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

**Victor Chinomso Ujor**

School of Life Sciences

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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 \textit{S. commune}. Proteins involved in glycolysis and heat shock response were predominantly down-regulated in the mycelia of \textit{S. commune} paired against \textit{T. viride}. Proteins associated with antagonism, cellular metabolism, glycolysis, and ATP generation and protein synthesis were up-regulated in the mycelia of \textit{T. viride} interacting with \textit{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 \textit{S. commune} paired against \textit{T. viride}. Similarly, the activities and protein levels of phenol-oxidising enzymes, namely laccase and manganese peroxidase increased in the confronted mycelial domain of \textit{S. commune}. Chitinase activity increased in mixed liquid cultures of both fungi. Protein, and gene expression patterns, in the confronted mycelia of \textit{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 \textit{S. commune} towards \textit{T. viride}, suggesting that the patterns reported herein, may be a response rather than an attack mechanism towards the latter.
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<th>Description</th>
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<tr>
<td>µl</td>
<td>microlitre</td>
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<tr>
<td>µM</td>
<td>micromolar</td>
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<tr>
<td>1D</td>
<td>1-Dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>2-Dimensional</td>
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<td>4-HNE</td>
<td>4-hydroxyalkenals</td>
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<td>ABTS</td>
<td>2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
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<td>ACP</td>
<td>Acyl carrier protein</td>
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<td>AKDH</td>
<td>Alpha –ketoglutarate dehydrogenase</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BCKAD</td>
<td>branched-chain α-ketoacid dehydrogenase</td>
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<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>DMP</td>
<td>2,6-dimethoxyphenol</td>
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<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate</td>
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<td>CP</td>
<td>Capping protein</td>
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<td>DNPH</td>
<td>2,4-dinitrophenyl hydrazine</td>
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<td>DIGE</td>
<td>Difference gel electrophoresis</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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<tr>
<td>eIF</td>
<td>Elongation initiation factor</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FAS</td>
<td>Fatty acid synthase</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
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<td>GC/MS</td>
<td>Gas Chromatography/Mass Spectrometry</td>
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<td>GT</td>
<td>Glycosyltransferase</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>Heat shock protein</td>
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<td>IEF</td>
<td>Isoelectric focusing</td>
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<td>IPG</td>
<td>Immobilised Ph gradient</td>
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<tr>
<td>kDa</td>
<td>Kilo dalton</td>
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<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
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<td>MnP</td>
<td>Manganese peroxidase</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MAPKK</td>
<td>Mitogen activated protein kinase kinase</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<td>MDH</td>
<td>Malate dehydrogenase</td>
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<tr>
<td>MudPIT</td>
<td>MultiDimensional Protein Identification Technology</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotnamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adnine dinucleotide phosphate</td>
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<td>NBT</td>
<td>Nitrotetrazolium</td>
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<tr>
<td>OFR</td>
<td>Open reading frame</td>
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<td>PDA</td>
<td>Potato dextrose agar</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PAL</td>
<td>Phenylalanine ammonia lyase</td>
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<tr>
<td>PD</td>
<td>Pyruvate decarboxylase</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RBB</td>
<td>Remazol Brilliant Blue</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcribed polymerase chain reaction</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse-phase</td>
</tr>
<tr>
<td>SAGE</td>
<td>Serial Analysis of Gene Expression</td>
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<tr>
<td>SCX</td>
<td>Strong Cation Exchange</td>
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<td>SSADH</td>
<td>Succinic semialdehyde dehydrogenase</td>
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<td>SCSC</td>
<td><em>Schizophyllum commune</em> + <em>Schizophyllum commune</em></td>
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<td>SCTR</td>
<td><em>Schizophyllum commune</em> + <em>Trichoderma viride</em></td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrilamide gel electrophoresis</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
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<tr>
<td>TEMED</td>
<td>Tetrametylenediamine</td>
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<td>TRSC</td>
<td><em>Trichoderma viride</em> + <em>Schizophyllum commune</em></td>
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<td><em>Trichoderma viride</em> + <em>Trichoderma viride</em></td>
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<tr>
<td>TIM</td>
<td>Triphosphate isomerase</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<tr>
<td>VOCs</td>
<td>Volatile organic compounds</td>
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<td>V</td>
<td>Volts</td>
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<td>WB</td>
<td>Woronin body</td>
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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 *Bjerkandera* 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; Schirmbröck *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 (LC50) 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.

![Cell membrane](image)

**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., 1994a, 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., 1994a, 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, 1992; 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, Pynnopus occineus, Ganoderma applanatum 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 cell 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, endogalacturonase). 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/disruption 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 sanguinoventus*, 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. sanguinoventus* 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; Alfатаfa & 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 Biofoods s.c., Walecz, 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 filamentososa* 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.
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 discoulouration 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.
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.

