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Proteomic response of *Trichoderma aggressivum* f. *europaeum* to *Agaricus bisporus* tissue and mushroom compost

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ABSTRACT

A cellular proteomic analysis was performed on *Trichoderma aggressivum* f. *europaeum*. Thirty-four individual protein spots were excised from 2-D electropherograms and analysed by ESI-Trap Liquid Chromatography Mass Spectrometry (LC/MS). Searches of the NCBInr and SwissProt protein databases identified functions for 31 of these proteins based on sequence homology. A differential expression study was performed on the intracellular fraction of *T. aggressivum* f. *europaeum* grown in media containing *Agaricus bisporus* tissue and Phase 3 mushroom compost compared to a control medium. Differential expression was observed for seven proteins, three of which were upregulated in both treatments, two were down regulated in both treatments and two showed qualitatively different regulation under the two treatments. No proteins directly relating to fungal cell wall degradation or other mycoparasitic activity were observed. Functions of differentially produced intracellular proteins included oxidative stress tolerance, cytoskeletal structure, and cell longevity. Differential production of these proteins may contribute to the growth of *T. aggressivum* in mushroom compost and its virulence toward *A. bisporus*.

Introduction

Trichoderma aggressivum is a filamentous fungus which causes severe economic losses in the cultivation of the edible mushroom *Agaricus bisporus* (Seaby 1987; Samuels *et al.* 2002; Mamoun *et al.* 200b). It was first isolated in Ireland in the late 1980s and has since been reported across Europe and North America (Muthumeenakshi *et al.* 1994; Fuente *et al.* 1998; Hermosa *et al.* 1999; Mamoun *et al.* 2000a; Kredics *et al.* 2010; Sobieralski *et al.* 2010). There are two subspecies, *T. aggressivum* f. *aggressivum* and *T. aggressivum* f. *europaeum* found in North America and Europe, respectively (Samuels *et al.* 2002).

Trichoderma aggressivum colonises the compost used as a growth substrate in mushroom cultivation (Largeteau & Savoie 2010). In later stages of colonisation green coloured conidia form in both the compost and the casing layer of infected areas which gives the condition its common names, green mould disease or *Trichoderma* compost mould (Seaby 1987; Fletcher & Gaze 2008). In areas colonised by *T. aggressivum* mushroom fruit body formation is retarded and fruit bodies that do form may be of poor quality due to damage or

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discolouration (Largeteau & Savoie 2010). The estimated losses in mushroom yield caused by *T. aggressivum* worldwide are in the tens of millions of dollars (Kredics *et al.* 2010).

The mechanisms of *T. aggressivum* colonisation of mushroom compost are not fully understood but it is thought to involve both mycoparasitic and saprotrophic components (Williams *et al.* 2003). *Trichoderma aggressivum* can colonise mushroom compost in the presence or absence of *A. bisporus*, but it sporulates more heavily when *A. bisporus* is present (Largeteau & Savoie 2010). Its growth is inhibited by compost microbiota, but less so than other *Trichoderma* species (Savoie *et al.* 2001a).

The tolerance of *T. aggressivum* to the presence of compost microbiota and *A. bisporus* and its ability to acquire nutrition from the mushroom compost and mushroom spawn have been proposed as the attributes that make it so harmful to mushroom agriculture (Savoie *et al.* 2001a; Williams *et al.* 2003; Fletcher & Gaze 2008). *Trichoderma aggressivum* is known to produce metabolites which are toxic to *A. bisporus* (Mumpuni *et al.* 1998; Krupke *et al.* 2003; Guthrie & Castle 2006), but other *Trichoderma* species, which do not cause severe green mould disease, are known to produce metabolites which may be as toxic (Mumpuni *et al.* 1998).

In this study, proteomic analysis was employed to study the response of *T. aggressivum* f. *europaeum* to mushroom compost and *A. bisporus* tissue *in vitro*. Previous studies (Savoie *et al.* 2001b; Williams *et al.* 2003; Guthrie & Castle 2006) have described the importance of proteins secreted by *T. aggressivum* (prev. *Trichoderma harzianum*) in its interaction with *A. bisporus*, however there is little information published on the intracellular protein fraction of *T. aggressivum*, which is the focus of this study.

