RESEARCH ARTICLE

Secretome analysis of the mycoparasitic fungus *Trichoderma harzianum* ALL 42 cultivated in different media supplemented with *Fusarium solani* cell wall or glucose

Marcelo Henrique Soller Ramada[1,2], Andrei Stecca Steindorff[1], Carlos Bloch Jr.[2] and Cirano José Ulhoa[3]

[1] Departamento de Biologia Celular, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Instituto de Ciências Biológicas, Brasília, DF, Brazil
[2] Laboratório de Espectrometria de Massa, Embrapa-Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Brasília, DF, Brazil
[3] Laboratório de Enzimologia, Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Goiás (ICB), Goiânia, GO, Brazil

Trichoderma harzianum is a fungus well known for its potential as a biocontrol agent against many fungal phytopathogens. The aim of this study was to characterize the proteins secreted by *T. harzianum* ALL42 when its spores were inoculated and incubated for 48 h in culture media supplemented with glucose (GLU) or with cell walls from *Fusarium solani* (FSCW), a phytopathogen that causes severe losses in common bean and soy crops in Brazil, as well as other crop diseases around the world. *Trichoderma harzianum* was able to grow in *Trichoderma* Liquid Enzyme Production medium (TLE) and Minimal medium (MM) supplemented with FSCW and in TLE+GLU, but was unable to grow in MM+GLU medium. Protein quantification showed that TLE+FSCW and MM+FSCW had 45- and 30- fold, respectively, higher protein concentration on supernatant when compared to TLE+GLU, and this difference was observable on 2D gel electrophoresis (2DE). A total of 94 out of 105 proteins excised from 2DE maps were identified. The only protein observed in all three conditions was epl1. In the media supplemented with FSCW, different hydrolases such as chitinases, β-1,3-glucanases, glucoamylases, α-1,3-glucanases and proteases were identified, along with other proteins with no known functions in mycoparasitism, such as npp1 and cys. *Trichoderma harzianum* showed a complex and diverse arsenal of proteins that are secreted in response to the presence of FSCW, with novel proteins not previously described in mycoparasitic-related studies.

Received: November 22, 2015
Revised: August 20, 2015
Accepted: November 30, 2015

Keywords:
De novo peptide sequencing / *Fusarium solani* / Gene analysis / Microbiology / Secretome / *Trichoderma harzianum*

Additional supporting information may be found in the online version of this article at the publisher's web-site

Correspondence: Professor Cirano José Ulhoa, Laboratório de Enzimologia, Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Goiás (ICB), 74690–900, Goiânia, GO, Brazil
E-mail: ulhoa@icb.ufg.br
Fax: +55-62-3521-1190

Abbreviations: **CAZymes**, carbohydrate active enzymes; **cdb**, carbohydrate binding domain; **DUF**, domains of unkown function; **FSCW**, *Fusarium solani* cell wall; **GH**, glycosyl hydrolase; **GLU**, glucose; **MM**, TLE medium not supplemented with nitrogen sources; **MW**, molecular weight; **MYG**, malt-yeast-glucose agar medium; **RNA-seq**, RNA sequencing; **SSCPs**, small secreted cysteine-rich proteins; **TLE**, *Trichoderma* liquid enzyme production medium

Colour Online: See the article online to view Fig. 3 in colour.

Significance of the study

Trichoderma harzianum ALL42 has a great potential for inhibiting the growth of *Fusarium solani* in vitro. Our research group has developed several approaches to study the interaction between *T. harzianum* ALL42 and *F. solani* by using methods based on EST libraries and subtractive library hybridization. The present work describes proteomic analyses of the *T. harzianum* ALL42 secretome based on 2D gel eletrophoresis combined with mass spectrometry. The variations in protein expression profile have been studied when *T. harzianum* ALL42 was grown in the presence of glucose or in the presence of *F. solani* cell walls (mycoparasitic-related secretome). We also discuss the possible functional roles of the identified proteins in the mycoparasitic-related conditions and analyze their gene expression by quantitative real-time RT-PCR (RT-qPCR). Our findings have revealed that *T. harzianum* ALL42 was able to secrete a rich repertoire of proteins potentially involved in mycoparasitism, nutrient acquisition and induction of defensive responses in plants, not previously observed on the RNA-based approaches regarding this interaction and in other mycoparasitic-related studies.

