



Characterisation of the interaction of *Pseudomonas putida* and *Pseudomonas tolaasii* with *Trichoderma aggressivum*

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Abstract Green mould disease is caused by *Trichoderma aggressivum* which colonizes mushroom compost and reduces yield. Two *Pseudomonas* species are associated with mushroom compost: *Pseudomonas putida*, which stimulates mushroom pinning, and *Pseudomonas tolaasii* which has a negative effect on crop production. The aim of this work was to characterize *T. aggressivum* – *Pseudomonas* interactions as these may be important factors in the development of green mould disease. *P. tolaasii* supernatant inhibited growth by 57% but *P. putida* stimulated growth of *T. aggressivum* by 44%. Tolaasin production was identified in *P. tolaasii* cultures with a peak at 96 h. Fluorescent microscopy examination of *T. aggressivum* hyphae revealed that exposure to *P. tolaasii* supernatant decreased mycelial formation while increasing the abundance of conidia. Label free proteomic analysis of changes in the abundance of *T. aggressivum* proteins indicated that exposure to *P. tolaasii* supernatant lead to an oxidative stress response and catabolic enzyme

activation (mitochondrial import inner membrane translocase complex (5.7-fold), oxidoreductase (5.2-fold), glucoamylase (5.1-fold)). Exposure of *T. aggressivum* to *P. putida* supernatant lead to an increase in the abundance of proteins associated with growth and development (structural constituents of ribosome (20-fold), H/ACA ribonucleoprotein complex subunit (18-fold), DNA binding and nucleosome assembly protein (5.3-fold), and prefoldin (5-fold)). These results indicate that exposure to *P. putida* can stimulate the growth of *T. aggressivum* and this interaction may be an important factor in increasing green mould disease in mushroom crops and so reducing yield.

Keywords *Agaricus* · *Trichoderma* · Proteomics · *Pseudomonas*

Abbreviations

FDR	False Discovery Rates
GO	gene ontology
SSDA	statistically significant differentially abundant
DEP	differentially expressed proteins (DEP)
NB	Nutrient broth
SN	supernatant

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Introduction

White button mushroom (*Agaricus bisporus* (Lange) Imbach) accounts for 15% of total mushroom production worldwide, with a production of 4.43 billion kg in

2013 and it is the most commonly cultivated mushroom in North America and Europe (Royse et al. 2017). In mushroom cultivation, naturally occurring bacteria and fungi coexist in the compost, breaking down nutrient rich straw and manure and preparing the substrate for *A. bisporus* growth (Chang and Miles 2004; Potocnik et al. 2012). Minor green mould diseases are caused by several species of the genus *Trichoderma* which have been present in mushroom industry for many years (Sinden and Hauser 1953; Fletcher and Gaze 2008). In the early 1980s a new species was identified in Ireland as *T. aggressivum* (Seaby 1987, 1996) and there have been many reports of its spread worldwide (Rinker 1993; Romaine et al. 1996; Geels 1997; Hermosa et al. 1999; Mamoun et al. 2000; Hatvani et al. 2007; Szczech et al. 2008; Romero-Arenas et al. 2009; Clift and Shamshad 2009; Hatvani et al. 2012; Kosanovic et al. 2013). This filamentous micro-fungus is a rapidly growing, aggressive competitor that has evolved and adapted to a specific mushroom growing niche (Kredics et al. 2010; Kosanovic et al. 2015). It colonizes the substrate and casing for *A. bisporus* growth, influences the mushroom yield and causes severe crop losses (O'Brien et al. 2017). It has been shown that the dilution level of *T. aggressivum* inoculum in *A. bisporus* compost directly correlates with reduction in mushroom yield. Crop loss of 2 to 6% has been found at the dilution level of 10^{-4} and 60 to 100% loss at the 10^{-1} level (O'Brien et al. 2017). Our unpublished data show that a 10^{-4} *T. aggressivum* inoculation (4.5×10^8 conidia/g) leads to 20 to 50% crop loss and a 10^{-3} inoculum (4.5×10^9 conidia/g) results in *A. bisporus* yield loss of 60 to 85%, indicating variability in crop responses. Strains of naturally occurring compost and casing organisms, *Arthrobacter terregens*, *Bacillus megaterium* and *Rhizobium* sp., have been found to stimulate fruiting of *A. bisporus* (Park and Agnihotri 1969). *Pseudomonas* bacteria, such as *Pseudomonas putida*, are significant promoters of mushroom fruiting body formation (Rainey 1991) since 10% of compost and sometimes more than 50% of casing bacteria belong to *Pseudomonas* genus (Godfrey et al. 2001). The exact mechanism that stimulates mushroom pinning has not been established, but it has been suggested that bacteria promote pinning by consumption of self-inhibitor compounds known as 1-octen-3-ol and 2-ethyl-1-hexanol produced by *A. bisporus* mycelium (Noble et al. 2009; Zarenejad et al. 2012). Other compost and casing *Pseudomonas* spp. are mycopathogens and these can cause