Materials and methods

Culture conditions

Stocks of *Trichoderma aggressivum* f. *europaeum* strain CBS 100526 were retrieved from liquid nitrogen storage and cultured for 2 d on malt extract agar (Fluka) at 25 °C in the dark. Plugs (5 mm) were excised from the growing edge of a culture and inoculated into liquid media (100 ml volume in a 250 ml conical flask) and incubated at 20 °C on an orbital shaker at 100 rpm in the dark. For proteome analysis the medium consisted of 2 % (w/v) malt extract (Fluka) and incubation was carried out for 3 d until hyphal mass was 4–5 g wet weight. For differential protein analysis three treatments were prepared: treatment 1 consisted of 1 % (w/v) malt extract with 1 % w/v mushroom cap tissue added prior to autoclave sterilisation, treatment 2 consisted of 1 % (w/v) malt extract with 1 % (w/v) lyophilised Phase 3 mushroom compost added prior to autoclave sterilisation, the control treatment consisted of 1 % malt extract only. Mushroom cap tissue was obtained from the commercial *Agaricus bisporus* strain Sylvan A15 harvested prior to opening, several fruit bodies were finely chopped and a random sample of fresh cap tissue was removed. Phase 3 compost was obtained by inoculating Phase 2 mushroom compost with Sylvan A15 spawn and incubating under standard industrial spawn-run conditions until *A. bisporus* colonisation was complete, compost was lyophilised for storage, and rehydrated in culture medium prior to autoclaving. Cultures for differential protein analysis were incubated for a total of 7 d and wet weights of 3–4 g hyphae were attained. Malt extract was used as the base medium to ensure a consistent level of hyphal growth and adequate protein yields and because growth on more minimal media causes the induction of mycoparasitism related genes in *Trichoderma* species due to starvation (Viterbo *et al.* 2002).

Protein extraction

After the incubation period the whole mycelial mass was filtered through a double layer of autoclaved Miracloth and washed twice with sterile water to remove residual culture medium. Cells were lysed by grinding in liquid nitrogen and the lysate resuspended in homogenisation buffer (0.4 M NaCl, 10 mM tris–HCl, 2 mM Ethylenediaminetetraacetic Acid (EDTA), 10 μg pepstatin A ml^{-1}, Tosyllysine Chloromethyl Ketone (TLCK), leupeptin and aprotinin, pH 8.0). Resuspended lysate was transferred to 1.5 ml Eppendorf tubes and centrifuged at 1000 rcf at 4 °C for 5 min. The supernatant containing cellular protein was removed to a fresh Eppendorf tube and the pellet discarded. Protein concentration was determined with the Biorad Bradford assay kit using a Biophotometer spectrophotometer (Eppendorf) calibrated at a range of 100–1500 mg ml^{-1} against BSA standards (Bradford, 1976). Protein samples (350 mg) were acetone precipitated, concentrated by centrifugation, and resuspended in 250 ml isoelectric focussing (IEF) buffer (8 M urea, 1 % triton X100, 4 % 3[(3-Chloamidopropyl) dimethy (ammanio)] Propanesulfonate (CHAPS), 10 mM tris–HCl, 2 M thiourea, 65 mM Diothiothreitol (DTT), and ~0.01 g bromophenol blue). Protein for differential analysis was prepared from two biological replicates per treatment and pooled within each treatment prior to IEF.

IEF and 2-D PAGE

IEF was carried out using 13 cm Immobiline Drystrip pH 4–7 gradient strips (GE Healthcare) in a coffin system Ettan IPGPhor II (Amersham bioscience). Samples were added directly to the strip by active rehydration followed by IEF. Rehydration focussing protocol was as follows: 50 V for 10 h, 0.25 V for 30 min, 8000 V gradient for 5 h, 8000 V for 8 h. After focussing, strips were equilibrated for 10 min in reducing buffer (30 % glycerol, 2 % SDS, 6 M urea, 50 mM tris–HCl, 1 % DTT, pH 6.8) followed by 10 min in alkylating buffer (30 % glycerol, 2 % SDS, 6 M urea, 50 mM tris–HCl, 12.5 % Indole acetic acid (IAA), pH 6.8).

