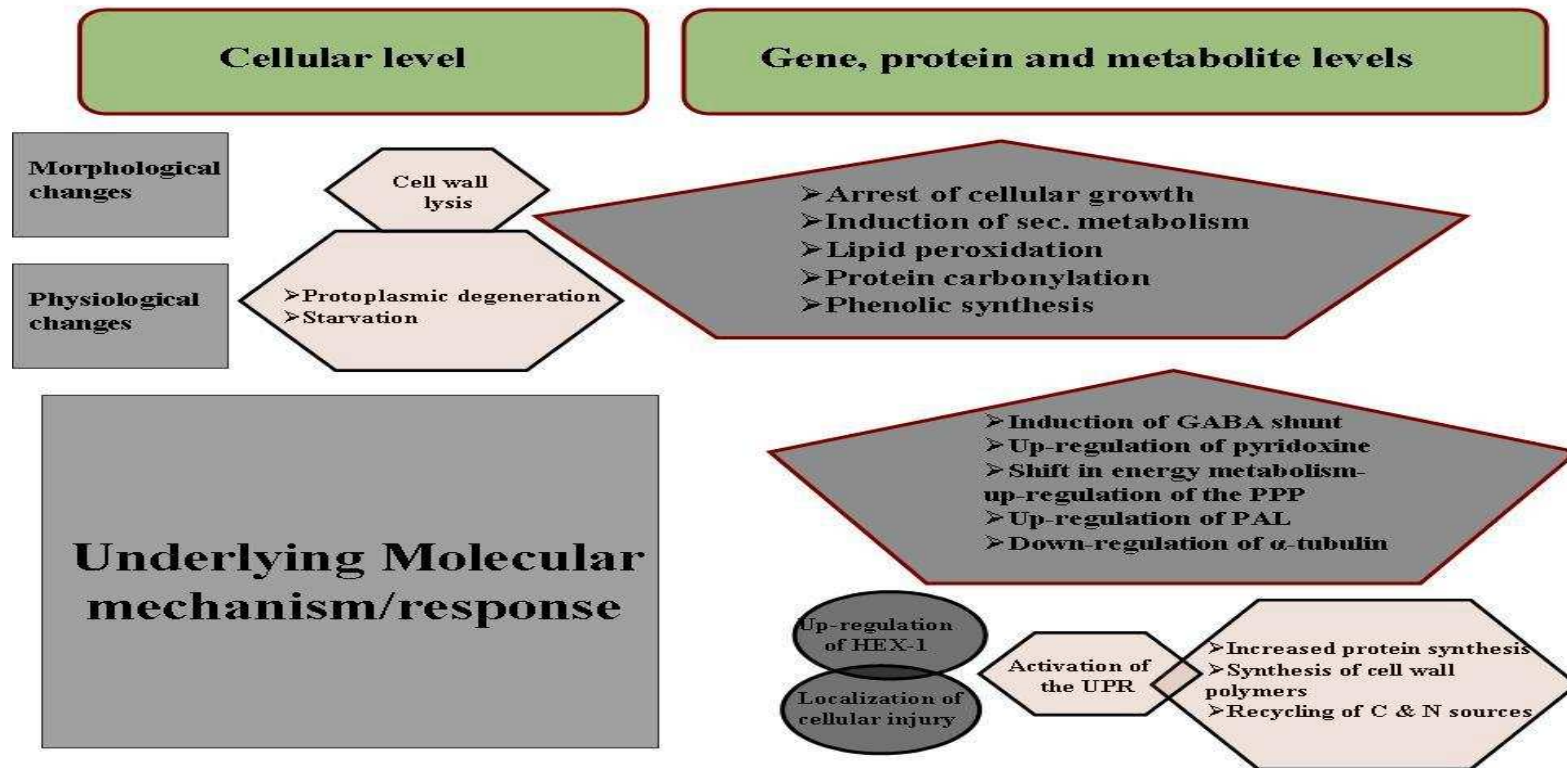
Figure 8.1 Hypothetical representation of morphological, physiological and biochemical changes occurring in the mycelia of *S. commune* following contact with the mycelia of *T. viride*. Different shapes and colours represent links between molecular and physiological/morphological responses to *T. viride*.

8.3 Future prospects

For experimental convenience, PDA was used throughout this work for interaction assays. However, the morphological, physiological and biochemical changes observed in this study are very likely to vary in the field. In view of this, combination of pairings on wood or other naturally occurring substrate(s) and on laboratory medium like PDA might be ideal for a more encompassing understanding of interactions between both fungi. For instance, the metabolite profiles of both species on naturally occurring substrates would possibly vary greatly from the profiles obtained on PDA. This therefore limits understanding of the chemical repertoire that is likely to be employed by each of these fungi during possible combat in the field.

Hynes *et al.* (2007) using a specially designed fermentation unit demonstrated the involvement of volatile compounds in the cascade of changes associated with interspecific mycelial combat. The approach employed in this study did not take into account the possible roles of such compounds either on their own or in combination with non-volatile compounds during interspecific mycelial combat. Furthermore, fungi are likely to encounter a wide range of organisms (fungal and non-fungal) and varying environmental conditions, which cannot be replicated in the laboratory, but however influence interactions in nature. However, it is worthy of mention that, it can be difficult interpreting results of interactions on natural, complex undefined substrates. Sterilization of substrates like wood would most likely alter their chemistry to levels that may affect experimental results. In addition, growth on natural substrates can be very slow, and sampling of interacting mycelia is far more challenging on wood or straw for instance.

As mentioned in chapter 7, the proteomic profile presented herein is only a fraction of the differentially expressed proteins. This is because, only proteins within the pH range of the IPG strip used, 3-10 were captured. Highly acidic or alkaline proteins, which may play crucial roles in the response of either fungus to the other, may have been missed thereby limiting understanding of the physiology and biochemistry of the response patterns studied. In addition, because of the cost of protein sequencing, the majority of the proteins that varied in volume between test and control samples were not sequenced.

Furthermore, an inherent limitation of 2-D gel electrophoresis-based studies is the inability to detect low-abundant proteins, which might perform crucial functions. A possible solution to this and other limitations of proteomic studies includes a bottom-up approach which employs online high performance liquid chromatography (HPLC) for the separation of protein samples, obviating the need for protein separation by gel electrophoresis (Rohrbough *et al*., 2007; Bhaduria *et al*., 2007).

The most common peptide separation technique by HPLC is reverse-phase (RP) LC (Rohrbough *et al*., 2007). This method separates proteins based on their hydrophobicity. Another LC method is strong cation exchange (SCX) chromatography, which separates peptides according to their charge (Rohrbough *et al*., 2007). Both LC methods can be used in combination in a method referred to as MultiDimensional Protein Identification Technology (MudPIT) (Rohrbough *et al*., 2007; Bhaduria *et al*., 2007). MudPIT employs columns that contain both RP and SCX phases allowing for easier and automated separation of biological mixtures (Rohrbough *et al*., 2007).

One of the major challenges in the proteomics aspect of this work was separation of *S. commune* protein samples to high resolution. This problem, in addition to poor detection of highly acidic and alkaline proteins, could be overcome by employing a more sensitive technique such as fluorescence difference gel electrophoresis (DIGE). 2D-DIGE is unparalleled compared to 2D-PAGE given its high throughput even with complex samples, timesavings and analytical power, although its reproducibility remains a major challenge (Bhaduria *et al*., 2007).

Understanding of protein functions can be greatly improved by detection of subcellular localization of protein(s) under conditions of growth (Silver, 1991). Up to 60% tagging of proteins can be achieved in eukaryotic systems by a combination of cloning strategies and mutagenesis allowing the detection of the subcellular localization of proteins during growth (Vogel *et al*., 2001). Such technique offers great promise for the determination of, and possible understanding of the direct roles of key proteins during fungus-fungus interactions.

One approach that would have made the proteomics study more robust would be to employ a complementary global gene expression technique. Examples of such techniques include serial analysis of gene expression (SAGE), microarray and mRNA differential display. These techniques allow the detection of levels of mRNA levels of a wide range of genes, which could be correlated to proteomics data to present a more profound picture of the physiological/biochemical changes associated with mycelial response to non-self. However, these techniques are primer based and both fungi employed in this study are not fully sequenced, making it impossible to design a wide range of primers to match functional genes. In the case of microarray technique, this limits the possibility of constructing an open reading frame (ORF)-specific microarray. Nevertheless, these techniques could still be employed, despite the associating challenges and limitations of performing them without specific primers. For instance, the shotgun approach based on random DNA fragments of unknown sequences arrayed on a glass slide could be used for microarray (Zaigler *et al.*, 2003).

A more specific approach involving the construction of cDNA libraries and subsequently using the expressed sequence tags (ESTs) for macroarray can also be applied. This also involves sequencing of the expressed genes, and it can be expensive (depending on the level of sequencing undertaken) and time-consuming. More over, it is limiting in the sense that the gene expression patterns may vary greatly between normal growth conditions, and during mycelial confrontations, such that certain differentially expressed genes under stress may not be present in the library. Notwithstanding, this technique has been successfully employed to study interactions between *Phlebiopsis gigantea* and *Heterobasidion parviporum* (Adomas *et al.*, 2006). Iakovlev *et al.* (2004) also employed mRNA differential display using oligo-dT primers to study interspecific interactions between *Heterobasidion annosum* and *Physisporinus sanguinolentus*. Although data in Chapter 6 show interesting changes in the patterns of selected *S. commune* genes in response to *T. viride*, real-time reverse transcription polymerase chain reaction (RT-qPCR) may have added more weight to the data from reverse transcription polymerase chain reaction (RT-PCR).

The detection of a rise in the levels of carbonylated proteins in the mycelia of *S. commune* confronted by *T. viride* implicates ER, oxidative and nutritional stresses in the response patterns of S. *commune*. Perhaps, this opens up a new channel in the study and understanding of antagonistic fungal interactions. This can be further investigated in time course experiments, using western blotting technique, which allows the detection of carbonylated proteins using specific antibodies. There is a need to better understand the possible disruption of mitochondrial functioning in paired fungi. Ultracentrifugation can be employed for the isolation of mitochondria from mycelial samples, and specific dyes such as Rhodamine, can be used to probe for the presence of intact and functioning mitochondria. This could provide powerful insight into the role of electron leakage in the physiological changes associated with antagonistic interactions.

The finding that PAL, ER stress and the UPR are possibly triggered in *S. commune* following mycelial contact with *T. viride* opens novel directions in the understanding of fungus-fungus interactions, thereby warranting further investigations. Specific profiling of the expression patterns of genes, involved in ER stress and the UPR in particular, as well as their protein products by combinations of western blotting, RT-qPCRC and possibly using gene knockdown/deletion/overexpression would make invaluable contributions to the understanding of the molecular biology of fungus-fungus interactions. Similarly, these techniques could be used to further probe the roles of PAL in the induction of secondary metabolism and the consequent secretion of phenolics in paired fungal mycelia and HEX1 in the localization of cellular injury.

References

Abraham, W. R. (2001). Bioactive sesquiterpenes produced by fungi: Are they useful for humans as well? *Current Medicinal Chemistry* **8**: 583-606.

Adachi, H. and Ishii N. (2000). Effects of tocotrienols on life span and protein carbonylation in *Caenorphadbitis elegans*. *Journal of Gerontology: Biological Sciences* **55**(6): B280 – B285.

Adler, L., Pederson, A., and Tunblad-Johansson, I. (1982). Polyol accumulation by two filamentous fungi grown at different concentrations of NaCl. *Physiologia plantarum* **56**: 139-142.

Adomas, A., Eklund, M., Johansson, M. and Asiegbu, F. O. (2006). Identification and analysis of differentially expressed cDNAs during non-self-competition interaction between *Phlebiopsis gigantea* and *Heterobasidion parviporum*. *FEMS Microbiology Ecology* **57**: 26-39.

Aguilaniu, H., Gustafsson, L., Rigoulet, M. and Nyström, T. (2003). Asymmetric inheritance of oxidatively damaged proteins during cytokinesis in *Saccharomyces cerevisiae*: a Sir2p dependent mechanism. *Science* **299**:1751-1753.

Ainsworth, A.M. and Rayner, A.D.M. (1991) Ontogenetic stages from coenocyte to basidiome and their relation to phenoloxidase activity and colonization processes in *Phanerochaete magnoliae*. *Mycological Research* **12**: 1414-1422.

Akhtar, M., Blanchette, R. A., Myers, G. C. and Kirk, T. K. (1998). An overview of biochemical pulping research. In: *Environmentally Friendly Technologies for the Pulp and Paper Industry*. Wiley, New York, Pp. 309-419.

Aluko, M. O. and Hering, T. F. (1970). Mechanisms associated with the antagonistic relationship between *Corticium solani* and *Gliocladium virens*. *Transactions of the British Mycological Society* **55**: 173-179.

Alfatafta, A. A., Gloer, J. B., Scott, J. A. and Malloch, D. (1994). Apiosporamide, a new antifungal agent from coprophilous fungus *Apiospora montagnei*. *Journal of Natural Products* **57**: 1696-1702.

Alymore, R. C., Wakely, G. E. and Todd, N. K. (1984). Septal sealing in the basidiomycete *Coriolus versicolor*. *Journal of General Microbiology* **130**: 2975-2982.

Anchel, M., Harvey, A. and Robbins, W. (1950). Antibiotic substances from basidiomycetes, VII *Clitocybe illudens*. *Proceedings of the National Academy of Sciences USA* **36**: 300-305.

Andrews, B. A. and Asenjo, J. A. (1987). Continuous culture studies of the synthesis and regulation of β-1,3-glucanase and protease enzymes from *Oerskovia xanthineolytica*, *Biotechnology and Bioengineering* **30**: 628-637

Anke, T. (1989). Basidiomycetes: A source of new bioactive secondary metabolites. *Progress in Industrial Microbiology* **27**: 51-66.

Arana, D. M., Nombela, C., Alonso, M. and Pla, J. (2005). The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen Candida albicans. *Microbiology* **151**: 1033-1049.

Arévalo-Rodriguez, M., Cardenas, M. E., Wu, X., Hanes, S. D. and Heitman, J. (2000). Cyclophilin A and Ess1 interact and regulate silencing by the Sin3-Rpd3 histone deacetylase. *EMBO Journal* **19**: 3739-37-49.

Aruoma, O. I., Halliwell, B., Laughton, M. J., Quinlan, G. J. and Gutteridge, J. M. C. (1989). The mechanisms of initiation of lipid peroxidation. Evidence against a requirement for an iron (II)-iron (III) complex. *Biochemical Journal* **258**: 617-620.

Ayer, W. A. and Miao, S. (1993). Secondary metabolite of the aspen fungus *Stachbotrys cyclindrospa*. *Canadian Journal of Chemistry* **71**: 487-493.

Baker, C J. and Orlandi, E. W. (1995). Active oxygen in plant pathogenesis. *Annual Review of Phytopathology* **33**: 299-321.

Baek, J M., Howell, C. R. Kenerly, C. M. (1999). The role of extracellular chitinase from *Trichoderma virens* GV29-8 in the biocontrol of *Rhizoctonia solani*. *Current Genetics* **35**: 41-50.

Baldrian, P. (2004). Increase of laccase activity during interspecific interactions of white-rot fungi. *FEMS Microbiology Ecology* **50**: 245-253.

Bao, W., Fukusjima, Y., Jensen Jr., K. A., Moen, M.A. and Hammel, K. E. (1994). Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. *FEBS Letters* **354**: 297-300.

Barak, R. and Chet, I. (1986). Determination by fluorscein diacetate staining, of fungal viability during mycoparasitism. *Soil Biology and Biochemistry* **18**: 315-319.

Beers, R. F. and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* **195:**130-140.

Bériault, R. Chénier, D., Singh, R., Middaugh, J., Maillox, R. and Appanna, V. (2005). Detection and purification of glucose 6-phosphate dehydrogenase, malic enzyme, and NADP-dependent isocitrate dehydrogenase by blue native polyacrylamide gel electrophoresis. *Electrophoresis* **26**: 2892-2897.

Bhadauria, V., Zhao, W., Wang, L., Zhang, Y., Liu, J. Y., Kong, L. and Peng, Y. (2007). Advances in fungal proteomics. *Microbiological Research* **162**: 193-200.

Bishop, D. K. (1994). *RecA* homologs *Dmc1* and *Rad51* interact to form multiple nuclear complexes prior to chromosome synapsis. *Cell* **79**: 1081-1092.

Bliss, D. E. (1951). The destruction of *Armillaria mellea* in citrus soils. *Phytopathology* **41**: 665-683.

Boddy, L. (1993). Saprotrophic cord-forming fungi: warfare strategies and other ecological aspects. *Mycological Research* **97**: 641-655.

Boddy, L (2000). Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbial Ecology* **31**: 185-194.

Boddy, L., Bardsley, D.W. and Gibbon, O.M. (1987). Fungal communities in attached ash branches. *New Phytolologist* **107**: 143-154.

Boddy, L., Gibbon, O.M. and Grundy, M.A. (1985). Ecology of *Daldinia concentrica*: effect of abiotic variables on mycelial extension and interspecific interactions. *Transactions of British Mycological Society* **85**: 201-211.

Bota, D. A. and Davies, K. J. (2002). Lon protease preferentially degrades oxidized mitochondrial aconitase by and ATP-stimulated mechanism. *Nature Cell Biology* **4**:674-680.