diseases in mushrooms such as brown (*P. tolaasii*) and ginger (*P. gingeri*) blotch of *Agaricus bisporus* (Tolaas 1915; Wong et al. 1982) and drippy gill disease or yellow (*P. agarici*) blotch of white button mushrooms (Young 1970; Soler-Rivas et al. 2000). *P. reactans* can cause brown discoloration on mushrooms (Wells et al. 1996) but *Pseudomonas tolaasii* which can produce tolaasin is the causative agent of mushroom brown blotch disease (Cho et al. 2007; Mohammad and Sabaa 2015) which is found worldwide (Saxon et al. 2014) and causes rapid decline in the quality of the mushroom crop (Soler-Rivas et al. 1999). Even though mushrooms with brown blotch disease do not cause health problems in humans, they cause economic losses due to reduced quality and consumers prefer to select entirely white mushrooms without brown spots on the mushroom caps (Soler-Rivas et al. 1999). The aim of this study was to characterize the interaction of *P. putida* and *P. tolaasii* with *T. aggressivum*, and to establish if they could increase the incidence and severity of green mould disease. Both *Pseudomonas* species are endemic for *Agaricus bisporus* compost beds, but one is considered beneficial and the other is a potential threat to yield excellence. Also, *P. tolaasii* can change from virulent to avirulent form (Saxon et al. 2014). Recent advances in proteomic technologies can produce new insights into microbial interactions. These technologies include large scale mass spectrometry-based proteomics capable of identifying and quantifying thousands of proteins across multiple samples in a single run (Zhang et al. 2013; Yates et al. 2009; Watson et al. 2015; Zhu et al. 2013; Barribeau et al. 2014). Label free quantitative (LFQ) mass spectrometry (MS) was used to compare proteomes of *T. aggressivum* treated with *P. putida* or *P. tolaasii* supernatant to gain a deeper understanding of any interactions by characterizing differences in the relative abundance of proteins. The mycopathogen *T. aggressivum* and the bacteria *P. putida* and *P. tolaasii* are present in the compost at the same time and fungal – bacterial interactions may be important factors in disease development in the crop.

Materials and methods

Culture conditions

T. aggressivum 100526 (acquired from the Royal Netherlands Academy of Arts and Science,

Utrecht, Netherlands), and *P. putida* and *P. tolaasii* isolated from mushroom compost (Teagasc, Ireland) and preserved in liquid nitrogen culture collection of Maynooth University were used in this study. *Trichoderma* cultures were grown in malt extract broth (MEB) (Oxoid) at 30 °C and 200 rpm for up to 2 days. Stocks were maintained on malt extract agar (MEA) (Oxoid). *Pseudomonas* cultures were grown in nutrient broth (NB) (Oxoid) at 30 °C for up to 5 days, and after 24 h, 48 h, 72 h, 96 h and 120 h centrifuged and filter-sterilized through 20 µm syringe filters to produce supernatants. Bacterial stocks were maintained on nutrient agar (NA) (Oxoid).

Virulence test

Virulence assay was performed by the inoculation of the harvested pilei of *A. bisporus* with bacteria suspension (1×10^{13} CFU ml⁻¹) prepared from an overnight culture of *Pseudomonas tolaasii* in nutrient broth. Stipes were removed from all basidiomes and top of the pilei were inoculated with 20 µl of bacteria suspension. Three replicates per each isolate were applied. Sterile water was used as a negative control. Inoculated pilei were kept at room temperature (22 ± 2 °C). Symptoms were rated as follows: 0 = no symptoms; 1 = brown blotch around inoculation site.

Zone of inhibition assay

Filter-sterilized *P. putida* and *P. tolaasii* supernatants were transferred onto 10⁴ *T. aggressivum* conidia inoculated potato dextrose (PDA) and malt extract (ME) agar plates. Plates were incubated at 30 °C for 48 h and examined for zones of inhibition around the wells containing 50 µl of 96 h bacterial supernatant.

Assessment of effect of bacterial supernatants on growth of *T. aggressivum*

Cultures (50 ml) of *T. aggressivum* 10⁴ conidia/ml grown for 48 h at 30 °C and 200 rpm with 25% v/v supernatants were harvested by centrifugation at 4143×g for 15 min. The supernatant was discarded, and wet mass was measured.