1 Introduction

Soil-borne pathogenic fungi are widely distributed and are responsible for serious damage to many agricultural and horticultural crops worldwide. The diseases caused by soil fungi are responsible for great losses in common bean (*Phaseolus vulgaris* L.) productivity in irrigated areas of the Southeast and Midwest regions of Brazil [1]. The diseases most commonly found in these regions are caused by *Rhizoctonia solani* Kühn, *Fusarium solani* f. sp. phaseoli and *Sclerotinia sclerotiorum* (Lib.) de Bary [2] and have previously been controlled through the use of chemical fungicides [1]. *Fusarium solani* (Mart.) Sacc. (teleomorph = *Nectria haematococca* (Berk. & Br.)) is a phytopathogenic fungus classified in the *Martiella* section, and it is an important causal agent of several crop diseases [3]. Despite the long-standing presence of this plant-pathogenic fungus in Brazil and extensive literature about the agronomic aspects of infection, information regarding the use of fungal biocontrol agents in this country is still lacking.

The genus *Trichoderma* (Ascomycetes, Hypocreales) was first described by Persoon more than 200 years ago, and consists of anamorphic fungi that are among the most commonly distributed fungi in nature, being found in ecosystems ranging from tundra to tropical [4]. The potential of *Trichoderma* as a biological control agent was first recognized in the early 1930s. Since then, the genus has been extensively investigated as an antagonist of soil-borne plant pathogens. The success of *Trichoderma* species as biological control agents is due to their high reproductive capacity, ability to survive under severe conditions, high efficiency in using nutrients, strong aggressiveness against plant pathogenic fungi and efficiency in promoting plant defense mechanisms [5]. Some *Trichoderma* strains have the ability to reduce the severity of plant diseases by inhibiting plant pathogens, mainly in the soil or plant roots, through their high antagonistic and mycoparasitic potential [6]. On the other hand, some *Trichoderma* rhizosphere-competent strains have a direct effect on plants, increasing their growth potential and nutrient uptake and stimulating plant defense mechanisms against biotic and abiotic damage [7].

All of the ecological traits of *Trichoderma* require, among other factors, secretion of key proteins probably involved in the *Trichoderma*–fungal host or *Trichoderma*–plant interaction. Proteomic analysis provides an excellent tool to study variations in protein profile during these complex processes. Secretome studies of *Trichoderma* during growth on cell walls of plant pathogenic fungi such as *Rhizoctonia solani*, *Macrophominia phaseolina, Fusarium sp*. and *Botrytis cinerea*, have previously been carried out [5, 8–10]. A recent study was used in in-silico analysis of the secretome of *T. reesei*, *T. atroviride* and *T. virens* together with data obtained from the genomes of these three species [11]. These studies have provided new information regarding the molecular physiology and ecology of these fungi. Most of the proteomic studies carried out have detected several cell wall-degrading enzymes such as chitinases, chitosanases, β-1,3/1,6-glucanase and many families of glycosyl hydrolases and proteases [12]. These proteins are important for interactions between the fungus and the environment, and some are necessary for the induction of the defensive response in plants [13].

Trichoderma species are readily isolated from Brazilian Cerrado soil by conventional methods and have been used in biotechnological exploitation of enzyme production and biological control [14, 15]. Previous results have shown that the isolate *T. harzianum* ALL42 has a great potential for inhibiting the growth of *F. solani* in-vitro [16] and for reducing disease impact in common bean (unpublished data). Our research group has developed several approaches to study the interaction between *T. harzianum* ALL42 and *F. solani* by using methods based on EST libraries [16] and subtractive library hybridization [17]. The present work describes proteomic analyses of the *T. harzianum* ALL42 secretome based on 2D gel eletrophoresis combined with mass spectrometry. The variations in protein expression profile have been studied when *T. harzianum* ALL42 was grown in the presence of glucose or in the presence of *F. solani* cell walls (mycoparasitic-related secretome). We also discuss the possible functional roles of the identified proteins in the mycoparasitic-related conditions and analyze their gene expression by quantitative real-time RT-PCR (RT-qPCR). Our findings have revealed that *T. harzianum* ALL42 was able to secrete a rich repertoire of

proteins potentially involved in mycoparasitism, nutrient acquisition and induction of defensive responses in plants, not previously observed on the RNA based approaches regarding this interaction and in other mycoparasitic-related studies.