Isoelectrically focussed proteins were resolved using Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE). Slab gels (12.5 %) were cast at 1.5 cm thickness in a Biorad Protean II multi-gel casting chamber. Focused and equilibrated IEF Drystrips were laid on top of the gel and sealed with agarose sealing solution (1 % agarose, 0.1 % bromophenol blue in 1× gel running buffer). Gels were run in triplicate for 18 h at a constant wattage of 1.5 W per gel in a Biorad Protean Plus Dodeca-Cell using system using 1× gel running buffer (190 mM tris–HCl, 0.19 M glycine, 0.1 % SDS) at 10 °C.

Protein visualisation and relative quantification

Proteins were visualised using the colloidal Coomassie staining method (Neuhoff *et al.* 1988; Candiano *et al.* 2004). Each gel was incubated in fixing solution (50 % (v/v) ethanol, 3 % (v/v) phosphoric acid) for 3 h on an orbital shaker, washed three times in distilled-deionised water and transferred to pre-incubation buffer (34 % (v/v) ethanol, 17 % (w/v) ammonium sulphate, 3 % phosphoric acid) for 20 min on an orbital shaker. Coomassie Brilliant blue G-250 (Serva) was added to the pre-incubation buffer (0.5 g 100 ml^{-1}) and the gels were stained at room temperature on an orbital shaker for 7 d. Gels were destained by transferring to a new container and washing with distilled-deionised water until no stain remained.

Gels were scanned as transparencies using an Epson ImageScanner III (Epson (UK) ltd., Hertfordshire, UK) as 16 bit greyscale images with a resolution of 600 dpi. For simple protein identification spots were selected across the pH and weight range based on good separation and resolution. For analysis of differential proteins Progenesis Samespots software version 3.3 (Nonlinear Dynamics) was used. Gels images were normalised, analysed by two-way analysis of variance (ANOVA) and protein spots which changed in intensity at the $P < 0.05$ level were selected and identified by Liquid Chromatography Mass Spectrometry (LC/MS).

Protein identification by LC/MS

Stained protein spots were excised and trypsin digested in gel as described in Shevchenko *et al.* (2006). Resuspended peptides were separated using an Agilent 1200 series nanoflow liquid chromatograph and analysed with an Agilent 6340 series ion trap mass spectrometer. Capillary voltage was set to 1800 V with a constant capillary flow rate of 2 μl min^{-1}. The mobile phase consisted of water and 90 % acetonitrile both containing 0.1 % (v/v) formic acid. Sample injection size was 5 μl.

Compounds were generated using the Agilent software and homologies were identified using the Mascot search function of Matrix Science (www.matrixscience.com) under the following parameters: trypsin enzyme, up to two missed cleavages, fixed carboxymethyl (C) modification, variable oxidation (M) modification, 2 Da peptide tolerance, 1 Da MS/MS tolerance, 1 + 2+ 3 + peptide charge, precursor *m/z* n/a, and ESI-TRAP instrument. Searches were carried out against all Fungi in the SwissProt and NCBInr Databases and protein matches with Mascot scores of >46 or >59 were taken to exhibit significant homology for each database, respectively. Functions were assigned to proteins based on sequence similarity using the UniProt database.

Results

Analysis of Trichoderma aggressivum *proteome*

LC/MS analysis allowed the identification of homologues for 27 *T. aggressivum* proteins. The position of each protein spot which was excised and analysed is indicated in Fig 1. The UniProt protein accession number, Mascot score, coverage and