Organic extraction of bacterial supernatants for HPLC analysis

Filter-sterilized *P. putida* and *P. tolaasii* supernatants were organically extracted by mixing in a 1:1 ratio with chloroform and shaken for 2 h. The organic phase was collected and evaporated on Rotavapor-R (Buchi, Switzerland) and re-suspended in methanol. Organic extracts were analyzed on a Shimadzu (SPD-M20A, Japan) with C-18 HPLC column using diode array detection with multiple wavelengths (220, 254, 280 nm). Flow rate was 1 ml/min with an injection of 20 µl per sample. Tolaasin standard was a kind gift from Dr. Yeong-Bae Yun (Cho et al. 2007) and was used to verify and quantify tolaasin in supernatants.

Fluorescent microscopy

T. aggressivum culture was grown for 24 h at 30 °C and 200 rpm and then supplemented with *P. putida* or *P. tolaasii* supernatants to final concentration of 25% v/v and incubated for additional 24 h under the same conditions. Hyphae were isolated, transferred to a glass slide, washed with PBS three times and stained with Calcofluor white (Sigma-Aldrich) for 5 min at room temperature. The samples were washed twice (PBS) and a cover slide was placed on top. Mycelium was viewed using an Olympus BX61 fluorescent microscope.

Label free quantitative proteomics of *T. aggressivum* treated with *P. putida*/*tolaasii* supernatants

Protein was extracted (Maher et al. 2018) from *T. aggressivum* mycelium which was grown for 24 h and then supplemented with *P. putida* or *P. tolaasii* supernatants ($n = 3$) for an additional 24 h. Bradford method was applied to quantify protein for acetone precipitation overnight. Samples were centrifuged at 14500×g for 10 min, and the pellet was re-suspended in 25 µl of 6 M urea, 2 M thiourea and 0.1 M Tris-HCl 150 buffer (pH 8.0). Protein was reduced with dithiothreitol (0.5 M DTT), alkylated with iodoacetamide (0.55 M IAA) and digested with sequence grade trypsin (Promega, Ireland) at a trypsin: protein ratio of 1:40, overnight at 37 °C. Tryptic peptides were purified for mass spectrometry using C18 spin columns (Medical Supply Company, Ireland) and 0.75 µg of peptide mix was eluted onto a

QExactive (ThermoFisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient from 2%–40% on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120-min reverse phase gradient at a low rate of 250 nl/min. A full MS scan of range 200–2000 was followed to select the 15 most intense ions prior to MS/MS. Protein identification from this data was performed using the Andromeda search engine in Max-Quant (version 1.2.2.5) to correlate against a *Trichoderma* spp. (proteome of *T. harzianum* was analysed since it is phylogenetically closest to *T. aggressivum* (Kosanovic et al. 2013) database downloaded from www.uniprot.org).

The MS proteomic data and MaxQuant search output files have been added to the ProteomeXchange Consortium (Côté et al. 2012) via the PRIDE partner repository with the dataset identifier PXD012656. The Perseus software package (v. 1.5.5.3) was used for results processing, statistical analyses and graphics generation. LFQ intensities were \log_2 -transformed and ANOVA of significance and t-tests between the treated groups was performed using a p value of 0.05 and significance was determined using FDR correction (Benjamini and Hochberg 1995). Proteins which had non-existent values (suggestive of absence or very low abundance in a sample) were also used in statistical analysis. Proteins found to be absent (below the level of detection) in one or more treatments and present (above the level of detection) in three or fewer treatments were also used in statistical analysis of the total differentially expressed group following imputation of the zero values with values that simulate low abundant proteins.

The Search Tool for the Retrieval of INteracting Genes/Proteins (STRING) v10.5 (<http://string-db.org/>) was used to map known and predicted protein:protein interactions. UniProt gene lists (extracted from Perseus) were inputted and analyzed in STRING using the medium confidence (0.5) setting to produce interactive protein networks for proteins increased and decreased in abundance.

These proteins were subjected to Gene Ontology (GO) analysis by Blast2GO software tool (<https://www.blast2go.com/>). Statistically enriched GO descriptors were examined to identify clusters of proteins enriched within statistically significant differentially abundant (SSDA) protein lists ($p < 0.05$).

Statistical analysis

All experiments were carried out on three independent occasions, and results were expressed as the mean \pm SE. Significant differences were considered at $p < 0.05$.

Results

Virulence test

In vitro pathogenicity testing was performed to assess the virulence of the *P. tolaasii* isolate used in the experimental work since *P. tolaasii* may undergo a change from virulent (pathogenic, non-fluorescent, smooth colony morphology on King's Medium B Agar) to avirulent (non-pathogenic fluorescent rough colony morphology) forms (Saxon et al. 2014). According to the results, *P. tolaasii* used in this work was a virulent form and caused brown spots on mushroom caps (Figure S1).