2 Materials and methods

2.1 Organisms and culture conditions

Trichoderma harzianum ALL42 (Enzymology Group collection, UFG-ICB) and *F. solani* (EMBRAPA-CNPAF) were grown on Malt-Yeast-Glucose (MYG) medium (0.5% (w/v) malt extract (Himedia, Milano, Italy) 0.25% (w/v) yeast extract (Biobrás, Montes Claros, Brazil) 1% (w/v) glucose (Sigma Chemical Co., St. Louis, USA) and 2% (w/v) agar (Bio-Rad, Marne-La-Coquette, France). 10^7 spores/mL of *T. harzianum* ALL42 were collected in sterile water, centrifuged at 500 × *g*, washed twice and inoculated in 1 L Erlenmeyer flasks containing 200 mL of *Trichoderma* Liquid Enzyme production medium (TLE) composed of: 0.1% (w/v) bactopeptone (Himedia), 0.03% (w/v) urea (VETEC, Duque de Caxias, Brazil), 0.2% (w/v) KH_2PO_4 (VETEC), 0.14% (w/v) $(NH_4)_2SO_4$ (VETEC), 0.03% (w/v) $MgSO_4.7H_2O$ (Sigma) and 2% (v/v) trace elements solution containing 0.025% (w/v) $FeSO_4$, 0.0085% (w/v) $MnSO_4$, 0.007% (w/v) $ZnSO_4$ and 0.01% (w/v) $CaCl_2$ (all from Sigma) or on Minimal Medium (MM) composed of: 0.2% (w/v) KH_2PO_4, 0.14% (w/v) $(NH_4)_2SO_4$, 0.03% (w/v) $MgSO_4.7H_2O$ and 2% (v/v) trace elements solution. Both media types were supplemented with 0.5 % (w/v) *F. solani* cell walls (FSCW) or with 2% (w/v) glucose (GLU), as a carbon source. The pH of all media was adjusted to 5.0 prior to inoculation. The cultures were grown in an orbital shaker at 180 rpm, at 28°C for 48 h. Five repetitions were performed for TLE+FSCW and MM+FSCW and 20 repetitions for TLE+GLU. The supernatants of the replicates were collected by filtering using Whatman® filter paper Grade 1 and were stored at -20°C until use. Mycelia were also collected and stored at -80°C until further analysis.

2.2 Protein preparation

Protein concentration was determined by the method of Bradford [18] using bovine serum albumin (Sigma) as a standard. The supernatants were concentrated by ultrafiltration using an Amicon (Millipore, Billerica, US) system with a 10 kDa cut-off membrane. The volume necessary to obtain 600 μg was lyophilized, recovered in 125 μL of deionized water and the proteins were precipitated using 2D Clean-up Kit (GE Healthcare). Each sample was re-suspended with 230 μL of De-Streak Rehydration Solution (GE Healthcare,Upsalla, Sweden), containing 0.5% (v/v) IPG buffer pH range 4–7 (GE Healthcare).

2.3 Protein separation by 2DE

The IPG strips (13 cm pH 4–7) were passively rehydrated with the samples containing 600 μg of protein for 14 h. Isoelectric focusing was performed in an IPGPhor III (GE Healthcare) system as follows: one step of 500 V for 1 h, one gradient to 1000 V for 1 h, one gradient to 8000 V for 4 h and one step of 8000 V for 6 h, accumulating a total of 67250 Vh. The IPG strips were than equilibrated in two steps, for 20 min each, using an equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M Urea, 30% (v/v) glycerol, 30% (w/v) SDS and trace amounts of bromophenol blue, all from GE Healthcare) containing 1% (w/v) DTT (GE Healthcare) or 2.5% (w/v) iodoacetamide (GE Healthcare). Equilibrated strips were submitted to the second dimension of electrophoresis on 12.5% (w/v) SDS-PAGE gels, according to Laemlli [19], in an Ettan Dalt Six cube system (GE Healthcare), using Electrophoresis Power Supply - EPS601 at 10 mA per gel for 1 h, and then at 40 mA per gel until complete. Temperature was controlled by a MultiTemp III (GE Healthcare) at 15°C. An Amersham Low Molecular Weight Kit for SDS Eletrophoresis (GE Healthcare) was used as molecular weight marker. Gels were stained using PhastGel™ Blue R (GE Healthcare) according to the manufacturer´s instructions.