Botsoglou, N. A., Fletouris, D. J., Papagergiou, G. E., Vassilopoulos, V. N., Mantis, A. J., Trakatellis, A. G. (1994). Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. *Journal of Agricultural and Food Chemistry* **42**: 1931-1937.

Bouché, N. Fiat, A., Bouchez, D., Møller, S. G. and Fromm, H. (2003). Mitochondrial succinic-semialdehyde dehydrogenase of the γ-aminobutyrate shunt is required to restrict the leves of reastive oxygen intermediates in plants. *Proceedings of the National Academy of Sciences, USA* **100**(11):6843-6848.

Bouché, N., Lacombe, B. and Fromm, H. (2003). GABA signalling: a conserved and ubiquitous mechanism. *Trends in Cell Biology* **13**(12): 607-610.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**:248-254.

Breton, C., Šnajdrová, L., Jeanneau, C., Koca, J. and Lamberty, A. (2006). Structures and mechanisms of glycosyltransferases. *Glycobiology* **16**(2): 29-37.

Brighton, C. T., Sennet, B. J., Farmer, J. C., Iannotti, J. P., Hansen, C. A., Williams, J. L. and Williamson, J. (1992). *Journal of Orthopaedic Research* **10**:385-393.

Briolat, V. and Rysset, G. (2002). Identification of *Clostridium perfringens* genes involved in the adaptive response to oxidative stress. *Journal of Bacteriology* **184**: 2333-2343.

Brody, S., Oh, C., Hoja, U. and Schweizer, E. (1997). Mitochondrial acyl carrier protein is involved in lipoic acid synthesis in *Saccharomyces cerevisiae*. *FEBS Letters* **408**: 217-220.

Brown, A. W. and Shelp, B. J. (1997). The metabolism and function of γ-aminobutyric acid. *Plant Physiology* **115**: 1-5.

Bruce, A and Highley, T. L. (1991). Control of growth of wood decay basidiomycetes by *Trichoderma* spp. and other potentially antagonistic fungi. *Forest Products Journal* **41**(2): 63-67.

Bruce, A., Srinivasan, U., Staines, H. J. and Highley, T. L. (1995). Chitinase and laminarase production in liquid culture by *Trichoderma* spp. and their role in biocontrol of wood decay fungi. *International Biodeterioration and Biodegradation* **35**(4): 337-353.

Brunner, K., Peterbauer, C., Mach, R. L., Lorito, M., Zeilinger, S. and Kubicek, C. P. (2003). The Nag1 N-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. *Current Genetics* **43**(4): 289-295.

Burton, E., Selhub, J. and Sakami, W. (1969). The substrate specificity of 5-methyltetrahydropteroyltriglutamate-Homocysteine methyltransferase. *Biochemical Journal* **111**: 793-795.

Busch, K. and Fromm, H. (1999). Plant succinic semialdehyde dehydrogenase. Cloning, puritification, localization in mitochondria, and regulation by adenine nucleotides. *Plant Physiology* **121**: 589-597.

Butchko, R. A. E., Platner, R. D. and Proctor, R. H. (2003). *FUM13* encodes a short chain dehydrogenase/reductase required for C-13 carbonyl reduction during fumonisin biosynthesis in Gibberella moniliformis. *Journal of Agricultural and Food Chemistry* **51**: 3000-3006.

Calvo, A. M., Wilson, R. A., Bok, J. W. and Keller, N. P. (2002). Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* **66**(3): 447-459.

Carbajal, E. M., Beaulieu, J., Nicole, L. M. and Tanguay, R. M. (1993). Intramitochondrial localization of the main 70-kDa heat-shock cognate protein in Drosophila cells. *Experimental Cell Research* **207**: 300-309.

Carbonneau, M. A., Peuchant, E., Sess, D., Canioni, P. and Clerc, M. (1991). Free and bound malondialdehyde measured as thiobarbituric acid adduct by HPLC in serum and plasma. *Clinical Chemistry* **37**: 1423-1429.

Carsolio, C., Gutierrez ,A., Jiménez, B., Van Montagu, M. and Herrera- Estrella, A. (1994). Characterization of *ech42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proceedings of National Academy of Sciences USA* **91**: 10903-10907.

Casadevall, A., Nosanchuk, J. D., Williamson, P. and Rodrigues, M. L. (2009). Vesicular transport across the fungal cell wall. *Trends in Microbiology* **17**(4): 158-162.

Castillo, L., Calvo, E, Martinez, A. I., Ruiz-herrera, J., Valentin, E., Lopez, J. A. and Sentadreu, R. (2008). A study of the *Candida albicans* cell wall proteome. *Proteomics* **8**: 3871-3881.

Chamot, D. and Kuhlemeier, C. (1992). Differential expression of genes encoding the hypusine-containing translation initiation factor, eIF-5A, in tobacco. *Nucleic Acid Research* **20**(4): 665-669.

Chang, E. C., Kosman, D. J. and Willsky, G. R. (1989). Arsenic oxide-induced thermotolerance in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **171**: 6349-6352.

Chen, Z., Nunes M. A., and Rodrigues, C. R. (2004). Appressorium turgour pressure of *Colletotrichum kahawae* might have a role in coffee cuticle protection. *Mycologia* **96**(6): 1199-1208.

Chet, I. (1987). *Trichoderma*-Application, mode of action and potential as a biocontrol agent of soilborne pathogenic fungi. In: Chet, I. (Eds.), *Innovative approaches to plant disease control*. John Wiley & Sons, New York, Pp. 137-160.

Chet, I., Harman, G. E. and Baker, R. (1981). *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *phythium* spp. fungal parasites. *Microbial Ecology* **7**: 29-38.

Chet, I., Inbar, J. and Hader, Y. (1997). Fungal antagonists and mycoparasites. In: Wicklow, D.T. and Söderström, B. (Eds.), *The Mycota IV. Environmental and microbial relationships*. Springer, Berlin, Pp. 165-184.

Chevrot, R., Rosen, R., Haudecoer, E., Cirou, A., Shelp, B. J., Ron, E. and Faure, D. (2006). GABA controls the level of quorum-sensing signal in *Agrobacterium tumafaciens*. *Proceedings of the National Academy of Science, USA* **103**(19): 7460-7464.

Chi, Y., Hatakka, A. and Maijala, P. (2006). Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? *International Biodeterioration and Biodegradation* **59**:32-39.

Claydon, N, Allan, M., Hanson, J. R. (1987). Antifungal alkyl pyrones of *Trichoderma harzianum*. *Transactions of British Mycological Society* **88**(4): 503-513.

Coleman, S. T., Fang, T. K., Rovinsky, S. A., Turano, F. J. and Moye-Roweley, W. S. (2001). Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **276**:244-250.

Comporti, M. (1985). Lipid peroxidation and cellular damage in toxic liver injury. *Laboratory investigation* **53**: 599-623.

Cooke, R. C. and Rayner, A. D. M. (1984). Ecology of Saprotrophic Fungi. Longman, London.

Cooper, J. A and Sept, D. (2008). New insights into mechanisms and regulation of actin capping protein. *International Review of Cell and Molecular Biology* **267**: 183-206.

Coote, P. G., Cle, M. B. and Jones, M. V. (1991). Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH. *Journal of General Microbiology* **137**: 1701-1708.

Cortes, C., Gutierrez, A., Olmedo, V., Inbar, J., Chet, I. and Herrera-Estrella, A. (1998). The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. *Molecular and General Genetics* **260**:**2**18-225.

Costeas, P. A. and Chinsky, J. M. (2000). Glucocorticoid regulation of branched-chain α-ketoacid dehydrogenase E2 subunit gene expression. *Biochemical Journal* **347**: 449-457.

Crowe, J. D. and Olsson, S. (2001). Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Applied and Environmental Microbiology* **67**(5): 2088-2094.

Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Wistrom, C. A. (1987). Stabilization of dry phospholipids bilayers and proteins by sugars. *Biochemical Journal* **242**: 1-10.

Davis, D. J., Burlak, C., and Money, N. P. (2000). Osmotic pressure of fungal compatible osmolytes. *Mycological research* **104**: 800-804.

de la Cruz, J., Kressler, D. and Linder, P. (1999). Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box and related families. *Trends in Biochemical Sciences* **24**: 192-198.

Delgado-Jarana, J., Sousa, S., González, F., Rey, M. and Llobell, A. (2006). ThHog1 controls hyperosmotic stress response in *Trichoderma Harzianum*. *Microbiology* **152**: 1687-1700.

Dennis, C. and Webster, J. (1971). Antagonistic properties of species-groups of *Trichoderma*. *Transactions of British Mycological Society* **57**(1): 25-39.

Denslow, S. A., Reuschhoff, E. E. and Daub, M. E. (2007). Regulation of the *Arabidopsis thaliana* vitamin B_6 biosynthesis genes by abiotic stress. *Plant Physiology and Biochemistry* **45**:152-161.

Denslow, S. A., Walls, A. A. and Daub, M. E. (2005). Regulation of biosynthetic genes and antioxidant properties of B_6 during plant defense response. *Physiological and Molecular Plant Pathology* **66**: 244-255.

Demethon, K., Paoletti, M., Pinan-Lucarré, B., Loubradou-Bourges, N., Sabourine, M., Saupe, S. J., and Clavé, C. (2003). Rapamycin mimics the incompatibility reaction in the fungus *Podospora anserine*. *Eukaryotic Cell* **2**(2): 238-246.

Diano, A., Bekker-Jensen, S., Dynesen, J. and Nielsen, J. (2006). Polyol synthesis in Aspergillus niger: influence of oxygen availability, carbon and nitrogen sources on the metabolism. *Biotechnology and Bioengineering* **94**: 899-908.

Döhlemann, G., Berndt, P. and Hahn, M. (2006). Trehalose metabolism is important for heat stress tolerance and spore germination of *Botrytis cinerea*. *Microbiology* **152**: 2625-2634.

Dolashka-Angelova, P., Angelov, M., Genova, L., Stoeva, S. and Voelter, W. (1999). A novel Cu,Zn superoxide dismutase from the fungal strain *Humicola lutea* 110: isolation and physico-chemical characterization. *Spectrochimica Acta Part A* **55**: 2249-2260.

Donelly, D. P. and Boddy, L. (2001). Mycelial dynamics during interactions between *Strophiria caerulea* and other cord-forming saprotrophic basidiomycetes. *New Phytologist* **151**: 691-704.

Dowson, C.G., Rayner, A.D.M. and Boddy, L. (1988).The form and outcome of mycelial interactions involving cord-forming decomposer basidiomycetes in homogenous and heterogeneous environments. *New Phytology* **109**: 423-432.

Dukan, S., Farewell, A., Ballesteros, M., Taddei, F., Radman, M. and Nyström, T. (2000). Protein oxidation in response to increased transcriptional or translational errors. *Proceedings of the National Academy of Sciences USA* **97**(11): 5746-5749.

Dunn, W. B., Baily, N. C. J. and Johnson, H. E. (2005). Measuring the metabolome: Current analytical technologies. *Analyst* **130**: 606-625.

Duran, A. and Nombela, C. (2004). Fungal cell wall biogenesis: building a dynamic interface with the environment. *Microbiology* **150**: 3099-3103.

Edelmann, P. and Gallant, J. (1977). On the translational error theory of aging. *Cell* **10**:131-137.

Eggert, C., Temp, U., Dean, J. F. D. and Eriksson, K. E. L. (1996). A fungal metabolite mediates degradation of non-lignin structures and synthetic lignin. *FEBS Letters* **391**: 144-148.

Eis, C. and Nidetzky, B. (1999). Characterization of trehalase phosphorylase from *Schizophyllum commune. Biochemical Journal* **341**: 385-393.

Ehrenshaft, M. and Daub, M. E. (2001). Isolation of PDX2, a second novel gene in the pyridoxine biosynthesis pathway of Eukaryotes, Archaebacteria and a subset of Eubacteria. *Journal of Bacteriology* **183**(11): 3383-3390.

Elad, Y., Chet, I., Boyle, P. and Henis, Y. (1983). Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii* – SEM studies and fluorescence microscopy. *Phytopathology* **73**: 85-88.

Elad, Y., Chet, I and Henis, Y. (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum. Canadian Journal of Microbiology* **28**: 719-725.

Elf, J., Berg, O. G. and Ehrenberg, M. (2001). Comparison of repressor and transcriptional attenuator system for control of amino acid biosynthetic operons. *Journal of Molecular Biology* **313**: 941-954.

El Hajj, M., Rebuffat, S., Le Doan, T., Klein, G., Satre, M. and Bodo, B. (1989). Interaction of tricorzianines A and B with model membranes and with *Amoeba dictyostelium. Biochimica et Biophysica Acta* **978**: 97-104.

Enjalbert, B., Nantel, A. and Whiteway, M. (2003). Stress-induced gene expression in *Candida albicans*: absence of a general stress response. *Molecular Biology of the Cell* **14**: 1460-1467.

Eroglu, A., Russo, .M J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H. and Torner, M. (2000). Intracellular trehalose improves the survival of criopreserved mammalian cells. *Nature Biotechnology* **18**: 163-167.

Esterbauer, H., Schaur, R. J. and Zollner, H. (1991). Chemistry and Biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine* **11**: 81-128.

Eyler, D. E., Green, R. and Dever, T. E. (2009). Hypusine-containing protein eIF5A promotes elongation. *Nature* **459**:118-121.

Fernández, J., Soto, T., Franco, A., Vincente-Soler, J., Casando, J. and Gacto, M. (1998). *Fungal Genetic and Biology* **25**: 79-86.

Fink-Boots, M., Malarczyk, M. and Leonowicz, A. (1999). Increased enzymatic activities and levels of superoxide anion and phenolic compounds in cultures of basidiomycetes after temperature stress. *Acta Biotechnology* **4**: 319-330.

Florianowicz, T. (2000). Inhibition of growth and sporulation of Penicillium expansum by extracts of selected basidiomycetes. *Acta Societatis Botanicorum Poloniae* **69**: 263-267.

Fokkema, N. J. (1973). The role of saprotrophic fungi in antagonism against *Drechslera sorokiniana* (*Heliminthosporium sativum*) on agar plates and on rye leaves with pollen. *Physiological Plant Pathology* **3**: 195-205.

Freimoser, F. M., Hu, G. and St Leger (2005). Variations in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation in vitro. *Microbiology* **151**: 361-371.

Freitag M. and Morrell, J. J. (1992). Changes in selected enzyme activities during growth of pure and mixed cultures of the white-rot decay fungus Trametes versicolor and the potential biocontrol fungus *Trichoderma Harzianum. Canadian Journal of Microbiology* **38**: 317-323.

Gadd, G. M. (1995). Signal transduction in fungi, pp. 183-210. In: Gow, N. A. R. and Gadd, G. M. (Eds), *The Growing Fungus*. Chapman and Hall, London.

Game, J. (1993). DNA double strand breaks and RAD50-RAD57 genes in *Saccharomyces. Seminars in Cancer Biology* **4**: 73-83.

García, I., Lora, J. M., De La Cruz, J., Benítez, T., Llobell, A and Pintor-Toro, J. A. (1994). Cloning and characterization of a chitinase (CHIT42) cDNA from the mycoparasitic fungus *Trichoderma harzianum. Current Genetics* **27**(1): 83-89.

Garre, V., Tenberge, K. B. and Eising, R. (1998). Secretion of fungal extracellular catalase by *Claviceps purpurea* during infection of rye: Putative role in pathogenicity and suppression of host defense. *Biochemistry and Cell Biology* **88**(8): 743-753.

Gasser, B., Saloheimo, M., Rinas, U., Dragosits, M., Rodriguez-Carmona, E., Bauman, K., Guiliani, M., Parilli, E., Branduardi, P., Lang, C., Porro, D., Ferre, P., Tutino, M. L., Mattanovich, D. and Villaverde, A. (2008). Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. *Microbial Cell Factories* **7**: 11-29.

Gererwick, W. H., Bernart, W., Moghaddam, M. F., Jiang, Z. D., Solem, M. L. and Nagle, D. G. (1990). Eicosanoids from the Rhodophyta: new metabolism in algae. *Hydrobiologia* **204/205**(1): 621-628.

Glass, N. L. and Demethon, K. (2006). Non-self recognition and programmed cell death in filamentous fungi. *Current Opinion in Microbiology* **9**: 553-558.

Gloer, J. B. (1995). The chemistry of fungal antagonism and defense. *Canadian Journal of Botany* **73**(1): 1265-1274.

Gloer, J. B. and Trockenbrod (1988). Interference competition among coprophilous fungi: Production of (+)-Isoepoxydon by *Poronia punctata*. *Applied and Environmental Microbiology* **54**: 861-864.

Gómez-Vásquez, R., Day, R., Buschmann, H., Randles, S., Beeching, J. R. and Cooper, R. M. (2004). Phenylpropanoids, phenylalanine ammonia lyase and peroxidases in elicitor-challenged cassava (*Manihot esculenta*) suspension cells and leaves. *Annals of Botany* **94**: 87-97.

Gong, Z., Dong, C., Lee, H., Zhu, J., Xiong, L., Gong, D., Stevenson, B. and Zhu, J. (2005). A DEAD BOX RNA helicase is essential for mRNA export and import for development and stress response in *Arabidopsis*. *The Plant Cell* **17**: 256-267.