![Timeline of agar plate interaction assay between *S. commune* and *T. viride* over 120 hours](image)

**Figure 2.3** Timeline of agar plate interaction assay between *S. commune* and *T. viride* over 120 hours. A: 0 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. commute 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. commute 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-containing 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
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](image)

**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, protoplastic 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 *Leptosphaeria oraeamaris* (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 single 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.
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*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Metabolite identity</th>
<th>Source (sample)</th>
<th>% Increase in peak area</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3-Hydroxypropanoic acid</td>
<td>SCTR</td>
<td>60</td>
<td>0.0198</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRSC</td>
<td>74</td>
<td>0.0001</td>
</tr>
<tr>
<td>9</td>
<td>GABA</td>
<td>SCTR</td>
<td>99.8</td>
<td>0.0005</td>
</tr>
<tr>
<td>10</td>
<td>Phosphoric acid</td>
<td>SCTR</td>
<td>55</td>
<td>0.0001</td>
</tr>
<tr>
<td>19</td>
<td>Erythritol/isomer</td>
<td>SCTR</td>
<td>60</td>
<td>0.0001</td>
</tr>
<tr>
<td>28</td>
<td>Malic acid</td>
<td>SCTR</td>
<td>41</td>
<td>0.0003</td>
</tr>
<tr>
<td>33</td>
<td>Citramalic acid</td>
<td>SCTR</td>
<td>72</td>
<td>0.0016</td>
</tr>
<tr>
<td>38</td>
<td>Mandelic acid</td>
<td>SCTR</td>
<td>77</td>
<td>0.0191</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRSC</td>
<td>71</td>
<td>0.0001</td>
</tr>
<tr>
<td>40</td>
<td>2-hydroxyglutaric acid</td>
<td>TRSC</td>
<td>90</td>
<td>0.0047</td>
</tr>
<tr>
<td></td>
<td>Metabolite</td>
<td>Source</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
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<td>-----------------------------------</td>
<td>--------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Xylitol</td>
<td>TRSC</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>4-Hydroxyphenyl ethanol</td>
<td>TRSC</td>
<td>0.0046</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>2-Furancarboxylic acid</td>
<td>SCTR</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRSC</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>Tropic acid</td>
<td>SCTR</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>Tropic acid</td>
<td>TRSC</td>
<td>0.0212</td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>Pyridoxine</td>
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<td>81</td>
<td>Myo-inositol phosphate</td>
<td>SCTR</td>
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<tr>
<td>76</td>
<td>N-acetylglucosamine</td>
<td>SCTR</td>
<td>0.0002</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>TRSC</td>
<td>0.0012</td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>Galactosylglycerol</td>
<td>TRSC</td>
<td>0.0013</td>
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</tr>
<tr>
<td>44</td>
<td>Sugar alcohol</td>
<td>TRSC</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>Sugar alcohol</td>
<td>TRSC</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>2,3,4-trihydroxybutanal</td>
<td>TRSC</td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>Hexanetetrol</td>
<td>SCTR</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>Unidentified</td>
<td>SCTR</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRSC</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

* Metabolites, which were detected only in test but not in control samples. **SCTR**: *S. commune* paired against *T. viride*; **TRSC**: *T. viride* paired against *S. commune*. **SCSC**: Self-paired *S. commune*; **TRTR**: 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*

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

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 osmolytes 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; González 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 protoplastic 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, protoplastic 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 rahl (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 protoplastic 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 (\(^{0}\_2\)\(^-\)) 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 malondialdehyde (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 \( \mu L \) 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 \( \mu l \) of sample was mixed with 650 \( \mu l \) 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 \( \mu l \) 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 \( \mu M \) to plot a standard curve (see Appendix II for MDA standard curve).

4.2.2 Protein carbonylation assay

Protein carbonyl content was measured in \textit{S. commune} mycelia over the same period as lipid peroxidation, according the method described by Reznick \textit{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 \( \mu L \) of fungal protease inhibitor cocktail (Sigma-Aldrich, UK). 100 \( \mu g \) of protein homogenate was mixed with 500 \( \mu L \) of 10 mM 2,4-dinitrophenyl hydrazine (DNPH), dissolved in 2 M HCL and incubated at 37°C for 1 hour. Proteins were then precipitated with 20% trichloroacetic acid for 20 mins at 4°C, 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 (\( \epsilon \)) 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°C. 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).

\[
\text{ATP} + \text{Luciferin} \overset{\text{Mg}^{++}}{\rightleftharpoons} \text{Adenyl-luciferin} + \text{PPi} \quad (1)
\]

\[
\text{Adenyl-luciferin} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{CO}_2 + \text{Light} \quad (2)
\]

### 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*. 
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.
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 4.4 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.
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.

\[
\text{L-H} + \text{OH}^- \rightarrow \text{H}_2\text{O} + \text{L}^-
\]

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, ISOPROANOL 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 DEGRADATES 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 MYCEIAL
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*.