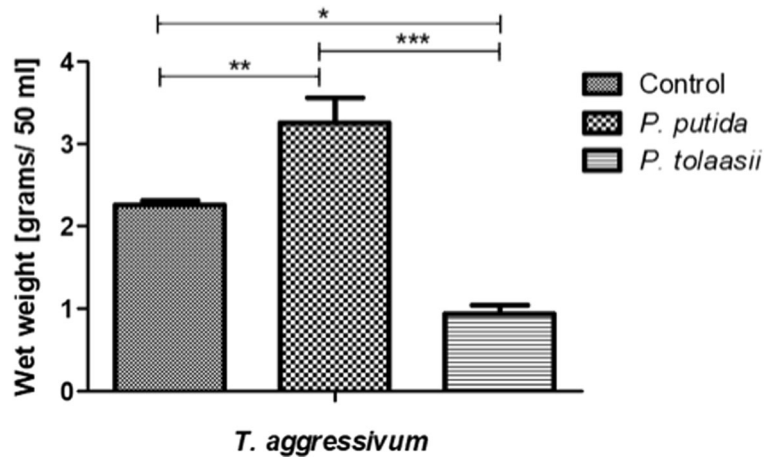
Assessment of effect of *P. tolaasii* and *P. putida* supernatants on *T. aggressivum* growth

The effect of *P. tolaasii* and *P. putida* supernatants on *T. aggressivum* growth was assessed after 48 h. *P. tolaasii* supernatant was found to significantly ($p = 0.05$) inhibit growth, while *P. putida* supernatant significantly ($p = 0.01$) stimulated growth of *T. aggressivum* (Fig. 1). It was found that in samples containing 25% v/v *P. putida* supernatant mycelial growth was increased by 44%, whereas 25% v/v *P. tolaasii* supernatant inhibited the growth of *T. aggressivum* by 57%. *P. tolaasii* 96 h supernatant induced zones of inhibition on PDA and ME agar plates inoculated with 10^4 *T. aggressivum* conidia for 48 h at 30 °C (Figure S2a, b). In contrast *P. putida* 96 h supernatant produced no zone of inhibition on PDA or ME agar plates (Figure S2c, d).

Detection and quantification of tolaasin in *P. tolaasii* supernatant

P. tolaasii and *P. putida* were cultured at 30 °C to produce supernatants at different time points. Supernatants were organically extracted as described and quantified by HPLC to the presence of tolaasin in *P. tolaasii* cultures. The retention time for tolaasin was found to be 17.41 min (Figure S3). Tolaasin was detected only in *P. tolaasii* 96-

Fig. 1 *T. aggressivum* growth [g] in MEB after 48 h incubation at 30 °C, containing 25% v/v of either: Nutrient Broth, *P. putida* 96 h supernatant or *P. tolaasii* 96 h supernatant. * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$



and 120-h supernatants, with the highest concentration being present at 96 h. The tolaasin concentration at 120 h was 29.88 μg in 50 ml *P. tolaasii* supernatant, whereas a total of 154.08 μg of tolaasin was present in 50 ml of *P. tolaasii* supernatant at 96-h culture.

Fluorescent microscopic examination of *Trichoderma* hyphae

Fluorescent microscopy examination of *T. aggressivum* hyphae stained with calcofluor revealed that exposure to *P. tolaasii* supernatant (Fig. 2g, h, i) decreased mycelial formation while increasing the abundance of conidia compared to *P. putida* supernatant exposure (Fig. 2d, e, f) and control groups (Fig. 2a, b, c). Elongated, branched *T. aggressivum* hyphae with septa dividing cells were observed in *P. putida* supernatant treated and control groups. In contrast fewer hyphae were observed in *P. tolaasii* treated group and these were short and rounded, with few septa dividing cells due to disruption of mycelial growth. Additionally, *P. tolaasii* treated *T. aggressivum* samples displayed abundant spore formation, compared to other two groups (Fig. 2g, h, i).

Proteomic analysis of response of *Trichoderma* after exposure to *P. putida* and *P. tolaasii*

In total 21,881 peptides were identified, representing 2820 proteins. Results of label free proteomic investigation of *T. aggressivum* proteins after exposure to *P. tolaasii* or *P. putida* supernatant indicated that exposure to *P. putida* supernatant lead to a 30% increase in different proteins compared to the ones exposed to *P. tolaasii* supernatant and the control group.

According to Perseus analysis a total of 161 *T. aggressivum* proteins in *P. tolaasii* treatment and 167 proteins in *P. putida* treatment were determined to be differentially abundant (analysis of variance [ANOVA], $P < 0.05$), with a fold change value of > 2 . A total of 768 (*P. putida*) and 286 (*P. tolaasii*) *T. aggressivum* proteins were deemed exclusive (i.e., with LFQ intensities present in all three replicates of one treatment and absent in all three replicates of the other two treatments). These proteins were also used in statistical analysis of the total levels corresponding to the differentially expressed group members following imputation of the zero values as described above. After data imputation, these proteins were included in the subsequent statistical analysis.