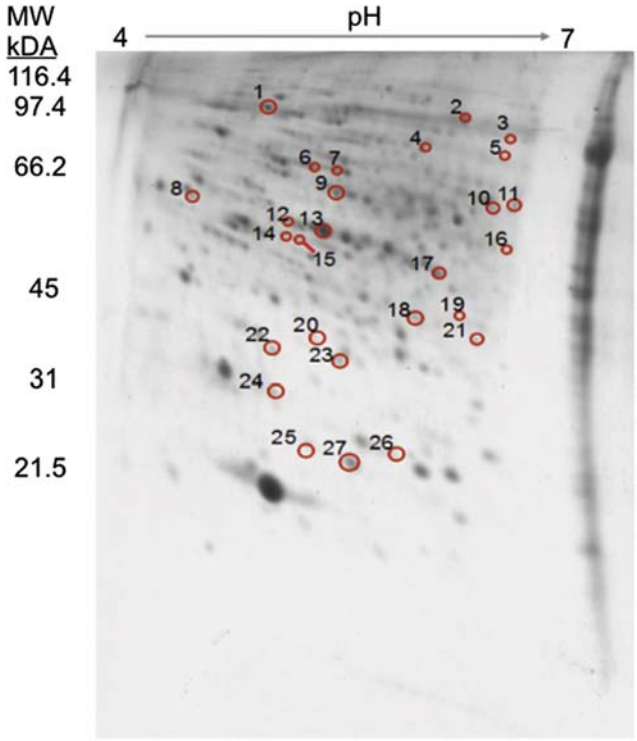

Fig 1 – Intracellular protein from *T. aggressivum* grown on 2 % malt extract, and stained with colloidal Coomassie. Proteins analysed by LC/MS are labelled 1–27 and described in Table 1.

assigned functions for each protein returned from searches of the NCBInr protein database are shown in Table 1. The majority of proteins were matched to the *Trichoderma virens*, *Trichoderma reesei*, or *Trichoderma harzianum* genomic databases (Martinez *et al.* 2008; Kubicek *et al.* 2011) with others coming from less closely related fungal species.

Analysis of proteomic response of Trichoderma aggressivum *to* Agaricus bisporus *tissue and Phase 3 mushroom compost*

Seven proteins were identified which showed a statistically significant ($P < 0.05$) change in abundance in either the mushroom compost or *A. bisporus* tissue treatment or both, relative to the control. The position from which each of these proteins was excised from the 2-D gel is shown in Fig 2, labelled spots A–G. The relative intensity of each spot and results returned from LC/MS analysis and Mascot search for each protein is given in Table 2.

Discussion

Trichoderma aggressivum is unique among *Trichoderma* species in terms of the damage it causes in the cultivation of *Agaricus bisporus*. This study is the first 2-D proteomic study on *T. aggressivum* and the first to assess the impact of *A. bisporus* tissue and mushroom compost on intracellular protein abundance in *T. aggressivum*. In this study 34 protein homologues

Table 1 – Mascot search results for LC/MS analysed intracellular proteins from *T. aggressivum* grown on malt extract. Spot number refers to gel position indicated in Fig 1.

Spot label	UniProt accession	Protein function	Species	Mascot score	Coverage %
1	G9NAQ0	Aldehyde dehydrogenase	*Trichoderma virens*	122	12
2	A3LYZ4	Lysophospholipase NTE1	*Scheffersomyces stipitis*	71	2
3	G9N9F6	Transketolase	*Trichoderma virens*	72	3
4	G9N370	Glucose-methyl-choline oxido reductase	*Trichoderma virens*	598	22
5	G0RFA6	Dehydratase	*Trichoderma reesei*	352	19
6	E9DS21	Aldehyde dehydrogenase	*Metarhizium acridum*	97	7
7	G9MTN9	Rab GDP-dissociation inhibitor	*Trichoderma virens*	636	41
8	G9N455	Translation elongation factor	*Trichoderma virens*	216	28
9	D6C5B7	Actin	*Cleistogenes songorica*	676	46
10	G9MVH9	6-phosphogluconate dehydrogenase (decarboxylating)	*Trichoderma virens*	370	16
11	G9NAQ0	Aldehyde dehydrogenase	*Trichoderma virens*	943	48
12	G0RNE6	Thiolase	*Trichoderma reesei*	357	29
13	F0XBV7	Pyruvate dehydrogenase e1 subunit	*Grosmannia clavigera*	178	9
14	G9MRQ8	Uncharacterised	*Trichoderma virens*	518	29
15	G9NAQ0	Aldehyde dehydrogenase	*Trichoderma virens*	206	10
16	G9NMQ9	Norsolorinic acid reductase	*Trichoderma atroviride*	154	7
17	G9MU97	Methyltransferase	*Trichoderma virens*	98	3
18	A1D8L8	Secretory lipase	*Aspergillus fischerianus*	60	4
19	Q00640	Glyceraldehyde-3-phosphate dehydrogenase	*Erysiphe graminis*	115	6
20	G9N428	Proteasome subunit alpha type decarboxylating	*Trichoderma virens*	415	41
21	G9MEX6	Glycolysis	*Trichoderma virens*	99	2
22	Q6FS57	Uncharacterised	*Torulopsis glabrata*	61	3
23	G9N2A8	Transcription regulation factor	*Trichoderma virens*	151	18
24	G9MQT2	Oxidoreductase	*Trichoderma virens*	114	18
25	E0YRV9	Hex1	*Trichoderma harzianum*	623	57
26	G0RAL0	Uncharacterised	*Trichoderma reesei*	219	39
27	G0R881	Uncharacterised	*Trichoderma reesei*	132	17