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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 multicycle 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 C1 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 (Staszczyk, 2008). Similarly, protein folding and transport by cyclophilins proceeds in an ATP-dependent manner
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 (Sigma). 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°C 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 $\epsilon = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. 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 $\epsilon = 2.75 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. 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-dimethylaminobenzaldehyde [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. commute 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:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl buffer (pH 8.8)</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>40% Bis acrylamide</td>
<td>3 ml</td>
</tr>
<tr>
<td>N, N, N’, N’-tetramethylethylenediamine (TEMED)</td>
<td>10 µl</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

**Stacking gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>3.65 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl buffer (pH 6.8)</td>
<td>625 µl</td>
</tr>
<tr>
<td>40% Bis acrylamide</td>
<td>500 µl</td>
</tr>
<tr>
<td>N, N’, N’-tetramethylethylenediamine (TEMED)</td>
<td>10 µl</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

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...
(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.](image-url)
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 \textit{S. commune} and \textit{T. viride} respectively, while the pH of their co-cultures showed the same pattern as \textit{T. viride} cultures from 7 days post mixing (figure 5.12).

![Mixing of cultures](image)

**Figure 5.12** Changes in medium pH of mixed cultures of \textit{S. commune} and \textit{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 3 and 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 (Tsuijyama & 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 (Tsuiyama & 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 \textit{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 \textit{S. commune} mycelia after 48 hours of contact with \textit{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 \textit{et al}., 1992; Liu \textit{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 \textit{S. commune} after 24 hours of contact with \textit{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 \textit{S. commune} paired against \textit{T. viride}.

It is worth mentioning that increase in the activity of laccase and manganese peroxidase was very likely as a result of \textit{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 \textit{S. commune} laccase gene was up-regulated in response to \textit{T. viride}. To this end, it could be concluded that laccase and manganese peroxidase were transcriptionally activated during mycelial confrontation with \textit{T. viride}. However, contrary to extensive reports, \textit{S. commune} appeared to have employed more of manganese peroxidase than laccase during interaction with \textit{T. viride}, considering the patterns of activity and protein levels detected for both enzymes (figures 5.5, 5.6 and 5.11). \textit{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 \textit{et al}., 2007). Although there is not enough evidence to confirm this, it does appear that \textit{Schizophyllum} species might preferentially synthesize more of manganese peroxidase for detoxification or homeostatic functions.
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 ($\text{O}_2^-$) and hydrogen peroxide ($\text{H}_2\text{O}_2$) which are scavenged by SOD and...
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 C1 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.
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. commute 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).
*commune* during confrontation. The PPP is a major source of NADPH, which provides reducing power for antioxidant enzymes and oxidoreductases.

![Diagram of GABA shunt](image)

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™ (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™. Tri reagent™-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™ 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™ 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.
Figure 6.1 Schematic representation of the stages of RNA isolation from ground mycelia of *S. commune*. 

- **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; 4°C)
  - 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; 4°C)
  - Collect aqueous phase