2.4 Gel analysis and in-gel digestion

The stained gels were scanned and analysis was performed using the ImageMaster 2D Platinum v.7.0 system (GE Healthcare). The best three replicates of each condition were selected for further analysis. Three landmarks were set to adjust for deviations in the analysis of the replicates themselves, followed by analysis between conditions. Spots different from the control condition (TLE+GLU), and only present in this condition were excised and digested with Trypsin Gold-Mass V582A (Promega, Madison, USA) according to the method described by [20], with modifications. Briefly, 100 μL of 50% (v/v) acetonitrile (J.T. Baker, Xalostoc, Mexico) /25 mM NH_4HCO_3 (VETEC) were added to triturated spots on 0.5 mL microtubes under agitation for 20 min. This procedure was repeated twice. Spots were dehydrated with acetonitrile for 5 min under agitation and then vaccum dried for 15 min. Spots were rehydrated with 15 μL of digestion buffer (25 mM NH_4HCO_3 containing 10 ng/μL of trypsin) for 15 min. Exceeding volume was discarded and then 80 μL of 25 mM NH_4HCO_3 was added to rehydrated gel spots. Digestion was performed at 37°C for 16 h. After digestion, the supernatant was collected on new microtubes. Gel pieces were agitated with 50 μL of 50% (v/v) acetonitrile/miliQ water containing 5% (v/v) of trifluoracetic acid (J.T. Baker) for 10 min. The supernatant was collected and added to the digested peptides previous collected. This procedure was repeated once. Tryptic peptides were vacuum dried and stored until mass spectrometric analysis.

2.5 MS

The resulting peptides from each spot were submitted to mass spectrometric analysis, which was carried using an UltraFlex III MALDI-TOF/TOF spectrometer (Bruker Daltonics, Bremen, Germany), controlled by the FlexControl 3.0 software (Bruker Daltonics). The samples were mixed with α-cyano-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) matrix solution (3:1, v/v) directly on an MTP AnchorChip 400/384 target plate (Bruker Daltonics) and dried at room temperature. Peptide monoisotopic masses were obtained in reflector mode over a range of 700–4500 *m/z* with external calibration using Peptide Calibration Standard II (Bruker Daltonics). Peptide MS/MS spectra were obtained by means of LIFT fragmentation after analyzing the obtained MS spectra and selection of precursor ions for fragmentation. The software FlexAnalysis 3.0 (Bruker Daltonics) and PepSeq (Waters, London, UK) were used for mass spectrometric data analysis. Peptide primary structures were inferred by means of manual de novo interpretation of fragmentation. MS spectra were analyzed using MASCOT Peptide Mass Fingerpring tool (www.matrixscience.com). MS/MS spectra were also identified using MS/MS Ion search tool (www.matrixscience.com). The three methods were compared for protein identification. The obtained sequences from manual de novo interpretation were then searched against the NCBI-nr protein database (www.ncbi.nlm.nih.gov) and against the *Trichoderma harzianum* CBS226.95 genome v.10 (http://genome.jgi.doe.gov/Triha1/Triha1.home.htmL) using the algorithm blastp. The identified protein sequences from *T. harzianum* genome were than submitted to PeptideMass tool from Expasy (www.expasy.org) to determine the theoretical isoeletric point and molecular weight, to WoLF PSORT [21] to determine whether a signal peptide could be identified, thus indicating secretion, and Pfam database analysis to indicate protein functions.

2.6 Enzyme assays

Enzyme activities of β-1,3-glucanase, glucoamylase, endoglucanase, exoglucanase, β-glucosidase, chitinase, α-L-arabinofuranosidase, α-mannosidase and proteases were determined according to [15, 22–24] and the descriptions provided in Supporting Information Material 1. All activities were expressed as specific activity (U/mg of protein).