were identified for *T. aggressivum* proteins (Figs 1 and 2), 31 of them with known or inferred functions (see Tables 1 and 2). These homologues were drawn from a wide range of species, most commonly from the genus *Trichoderma*. The identified proteins can be divided into metabolic, informational, structural, and stress response functions.

Metabolic proteins are involved in the uptake of nutrients and the production of energy and the majority of proteins identified in this study were from this category. Of the analysed proteins 11 had functions relating to the degradation or modification of proteins (no. 5, 6, 12, 20), carbohydrates (no. 4, 13), and lipids (no. 2, 18) or the glycolytic pathway (no.

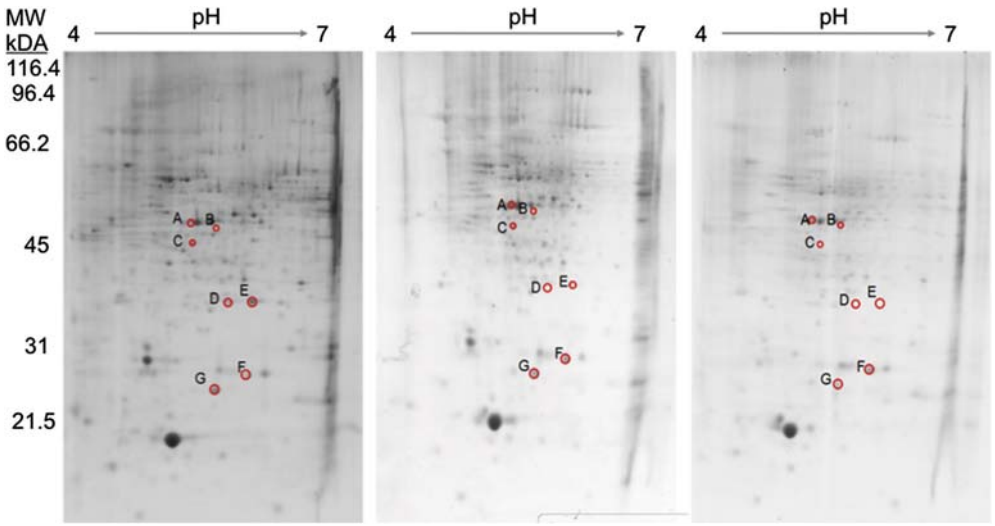

Fig 2 – *Trichoderma aggressivum* protein spots showing significant differential expression in media containing mushroom compost and/or *A. bisporus* tissue based on Progenesis Samespots analysis. From left to right the treatments are control, *A. bisporus* tissue and mushroom compost. LC/MS and densitometry results for each spot are given in Table 2.

Table 2 – Mascot search results for LC/MS analysed *T. aggressivum* proteins showing differential expression in media containing *A. bisporus* tissue or Phase 3 mushroom compost. Letters refer to spot position given in Fig 2 and fold changes are expressed relative to the control treatment.