A principal-component analysis (PCA) performed on all filtered proteins distinguished the control and *P. putida*/*tolaasii*-treated samples, indicating a clear difference between the proteomes (Figure S4). Proteins that increased in relative abundance in *P. putida*-treated *T. aggressivum* compared to the control were structural constituents of ribosome (20-fold), H/ACA ribonucleo-protein complex subunit (18-fold), cyanide-degrading nitrilase (11-fold), signal recognition particle 54 kDa protein (6.4-fold), DNA binding and nucleosome assembly protein (5.3-fold), prefoldin subunit 3 (5-fold), and integral component of membrane (4.8-fold) (Fig. 3a). Proteins decreased in relative abundance in *P. putida*-treated *T. aggressivum* were O-methyltransferase activity protein (17.5-fold), mitochondrial 2-methylisocitrate lyase (14.6-fold), GMC oxidoreductase (13.5-fold), nitrate assimilation protein (12.4-fold), and nucleoside catabolic process unit (4.8-fold) (Fig. 3a).

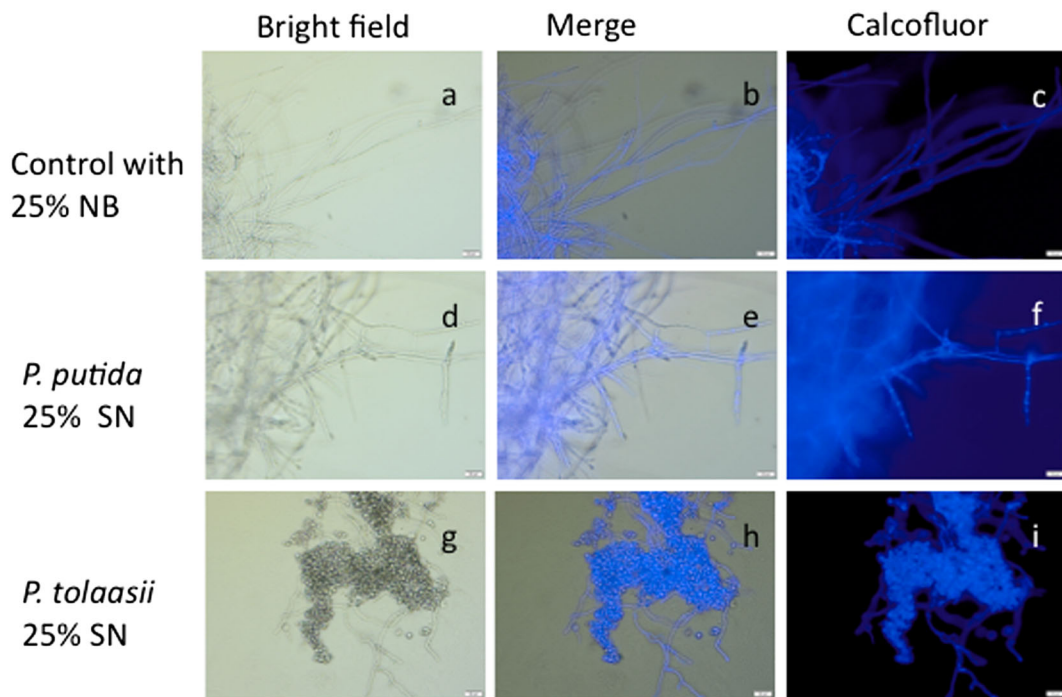


Fig. 2 Fluorescent microscopy 20x magnification, 48 h at 30 °C. Photomicrograph of calcofluor stained hyphae showing that *P. tolaasii* (g, h, i) 96 h supernatant (SN) added in 25% to

T. aggressivum growing culture decreases mycelial formation while increasing the conidia abundance compared to the same percent supernatant of *P. putida* (d, e, f) and control (a, b, c) groups

Proteins that increased in relative abundance in *P. tolaasii*-treated *T. aggressivum* compared to the control were mitochondrial import inner membrane translocase complex protein (5.7-fold), pseudouridine synthesis (5.5-fold), protein folding (5.4-fold), prefoldin complex (5.4-fold), oxidoreductase (5.2-fold), mitochondrial respiratory chain complex III assembly (5.2-fold), and glucoamylase (5.1-fold) (Fig. 3b). Proteins decreased in relative abundance in *P. tolaasii*-treated *T. aggressivum* were O-methyltransferase activity protein (8-fold), carboxylic acid metabolic process protein (6.8-fold), isocitrate lyase activity (6.8-fold), fatty-acyl-CoA binding protein (6-fold), serine-type endopeptidase activity (5.2-fold), hydrolases (4.6-fold), tricarboxylic acid cycle protein (4.4-fold), malate synthase (4.3-fold), and nucleoside diphosphate kinase (4-fold) (Fig. 3b).