González, E., Danehower, D. and Daub, M. E. (2007). Vitamer levels, stress response, enzyme activity, and gene regulation of Arabidopsis lines mutant in the pyridoxine/pyridoxamine 5′-phosphate oxidase (*PDX3*) and pyridoxal B_6 salvage pathway. *Plant Physiology* **145**: 985-996.

Gonzalez, Del Val, A., Platas, G., Arenal, F., Orihuela, J. C., Garcia, M., Hernandez, P., Royo, I., De Pedro, N., Silver, L. L., Young, K., Vincent, M. F. and Palaez, F. (2003). Novel illudins from *Coprinopsis epicopalis* (syn. *Coprinus episcopalis),* and distribution of illudin-like compounds among filamentous fungi. *Mycological Research* **107**(10): 1201-1209.

Gooday, G. W. (1995). The architecture of fungal cells. In: Gow, N. A. R. and Gadd, G. M. (Eds.), *The Growing Fungus*. Chapman and Hall, London, Pp. 43-63.

Goodcare, R., Vaidyanathan, S., Dunn, W. B., Harrigan, G. G. and Kell, D. B. (2004). Metabolomics by numbers: acquiring and understanding the global metabolite data. *Trends in Biotechnology* **22**: 245-258.

Gothel, S. F., Marahiel and M. A. (1999). Peptidyl-propyl *cis-trans* isomerases, a superfamily of ubiquitous folding catalysts. *Cellular and Molecular Life Sciences* **55**: 423-436.

Gregorio, A. P. F. (2007). Studies on mycelial interactions between two species of tropical higher fungi. PhD Thesis. University of Westminster UK.

Gregorio, A. P. F., Da Silva, I. R., Sedarati, M. R. and Hedger, J. (2006). Changes in lignin degrading enzymes during interactions between mycelia of the tropical decomposer basidiomycetes *Marasmiellus troyanus* and *Marasmius pallescens*. *Mycological Research* **110**: 161-168.

Grein, S. and Pyerin, W. (1999). BTF3 is a potential new substrate for protein kinase CK2. *Molecular and Cellular Biochemistry* **191**: 121-128.

Griffith, G.S. and Boddy, L. (1991). Fungal decomposition of attached angiosperm twigs. IV. Effect of water potential on interactions between fungi on agar and in wood. *New Phytologist* **117**: 633-641.

Griffith, G.S., Rayner, A.D.M. and Wildman, H.G. (1994a). Interspecific interactions and mycelial morphogenesis of *Hypholoma fasciculare* (Agaricaceae). *Nova Hedwig* **59**: 47-75.

Griffith, G.S., Rayner, A.D.M. and Wildman, H.G. (1994b). Interspecific interactions, mycelial morphogenesis and extracellular metabolite production in *Phlebia radiata* (Aphyllophorales). *Nova Hedwig* **59**: 331-344.

Griffith, G. S., Rayner, A. D. M. and Wildman, H. G. (1994c). Interspecific interactions, mycelial morphogenesis and extracellular metabolite production in *Phlebia radiata* (Aphylophorales) *Nova Hedwigia* **59**: 311-329.

Grune, T., Jung, T., Merker, K. and Davies, K. J. A. (2004). Decreased proteolysis cause by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging and disease. *The International Journal of Biochemistry and Cell Biology* **36**:2519-2530.

Guettler, S., Jackson, E.N., Lucchese, S.A., Honaas, L., Green, A., Hittinger, C.T., Tian, Y., Lilly, W.W. and Gathman, A.C. (2003). ESTs from the basidiomycete *Schizophyllum commune* grown on nitrogen-replete and nitrogen-limited media. *Fungal Genetics and Biology* **91**: 191-198.

Guillen, F., Martínez, A. P. and Evans, C. S. (1994). Hydrogen peroxide producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl-alcohol oxidase. *Applied Microbiology and Biotechnology* **41**(4): 465-470.

Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y. and Aebersold, R. (2000). Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proceedings of the National Academy of Sciences, USA* **97**(17): 9390-9395.

Hahlbrock, K. and Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 347-369.

Halliwell, B. and Chirico, S. (1993). Lipid peroxidation: its mechanism, measurement and significance. *American Journal of Clinical Nutrition* **57**: 715S-725S.

Halliwell, B. and Gutteridge, J. M. C. (1989). Free radicals in biology and medicine. Clarendon Press, Oxford,

Hammel, K. E., Kapich, A. N., Jensen, Jr., K. A. and Ryan, Z. C. (2002). Reactive oxygen species as agents of wood decay by fungi. *Enzyme and Microbial Technology* **30**: 445-453.

Hashimoto, K., Tokimatsu, T., Kawano, S., Yoshizawa, A. C., Okudo, S., Goto, S. and Kanehisa, M. (2009). Comprehensive analysis of glycosyltransferases in eukaryotic genomes for structural and functional characterization of glycans. *Carbohydrate Research* **344**: 881-887.

Hatakka, A. (2001). Biodegradation of lignin. In: Hofritcher, M and Steinbüchel, A. (Eds.), *Biopolymers, Biology, Chemistry, Biotechnology, Applications, Vol. 1. Lignin, humic substances and coal*. Wiley-VCH, Weinheim, Pp. 129-180.

Hatvani, N., Kredics, L., Antal, Z. and Mécs, I. (2002). Changes in the activity of extracellular enzymes in dual cultures of *Lentinula edodes* and mycoparasitic *Trichoderma* strains. *Journal of Applied Microbiology* **92**: 415-423.

Heath, I. B. (1995). The cytoskeleton. In: Gow, N. A. R. and Gadd, G. M. (Eds.), *The Growing Fungus*. Chapman and Hall, London, Pp. 99-125.

Heaton, M. P. and Neuhaus, F. C. (1994). Role of the D-alanyl carrier protein in the biosynthesis of D-alanyl-lipoteichoic acid. *Journal of Bacteriology* 1**76**: 681-690.

Heilmann-Clausen, J. and Boddy, L. (2005). Inhibition and stimulation effects in communities of wood decay fungi: exudates from colonized wood influence growth by other species. *Microbial Ecology* **49**:399-406.

Heilmann-Clausen, J. and Christensen, M. (2003). Fungal diversity on decaying beech log – implications for sustainable forestry. *Biodiversity and conservation* **12**: 953-973.

Helenius, A. and Aebi, M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. *Annual Review of Biochemistry* **73**: 1019-1049.

Highley, T. L. and Ricard, J. (1988). Antagonism of *Trichoderma* spp. and *Gliocladium virens* against wood decaying fungi. *Material und Organismen* **23**(3): 157-169.

Hjeljord, L. and Tronsmo, A. (1998). *Trichoderma* and *Gliocladium* in biological control: an overview. In: Harman, G. E. and Kubicek, C. P. (Eds.), *Trichoderma and Gliocladium*. Taylor & Francis, London, Pp. 131-152.

Holdenrieder, O. and Greig, B.J.W. (1998). Biological methods of control. In: Woodward, S., Stenlid, J., Karjalainen, R. and Huttermannn, A. (Eds.), *Heterobasidion annosum Biology, Ecology, Impact and Control*. CAB International, Wallingford, Pp. 235-258.

Holmer, L., Renvall, P., Stenlid, J. (1997). Selective replacement between species of wood-rotting basidiomycetes, a laboratory study. *Mycological Research* **101**: 714-720.

Homma, K., Terui, S., Minemura, M., Qadota, H., Anraku, Y., Kanaho, Y. and Ohya, Y. (1998). Phosphatidylinositol-4-phosphate 5-kinase localized on the plasma membrane is essential for yeast cell morphogenesis. *The Journal of Biological Chemistry* **273**: 25779-15786.

Horie, K., Rakwal, R., Hirano, M., Shibato, J., Nam, H. W., Kim, Y. S., Kouzuma, Y., Agrawal, G. K., Masuo, Y. and Yonekura, M. (2008). Proteomics of two cultivated mushrooms *Sparassis crispa* and *Hericium erinaceum* provides insight into their numerous functional protein components and diversity. *The Journal of Proteome Research* **7**: 1819-1835.

Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Disease* **87**(1): 4-10.

Howell, C. R. (1987). Relevance of mycoparasitism in the biological control of Rhizoctonia solani by *Gliocladium virens*. *Phytopathology* **77**: 992-994.

Hulme, M. A. and Shields, J. K. (1970). Biological control of decay in wood by competition for non-structural carbohydrates. *Nature* **227**: 300-301.

Humphries, S. N., Bruce, A., Buultjens, E. and Wheatley, R. E. (2002). The effects of volatile microbial secondary metabolites on protein synthesis in *Serpula lacrymans*. *FEMS Microbiology Letters* **210**: 215-219.

Humphries, S. N., Wheatley, R. E. and Bruce, A. (2001). The effects of specific volatile compounds produced by *Trichoderma* spp. On the growth of wood decay basidiomycetes. *Holzforschung* **55**: 233-237.

Hynes, J., Müller, C. T., Jones, T. H. and Boddy, L. (2007). Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. *Journal of Chemical Ecology* **33**: 43-57.

Iakovlev, A. and Stenlid, J. (2000). Spatiotemporal patterns of laccase activity in interacting mycelia of wood-decaying basidiomycete fungi. *Microbial Ecology* **39**: 236-245.

Iakovlev, A., Olson, Å., Elfstrand, M. and Stenlid, J. (2004). Diefferential gene expression during interactions between *Heterobasidion annosum* and *Physisporinus sanguinolentus*. *FEMS Microbiology Letters* **241**: 79-85.

Ikediugwu, F. E. O. (1976). The interface in hyphal interference by *Peniophora gigantea* against *Heterobasidion annosum*. *Transactions of the British Mycological Society* **66**: 291-296.

Ikediugwu, F. E. O., Dennis, C. and Webster, J. (1970). Hyphal interference by *Peniophora gigantea* against *Heterobasidion annosum*. *Transactions of the British Mycological Society* **54**: 307-309.

Inbar, J. and Chet, I. (1995). The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. *Microbiology* **141**: 2823-2829.

Inbar, J. and Chet, I. (1992). Biomimics of fungal cell-cell recognition by the use of lectin-coated nylon fibers. *Journal of Bacteriology* **174**(3): 1055-1059.

Ivanov, E., Angelov, M., Slokoska, L., Pashova, S., Toshkova, R., Dolashka-Angelova, P., Dimitrova, P. and Voelter, W. (2002). Effect of Cu/Zn-superoxide dismutase from the fungal strain *Humicola lutea* 103 on antioxidant defense of graffi tumor-bearing hamsters. *Zeitschrift für Naturforschung* **57**: 197-204.

Jaworski, A., Kirschbaum, J. and Brückner, H. (1999). Structures of trichovirin II, peptaibol antibiotics from the mold *Trichoderma viride* NRRL 5243. *Journal of Peptide Science* **5**: 341-351.

Jazwinski, S. M. (2004). Yeast replicative life span – the mitochondrial connection. *FEMS Yeast Research* **5**:119-125.

Jeffries, P. (1997). Mycoparasitism. In: Wicklow, D.T. and Söderström, B. (Eds.), *The Mycota IV. Environmental and Microbial Relationships*. Springer, Berlin, Pp. 149-164.

Jewett, M. C., Hoffman, G. and Nielsen, J. (2006). Fungal metabolite analysis in genomics and phenomics. *Current Opinion in Microbiology* **17**: 191-197.

Joliffe, I. T. (1986). Principal Components analysis. Springer-Verlag, New York.

Jones, D. H. (1984). Phenylalanine ammonia-lyase: regulation of its induction and its role in plant development. *Phytochemistry* **23**: 1349-1359.

Jones, P. and Vogt, T. (2001). Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. *Planta* **213**: 164-174.

Jones, P. G., Mitta, M., Kim, Y., Jiang, W. and Inouye, M. (1996). Cold shock induces major ribosomal-associated protein than unwinds double-stranded RNA in *Escherichia coli. Proceedings of the National Academy of Sciences USA* **93**: 76-80.

Kalgatgi, K. K. and Rao, P. V. S. (1976). Regulation of L-phenylalanine ammonia-lyase from *Rhizoctonia solani. Journal of Bacteriology* **126**(2): 568-578.

Kallberg, Y., Oppermann, U., Jörnvall, H. and Persson, B. (2002). Short-chain dehydrogenases/reductases (SDRs) coenzyme-based functional assignments in completed genomes. *European Journal of Biochemistry* **269**: 4409-4417.

Kang, S. C., Park, S. and Lee, D. G. (1999). Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae. Journal of Invertebrate Pathology* **73**: 276-281.

Kang, S., Jumg, Y., Kim, C. and Song C. (1998). Extracellular and cytosolic iron superoxide dismutase from *Mycobacterium bovis* BCG. *Clinical and Diagnostic Laboratory Immunology* **5**(6):784-789.

Kauss, H. (1973). Turnover of galactosylglycerol and osmotic balance in Ochromonas. *Plant Physiology* **52**: 613-615.

Ketela, T., Green, R. and Bussey, H. (1999). *Saccharomyces cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. *Journal of Bacteriology* **181**(11): 3330-3340.

Kettering, M., Anke, H, Valdivia, C. and Sterner, O. (2005). Heptemerones A-G, seven novel iditerpenoids from *Coprinus heptemerus*: producing organisms, fermentation and biological activities. *Journal of Antibiotics (Tokyo)* **58**(6): 390-396.

Khan, A., Williams, K., Soon, J. and Nevalainen, H. K. M. (2008). Proteomic analysis of the knob-producing nematode-trapping fungus *Monacrosporium lysipagum. Mycological Research* **112**: 1447-1452.

Kim, S. H., Kronstad, J. W. and Ellis, B. E. (2001). Induction of phenyl alanine ammonia-lyase activity by tryptophan in *Ustalgo maydis*. *Phytochemistry* **58**: 849-857.

Kimura, K., Yamaoka, M. and Kamisaka, Y. (2003). Rapid estimation of lipids in oleaginous fungi and yeast using nile red fluorescence. *Journal of Microbiological Methods* **56**: 331-338.

Kobayashi, H., Suzuki, T. and Unemot, T. (1986). Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton-translocating ATPase. *Journal of Biological Chemistry* **261**: 627-630.

Korhonen, K., Lipponen, K., Bendz, M., Johansson, M., Ryen, I., Venn, K., Seiskari, P. and Niemi, M. (1993). Control of *Heterobasidion annosum* by stump treatment with 'rotstop,' a new commercial formulation of *Phlebiopsis gigantea*. 8th International Conference on Root and Butt Rot (Johansson, M. and Stenlid, J. Eds.), University of Agriculture, Uppsala, pp. 675-685.

Kruskal, W. H. and Wallis, W. A. (1952). Use of ranks in one criterion variance analysis. *Journal of the American Statistical Association* **47**: 583-621.

Krysan, D. J. (2009). The cell wall and endoplasmic reticulum stress responses are co-ordinately regulated in *Saccharomyces cerevisiae*. *Communicative and Integrative Biology* **2**(3): 233-235.

Kubicek, C. P., Mach, R. L., Peterbauer, C. K. and Lorito, M. (2001). *Trichoderma*: From genes to Biocontrol. *Journal of Plant Pathology* **83**(2): 11-23.

Kumar, S. and Punekar, N. S. (1997). The metabolism of 4-amino butyrate (GABA) in fungi. *Mycological Research* **101**(4): 403-409.

Kunze, M., Pracharoenwattana, I., Smith, S. M. and Hartig, A. (2006). A central role for the peroxisomal membrane in glyoxylate cycle function. *Biochimica et Biophysica Acta* **1763**: 1441-1452.

Kupka, J., Anke, T., Muzimoto, K., Ginetti, B. M. and Steglich, W. (1983). Antibiotics from basidiomycetes, XVII. The effect of marasmic acid on nucleic acid metabolism. *Journal of Antibiotics* **34**: 155-160.

Kurita, O. (2003). Overexpression of peroxisomal malate dehydrogenase *MDH3* gene enhances cell death on H2O2 stress in the *ald5* mutant of *Saccharomyces cerevisiae*. *Current Microbiology* **47**: 192-197.

Kurosaki, F., Tashiro, N. and Nishi, A. (1986). Induction of chitinase and phenylalanine ammonia-lyase in cultured carrot cells treated with fungal mycelial walls. *Plant and Cell Physiology* **27**(8): 1587-1591.

Kurova, A. V., Kupletskaya, M. B., Skrynnikova, E. V. and Somova, N. G. (2001). Search for micromycetes producing extracellular catalase and study of conditions of catalase synthesis. *Applied Biochemistry and Microbiology* **37**(1): 59-64.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Larson, T. G., Choi, G. H. and Nuss, D. L. (1992). Regulatory pathways governing modulation of fungal gene expression by a virulence-attenuating mycovirus. *EMBO Journal* **11**: 4539-4584.

Le Doan, T., El Hajj, M., Rebuffat, S., Rajeswari, M. R. and Bodo, B. (1986). Flueorescein studies of interaction of trichorzianine A IIIc with model membranes. *Biochimica et Biophysica Acta* **858**: 1-5.

Lee, B., Yoshida, Y. and Hasunuma, K. (2009). Nucleoside diphosphate kinase-1 regulates hyphal development via the transcriptional regulation of catalase in *Neurospora crassa*. *FEBS Letters* **583**:3291-3295.