Spot label	Accession number	Protein function	Species	Mascot score	% Coverage	Expression – tissue	Expression – compost
A	G9ND76	60S acidic ribosomal protein	*Trichoderma viride*	242	27	1.91	1.76
B	G9N1G1	Superoxide dismutase (Fe–Mn)	*Trichoderma viride*	146	17	1.15	2.89
C	G9N9E1	Spermine/spermidine synthase	*Trichoderma viride*	157	15	−1.31	−1.58
D	C5E1C0	Maintenance of telomere capping protein 2	*Zygosaccharomyces rouxii*	64	5	−1.93	−1.82
E	F0X7L2	Guanylate kinase	*Grosmannia clavigera*	80	5	2.05	−2.03
F	A8BA83	Superoxide dismutase [Cu–Zn]	*Trichoderma harzianum*	478	75	1.93	3.04
G	G0R881	Actin depolymerase	*Trichoderma reesei*	132	17	1.62	2.29

10, 19, 21) while six were identified as transferases (no. 3, 17) and aldehyde dehydrogenases (no. 1, 6, 11, 15) with unknown catalytic activity (Table 1). Specific proteins involved in the degradation of pentose sugars (no. 10, 13) may be relevant to the degradation of carbohydrates liberated during degradation of fibrous components of mushroom compost, however there was no significant change to these proteins in either experimental treatment (Table 2).

No proteins directly involved in the degradation of fungal cell wall components were identified. This may be partly due to the fact that the fungus was grown under non-inducing conditions. Previous studies have shown the secretion of fungal cell wall associated depolymerising enzymes by *T. aggressivum* in the presence of *A. bisporus* (Williams *et al.* 2003; Guthrie & Castle 2006) and the production of these enzymes is considered to be indicative of mycoparasitic activity. However, in both studies the depolymerising enzymes were secreted into the extracellular space of *T. aggressivum* and so it may be that these proteins are insensitive to change or are present in relatively low abundance in the intracellular protein fraction.

Informational proteins are those that are involved in DNA duplication, transcription, and translation. These proteins are conserved between species and may be constitutively produced. Two transcription factors (no. 23, 25) and one protein which is a component of the ribosome (Table 2, spot A) were identified. One transcription factor (no. 23) has no known function or associated genes. The other, Hex 1 (no. 25) has homologues in all *Trichoderma* species (Table 1). Hex 1 is a major component of the woronin body complex as well as a transcription factor. Woronin bodies are present at the periphery of fungal hyphae where they prevent cellular leakage by blocking septal pores when hyphae are sheared (Lew 2011). Sequence analysis of Hex 1 from reveals that it has a peroxisome targeting signal and a high level of conservation between fungal species (Curach *et al.* 2004). In other *Trichoderma* species it has been shown to account for a significant amount of the total protein associated with the cell envelope (Lim *et al.* 2001) and is differentially produced in response to different carbon sources (Curach *et al.* 2004) and under biological control conditions (Marra *et al.* 2006) but no change was observed in Hex 1 abundance in either experimental treatment in this study.

There was a statistically significant down-regulation of the ribosome component 60S acidic ribosomal protein P0 (Table 2, spot A). Abundance of this protein was observed to change in both treatments. The 60S acidic ribosomal protein P0 is a protein conserved in all eukaryotes which is the orthologue of the *Escherichia coli* protein L10 (Rich & Steitz 1987). Differential expression and regulation of 60S ribosomal protein P0 have been shown to be related to the cellular response to various stresses, including oxidative stress (Abramczyk *et al.* 2003; Maniratanachote *et al.* 2006).

Structural proteins are involved in cell stability, compartmentalization, and growth and they are typically constitutively produced (Klemsdal *et al.* 1996). No change was observed in the production of Rab GDP-dissociation inhibitor (no. 7) or Actin (no. 9) (Table 1), proteins involved in protein transport, and the cytoskeleton, respectively. A protein with an actin binding and depolymerising function was identified (Table 2, spot G) which was upregulated in response to both experimental treatments which indicates that there may be an alteration of cytoskeletal structure in *T. aggressivum* exposed to *A. bisporus* and mushroom compost.

The differentially produced proteins identified in this experiment can be divided into four groups: proteins increased in response to both treatments, proteins decreased in response to both treatments, proteins increased only in one treatment, and proteins increased in one treatment while reduced in the other.