- **Precipitation**: Mix with 200 µL of isopropanol
  - Incubate at 120°C (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
<table>
<thead>
<tr>
<th>Gene name/putative function</th>
<th>NCBI Accession number</th>
<th>Primers (Forward and reverse)</th>
<th>Predicted size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>BG673933</td>
<td>AATAATGGGCCGTGAAGGTG</td>
<td>428</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGACGGTGTCGTA CTTGAA</td>
<td></td>
</tr>
<tr>
<td>18SrRNA *</td>
<td>EU520239</td>
<td>CAACGGATCTCTTGGCTCTC</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCCAGCAGACCTCCACTTC</td>
<td></td>
</tr>
<tr>
<td>Polyubiquitin **</td>
<td>U74318</td>
<td>AGGTCCAATCCTCTGACACG</td>
<td>1079</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCAGAGACCACCCGGAGAC</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial ATP Synthase</td>
<td>BG713751</td>
<td>CCCCATCCAGATTCTGTC</td>
<td>540</td>
</tr>
<tr>
<td>(beta chain)</td>
<td></td>
<td>CCTCCGAAGAGACCGATCTTG</td>
<td></td>
</tr>
<tr>
<td>Vacuolar ATP Synthase (C</td>
<td>BQ102562</td>
<td>CACCAAGAACCTCATCAGCA</td>
<td>486</td>
</tr>
<tr>
<td>subunit)</td>
<td></td>
<td>GTGATGCCAACACAGACACC</td>
<td></td>
</tr>
<tr>
<td>Serine/threonine protein</td>
<td>BQ134634</td>
<td>ACGCCTACGAATCTGAGCAT</td>
<td>541</td>
</tr>
<tr>
<td>kinase kinase (MAPKK)</td>
<td></td>
<td>GAGGATATTTGTCGCGCTTCA</td>
<td></td>
</tr>
<tr>
<td>Serine/threonine protein</td>
<td>AB218429</td>
<td>ATCTACGCCTCAAGACG</td>
<td>2054</td>
</tr>
<tr>
<td>kinase (MAPK)**</td>
<td></td>
<td>GAAGTTCCGCGCTGAGA</td>
<td></td>
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<tr>
<td>Glutamine synthetase</td>
<td>BQ173853</td>
<td>ATGCCAAAAAGACGATGG</td>
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<td></td>
<td></td>
<td>GGTAGTGGGCCGCTCAATCAGA</td>
<td></td>
</tr>
<tr>
<td>Alpha tubulin</td>
<td>BF942492</td>
<td>CGACCTGCTCTACTC CAA</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCGACAGAGAAAGCGTAACC</td>
<td></td>
</tr>
<tr>
<td>Heat shock cognate protein</td>
<td>BG673919</td>
<td>CAACGGCTTTGGAAGGTG</td>
<td>404</td>
</tr>
<tr>
<td>(sks2)</td>
<td></td>
<td>GTGGATGTCGCGCTC AAGGT</td>
<td></td>
</tr>
<tr>
<td>Gene Name</td>
<td>Accession</td>
<td>5' Primer Sequence</td>
<td>3' Primer Sequence</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Laccase **</td>
<td>AB015758</td>
<td>GCAAGACCAGCTCAATGACA</td>
<td>GCCGAACCTCGTAGACGAAAG</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>BG713762</td>
<td>ACGTCGTAACACGGTCAACAA</td>
<td>TCCCGATTATCATGCACAAAA</td>
</tr>
<tr>
<td>2-Oxoglutarate dehydrogenase</td>
<td>BI135399</td>
<td>GACTCCGTCGACCAGATGAT</td>
<td>GCAGCACCACAAGGGTAAGT</td>
</tr>
<tr>
<td>(E1 component)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acyl carrier protein</td>
<td>BG739705</td>
<td>TCGTATCGAAATCCCTGAC</td>
<td>AACTACTGTGCGCGGTGATG</td>
</tr>
<tr>
<td>Plasma membrane H⁺ATPase</td>
<td>BI324797</td>
<td>TACCAACCAACAAGCTACACCA</td>
<td>AGTCGAGGAGCTTGATGAC</td>
</tr>
<tr>
<td>Chitin synthase *</td>
<td>M82956</td>
<td>GTGTGCAATAGTGTCGAGATG</td>
<td>CGTGACAGGCTTCTTGTA</td>
</tr>
<tr>
<td>Neutral trehalase</td>
<td>BQ102603</td>
<td>CTGACAAGACGGAAGATG</td>
<td>ACGAGTTGACACACAGGCTT</td>
</tr>
<tr>
<td>Cytochrome C₁</td>
<td>BQ102564</td>
<td>AAATGTTCTCGAGGGCTGTCG</td>
<td>GATAGACCCGACCGAGACAA</td>
</tr>
<tr>
<td>Ribosomal protein S8</td>
<td>BI324810</td>
<td>CCGCAAAAAGAGAAAGTTTCG</td>
<td>TTGTACGAGAAGCTCGTTG</td>
</tr>
<tr>
<td>Actin (control) **</td>
<td>AF156157</td>
<td>CACCGTATCGTCACCAACTG</td>
<td>TGATCTGCGTCATCTTCTGC</td>
</tr>
</tbody>
</table>

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](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, \(\alpha\)-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*.
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*.

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

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*.

<table>
<thead>
<tr>
<th>Gene function/name</th>
<th>( P ) value</th>
<th>Fold decrease in band volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relative to SC</td>
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<tr>
<td></td>
<td>24 hours of contact</td>
<td>48 hours of contact</td>
</tr>
<tr>
<td>GAPDH</td>
<td>&lt; 0.0001</td>
<td>4.3</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>repressed</td>
<td>repressed</td>
</tr>
<tr>
<td>Glutamine synthetase*</td>
<td>&lt;0.0001</td>
<td>2.0</td>
</tr>
<tr>
<td>Vacuolar ATP synthase</td>
<td>0.0007</td>
<td>3.5</td>
</tr>
<tr>
<td>2-Oxoketoglutarate dehydrogenase</td>
<td>repressed</td>
<td>repressed</td>
</tr>
<tr>
<td>( \text{H}^+ )-transporting ATPase</td>
<td>repressed</td>
<td>repressed</td>
</tr>
<tr>
<td>Alpha tubulin</td>
<td>repressed</td>
<td>repressed</td>
</tr>
<tr>
<td>Neutral trehalase</td>
<td>0.0008</td>
<td>5.4</td>
</tr>
<tr>
<td>Cytochrome C1*</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial ATP Synthase (( \beta )-chain)</td>
<td>&lt; 0.0001</td>
<td>6.3</td>
</tr>
</tbody>
</table>