Lee, J., Jung, H. and Kim, S. (2003). 1,8-dihydroxynaphthalene (DHN)-melanin biosynthesis inhibitors increase erythritol production in *Torula coralline*, and DHN-melanin inhibits erythrose reducatse. *Applied and Environmental Microbiology* **69**(6): 3427-3434.

Levin, D. E. (2005). Cell wall integrity signalling in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews* **69**(2): 262-291.

Levine, R. L. (1983). Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. *Journal of Biological Chemistry* **258**:11823-11827.

Levine, R. L. (2002). Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radical Biology and Medicine* **32**:790-796.

Li, D., Alic, M., Brown, J. A. and Gold, M. H. (1995). Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. *Applied and Environmental Microbiology* **61**: 341-345.

Li, Q., Harvey, L. M. and McNeil, B. (2008). Oxygen enrichment effects on protein oxidation, proteolytic activity and energy status of submerged batch cultures of *Aspergillus niger* B1-D. *Process Biochemistry* **43**:238-243.

Li, Q., Abrashev, R., Harvey, L. M. and McNeil, B. (2008). Oxidative stress-associated impairment of glucose and ammonia metabolism in the filamentous fungus, *Aspergillus niger* B1-D. *Mycological Research* **112**: 1049-1055.

Li, Q., Harvey, L. M. and McNeil, B. (2008). The effects of elevated process temperature on the protein carbonyls in the filamentous fungus, *Aspergillus niger* B1-D. *Process Biochemistry* **43**: 877-881.

Li, W. and Ye, Y. (2008). Polyubiquitin chains: functions, structures, and mechanisms. *Cellular and Molecular Life Sciences* **65**: 2397-2406.

Lim, E. and Bowles, D. J. (2004). A class of plant glycosyltransferases involved in cellular homeostasis. *The EMBO Journal* **23**: 2915-2922.

Liu, F., Ng, S. K., Lu, Y., Low, W., Lai, J. and Jedd, G. (2008). Making two organelles from one: Woronin body biogenesis by peroxisomal protein sorting. *Journal of Cell Biology* **180**: 325-329.

Liu, X., Villalta, P. W. and Sturla, S. J. (2009). Simultaneous determination of inositol and inositol phosphates in complex biological matrices: quantitative ion-exchange chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **23**: 705-712.

Liu, Y., Wu, R., Wan, Q., Xie, G. and Bi, Y. (2007). Glucose-6-phosphate dehydrogenase plays a pivotal role in nitric oxide-involved defense against oxidative stress under salt stress in red kidney been roots. *Plant Cell Physiology* **48**(3): 511-522.

Lorito, M., Woo, S. L., D'Ambrosio, M., Harman, G. E., Kubicek, C. P and Scala, F. (1996). Synergistic interaction between cell wall degrading enzymes and membrane affecting compounds. *Molecular plant-microbe interactions* **9**: 206-213.

Lu, K. P., Hanes, S. D. and Hunter, T. (1996). A human Peptidyl-propyl isomerase essential for regulation of mitosis. *Nature* **380**: 544-547.

Lu, Z., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M. and Jansson, J. K. (2004). *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter system. *Applied and Environmental Microbiology* **70**(5): 3073-3081.

Lyr, H (1958). Die Inuktion der laccase-bildung bei *Collybia velutipes* Curt. *Archiv Für Microbiologie* **28**: 310-324.

Lyr, H. (1963). Enzymatiscjes detoxifikation chlorierter phenole. *Phytopathologie Zeitscchrift* **38**: 342-354.

Mach, R. L., Peterbauer, C. K., Payer, K., Jaksits, S., Woo, S. L., Zeilinger, S., Kullnig, C M., Lorito, M. and Kubicek, C. P. (1999). Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. *Applied and Environmental Microbiology* **65**: 1858-1863.

Magnuson, J. K. and Lasure, L. L. (2004). Organic acid production by filamentous fungi. In: Lange, J. and Lange, J. (Eds.), Advance in fungal Biotechnology for industry, Agriculture and Medicine. Washington; Kluwer Academic/ Plenum Publishers, pp. 307-340.

Magnuson, K., Jackowski, S., Rock, C. O. and Cronan, J. E., Jr. (1993). Regulation of fatty acid biosynthesis in *Escherichia coli*. *Microbiological Reviews* **57**: 522-542.

Margolles-Clark, E., Harman, G. E., Penttilä, M. (1996). Enhanced expression of endochitinase in *Trichoderma harzianum* with cbh1 promoter of *Trichoderma reesei*. *Applied and Environmental Microbiology* **62**: 2152-2155.

Malarczyk, E., Korszen-Pilecka, I. and Leonwicz, A. (1989). Production of ferro-phenolic complex by *Norcadia opaca* and metabolism of phenolic acids in bacterial cultures. *Phytochemistry* **28**: 415-418.

Malarczyk, E., Zinko, E., Nowak, G., Ziaja, J., Kochmánska-Rdest, J. and Leonwicz, A. (1997). The relation between the concentration of phenolics and the activity of manganese peroxidase in cultures of two species of *Pleurotus* during fruit body formation. Proceedings of the TAPPI Biological Sciences Symposium, San Francisco, pp. 391-393.

Malhotra, J. D. and Kaufman, J. R. (2007). The endoplasmic reticulum and the unfolded protein response. *Seminars in Cell and Developmental Biology* **18**: 716-731.

Managbanag, J. R. and Torzilli, A. P. (2002). An analysis of trehalase, glycerol and mannitol accumulation during heat and salt stress in a marsh isolate of *Aureobasidium pullulans*. *Mycologia* **94**(3): 384-391.

Marklund, S. L. (1984). Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochemical Journal* **222**:649-655.

Marklund, S. and Marklund, G. (1974). Involvement of the superoxide anion radical in autoxidation of pyrogalol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry* **47**: 469-474.

Markovich, N. A. and Kononova, G. L. (2003). Lytic enzymes of *Trichoderma* and their role in plant defense from fungal disease: A Review. *Applied Microbiology and Microbiology* **39**(4): 341-351.

Mashego, R., Rumbold, K., De May, M., Vandamme, E., Soetaert, W. and Heijnen, J. J. (2007). Microbial metabolomics: past, present and future methodologies. *Biotechnology Letters* **29**: 1-16.

Matouschek, A., Rospert, S., Schmid, K., Glick, B. S. and Schatz, G. (1995). Cyclophilin catalyses protein folding in yeast mitochondria. *Proceedings of the National Academy of Sciences USA* **92**: 6319-6323.

Mayer, D. C. G., Jiang, L., Achur, R. N., Kakizaki, I., Gowda, D. C. and Miller, L. H. (2006). The glycophorin C N-linked glycan is a critical component of the ligand for the *Plasmodium falciparum* erythrocyte receptor BAEBL. *Proceedings of the National Academy of Sciences USA* **103**: 2358-2362.

Mazzucotelli, E., Tatari, A., Cattivelli, L. and Forlan, G. (2006). Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. *Journal of Experimental Botany* **57**(14): 3755-3766.

Mikosch, T. S. P., Sonnerberg, A. S. M. and Van Griensven, L. J. L. D. (2001). Isolation characterization and expression of patterns of DCM1 homolog from the basidiomycete *Pleurotus ostreatus*. *Fungal genetics and Biology* **33**: 59-66.

Miyashita, Y. and Good, A. G. (2008). Contribution of GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*. *Plant Cell Physiology* **49**(1): 92-102.

Momany, M. and Jedd, G. (2002). Mapping woronin body position in *Aspergillus nidulans*. *Mycologia* **94**(2): 260-266.

Montero-Lomeli, M., Morais, B. L., Figueiredo, D. L., Neto, D. C., Martins, J. R. and Masuda, C. A. (2002). The initiation factor Eif4A is involved in the response to lithium stress in Saccharomyces cerevisiae. *Journal of Biological Chemistry* **277**: 21542-21548.

Morlang, S., Weglohner, W. and Franeschi, F. (1999). Hera from *Thermus thermophilus*: the first thermostable DEAD-box helicase with an R Nase P protein motif. *Journal of Molecular Biology* **294**: 795-805.

Nakano, K. and Mabuchi, I. (2006). Atin-capping protein is involved in controlling organization of actin cytoskeleton together with ADF/cofilin, profiling and F-actin crosslinking proteins in fission yeast. *Genes to Cells* **11**: 893-905.

Nathan, C., and Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proceedings of the *National Academy of Sciences USA* **97**: 168841-168848.

Nehls, U., Ecke, M. and Hampp, R. (1999). Sugar- and nitrogen-dependent regulation of an *Amanita muscaria* phenylalanine ammonium lyase gene. *Journal of Bacteriology* **181**(6): 1931-1933.

Niemelä, T., Renvall, P. and Pentillä, R. (1995). Interactions of fungi at late stages of wood decomposition. *Annales Botanici Fennici* **32**:141-152.

Noltman, E. A., Gulber, C. J. and Kuby, S. A. (1961). Glucose 6-phosphate dehydrogenase (zwischenferment). I. Isolation of the crystalline enzyme from yeast. *Journal of Biological Chemistry* **231**:1225-1230.

Nyström, T. (2004). Role of oxidative carbonylation in protein quality control and senescence. *EMBO Journal* **24**(7): 1311-1317.

Oćon, A., Hampp, R. and Requena, N. (2007). Trehalose turnover during abiotic stress in arbuscular mycorrhizal fungi. *New Phytologist* **174**: 879-891.

O'Farell, P. Z., Goodman, H. M. and O'Farell, P. H. (1997). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* 12: 133-141.

O'Hagan, S., Dunn, W. B., Brown, M., Knwoles, J. D. and Kell, D. B. (2005). Closed-loop multiobjective optimization of analytical instrumentation: Gas Chromatography/Time-of-Flight Mass Spectrometry of metabolomes of human serum and of yeast fermentations. *Analytical Chemistry* **77**: 290-303.

Oliver, C. N., Ahn B. W., Moerman, E. J., Goldstein, S. and Stradtman, E. R. (1987). Age-related changes in oxidized proteins. *Journal of Biological Chemistry* **262**:5488-5491.

Owens, E. M., Reddy, C. A. and Grethlein, H. E. (1994). Outcome of interspecific interactions among brown-rot and white rot wood decay fungi. *FEMS Microbiology Ecology* **14**: 19-24.

Padmanabha, R., Chen-Wu, J. L., Hanna, D. E. and Glover, C. V. (1990). Isolation, sequencing and distribution of yeast CKA2 gene: Casein Kinase II is essential for viability in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10**: 4089-4099.

Panagiotou, G., Villas-Bôas, S. G., Christakopoulos, P., Nielsen, J. and Olsson, L. (2005). Intracellular metabolite profiling of *Fusarium oxysporum* converting glucose to ethanol. *Journal of Biotechnology* **115**: 425-434.

Panaretou, B. and Piper, W. (1992). Plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and entry into stationary phase. *European Journal of Biochemistry* **206**: 635-640.

Pandey, A. K., Jain, P., Podila, G. K., Tudzynski, B. and Davis, R. M. (2009). Cold induced *Botrytis cinerea* enolase (*BcEnol-1*) functions as a transcriptional regulator and is controlled by cAMP. *Molecular Genetics and Genomics* **281**: 135-146.

Park, J., Aravind, L., Wolff, E. C., Kaevel, J., Kim, Y. S. and Park, M. H. (2006). Molecular cloning, expression and structural prediction of deoxyhypusine hydroxylase: A HEAT-Repeat-containing metaloenzyme. *Proceedings of the National Academy of Sciences USA* **103**(1): 51-56.

Pearce, M.H. (1990). In vitro interactions between *Armillaria luteobubalina* and other wood decay fungi. *Mycological Research* **94**: 753-761.

Pearce, M.H., Nelson, E.E. and Malajczuk, N. (1995). Effect of the cord-forming saprotrophs *Hypholoma australe* and *Phanerochaete filamentosa* and of ammonium sulphamate on establishment of *Armillaria luteobubalina* on stumps of *Eucalyptus diversicolor*. *Mycological Research* **99**: 951-956.

Peberdy, J. F. (1990). Fungal cell walls-a review. In: Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W. and Copping, L. G. (Eds.), *Biochemistry of cell walls and membranes in fungi*. Springer-Verlag, Berlin, Pp. 5-30.

Peiris, D., Dunn, W. B., Brown, M., Kell, D. B., Roy, I and Hedger, J. N. (2008). Metabolite profiles of interacting mycelial fronts differ for pairings of wood decay basidiomycete fungus, *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminatus*. *Metabolomics* **4**: 52-62.

Peiris, D. G. (2009). Interspecific interactions between decomposer fungi. PhD Thesis. . University of Westminster UK.

Perera, N. M., Michell, R. H. and Dove, S. K. (2004). Hypo-osmotic stress activates Plc1p-dependent phosphatidylinositol 4,5-biphosphate hydrolysis and inositol hexakiphosphate accumulation in yeast. *The Journal of Biological Chemistry* **279**(7): 5216-5226.

Peters, S., Draeger, S., Aust, H.-J., Schulz, B. (1998). Interactions in dual cultures of endophytic fungi with host and nonhost plant calli. *Mycologia* **90**:360-367.

Pham, X. H., Reddy, M. K., Ehtesham, N. Z., Matta, B. and Tuteja, A. (2000). DNA helicase from *Pisum sativum* is homologous to translation initiation factor and stimulates topoisomerase I activity. *The Plant Journal* **24**: 219-229.

Pinan-Lucarré, B., Paoletti, M. and Clavé, C. (2007). Cell death by incompatibility in the fungus *Podospora*. *Seminars in Cancer Biology* **17**: 101-111.

Piper, P. W. (1995). The heat shock and ethanol stress responses in yeast exhibit similarity and function overlap. *FEMS Microbiology Letters* **134**: 121-127.

Popov, V. N., Volvenkin, S. V., Kosmatykh, T. A., Saud, A., Schnarrenberger, C. and Epritcev, A. (2001). Induction of a peroxisomal malate dehydrogenase isoform in liver of starved rats. *Biochemistry (Moscow)* **66**(5): 496-501.

Prescott, L. M., Harley, J. P. and Klein, D. A (1999). Microbiology, (4th Edition). McGraw Hill, New York, Pp. 48-50; 123-124; 125; 242; 909.

Qin, G., Tian, Z., Chan, Z. and Li, B. (2007). Crucial role of antioxidant proteins and hydrolytic enzymes in pathogenicity of *Penicillium expansum*. *Molecular and Cellular Proteomics* **6.3**: 425-438.

Ramirez, M. L., Chulze, S. N., and Magan, N. (2004). Impact of osmotic and matric water stress on germination, growth, mycelial water potentials and endogenous accumulation of sugars and sugar alcohols in *Fusarium graminearum. Mycologia* **96**(3): 470-478.

Ramos, H., Attias-De-Murciano, A., Cohen, B. E. and Bolard, J. (1989). The polyene antibiotic amphotericin B acts as a calcium ionophore in sterol-containing liposomes. *Biochimica et Biophysica Acta* **982**: 303-306.

Rayner, A. D. M. and Todd, N. K. (1979). Population and community structure and dynamics of fungi in decaying wood. *Advances Botanical Research* **7**:333-420.

Rayner, A.D.M. and Webber, J.F. (1984). Interspecific mycelial interactions- an overview. In: Jennings, D.H. and Rayner A.D.M. (Eds.), *The ecology and physiology of the fungal mycelium*. Cambridge University Press, Cambridge, Pp. 383-417.

Rayner, A. D. M. and Boddy, L. (1988). Fungal decomposition of wood; its Biology and Ecology. John Wiley & Sons, Chichester.

Rayner, A. D. M. and Boddy, L. (1988). Fungal communities in the decay of wood. *Advances in Microbial Ecology* **10**: 115-166.

Rayner, A. D. M. (1991). The challenge of the individualistic mycelium. *Mycologia* **83**: 48-71.

Rayner, A. D. M. (1991). Conflicting flows – the dynamics of mycelia territoriality. *McIlvainea* **10**: 24-35

Rayner, A. D. M., Griffith, G. S. and Wildman, H. G. (1994). Induction of metabolic and morphogenetic changes during mycelial interactions among species of higher fungi. *Biochemical Society Transactions* **22**: 2389-2394.

Rayner, A.D.M., Griffith, G.S. and Ainsworth, A.M. (1995). Mycelial interconnectedness. In: Gow, N.A.R. and Gadd, G.M. (Eds.), *The growing fungus*. Chapman and Hall, London, Pp. 20-40.

Rayner, A. D. M. (1997). Degrees of freedom. Living in dynamic boundaries. Imperial College Press, London.

Reed, R. H., Collins, J. C., and Russel, G. (Effect of salinity upon galactosyl-glycerol content and concentration of marine red alga *Porphyra purpurea* (Roth) C. Ag. *Journal of Experimental Botany* **31**(125): 1539-1554.

Reinhammar, B., Ma;strom, B. G. (1981). "Blue" copper-containing oxidases. In: Spiro, T. G. (Ed.), *Copper proteins (Metal ions in Biology, Vol. 3)*. Wiley, New York, Pp. 109-149.

Renvall, P. (1995). Community structure and dynamics of wood-rotting basidiomycetes on decomposing conifer trunks in northern Finland. *Karstenia* **35**:1-51.

Reznick, A. Z. and Packer, L. (1994). Oxidative damage of proteins: spectrophotometric method for carbonyl assay. *Methods in Enzymology* **233**: 357-363.