Proteins increased in both treatments include: 60S acidic ribosomal protein (Fig 2, spot A), Fe–Mn superoxide dismutase (Table 2, spot B), and an actin depolymerising enzyme from *Trichoderma reesei* (Table 2, spot G). For spots B and G the increase was greater in the mushroom compost treatment than the *A. bisporus* treatment.

The 60S acidic ribosomal protein and Fe–Mn superoxide dismutase are involved directly or indirectly in the oxidative stress response, and an increase in their production may result from components of *A. bisporus* tissue and mushroom compost which exert oxidative stress on *T. aggressivum*, or which induce changes in the metabolism of *T. aggressivum* leading to an increase of intracellular reactive oxygen species.

Actin depolymerising enzyme can modify the structure of the actin cytoskeleton, which may lead to changes in hyphal growth. It has been previously shown that *A. bisporus* metabolites increase the rate of extension of *T. aggressivum* hyphae (Muthumeenakshi *et al.* 1998) and that and saprotrophic growth in mushroom compost is key to its survival strategy

(Williams et al. 2003). The re-arrangement of the actin cytoskeleton may be an aspect of both of these behaviours.

Proteins decreased in both treatments: two proteins (Table 2, spots C and D) were down regulated in both experimental treatments. Protein C was identified as spermine synthase and was only slightly reduced in the *A. bisporus* treatment with a more substantial decrease in abundance being observed in the mushroom compost treatment. Protein D was homologous with maintenance of telomere capping protein 2 from *Zygosaccharomyces rouxii* and was decreased in production to an approximately equal extent in both treatments.

Spermidine synthase catalyses the production of the polyamine spermidine from spermine (Pegg 1986). A reduction in the amount of spermidine synthase may relate to changes in the oxidative stress response as both spermidine and its precursor spermine are anti-oxidants which are also transcription factors for other genes involved in the degradation of reactive oxygen species (Kuznetsov et al. 2006; Tkachenko & Fedotova 2007; Chattopadhyay et al. 2009).

Deletion of the gene encoding maintenance of telomere capping protein 2 in *Z. rouxii* results in a phenotype more susceptible to telomere-shortening related cell-cycle arrests when exposed to heat stress (Addinall et al. 2008). The mechanism by which this protein protects the telomere cap is not known, but if it serves the same function in *T. aggressivum* then a decrease in the amount of this protein may lead to reduced cell longevity.

Proteins increased only in one treatment: copper–zinc superoxide dismutase (Table 2, spot F) was upregulated in the mushroom compost treatment, but not substantially altered in response to *A. bisporus* tissue. This protein serves a similar function as Fe–Mn superoxide dismutase. The production of this protein may indicate that oxidative stress resulting from exposure to mushroom compost is more significant than that experienced by *T. aggressivum* growing in medium containing *A. bisporus* tissue.

Proteins increased in one treatment while reduced in the other: guanylate kinase (Table 2, spot E) was increased twofold in the *A. bisporus* tissue treatment, but reduced by a similar amount in the mushroom compost treatment. This protein is essential in the production of the purine base guanine, and as such is vital to cell replication (Konrad 1992). The product of guanylate kinase, guanosine diphosphate, is also an intermediary in the cyclic GMP signalling pathway. In fungi the cyclic GMP pathway is part of processes such as conidiation, cell division (Eckstein 1988) and hyphal extension, and branching (Robson et al. 1991). It is difficult to assess what exact role guanylate kinase may be playing in this experiment but it is interesting that it is the only protein to have shown the opposite response to the mushroom compost and *A. bisporus* tissue treatments.

Conclusion

The results presented here give new insight into the proteome of *Trichoderma aggressivum* f. *europaeum* and its response to *Agaricus bisporus* and mushroom compost. Several proteins were shown to be differentially produced in the presence of *A. bisporus* tissue and/or Phase 3 mushroom compost with functions relating to stress tolerance, cell signalling, longevity, and structure. These functions may be part of the capability of *T. aggressivum* to grow uninhibited in commercial mushroom composts, displacing *A. bisporus*, and ultimately reducing mushroom yields.

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