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 C₁ 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 C1, 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 (Goody, 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 $\gamma$-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 synthetase 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 (Dohlemann 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 interspecific 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](image)

**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 of 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, 0.5 % (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

<table>
<thead>
<tr>
<th>IEF steps</th>
<th>Voltage</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1</strong></td>
<td>150 V (for proteins from both fungi)</td>
<td>30 mins (SCSC/SCTR)</td>
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<td></td>
<td></td>
<td>5 mins (TRTR/TRSC)</td>
</tr>
<tr>
<td><strong>Step 2</strong></td>
<td>300 V (for proteins from both fungi)</td>
<td>30 mins (SCSC/SCTR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 mins (TRTR/TRSC)</td>
</tr>
<tr>
<td><strong>Step 3</strong></td>
<td>600 V (SCSC/SCTR); 800 V (TRTR/TRSC)</td>
<td>30 mins (for proteins from both fungi)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Step 4</strong></td>
<td>3500 V (for proteins from both fungi)</td>
<td>3 hrs 30 mins (SCSC/SCTR)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 hrs 10 mins (TRTR/TRSC)</td>
</tr>
</tbody>
</table>

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 analysis. 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-polyacrylamide 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 \textit{S. commune}; SCTR: \textit{S. commune} confronted by \textit{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*.

<table>
<thead>
<tr>
<th>Protein spot</th>
<th>NCBI Ac. No.</th>
<th>P-value (ANOVA)</th>
<th>Fold Increase</th>
<th>Protein identity</th>
<th>Mascot Score</th>
<th>Sequence Coverage (%)</th>
<th>Predicted mol. wt. (kDa)</th>
<th>Predicted pl</th>
<th>No of matched peptides*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC1</td>
<td>gi</td>
<td>189191156</td>
<td>1.679E-10</td>
<td>19.8</td>
<td>Hypothetical protein</td>
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<td>1</td>
<td>121</td>
<td>5.7</td>
</tr>
<tr>
<td>SC2</td>
<td>gi</td>
<td>169779443</td>
<td>4.242E-6</td>
<td>17.5</td>
<td>Hypothetical protein</td>
<td>84</td>
<td>15</td>
<td>18</td>
<td>5.9</td>
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<td>SC6</td>
<td>gi</td>
<td>38567258</td>
<td>1.938E-5</td>
<td>11.5</td>
<td>transcription factor BTF3a</td>
<td>79</td>
<td>15</td>
<td>16</td>
<td>7.6</td>
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<tr>
<td>SC8</td>
<td>gi</td>
<td>209446855</td>
<td>2.294E-11</td>
<td>10.3</td>
<td>phenylalanine ammonia-lyase</td>
<td>61</td>
<td>1</td>
<td>80</td>
<td>5.7</td>
</tr>
<tr>
<td>SC20</td>
<td>gi</td>
<td>34014958</td>
<td>6.405E-7</td>
<td>8.2</td>
<td>HEX1- Woronin body component</td>
<td>66</td>
<td>8</td>
<td>25</td>
<td>6.6</td>
</tr>
<tr>
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<td>gi</td>
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<td>2.739E-5</td>
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<td>202</td>
<td>9</td>
<td>35</td>
<td>9.6</td>
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<td>SC39</td>
<td>gi</td>
<td>8563739</td>
<td>2.211E-4</td>
<td>7.2</td>
<td>RNA polymerase II, 2nd largest subunit</td>
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<td>4</td>
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<td>9.6</td>
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<td>gi</td>
<td>187932846</td>
<td>7.598E-13</td>
<td>7.1</td>
<td>glycosyl transferase</td>
<td>29</td>
<td>2</td>
<td>49</td>
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<td>SC55</td>
<td>gi</td>
<td>409567</td>
<td>0.006</td>
<td>6.4</td>
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<td>7</td>
<td>18</td>
<td>5.4</td>
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<td>SC75</td>
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<td>225708148</td>
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<td>5.6</td>
<td>Probable E3 ubiquitin protein ligase</td>
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<td>13</td>
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<td>SC100</td>
<td>gi</td>
<td>51247011</td>
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<td>3</td>
<td>43</td>
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<td>169865690</td>
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<td>9.6</td>
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<td>SC150</td>
<td>gi</td>
<td>45610189</td>
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<td>4.0</td>
<td>Mitochondrial ATP synthase-D chain</td>
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<td>21</td>
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<td>3.6</td>
<td>Cyclophilin A-1</td>
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<td>9.6</td>
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<td>409567</td>
<td>0.002</td>
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<td>Translational initiation factor 5a</td>
<td>104</td>
<td>7</td>
<td>18</td>
<td>5.4</td>
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</tbody>
</table>