Richie, D. L., Hartl, L., Aimanianda, V., Winters, M. S., Fuller, K. F., Miley, M. D., White, S., Mccarthy, J. W., Latge, J., Feldmesser, M., Rhodes, J. C. and Askew, D. S. (2009). A role of the unfolded protein response (UPR) in virulence and antifungal susceptibility in *Aspergillus fumigatus*. *PLoS Pathogens* **5**(1): 1-17.

Rishbeth, J. (1971). In: Nordin, V. J. (Ed.), *Biological control of Forest Diseases*. Canadian Forest Service Ottawa, Pp. 29-35.

Ristilä, M., Matxain, J. M., Strid, A. and Eriksson, L. (2006). pH-dependent electronic and spectroscopic properties of pyridoxine (Vitamin B_6). *Journal of Physical Chemistry* **110**: 16774-16780.

Rohrbough, J. G., Galgiani, J. N. and Wysocki, V. H. (2007). The application of proteomic techniques to fungal protein identification and quantification. *Annals of the New York Academy of Science* **1111**: 133-146.

Ron, D. and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular Cell Biology* 8: 519-529.

Rosa, L. H., Cota, B. B., Machado, K. M. G., Rosa, C. A. and Ziani, C. L. (2005). Antifungal and other biological activities of from *Oudemansiella canarii*. *World Journal of Microbiology and Biotechnology* **21**(6-7): 983-987.

Ross, J., Li, Y., Lim, E-K., and Bowles, D. J. (2001). Higher plant glycosyltransferases. *Genome Biology* **2**: 3004.1-3004.6.

Roy, G., Laflamme, G., Brussieres, G. and Dessureault, M. (2003). Field tests and biological control of *Heterobasidion annosum Phaeotheca dimorphospora* in comparison with *Phlebiopsis gigantea*. *Forest Pathology* **33**: 127-140.

Ruiz-Dueñas, F. J., Guillén, F., Camarero, S., Pérez-Boada, M., Martínez, M. J. and Martínez, A. T. (1999). Regulation of peroxidation transcript levels in liquid cultures of the lignolytic fungus, *Pleurotus eryngii*. *Applied and Environmental Microbiology* **65**: 4458-4463.

Saini, P., Eyler, D. E., Green, R. and Dever, T. E. (2009). Hypusine-containing protein eIF5A promotes translation elongation. *Nature* **459**: 118-121.

Sakamoto, Y., Ando, A., Tamai, Y., Miura, K. and Yajima, T. (2002). Protein expression during fruit body induction of *Flammulina velutipes* under reduced temperature. *Mycological Research* **106**(2): 222-227.

Saloheimo, M., Niku-Paavola, M. L. and Knowles, J. K. C. (1991). Isolation and structural analysis of the laccase gene of the lignin-degrading fungus *Phlebia radiata*. *Journal of General Microbiology* **137**: 1537-1544.

Sanan-Mishra, N., Pham, X. H., Sopory, S. K. and Tuteja, N. (2005). Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. *Proceedings of the National Academy of Sciences USA* **102**: 509-514.

Sato, S., Liu, F., Koc, H. and Tien, M. (2007). Expression analysis of extracellular proteins from *Phanerochaete chrysosporium* grown on different liquid and solid substrates. *Microbiology* **153**: 3023-3033.

Saupe, S. J. (2000). Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. *Microbiology and Molecular Biology Reviews* **64**(3): 489-502.

Savoie, J.-M., Mata, G. and Billete, C. (1998). Extracellular laccase production during fungal interactions between *Trichoderma* sp. and Shitake, *Lentinula edodes*. *Applied Microbiology and Biotechnology* **49**: 589-593.

Savoie, J. M. and Mata, G. (1999). The antagonistic action of *Trichoderma* sp. hyphae to *Lentinula edodes* hyphae changes in lignocellulytic activities during cultivation in wheat straw. *World Journal of Microbiology and Biotechnology* **15**: 369-373.

Schweizer E. and Hoffman, J. (2004). Microbial fatty acid synthase (FAS): major players in a network of cellular FAS systems. *Microbiology and Molecular Biology Reviews* **68**(3):501-517.

Schirmböck, M., Lorito, M., Wang, Y., Hayes, C. K., Arisan-Atac, I., Scala, F., Harman, G. E. and Kubicek, C. P. (1994). Parallel formation of and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Applied and Environmental Microbiology* **60**(12): 4364-4370.

Schmid, J. and Harod, F. M. (1988). Dual roles of calcium ions in apical growth of *Neurospora crassa*. *Journal of General Microbiology* **134**: 2623-2631.

Schoeman, M. W., Webber, J. F. and Dickinson, D. J. (1996). The effects of diffusible metabolites of *Trichoderma harzianum* on in vitro interactions between basidiomycete isolates at two different temperature regimes. *Mycological Research* **100**:1454-1458.

Schönbrunner, E. R., Myer, S., Tropschugs, M., Fisher, G., Takashi, N. and Schmid, F. X. (1991). Catalysis of protein folding by cyclophilins from different species. *The Journal O Biological Chemistry* **266**(6): 3630-3635.

Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W. and Benink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**: 770-774.

Score, A.J., Bruce, A., King, B. and Palfreyman, J.W. (1998). The biological control of *Serpula lacrymans* by *Trichoderma* species. *Holzforschung* **52**: 124-132.

Score, A.J., Palfreyman, J.W. and White, N.A. (1997) Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions. *International Biodeterioration Biodegradation* **33**: 115-128.
Scott, J. H. and Schekman, R. (1980). Lyticase: Endoglucanase and protease activities that act together in yeast cell lysis. *Journal of Bacteriology* **142**(2): 414-423.

Scrimale, T., Didone, L., De Mesy Bentley, K. L. and Krysan, D. J. (2009). The unfolded protein response is induced by the cell wall integrity Mitogen-activated protein kinase pathway and required for cell wall integrity in Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews* **20**: 164-175.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carnici, P., Hayashizaki, Y. and Shinozaki, K. (2001). Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stress by using full-length cDNA microarray. *Plant Cell* **13**: 61-72.

Semighini, C. P., Hornby, J. B., Dumitru, R., Nickerson, K. W. and Steven, D. H. (2006). Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. *Molecular Microbiology* **59**(3): 753-764.

Serrano, R., Kielland-Brandt, M. C. and Fink, G. R. (1986). Yeast plasma membrane ATPase is essential for growth and has homology with (Na+ + K+), K+- and Ca2+-ATPases. *Nature* **319**: 689-693.

Shafqat, N., Shafqat, J., Eissner, G., Marschall, H. U., Tryggvasson, K. Erriksson, U. Gabrielli, F., Lardy, H., Jörnvall, H. and Oppernmann, U. (2006). Hep27, a member of the short-chain dehydrogenase/reductase family, is an NADPH-dependent carbonyl reductase in vascular endothelial tissue. *Cellular and Molecular Life Sciences* **63**: 1205-1213.

Shelp, B. J., Brown, A. W. and Faure, D. (2006). Extracellular γ-aminobutyrate mediates communication between plants and other organism. *Plant Physiology* **142**: 1350-1352.

Shen, B., Hohman, S., Jensen, R. G., and Bohnert, H. J. (1999). Roles of sugar alcohols in osmotic stress and adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. *Plant Physiology* **121**: 45-52.

Shenton, D. and Grant, C. M. (2003). Protein s-thiolation targets glycolysis and protein synthesis in response to oxidative stress in yeast *Saccharomyces cerevisiae*. *Biochemical Journal* 3**74**: 513-515

Shi, H., Xiong, L., Stevenson, B., Lu, T and Zhu, J. K. (2002). *Arabidopsis* salt overly sensitive 4 mutants uncover a critical role of vitamin B_6 in plant salt tolerance. *Plant Cell* **14**: 575-588.

Silberboth, S., Sterner, O., Erkel, G. and Anke, T. (2000). The irpexans, a new group of biologically active metabolites produced by basidiomycete, *Irpex* sp. 93025. *Journal of Antibiotics (Tokyo)* **53**(10): 1137-1144.

Silver, P. A. (1991). How proteins enter the nucleus. *Cell* **64**: 489-497.

Singer, M. A. and Linquist, S. (1998). Multiple effects of trehalose on protein folding in vitro and in vivo. *Molecular Cell* **1**: 639-648.

Singh, H., Derwas, N. and Poulos, A. (1987). Beta-oxidation of very-long-chain fatty acids and their coenzyme A derivatives by human skin fibroblasts. *Archives of Biochemistry and Biophysics* **254**(2): 526-33.

Singh, R., Mailloux, R. J., Puiseux-Dao, S. and Appanna, V. D. (2007). Oxidative stress evokes a metabolic adaptation that favours increased NADPH synthesis and decreased NADH production in *Pseudomonas fluorescens*. *Journal of Bacteriology* **189**(18): 6665-6675.

Slifkin, M. and Cumbie, R. (1988). Congo red fluorochrome for the rapid detection of fungi. *Journal of Clinical Microbiology* **26**(5): 827-830.

Snedden, W. A. and Fromm, H. (1999). Regulation of the γ-aminobutyrate-synthesizing enzyme, glutamate decarboxylase, by calcium-calmodulin: a mechanism for rapid activation in response to stress. In: Lerner, H. R. (Ed.), *Plant response to environmental stresses: From phytohormones to genome reorganization*. Marcel Dekker, New York, Pp 549-574.

Solomon, P. S. and Oliver, R. P. (2002). Evidence that γ-aminobutyric acid is a major nitrogen source during *Cladosporium fulvum* infection of tomato. *Planta* **214**: 414-420.

Sonnenbichler, J., Bliestle, I. M., Peipp, H. and Holdenrieder, O. (1989). Secondary fungal metabolites and their biological activities, I. Isolation of antibiotic compounds from cultures of *Heterobasidion annosum* synthesized in the presence of antagonistic fungi or host plant cells. *Biological Chemistry Hoppe-Seyler* **370**: 1295-1303.

Sonnenbichler, J., Jurgen, D. and Peipp, H. (1994) Secondary fungal metabolites and their biological activities, V. Investigations concerning the induction of the biosynthesis of toxic secondary metabolites in basidiomycetes. *Biological Chemistry Hoppe-Seyler* **375**: 71-79.

Sonnenbichler, J., Peipp, H. and Dietrich, J. (1993). Secondary fungal metabolites and their biological activities, I. Further metabolites from dual cultures of the antagonistic basidiomycetes Heterobasidion annosum and *Gleophyllum abietinum. Biological Chemistry Hoppe-Seyler* **374**: 467-473.

Soundarajan, S., Jedd, G., Li, X., Ramos-Pamplona, M., Chua, N. H. and Nagvi, N. I. (2004). Woronin body function in *Magnaporthe grisea* is essential for efficient pathogenesis and for survival during nitrogen starvation stress. *The Plant Cell* **16**: 1556-1574.

Srebotnik, E., Jensen Jr., K. A. and Hammel, K. E. (1994). Fungal degradation of recalcitrant non-phenolic lignin structures without lignin peroxidase. *Proceedings of the National Academy of Science, USA* **91**(26):12794–12797.

Stassen, N. Y., Logsdon,, Jr., J. M., Vora, G. J., Palmer., J. D. and Zolan, M. E. (1997). Isolation and characterisation of *rad51* ortologs from *Coprinus cinereus* and *Lycopersicon esculentum*, and phylogenetic analysis of eukaryotic *recA* homologs. *Current Genetics* **31**: 144-157.

Staszczak, M. (2008). The role of the ubiquitin-proteasome system in the response of the lignolytic fungus Trametes versicolor to nitrogen deprivation. *Fungal Genetics and Biology* **45**: 328-337.

Stradtman, E. R. (1992). The theory of glutamine synthetase regulation. *Journal of Biological Chemistry* **276**: 44357-44364.

Strongman, D. B., Miller, J. D., Calhoun, L., Findlay, J. A. and Whitney, N. J. (1987). The biochemical basis for interference competition among some lignocolous marine fungi. *Botanica Marina* **30**:21-26.

Strunz, G. M., Kakushima, M. and Stillwell, M. A. (1972). Scytalidin: a new fungitoxic metabolite produced by Scytalidium species. *Journal of Chemical Society, Perkin, Transactions* **1**: 2280-2283.

Sudman, V. and Näse, L. (1972). The synergistic ability of some wood-degrading fungi to transform lignins and lignosulfonates on various media. *Archives of Microbiology* **86**: 339-348.

Sujiyama, S.-I. and Minami, M. (2005). Production of phenol-oxidizing enzymes in the interaction between white-rot fungi. *Mycoscience* **46**: 268-271.

Svensson, M., Lundgren, L. N., Woods, C., Fatehi., J. and Stenlid, J. (2000). Pyrone and pyridone compounds in the liquid culture of *Physisporinus sanguinolentus*. *Phytochemistry* **56**(7): 747-751.

Sykes, K., Gething, M.-J. and Sambrook, J. (1993). Proline isomerases function during heat shock. *Proceedings of the National Academy of Sciences USA* **90**: 5853-5857.

Takahashi, S., Tatagiri, T., Hirayama, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2001). Hyperosmotic tress induces a rapid and transient increase in inositol-1,4,5-triphosphate independent of abscisic acid in *Arabidopsis cell* culture. *Plant Cell Physiology* **42**(2): 214-222.

Tanner, N. K. and Linder, P. (2001). DExD/H box RNA helicases: From generic motors to specific dissociation functions. *Molecular Cell* **8**: 251-262.

Teerapatsakul, C., Bucke, C., Para, R. Kehsavarz, T. and Chitradon, L. (2007). Dye decolourization by laccase entrapped in copper alginate. *World Journal of Microbiology and Biotechnology* **24**(8): 1367-1374.

Teixeira, M. C., Santos, P. M., Fernandes, A. R. and Sa-Correia, I. (2005). A proteome analysis of the yeast response to the herbicide 2,4-dichlorophenoxyacetic acid. *Proteomics* **5**:1889-1901.

Tey, W. K., North, A. L., Reyes, J. L., Lu, Y. F. and Jedd, G. (2005). Polarized gene expression determines woronin body formation at the leading edge of the fungal colony. *Molecular Biology of the Cell* **16**: 2651-2659.

Thines, E., Weber, R. W. S. and Talbot, N. J. (2000). MAP kinase and protein kinase A-dependent mobilization of triaglycerol and glycogen during appressorium turgour generation by Magnaporthe grisea. *Plant Cell* **12**: 1703-1718.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology* **140**: 19-26.

Tretter, L. and Adam-vizi, V. (2000). Effect of succinate on mitochondrial lipid peroxidation. The protective effect of succinate against functional and structural changes induced by lipid peroxidation. *Journal of Neuroscience* **20**: 8972-8979.

Trinci, A. P. and Collinge, A. J. (1974). Occlusion of the septal pores of damaged hyphae of *Neurospora crassa* by hexagonal crystals. *Protoplasma* **80**: 57-67.

Vashisht, A. A. and Tuteja, N. (2006). Stress responsive DEAD-box helicases: A new pathway to engineer plant stress tolerance. *Journal of Photochemistry and Photobiology B: Biology* **84**: 150-160.

Viaud, M. C., Balhadère, P. V. and Talbot, N. J. (2002). A *Magnaporthe* grisea cyclophilin acts as a virulence determinant during plant infection. *The Plant Cell* **14**: 917-930.

Viaud, M., Brunet-Simon, A., Brygoo, Y., Pradier, J. and Levis, C. (2003). Cyclophilin A and calcineurin functions investigated by gene inactivation, cyclosporine A inhibition and cDNA arrays approaches in the phytopathogenic fungus Botrytis cinerea. *Molecular Biology* **50**(5): 1451-1456.

Vogel, J., Drapkin, B., Oomen, J., Beach, D., Bloom, K. and Synder, M. (2001). Phosphorylation of gamma-tubulin regulates microtubule organization in budding yeast. *Developmental Cell* **1**: 621-31.

Walden, H., Taylor, G. L., Lorentzen, E., Pohl, E., Lilie, H., Schramm, A., Knura, T., Stubbe, K., Tjaden, B. and Hensel, R. (2004). Structure and regulation of a regulated Archaeal triosephosphate isomerise adapted to high temperature. *Journal of Molecular Biology* **342**: 861-875.

Wang, C., Duan, Z., and St. Leger, R. J. (2008). MOS1 osmosensor of *Metarhizium anisopliae* for adaptation to insect host hemolymph. *Eukaryotic Cell* **7**(2): 302-309.

Wang, P. and Heitman, J. (2005). The cyclophilins. *Genome Biology* **6**(7): 226.1-226.6

Webber, H. A., Baenziger, N. C. and Gloer, J. B. (1988). Podosporin A: a novel antifungal metabolite from *Podospora decipiens* Niessl. *Journal of Organic Chemistry* **53**: 4567-4569.

Webber, J. F. and Hedger, J. N. (1986). Comparisons of interactions between *Ceratocystis ulmi* and elm bark saprobes in vitro and in vivo. *Transactions of British Mycological Society* **86**: 93-101.

Wessels, J. G. H. (1986). Cell Wall synthesis in apical hyphal growth. *International Review of Cytology* **104**: 37-39.

Wheatley, R. E. (2002). The consequence of volatile organic compound mediated by bacterial and fungal interactions. *Antonie van Leeuwenhoek* **81**: 357-364.