According to STRING analysis (string-db.org) there was no significant enrichment of protein groups seen in *T. aggressivum* sample treated with *P. tolaasii*. However, proteins were found to have more interactions among themselves than expected, indicating that they are biologically connected as a

group. For the samples treated with *P. putida* supernatant functional enrichments were found in prefoldin (prefoldin, prefoldin beta-like) and aldo/keto reductase family (aldo/keto reductase/potassium channel subunit beta and NADP dependant oxidoreductase domain). Prefoldin promotes protein folding in cells and aldo/keto reductase family reduces organic compounds.

Proteins were subjected to Gene Ontology (GO) analysis by Blast2GO software tool. A number of GO terms belonging to biological process (small molecule metabolic process, response to stress, cellular component organization and biosynthetic process), molecular function (ion binding, oxidoreductase activity, structural constituent of ribosome and organic cyclic compound binding), cellular component (intracellular organelle, membrane-bounded organelle, intracellular and endomembrane system) were significantly enriched within the dataset, with ion binding, hydrolase and oxidoreductase activity enriched with *P. tolaasii* group (Figure S5). Hydrolases, oxidoreductases and transferases were the most enriched enzymes categories amongst proteins identified in *T. aggressivum* treated groups, with hydrolases being the most abundant in *P. tolaasii* treated group (Fig. 4).