*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*.

<table>
<thead>
<tr>
<th>Protein spot</th>
<th>NCBI Ac. No.</th>
<th>P-value (ANOVA)</th>
<th>Fold Increase</th>
<th>Protein identity</th>
<th>Mascot Score</th>
<th>Sequence Coverage (%)</th>
<th>Predicted mol. wt. (kDa)</th>
<th>Predicted pl</th>
<th>No of matched peptides*</th>
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<tr>
<td>SC33</td>
<td>gi</td>
<td>28317</td>
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<td>SC34</td>
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<td>15614217</td>
<td>2.250E-8</td>
<td>7.6</td>
<td>nucleoside diphosphate kinase</td>
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<td>8</td>
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<td>5.0</td>
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<td>SC62</td>
<td>gi</td>
<td>169856964</td>
<td>8.196E-10</td>
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<td>40S ribosomal protein S14</td>
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<td>SC83</td>
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<td>Triosephosphate isomerase</td>
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<td>Trehalose phosphorylase</td>
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<td>5</td>
<td>21</td>
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<td>SC444</td>
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<td>50288201</td>
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<td>Hypothetical protein</td>
<td>243</td>
<td>8</td>
<td>47</td>
<td>5.7</td>
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</tbody>
</table>

*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*.

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

<table>
<thead>
<tr>
<th>Protein spot</th>
<th>NCBI Ac. No.</th>
<th>P-value (ANOVA)</th>
<th>Fold Increase</th>
<th>Protein identity</th>
<th>Mascot Score</th>
<th>Sequence Coverage (%)</th>
<th>Predicted mol. wt. (kDa)</th>
<th>Predicted pI</th>
<th>No of matched peptides*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR3</td>
<td>gi</td>
<td>108800163</td>
<td>1.887E-6</td>
<td>6.0</td>
<td>short-chain dehydrogenase/reductase</td>
<td>17</td>
<td>8</td>
<td>30</td>
<td>6.1</td>
</tr>
<tr>
<td>TR5</td>
<td>gi</td>
<td>15401284</td>
<td>4.097E-14</td>
<td>5.8</td>
<td>Mitochondrial ATP synthase, beta chain</td>
<td>91</td>
<td>7</td>
<td>54</td>
<td>5.3</td>
</tr>
<tr>
<td>TR15</td>
<td>gi</td>
<td>72052427</td>
<td>1.916E-9</td>
<td>4.2</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>83</td>
<td>10</td>
<td>36</td>
<td>7.7</td>
</tr>
<tr>
<td>TR16</td>
<td>gi</td>
<td>72052427</td>
<td>0.002</td>
<td>4.2</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>130</td>
<td>6</td>
<td>36</td>
<td>7.7</td>
</tr>
<tr>
<td>TR22</td>
<td>gi</td>
<td>164658544</td>
<td>1.366E-10</td>
<td>3.9</td>
<td>Hypothetical protein</td>
<td>106</td>
<td>16</td>
<td>13</td>
<td>4.8</td>
</tr>
<tr>
<td>TR29</td>
<td>gi</td>
<td>27803030</td>
<td>4.548E-10</td>
<td>3.7</td>
<td>Hypothetical protein</td>
<td>92</td>
<td>5</td>
<td>55</td>
<td>10.1</td>
</tr>
<tr>
<td>TR44</td>
<td>gi</td>
<td>46136835</td>
<td>2.104E-4</td>
<td>3.2</td>
<td>Hypothetical protein</td>
<td>204</td>
<td>15</td>
<td>37</td>
<td>9.9</td>
</tr>
<tr>
<td>TR68</td>
<td>gi</td>
<td>72052427</td>
<td>2.213E-10</td>
<td>2.7</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>356</td>
<td>17</td>
<td>36</td>
<td>7.7</td>
</tr>
<tr>
<td>TR80</td>
<td>gi</td>
<td>256727588</td>
<td>3.041E-11</td>
<td>2.6</td>
<td>Hypothetical protein</td>
<td>158</td>
<td>3</td>
<td>93</td>
<td>6.2</td>
</tr>
<tr>
<td>TR150</td>
<td>gi</td>
<td>47027997</td>
<td>0.003</td>
<td>2.0</td>
<td>Aspartyl protease</td>
<td>134</td>
<td>9</td>
<td>43</td>
<td>5.2</td>
</tr>
</tbody>
</table>

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*.