Wheatley, R., Hacket, C., Bruce, A. and Kundezewicz, A. (1997). Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. inhibitory to wood decay fungi. *International Biodeterioration and Biodegradation* **39**: 199-205.

White, N. A. and Boddy, L. (1992). Extracellular enzyme localization during interspecific fungal interactions. *FEMS Microbiology Letters* **98**: 75-79.

Whyte, A. C., Gloer, J. B., Scott, J. M. and Malloch, D. (1996). Cercophorins A-C: Novel antifungal and cytotoxic metabolites from the coprophilous fungus *Cercophora areolata*. *Journal of Natural Products* **59**: 765-769.

Wold, H. (1966). Estimation of Principal Components and Related Models by Iterative Least Squares. In: Krishnaiah, P. R. (Eds.), *Multivariate analysis*. Academy Press, New York, Pp. 391-420.

Woodward, S. and Boddy, L. (2008). Interactions between saprotrophic fungi. In: Boddy, L., Frankland, J. C. and Van West, P. (Eds.), *Ecology of Saprotrophic Basidiomycetes*. Elsevier, London, Pp. 125-153.

Woronin, M. (1864). Zur Entwicklungsgeschichte der *Ascobolus pulcherimus* Cr. Und einiger pezzizen. Abh. Senkenb. *Naturforsch* **5**: 333-344.

Wu, S., Sampson, M. J., Decker, W. K. and Craigen, W. J. (1999). Each mammalian mitochondrial outer membrane porin protein is dispensable: effects on cellular respiration. *Biochimica et Biophysica Acta* **1452**: 68-78.

Xiaobin, C., Rong, J., Pingsheng, L., Shiqian, T., Qin, Z., Wenzhong, T. and Xudong, L. (2007). Purification of a new manganese peroxidase of the white-rot fungus Schizophyllum sp. F17, and decolourization of azo dyes by the enzyme. Enzyme and *Microbial Technology*, **41**: 258-264.

Yanai, K., Takaya, N., Kojima, N., Horiuchi, H., Ohta, A. and Takagi M. (1992). Purification of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. *Journal of Bacteriology* **174**(22): 7398-7406.

Yan, L. J. and Sohal, R. S (1998). Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proceedings of the National Academy of Sciences USA* **421**: 12896-12901.

Yan, L. J., Levine, R. L. and Sohal, R. S. (1997). Oxidative damage during aging targets mitochondrial aconitase. *Proceedings of the National Academy of Sciences USA* **94**:1168-1172.

Yuen, T. K., Hyde, K. D. and Hodgkiss, I. J. (1999). Interspecific interactions among tropical and subtropical freshwater fungi. *Microbial Ecology* **37**:257-262.

Yumoto, I., Ichihasi, D., Iwata, H., Istokovics, A., Ichise, N., Matsuyama, H., Okuyama, H. and Kawasaki, K. (2000). The purification and characterization of a catalase from the facultatively psychrophylic bacterium *Vibiro rumoiensis* S-1[T] exhibiting high catalase activity. *Journal of Bacteriology* **182**(7): 1903-0909.

Zaigler, A., Schuster, S. and Soppa, J. (2003). Construction and usage of one-fold coverage shotgun DNA microarray to characterize the metabolism of the archaeon *Haloferax volcanii*. *Molecular Microbiology* **48**: 1089-1105.

Zeilinger, S., Galhaup, C., Payer, K., Woo, S. L., Mach, R. L., Fekete, C., Lorito, M. and Kubicek, C. P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genetics and Biology* **26:**131-140.

Zeilinger, S. and Omann, M. (2007). *Trichoderma* Biocontrol: Signal transduction pathways involved in host sensing and mycoparasitism. *Gene Regulation and Systems Biology* **1**: 227-234.

Zhang, H., Hong, Y. Z., Xiao, Y. Z., Yuan, J., Tu, X. M. and Zhang, X. Q. (2006). Efficient production of laccases by *Trametes* sp. AG28-2 in cocultivation with a *Trichoderma* strain. *Applied Microbiology and Biotechnology* **73**: 89-94.

Zheng, X M., Moncollin, V., Egly, J. M. and Chambon, P. (1987). A general transcription factor forms a stable complex with RNA polymerase B (II). *Cell* **50**: 361-368.

Zocher, R., Keller, U., Lee, C. and Hoffman, K. (1991). A seventeen kilodaltons Peptidyl-propyl *cis-trans* isomerase of the cyclosporine-producer *Tolypocladium inflatum* is sensitive to cycloporin A. *The Journal of Antibiotics* **45**(2): 265-268.

Cited web references

www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi

http://biotools.umassmed.edu/bioapps/primer3_www.cgi

http://www.nist.gov/srd/nistla.htm

http://csbd.mpimp_golm.mpg.de/csbdb/gm/msri/gmd_conributuions.html

http://www.mathworks.com

http://www.ncbi.nlm.nih.gov/

Appendices

Appendix I

GC-TOF-MS analysis was carried out at the School of Chemistry, University of Manchester, UK. Ten replicates of dried extracts for each of the interactions pairs (self and non-self) were reconstituted in 700 μl of methanol, out of which 150 μl was dried lyophilised in a HETO VR MAXI vacuum centrifuge (Thermo life Sciences, Basingstoke, UK). Internal standard solution comprising of 1.73 mg/ml of succinic d_4 acid, glycine d_5 and malonic d_2 acid was added to the samples before lyophylisation. Two-stage chemical derivatization was carried out, involving, Oximation (stage one) (heating samples with O-methylhydroxylamine (50 μl; 20 mg/ml in pyridine for 80 mins at 40ºC), and trimethysilylation with N-acetyl-N-(trimethylsilyl)-trifluoroacetamide (50 μl) for 80 mins at 40ºC.

After derivatisation, GC-TOF-MS was performed with Agilent 6890 Gas Chromatograph (Stockport, UK), coupled to a Leco Pegasus III Mass Spectrometer (St Joseph, USA). Control was achieved with a ChromaTof software v2.15. Sample analysis was randomized and three machine replicates were performed for each sample. Analytical conditions described by O'Hagan *et al.* (2005) were employed for the analysis. Three random samples from each sample class were chosen for peak deconvolution with the Leco ChromaTof software (peak = 3s, baseline = 1, smoothing = 3). Metabolite peaks, retention index (RI) and mass spectrum for all 108 metabolite peaks were entered in the database for peak matching (RI ±, mass spectral match > 700).

Data were exported as ASCII files to Microsoft Excel for further analysis. The internal standard, succinic d_4 acid was employed for peak normalisation (peak area of metabolite/peak area of standard). Three different libraries were used for peak matching and identification;

> ➢ NIST/EPA/NIH02 (http://www.nist.gov/srd/nistla.htm)

- MPI-Golm prepared mass spectra/RI library (http://csbd.mpimp_golm.mpg.de/csbdb/gm/msri/gmd_conributuions.html)
- Authors' (WD) prepared spectra/RI library containing 500 entries.

Preliminary identification was confirmed by mass spectral match > 700 and further confirmed by analysis of authentic metabolite standards, purchased from Sigma-Aldrich (Gillingham, UK) or from ACROS Chemicals (Loughborough, UK), applying the same analytical conditions.

All data were imported into Matlab® Version 7.1 (http://www.mathworks.com) running under Windows XP on an IBM-compatible PC. Within Matlab®, exploratory analysis using principal components analysis (PCA) (Joliffe, 1986), and NIPALS algorithm (Wold, 1966) on all data sets t reduce dimensionality of multivariate data and preserve most of the variance. Univariate statistical analysis was performed using Kruskal-Wallis test (Kruska and Wallis, 1952) to determine the statistical difference for any given peak between sample classes.

Section 2: Mass spectra of selected metabolite peaks (see Chapter 3; section 3.3.2)
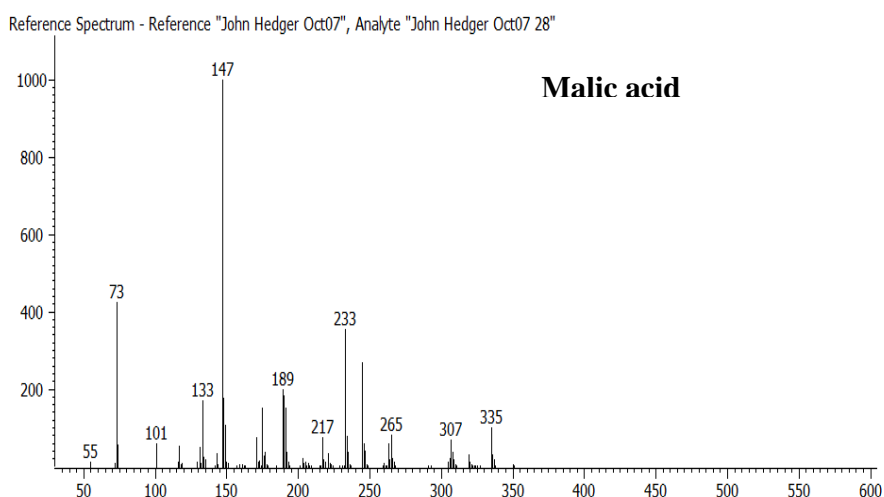
Figure 1: Mass spectrum for malic acid
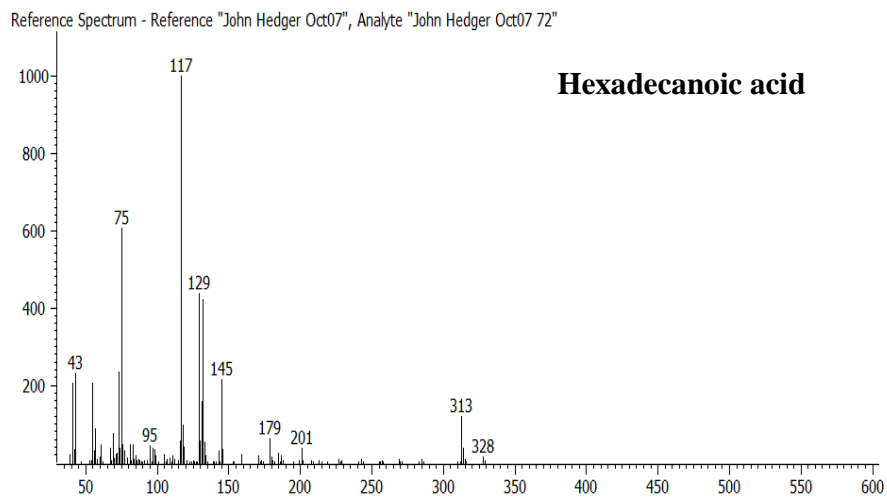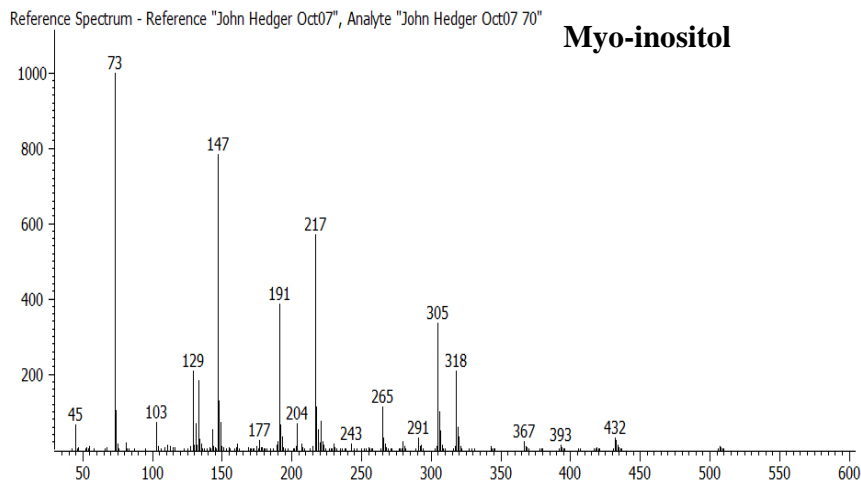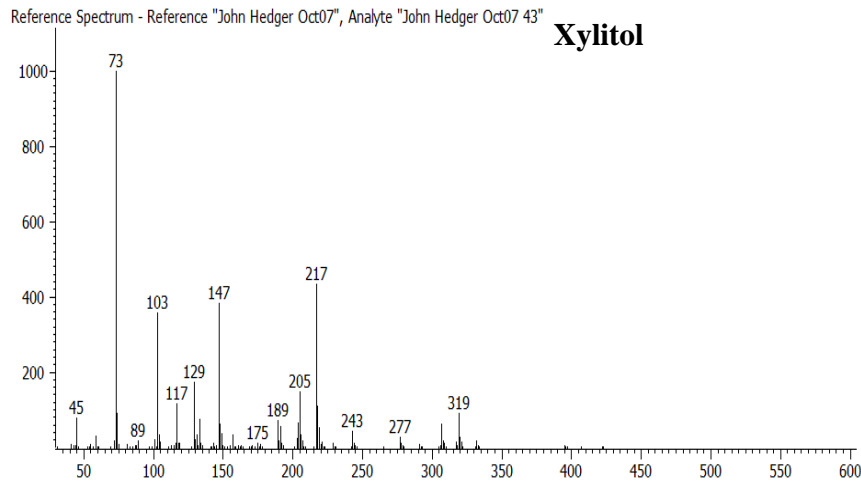
Figure 2: Mass spectra for xylitol, myo-inositol, and hexadecanoic acid

Figure 3: Comparative plot of designated metabolite peak areas between self- and non-self interacting cultures for both *S. commune* and *T. viride*.
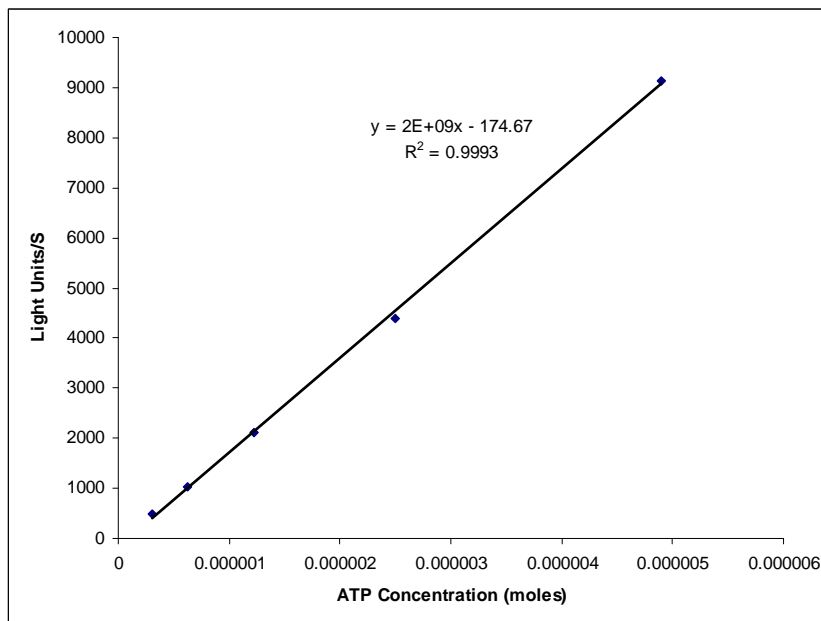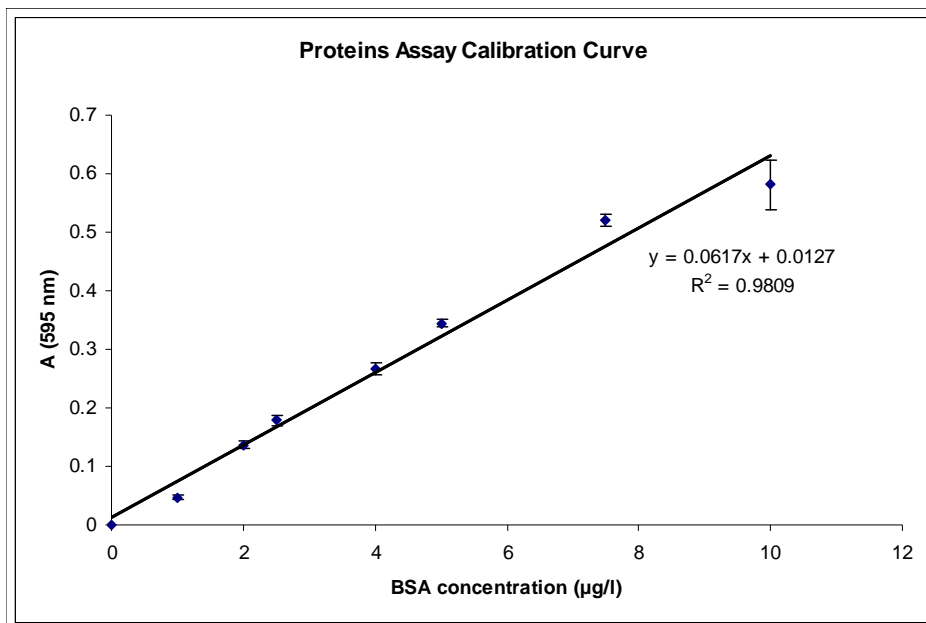
Appendix II

Standard graphs

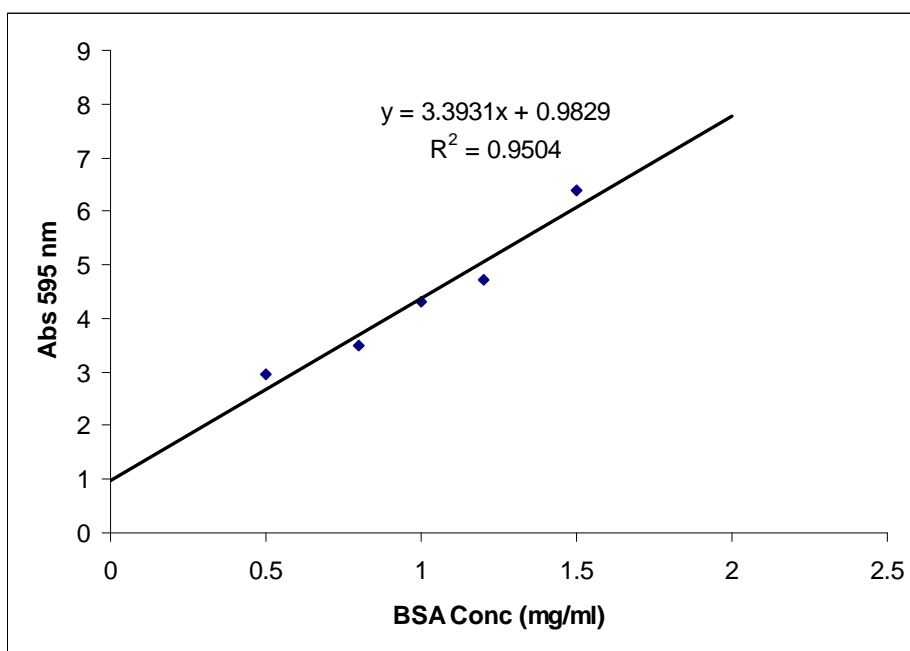The method of Bradford (1970) was used for protein quantification, in all cases.