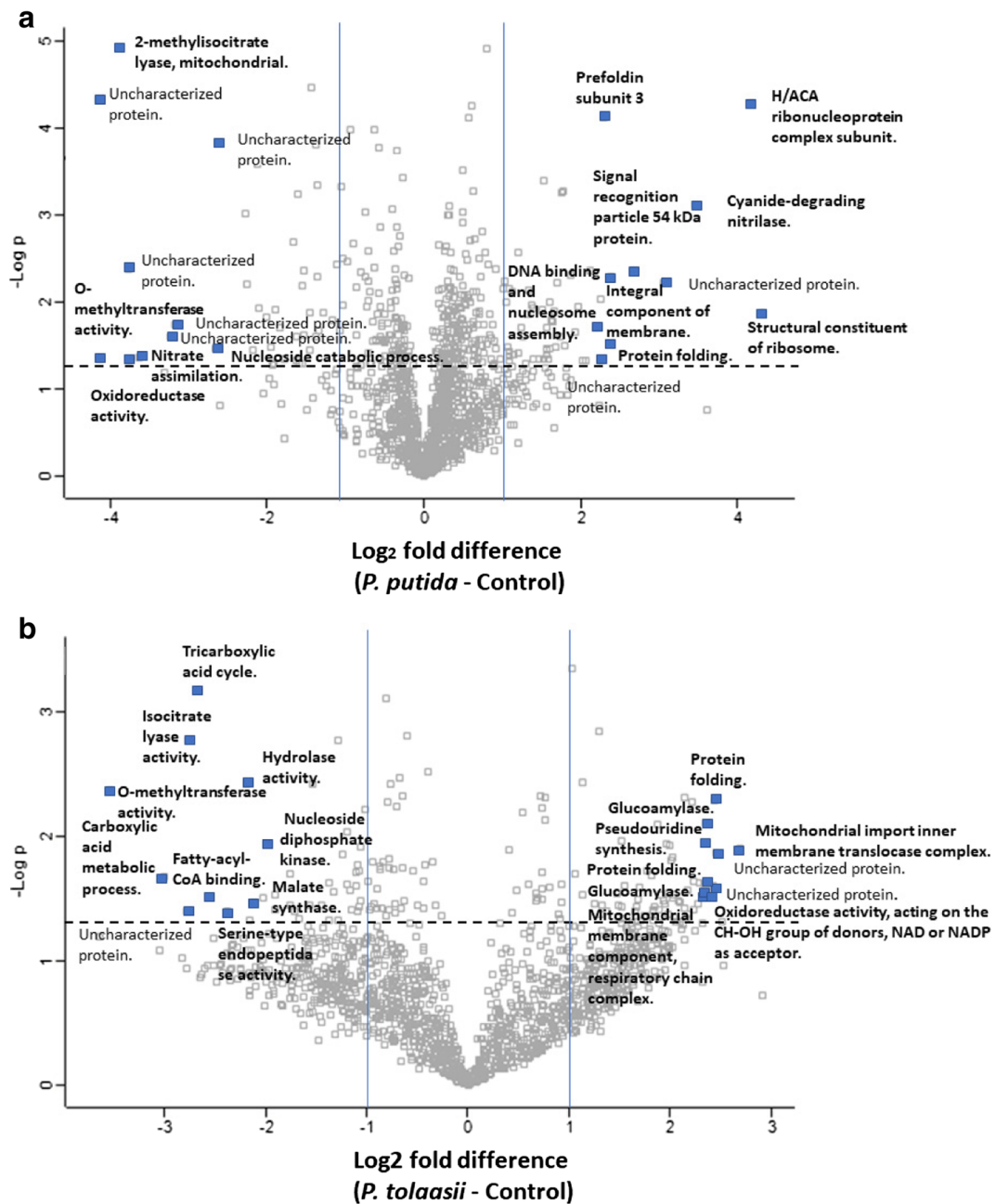


Fig. 3 Proteomic responses of *T. aggressivum* following 24 h incubation with 25% v/v 96 h supernatant of *P. putida* (a) or *P. tolaasii* (b). Volcano plots represent protein intensity difference ($-\log_2$ mean intensity difference) and significance in differences ($-\log P$ value) based on a two-sided t-test. Proteins above the line

are considered statistically significant ($p < 0.05$) and those to the right and left of the vertical lines indicate relative fold changes > 2 . Annotations are given for the most differentially abundant proteins identified. These plots are based upon post imputed data

Discussion

Green mould disease is characterized by white mycelium that colonises *A. bisporus* compost and casing and turns

green due to sporulation. Brown spots on mushroom bodies are associated symptoms and in severe outbreaks no crop is produced (Seaby 1996). Many *Trichoderma* species, such as *T. harzianum*, *T. atroviride*, *T. koningii*,

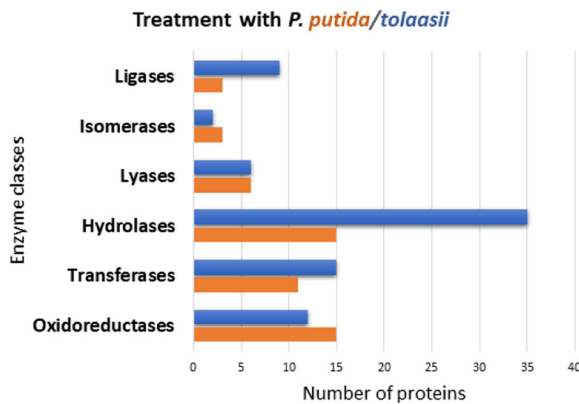


Fig. 4 Classes of enzyme identified in *T. aggressivum* following exposure to *P. putida* or *P. tolaasii* supernatants

T. vires can be present in mushroom growing environment but not all of these have the same capacity to induce diseases (Kosanovic et al. 2013). Before the 1990s, green mould disease was a relatively minor problem on mushroom farms (Samuels et al. 2002). However since then *T. aggressivum* induced green mould disease has emerged as a major problem for mushroom growers and has spread all over the world (Seaby 1996; Geels 1997; Hermosa et al. 1999; Mamoun et al. 2000; Rinker 1993; Romaine et al. 1996; Hatvani et al. 2007; Szczech et al. 2008; Romero-Arenas et al. 2009; Cliff and Shamshad 2009; Hatvani et al. 2012; Kosanovic et al. 2013). *T. aggressivum* reduces yield in mushroom facilities by being a compost competitor of *A. bisporus*, and not a direct pathogen of *Agaricus* (Krupke et al. 2003; O'Brien et al. 2017). A number of antifungal compounds (e.g. 3,4-dihydro-8-hydroxy-3-methylisocoumarin) are produced by *T. aggressivum* and these metabolites are not produced by other, non-aggressive *Trichoderma* spp. (Krupke et al. 2003). Additionally, red-pepper mites, which feed on *Trichoderma* spores, frequently infest mushroom crops with green mould disease and so make the product unmarketable (Krupke et al. 2003).

The casing layer is an ideal environment for bacterial migration from compost, being porous and wet, thus *Pseudomonas* spp. are known to migrate and to exhibit chemotaxis toward *A. bisporus* mycelium exudates that provide growth substrates for them (Grewal and Rainey 1991). *Pseudomonas* genus makes up to 10% of compost for *A. bisporus* cultivation and sometimes more than 50% of casing bacteria (Godfrey et al. 2001). *P. putida* is a promoter of mushroom pinning and thus considered beneficial for mushroom production, even though the exact mechanism is unknown, it has been