<table>
<thead>
<tr>
<th>Protein spot</th>
<th>NCBI Ac. No.</th>
<th>P-value (ANOVA)</th>
<th>Fold Increase</th>
<th>Protein identity</th>
<th>Mascot Score</th>
<th>Sequence Coverage (%)</th>
<th>Predicted mol. wt. (kDa)</th>
<th>Predicted pI</th>
<th>No of matched peptides*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR47</td>
<td>gi</td>
<td>84702107</td>
<td>9.263E-6</td>
<td>3.0</td>
<td>signal recognition particle-docking protein</td>
<td>37</td>
<td>2</td>
<td>48</td>
<td>4.4</td>
</tr>
<tr>
<td>TR59</td>
<td>gi</td>
<td>73960395</td>
<td>1.740E-12</td>
<td>2.8</td>
<td>Hypothetical protein</td>
<td>29</td>
<td>9</td>
<td>18</td>
<td>10.3</td>
</tr>
<tr>
<td>TR103</td>
<td>gi</td>
<td>409567</td>
<td>9.024E-12</td>
<td>2.3</td>
<td>Initiation factor 5a</td>
<td>96</td>
<td>7</td>
<td>18</td>
<td>5.4</td>
</tr>
<tr>
<td>TR115</td>
<td>gi</td>
<td>145608614</td>
<td>2.203E-9</td>
<td>2.2</td>
<td>Hypothetical protein</td>
<td>84</td>
<td>7</td>
<td>32</td>
<td>9.4</td>
</tr>
</tbody>
</table>
Table 7.7 Conserved domains and possible functions of *T. viride* proteins up-/down-regulated during interactions with *S. commune*.

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

**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 \( \text{cis} \leftrightarrow \text{trans} \) isomerisation of peptide bonds preceding proline residues; hence, they are also referred to as peptidyl-prolyl \( \text{cis} \rightarrow \text{trans} \) isomerase (PPIases) (Viaud *et al*., 2003; Schonbrunner *et al*., 1991; Wang & Heitman, 2005). The peptidyl-prolyl \( \text{cis} \rightarrow \text{trans} \) 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-Rodríguez *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 secretory 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 cyclophilin 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 microscopic 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 archaesa, 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. eIF-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 eIF-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 eIF-5A in *S. cerevisiae* resulted in increased ribosomal transit time; however, addition of recombinant eIF-5A from yeast expedited the rate of tripeptide synthesis *in vitro* (Saini *et al.*, 2009).

The same authors also observed that inactivation of eIF-5A mimicked eEF2 inhibitor, sordarin. eEF2 transports aminoacyl-tRNAs to the ribosome and enhances translocation (Preeti *et al.*, 2009). Based on these findings, Saini *et al.* (2009) inferred that eIF-5A might function jointly with eEF2 to support ribosomal translocation. Given the strong up-regulation observed for 3 proteins in *S. commune* identified as eIF-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 eIF-5A. Nonetheless, this does not exclude the possibility that eIF-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; Staszcak, 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 (Staszcak, 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 eIF-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 organelar 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 stress (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 recognize 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, asparagine, 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 isocitrate 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 diphosphates (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-phosphate (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.](image)

**Figure 7.11** Hypothetical schemes of biochemical events following the up-regulation of PAL, MDH, BCKAD and HEX.

Down-regulation of 5-methyltetrahydropteroylglutamate—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; Dohlemann 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. commute*. 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 C₁ 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, eIF5a, 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 *et al*., 1994; Griffith *et
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. Moreover, 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-qPCR 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


**Cited web references**


http://biotools.umassmed.edu/bioapps/primer3_www.cgi


http://csbd.mpimp-golm.mpg.de/csbdb/gm/msri/gmd_conributuions.html

http://www.mathworks.com

Appendices
Appendix I

Section 1: Gas Chromatography-Time of Flight-Mass Spectrometry (GC-TOF-MS), see Chapter 3; section 3.2.3

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 d₄ acid, glycine d₅ and malonic d₂ acid was added to the samples before lyophilisation. 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 40°C), and trimethsilylation with N-acetyl-N-(trimethylsilyl)-trifluoroacetamide (50 µl) for 80 mins at 40°C.

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 d₄ 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_conributions.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 to 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
Section 3: Comparative plot of metabolite peak areas between self- and non-self paired cultures (see Chapter 3: section 3.3.2)

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.