$y = 0.1569x + 0.1057$
$R^2 = 0.839$
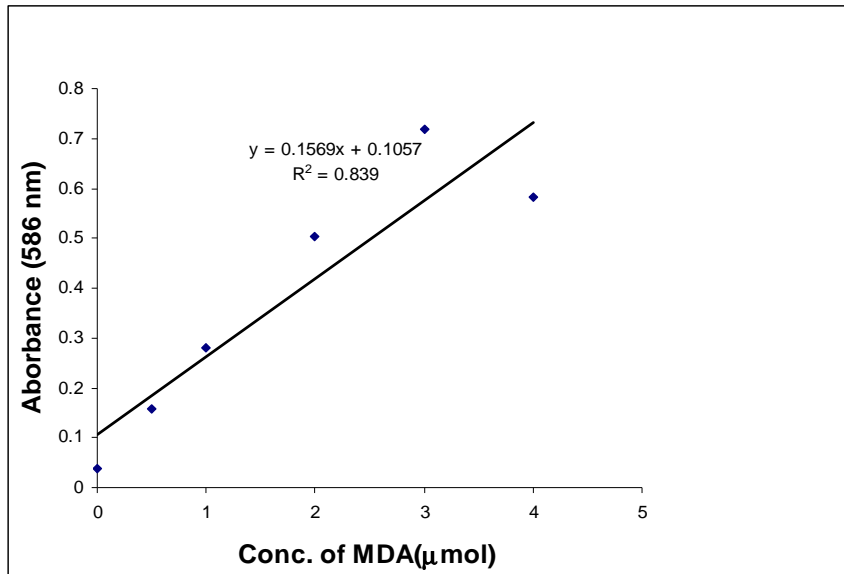
Figure 4: Malondialdehyde standard graph for lipid peroxidation assay.

$y = 2E+09x - 174.67$
$R^2 = 0.9993$

Figure 5: ATP standard graph for ATP assay.

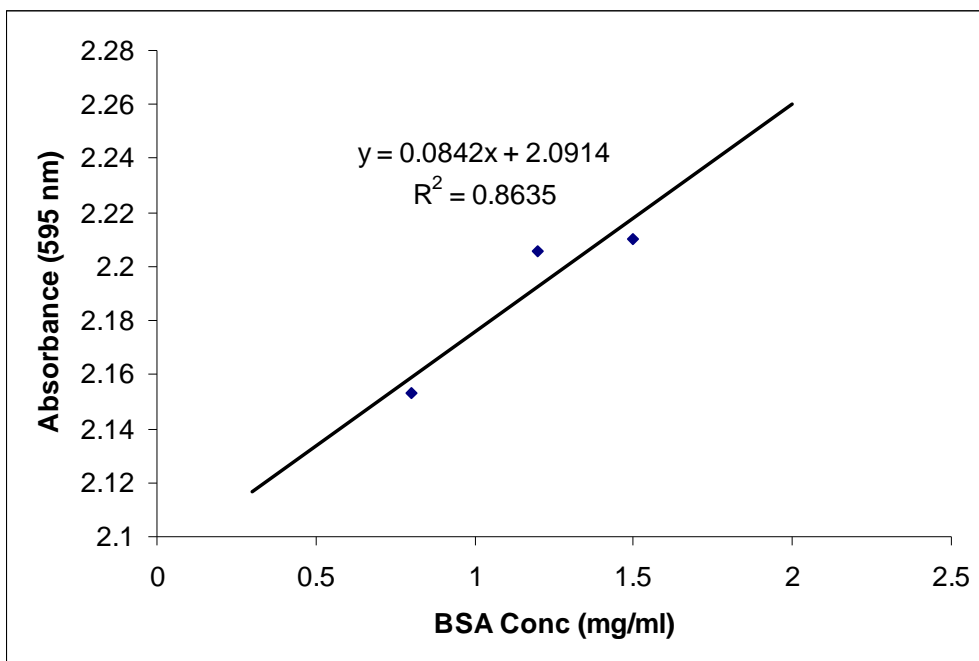**Figure 6: Protein standard graph for protein carbonylation assay**

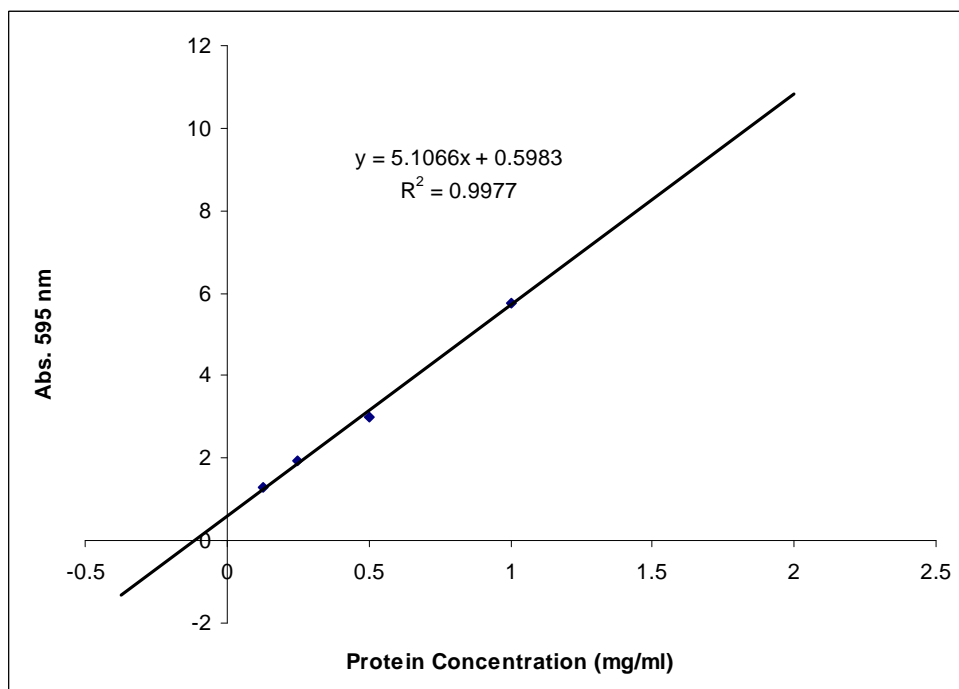**Figure 7: Protein standard graph for superoxide dismutase assay**
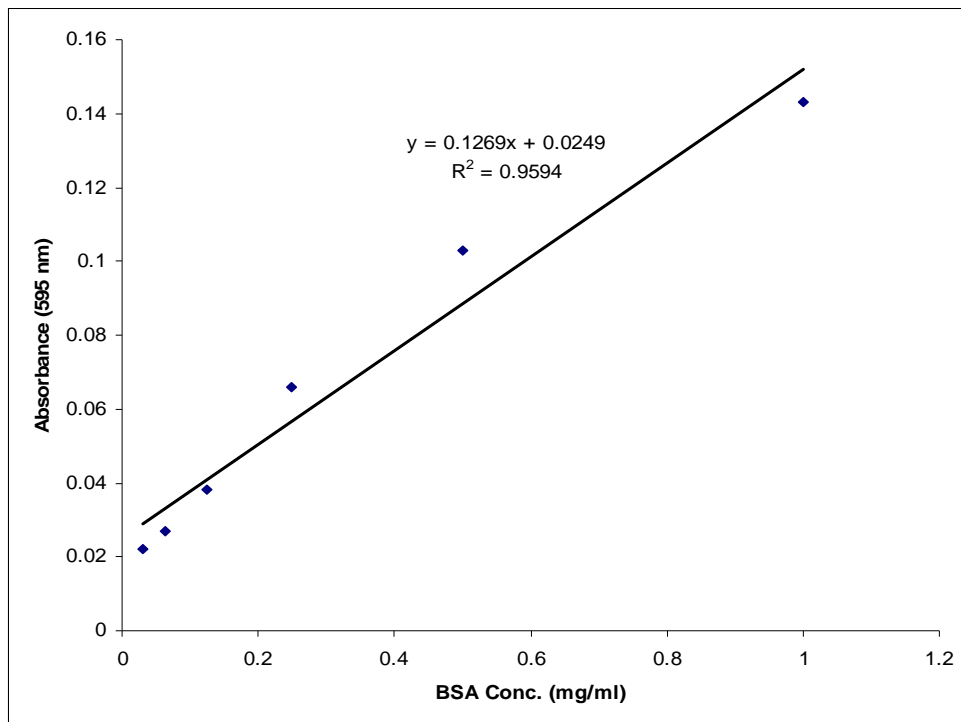
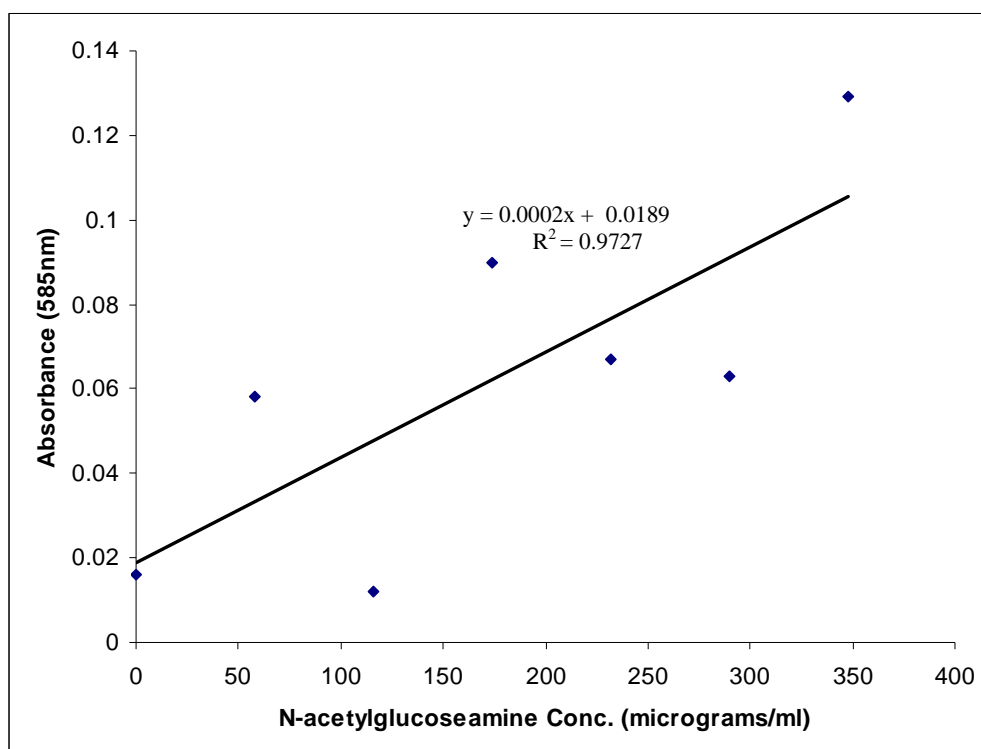**Figure 8: Protein standard graph for catalase assay**
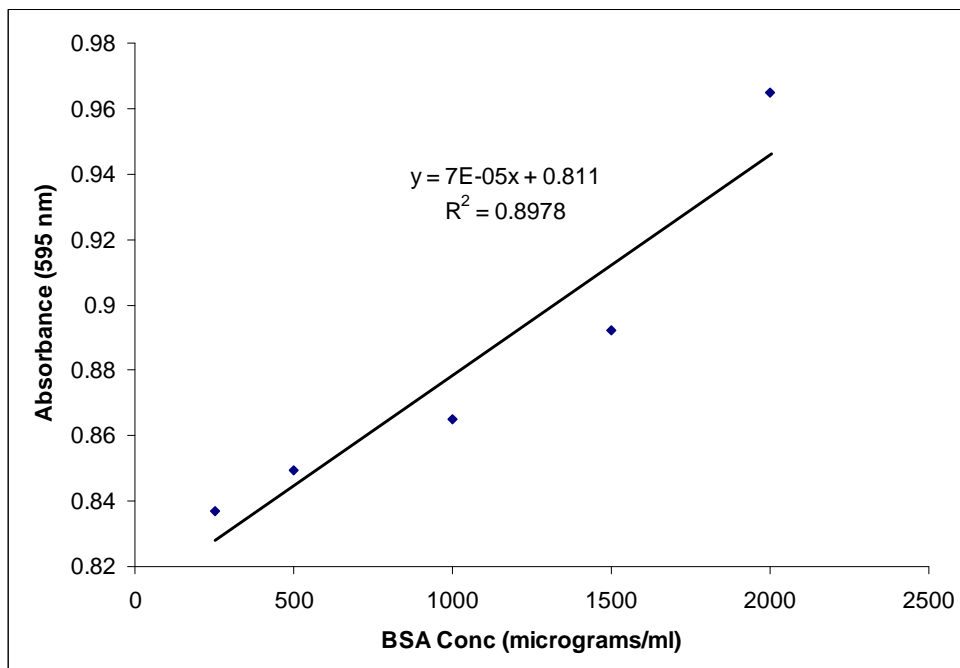
Figure 9: protein standard graph for Glucose-6-phosphate dehydrogenase assay

Figure 10: Protein standard curve for laccase and manganese peroxidase in-gel activity staining.

Figure 11: protein standard graph for the quantification of proteins used for 2-D gel electrophoresis.

Figure 12: N-acetylglucoseamine standard graph for chitinase assay.

Appendix III

Buffer recipes

<u>1X Phosphate buffered saline (PBS)</u>

1. The following were dissolved in 800 ml of distilled water;

- 8 g of NaCl
- 0.2 g of KCl
- 1.44 g of Na_2HPO_4
- 0.24 g of KH_2PO_4

2. pH was adjusted to 6.5

3. The volume was made up to 1L with additional distilled water

4. Buffer solution was sterilized by autoclaving at 121ºC for 15 mins.

10 X Tris-Glycine SDS-PAGE running buffer

- Tris-base 30.3 g

- Glycine 144 g

- Sodium dodecyl sulphate (SDS)10 g
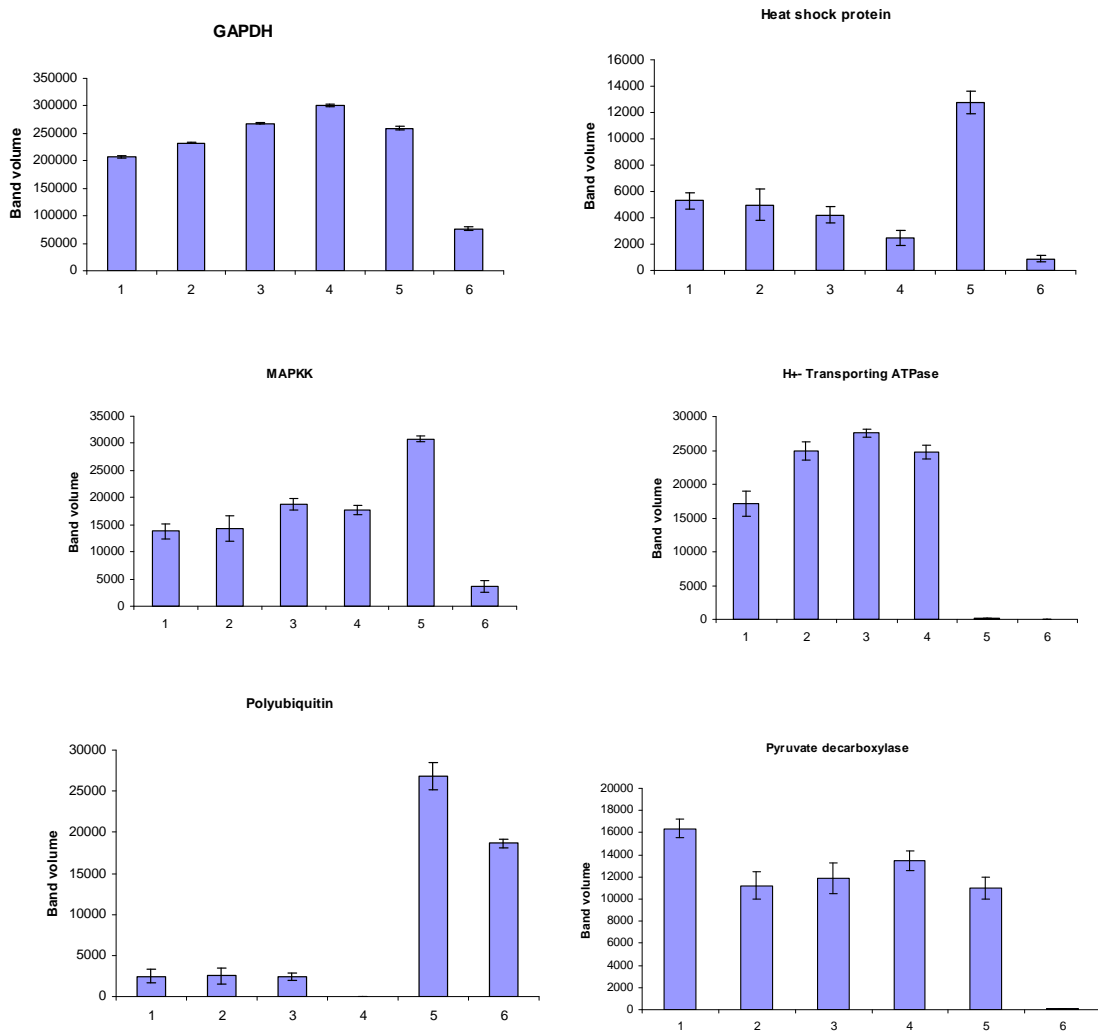
- Milli Q water Final volume of 1L.