suggested that bacteria consume 2-ethyl-1-hexanol and 1-octen-3-ol – a self-inhibitory compounds of *A. bisporus* growth (Noble et al. 2009), secrete hormone-like compounds that might stimulate pinning, and produce siderophores that control pathogens (Zarenejad et al. 2012). In contrast, *P. tolaasii*, which is also a mushroom substrate bacterium, is known as a causal agent of brown blotch disease and so considered a major problem in mushroom production (Cho et al. 2007; Osdaghi et al. 2019). *P. tolaasii* can produce an antimicrobial toxin, tolaasin, under certain humidity and temperature conditions in mushroom farms. Tolaasin is a peptide containing 18 amino acids with a β -hydroxyoctanoic acid on the N-terminus and molecular mass of 1.985 KDa (Nutkins et al. 1991). Tolaasin causes lysis of *A. bisporus* mycelium (Mohammad and Sabaa 2015), disrupts cells by inducing pore formation in plasma membrane and displays biosurfactant properties (Hutchison and Johnstone 1993). Brown spots on mushrooms are caused by melanin production as a result of defence response to the toxin, tolaasiin (Saxon et al. 2014). Even though brown blotch disease does not cause any ill effects in humans, brown spotting symptoms on mushroom caps are not attractive to the consumer (Cho et al. 2007). Previous work has shown that *P. putida*, isolated from compost casing, increased mushroom yield up to 26.6% compared to the non-inoculated control and *P. tolaasii* inhibited growth, and significantly reduced mushroom yield (– 72.5%) (Mohammad and Sabaa 2015). There is a constant search for new, effective products to be used in mushroom production. *B. subtilis* has been shown to promote mushroom yield and to suppress green mould disease when applied in lower concentrations than the commercial bio-fungicides based on *B. velezensis* (Serenade® WP (AgraQuest, Canada) (Potocnik et al. 2019). Pandin et al. (2018) demonstrated how *B. velezensis* QST713 gave protection to *A. bisporus* crops against green mould disease caused by *T. aggressivum*. Further investigations of the potential of different beneficial, *A. bisporus* compost and casing bacteria and their secondary metabolites are warranted. Screening for a disease control agent that promotes *A. bisporus* yield and suppresses the growth of mycopathogens such as *T. aggressivum* and others are needed. Many chemicals are no longer in use since fungicides reduce the growth of *A. bisporus* to some extent, change the casing microflora, have toxic effects and pathogens are developing resistance to them. Thus, there is an increased need for

biorational agents that can control mushroom diseases rather than using chemicals (Grogan 2008).

In the work presented here, *P. putida* and *P. tolaasii* were cultured in nutrient broth for different periods of time, harvested and filter-sterilized to yield culture supernatants (also known as culture filtrates). Such supernatants enable a characterization of the interaction of the microbial secretome with target organisms and are a good representation of the range of toxins, enzymes and other products secreted by microbes that may play a role in pathogenesis (Mc Namara et al. 2017).

Exposure of *T. aggressivum* to *P. tolaasii*-supernatant produced growth inhibition on agar plates, significantly reduced biomass accumulation, disrupted hyphal formation and supernatants demonstrate the presence of tolaasin by HPLC. Exposure of *T. aggressivum* to *P. tolaasii* supernatant lead to an increase in the abundance of proteins with hydrolase, and oxidoreductase activity from Volcano plot analysis. An increase in the abundance of drug and ion binding proteins was also found. All these results indicate that in the presence of *P. tolaasii* supernatant there is growth inhibition and the induction of an oxidative stress response by the fungal cells.

Exposure to *P. putida* supernatant, in contrast, did not inhibit growth of *T. aggressivum* on agar plates and did not influence chitin structure and formation in mycelial wall, and hyphae elongation. Yet, it significantly stimulated *T. aggressivum* biomass production and -led to an increase in proteins associated with ribosome biosynthesis and protein development such as structural constituents of ribosome (20-fold), H/ACA ribonucleoprotein complex subunit (18-fold), signal recognition particle 54 kDa protein (6.4-fold), DNA binding and nucleosome assembly protein (5.3-fold), prefoldin subunit 3 (5-fold) (Fig. 4a). These results clearly indicate that exposure of *T. aggressivum* to *P. putida* supernatant resulted in a growth stimulation response. The mechanism of this growth stimulation is as yet unknown. It may be that the bacterium degrades an inhibitor of the fungus or metabolises a substrate into a form more amenable to use by *T. aggressivum*.

The results presented here indicate that exposure to *P. tolaasii*, even though it can cause brown spotting of mushrooms, decreases *T. aggressivum* growth. However, exposure to *P. putida*, which promotes pinning of mushrooms, can stimulate the growth of *T. aggressivum* and thus may be an important factor in increasing the incidence and severity of green mould disease in

mushroom crops and so reducing yield. Further study is planned on the possible interactive effects between *P. tolaasii* and *P. putida* both *in vitro* and in compost and casing where a dynamic and diverse microbiome environment is present. Different application of *Pseudomonas* supernatants should be further investigated regarding better yield and efficacy of disease control of *A. bisporus*.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Human or animals participants This article does not contain any study with human participants or animals performed by the authors.

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