Figure 4: Malondialdehyde standard graph for lipid peroxidation assay.

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

1X Phosphate buffered saline (PBS)

1. The following were dissolved in 800 ml of distilled water;
   - 8 g of NaCl
   - 0.2 g of KCl
   - 1.44 g of Na₂HPO₄
   - 0.24 g of KH₂PO₄
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 121°C 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

Section 1: A plot of the band volumes of DNA products after RT-PCR in the mycelia of *S. commune* paired against *T. viride*, compared to self-paired mycelia of the former (Chapter 6; section 6.3)

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 50 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*.

<table>
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<th>Spot</th>
<th>No. of peptide sequences</th>
<th>Peptide sequences</th>
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<td>1</td>
<td>QRDQIDQWMQGIIR</td>
</tr>
<tr>
<td>SC2</td>
<td>2</td>
<td>KGFGYKGSSFHVR, RVIPQFMLQGFDFTQG</td>
</tr>
<tr>
<td>SC6</td>
<td>2</td>
<td>KLAESYQNMQKN, KSDGNVIIHAAFPKV</td>
</tr>
<tr>
<td>SC8</td>
<td>1</td>
<td>RKSEEAEIILRL</td>
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<td>2</td>
<td>RLQQAFESGRG, RYLGVDLFTKQ</td>
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<tr>
<td>SC41</td>
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<td>RRMQFLGEIOVVRG</td>
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<td>SC75</td>
<td>1</td>
<td>KAYDLHVLKR</td>
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<tr>
<td>SC97</td>
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<td>KVHLVAIDIFTGKK</td>
</tr>
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<td>1</td>
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<tr>
<td>SC100</td>
<td>1</td>
<td>RGEKVPVGVTLATITRT</td>
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<tr>
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<td>KHVVFGEVVEGMDVVKN</td>
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<tr>
<td>SC394</td>
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<td>KVHLVAIDIFTGKK</td>
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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*.

<table>
<thead>
<tr>
<th>Spot</th>
<th>No. of peptide sequences</th>
<th>Peptide sequences</th>
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<tbody>
<tr>
<td>SC33</td>
<td>2</td>
<td>KHGNSHQGEPRD, RLKYENEVALRQ</td>
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<td>KTPGPAQSAKLRA, RCVGITALHIKL, RIEDVTVPDSTR, RDESSPYAAMLAAQDVAARC</td>
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<td>SC302</td>
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<td>SC313</td>
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<td>GIPNVIDSYAR, VRPELPIIYR, AVFVPEDVIER, IHLIDDEYRK, SGFLTEPGDNAQVAR, NNHNILQGVASPDLR</td>
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<tr>
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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*.

<table>
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<td>TR15</td>
<td>3</td>
<td>YDSSHGSFK, AASYEEITNAIK, VPTANVSVIDLTVR</td>
</tr>
<tr>
<td>TR16</td>
<td>2</td>
<td>YDSSHGSFK, VPTANVSVIDLTVR</td>
</tr>
<tr>
<td>TR22</td>
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<td>YEDISPSTHNMDVPNVT, KYEDISPSTHNMDVPNVT</td>
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<tr>
<td>TR29</td>
<td>2</td>
<td>VEQAEIQQEPSGR, NAIQPFNGYDWQGR</td>
</tr>
<tr>
<td>TR44</td>
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</tr>
<tr>
<td>TR68</td>
<td>5</td>
<td>YDSSHGSFK, VLDLLAHVAK, AASYEEITNAIK, VPTANVSVIDLTVR, LVSWYDNEWGYSR</td>
</tr>
<tr>
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<td>DSVSVGFQWASR, VLADDFGWVDIDAR</td>
</tr>
<tr>
<td>TR150</td>
<td>2</td>
<td>GHTLYNPTK, VSSEFVSDTNSGGLGLALDSINTVSPK</td>
</tr>
</tbody>
</table>

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*.

<table>
<thead>
<tr>
<th>Spot</th>
<th>No. of peptide sequences</th>
<th>Peptide sequences</th>
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</thead>
<tbody>
<tr>
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<td>TR59</td>
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<td>APPSRAPPASPPAR</td>
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<tr>
<td>TR103</td>
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<td>VHLVAIDIFTGK</td>
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<tr>
<td>TR115</td>
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<td>VDVHYYEDGNVR</td>
</tr>
</tbody>
</table>
Appendix VI

Identification of *T. viride* by PCR

**Primer sequences:**

Forward - CGTCATTTCAACCCTCGAAC  
Backward – CCTACCTGATCCGAGGTCAA

**NCBI Accession number:** HM438946