Final concentration: 125mM Tris-base, 192mM Glycine, 0.1 % SDS, pH 8.3.

50X TAE DNA gel running buffer

1. The following were dissolved in 900 ml of distilled water

   - 242 g of Tris base

   - 57.1 ml glacial acetic acid

   - 18.6 g EDTA

2. The volume was adjusted to 1L with additional distilled water.

Appendix IV

Figure 13: Comparative plot of DNA band volumes. 1 & 2: mRNA from unpaired mycelia of *S. commune* corresponding to 24- and 48-hour contact periods respectively in paired cultures; 3 & 4: mRNA from self-paired mycelia of *S. commune* after 24 and 48 hours of contact respectively; 5 & 6: mRNA from *S. commune* mycelia paired against *T. viride*, after 24 and 48 hours of contact respectively.
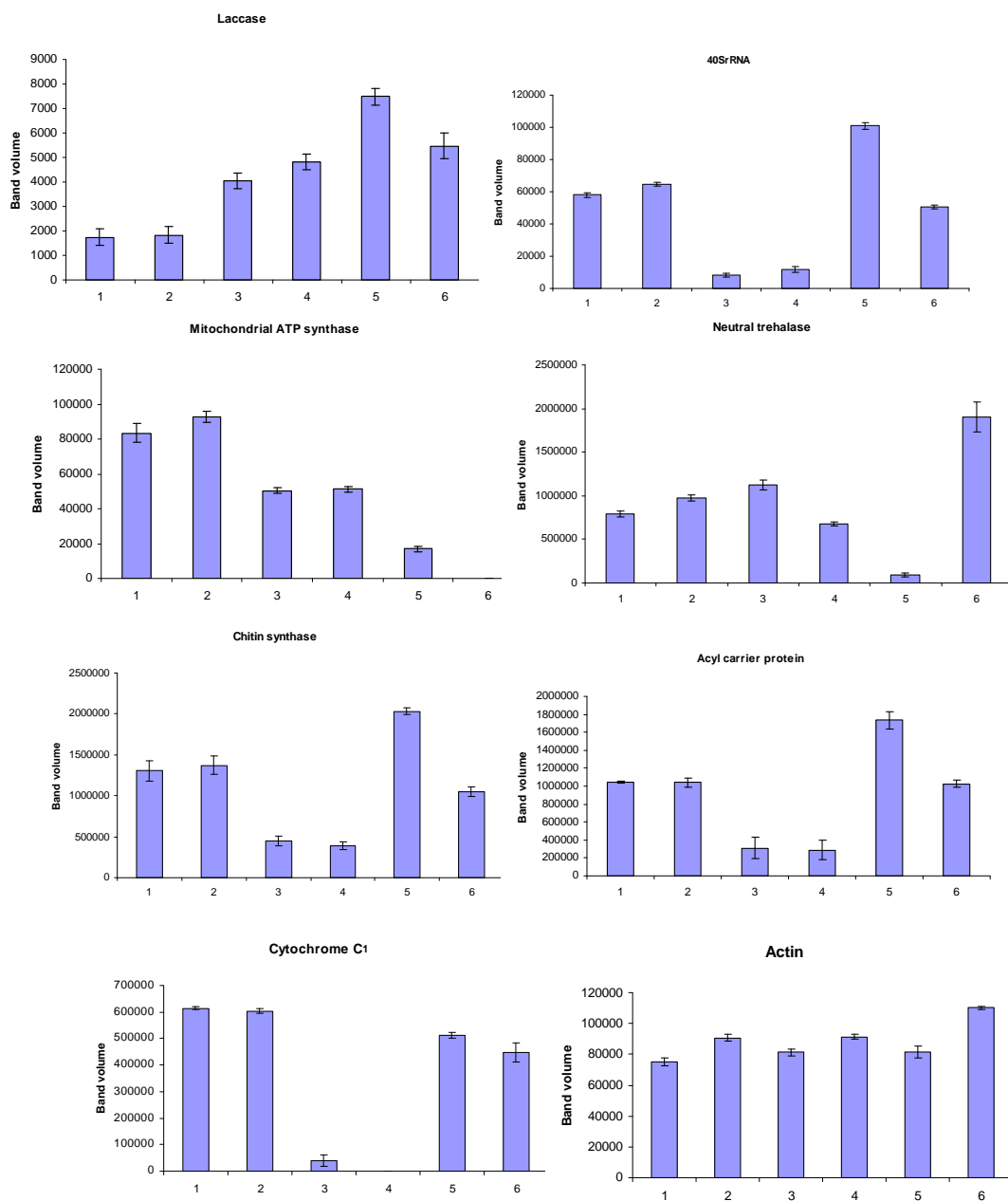
Figure 14: Comparative plot of DNA band volumes (see key to 1-6 in figure 13).

Section 2: Expression patterns of chitinase and calmodulin genes of *T. viride*.
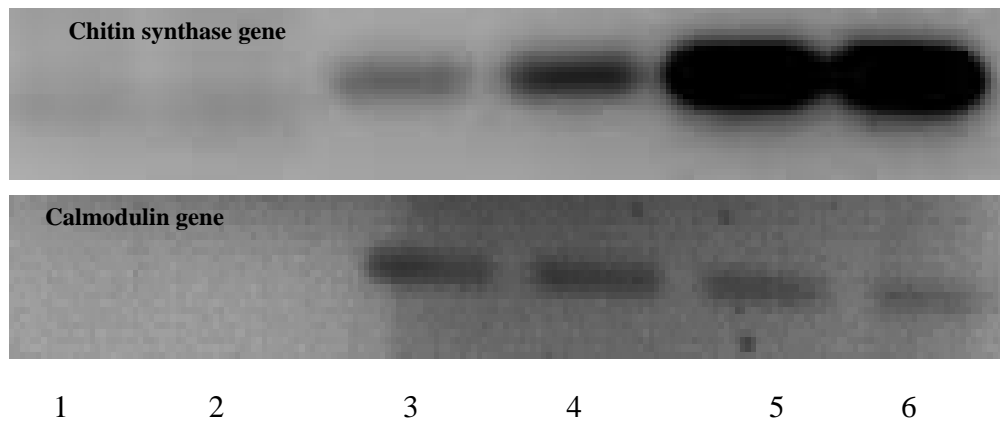
Figure 15: Expression patterns of the chitinase and calmodulin genes of *T. viride* in unpaired and self-paired cultures compared to its mycelia interacting with *S. commune*. 1-6: 1 & 2: mRNA from unpaired mycelia of *T. viride* corresponding to 24- and 48-hour contact periods respectively in paired cultures; 3 & 4: mRNA from self-paired mycelia of *T. viride* after 24 and 48 hours of contact respectively; 5 & 6: mRNA from *T. viride* mycelia paired against *S. commune*, after 24 and 48 hours of contact respectively.

Appendix V

Section 1 : LC/MS-MS & protein identification

LC/MS-MS and protein identification were carried out at the University of York. Protein spots were destained with Farmer's reagent before in-gel tryptic digestion. Destaining involved washing the spots in Farmer's reducing reagent (20 % aqueous sodium thiosulphate: containing 1% potassium ferricyanide), then washed two times with 50 % (v:v) acetonitrile containing 25 mM ammonium bicarbonate, and then once with acetonitrile, before drying in a vacuum concentrator for 20 mins. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in 5o mM acetic acid supplied by the manufacturer. The mixture was dilute 5-fold by adding 25 mM ammonium bicarbonate, resulting in a final trypsin concentration of 0.01 μg/ μl. Gel pieces were rehydrated in 10

µl of trypsin solution for 30 mins before adding 25 mM ammonium bicarbonate to submerge the samples. Digest mixtures were incubated overnight at 37ºC.

0.5 µl of each digest mixture was used for MALDI in the target plate with the addition of equal volume of freshly prepared solution (5 mg/ml) of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1 % trifluoroacetic acid (v:v). Positive-ion MALDI mass spectra were obtained using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of m/z 800-4000. Final mass spectra were internally calibrated using the tryptic autoproteolysis products at m/z 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data.

The strongest peaks were then subjected to CID-MS/MS applying Source collision energy of 1kV, with air as the collision gas. The precursor mass window was set to relative resolution of 50, and the metastable suppressor was enabled. The default calibration was used for MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points across a peak and polynomial order 4); peak detection used a minimum S/N of 5, local noise window of 50 m/z, and minimum peak width of 2.9 bins. Filters of S/N 20 and 30 were used for generating peak lists from MS and MS/MS spectra, respectively.

Mass spectral data obtained in batch mode were submitted to database searching using a locally running copy of the Mascot program (Matrix Science Ltd., version 2.1). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 3.6) to Mascot. Search criteria included: Maximum missed cleavages, 1; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M); Peptide tolerance, 100 pmm; MS/MS tolerance, 0.1Da.

Section 2: Peptide sequences

Table 1: Peptide sequences of proteins up-regulated in the mycelia of *S. commune* paired against *T. viride* compared to self-paired mycelia of *S. commune*.

Spot	No. of peptide sequences	Peptide sequences
SC1	1	QRDQIDQWMQGIR
SC2	2	KGFGYKGSSFHRV, RVIPQFMLQGGDFTRG
SC6	2	KLAESYQNMQKN, KSDGNVIHFAAPKV
SC8	1	RKSEEALEILRL
SC20	2	RLQQAFESGRG, RYLGVDLFTKQ
SC28	3	KVVVIPAGVPRK,RVFGVTTLDVVRA, RDDLFNTNASIVRD
SC39	1	KDDISPEISIVRD
SC40	1	RNISNEVLLDKSRG
SC41	1	RRMQFLGEIGGVRI
SC55	1	KVHLVAIDIFTGKK
SC75	1	KAYDLLHVLKR
SC87	1	KVHLVAIDIFTGKK
SC93	1	RFFVPSDSSRD
SC100	1	RGEKVPVGTVLATIRT
SC114	4	KVVVIPAGVPRK,RVFGVTTLDVVRA,RDDLFNTNASIVRD, RGAPGVAADVSHVDTASEVKG
SC150	1	KAIDAFEVEAVKN
SC195	1	KHVVFGEVVEGMDVVKN
SC394	1	KVHLVAIDIFTGKK

Table 2: Peptide sequences of proteins down-regulated in the mycelia of *S. commune* paired against *T. viride* compared to self-paired mycelia of *S. commune*.

Spot	No. of peptide sequences	Peptide sequences
SC33	2	KHGNSHQGEPRD, RLKYENEVALRQ
SC34	1	RTYLMIKPDGVQRN
SC62	4	KTPGPGAQSALRA,REVGITALHIKL, RIEDVTPVPTDSTRR, RDESSPYAAMLAAQDVAARC
SC69	2	LNVQPIAGVEEVNMFR, ELTELVPGILNQLGPDSLASLR
SC83	1	VATPAQAQEVHAAIR
SC302	4	DAGAIAGLDVLR,IQALVSEYFGGR, AVVTVPAYFNDSQR, IINEPTAAAIAYGLDR
SC313	6	GIPNVIDSYAR,VRPELPIIYR,AFVPEDVPIER, IHLIDDIDEYRK,SGFLTEPGDNAQVAR, NNHNILQGVASPDLR
SC345	3	IGTETYHTLK,IALDVASSEFYK, SGIQIVGDDLTVTNPLR
SC350	1	LLVAHEAGMR
SC369	1	TGYLNVALFGVDSR
SC412	1	NHAIADAVLLSGDEDVR
SC438	3	IGTETYHTLK,IALDVASSEFYK,LGANAILGVSIAVAEAGAAEK
SC444	4	FELSGIPPAPR,DAGTIAGLNVLR,LVNHFVQEFK, ATAGDTHLGGEDFDNR
SC465	3	IGTETYHTLK, IALDVASSEFYK, SGIQIVGDDLTVTNPLR

Table 3: Peptide sequences of proteins up-regulated in the mycelia of *T. viride* paired against *S. commune* compared to self-paired mycelia of *T. viride*.

Spot	No. of peptide sequences	Peptide sequences
TR3	1	LRSNGHGGVINVASAASFGSAPR
TR5	1	AAMGKAMMYICSVFSQLER
TR15	3	YDSSHGSFK, AASYEEITNAIK, VPTANVSVIDLTVR
TR16	2	YDSSHGSFK, VPTANVSVIDLTVR
TR22	2	YEDISPSTHNMDVPNVTR, KYEDISPSTHNMDVPNVTR
TR29	2	VEQAEIQYEPSGR, NAIQQFNGYDWQGR
TR44	3	LEGLFNFLPATAAK, EGVTLGLGGSFDTQK, DFYHLSATTFEFK
TR68	5	YDSSHGSFK,VLDLLAHVAK,AASYEEITNAIK,VPTANVSVIDLTVR, LVSWYDNEWGYSR
TR80	2	DSVVSGFQWASR, VLADDFGWDVTDAR
TR150	2	GHTLYNPTK, VSSEFVSDTSNSGLLGLALDSINTVSPK

Table 4: Peptide sequences of proteins down-regulated in the mycelia of *T. viride* paired against *S. commune* compared to self-paired mycelia of *T. viride*.

Spot	No. of peptide sequences	Peptide sequences
TR47	1	ELMDELGKIVR
TR59	1	APPSRAPPPAEPPAR
TR103	1	VHLVAIDIFTGK
TR115	1	VDVHYYEDGNVR

Appendix VI

Identification of *T. viride* by PCR

500 bp →

5.8SrRNA/28SrRNA gene
566 bp
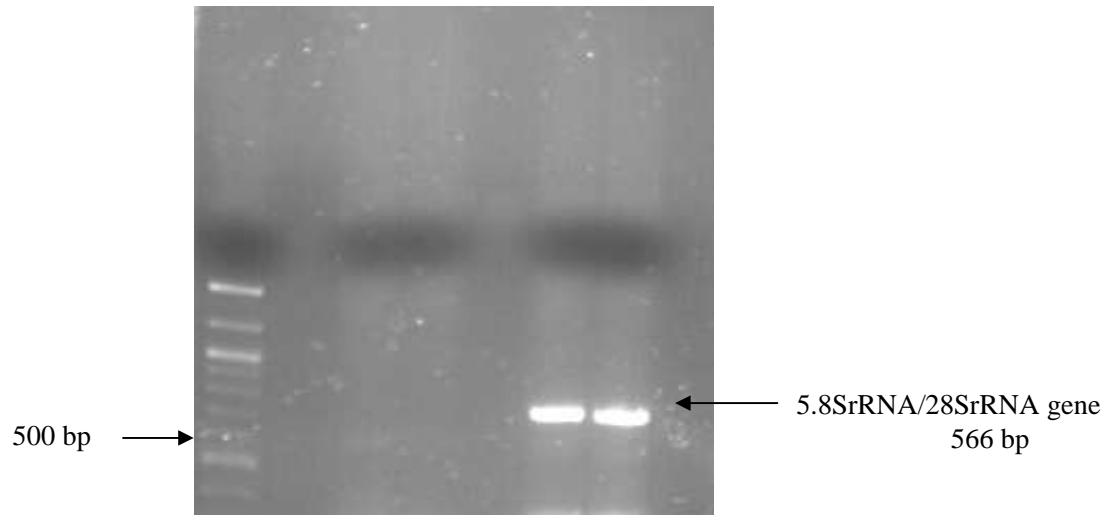
Primer sequences: Forward - CGTCATTTCAACCCTCGAAC

Backward – CCTACCTGATCCGAGGTCAA

NCBI Accession number: HM438946