2.7 Quantitative real-time RT-PCR analysis (RT-qPCR)

RT-qPCR was used to evaluate gene expression of the identified proteins of *T. harzianum* when grown in the different culture media. Primers were designed using PrimerQuest Advanced tool (www.idtdna.com) (Supporting Information Material 2). Total RNA was obtained from the mycelia grown in different media and digested with DNase I (Invitrogen, Carlsbad, USA). Total RNA (5 μg) from each pooled sample was reverse transcribed into cDNA using an oligo(dT) primer in a volume of 20 μL using the Revertaid™ First Strand cDNA synthesis kit (Fermentas, Vilnius, Fermentas). The synthesized cDNA was diluted with 80 μL of water and used as a template for real-time PCR. Reactions were performed in the iQ5 real-time PCR system (Bio-Rad, Hercules, USA). Each reaction (20 μL) contained 10 μL of MAXIMA® SYBR-green PCR Master mix (Fermentas), forward and reverse primers (500 nM each), cDNA template and nuclease free water. PCR cycling conditions were 10 min at 95°C (1 cycle), 15 s at 95°C followed by 1 min at 60°C (40 cycles) and a melting curve of 30 s at 60°C with a final ramp to 95°C with continuous data collection (1 cycle) to test for primer dimers and nonspecific amplification. The α-tubulin (HS574101) transcript was used as an internal reference to normalize the amount of total cDNA present in each reaction. The expression level of the genes was calculated from the quantitation cycle according to the quantitation cycle method [25]. The experiments were conducted with three repetitions for each sample and results were compared by one-way ANOVA with Dunnett's post-test ($\alpha = 5\%$) to analyze the differences between the different conditions and the control using GraphPad Prism version 5.00 for Windows.

3 Results and discussion

3.1 Protein preparation and 2DE

Proteomic approaches carried out on *Trichoderma* spp. have provided novel insights into the mechanisms of interaction with plant pathogenic fungi and plants [12, 26]. *Trichoderma* requires the secretion of proteins in order to break down polymeric organic molecules into a form that can be absorbed. In addition they also secrete proteins that can act as toxins or signals for communication with mutual partners. Therefore, the inventory of the secretome of such an organism may reveal its potential ecological adaptations [26].

This study describes the identification of the most abundant proteins secreted by *T. harzianum* ALL42 into growth media containing either glucose (TLE+GLU) or *F. solani* cell walls (MM+FSCW/TLE+FSCW). Growth in glucose-containing medium was used as a control condition, whereas growth in FSCW-containing media was used as a mycoparasitic-related condition [17]. *Trichoderma harzianum* ALL42 was able to grow and secrete proteins in TLE+FSCW, MM+FSCW and TLE+GLU media (Table 1), but was not able to grow on MM+GLU. Growth rate between conditions were comparable (data not shown). The striking difference in secretion levels (Table 1) is expected since glucose is a well-known catabolic repressor of many proteins in fungi, especially of glycosyl hydrolases [27, 28]. This difference also may be partly due to the difference in pH in culture media after 48 h (Table 1). A proteomic study related to lignocellulolytic enzymes of *Trichoderma reesei* strains showed

Table 1. Summary of the parameters observed from the reference bidimensional gels of each condition

Culture condition	Number of spots[a)]	R^{2}[b)]	Protein quantification ± SD (μg/mL)[c)]	pH
TLE+GLU	11	0.97/0.92	2.86 ± 0.13	2.3
TLE+FSCW	202	0.98/0.94	90.52 ± 0.40	6.2
MM+FSCW	194	0.99/0.97	60.37 ± 0.67	6.7

a) Number of spots detected by ImageMaster™ 2D Platinum v7.0 after processing. The same amount (600 μg) of protein was used for each gel.
b) Correlation between the reference map and its replicas using three different spots as landmarks for error correction.
c) Quantification of proteins from each supernatant and their standard deviations.

that the production and efficiency of these enzymes are significantly affected by pH in cellulosic culture media [29].

An SDS-PAGE experiment was performed prior to 2DE to evaluate the quality and profile of the samples (Supporting Information Material 3). The mycoparasitic conditions showed a very similar profile between them, almost undistinguishable, as TLE+GLU showed a very different profile. Protein profiles from total extracellular proteins from each culture supernatant were better visualized on 2DE. The resulting protein maps are shown in Fig. 1. Approximately 202, 194 and 11 protein spots were separated by 2DE from the supernatant of TLE+FSCW, MM+FSCW and TLE+GLU (Table 1), respectively. The high R^2 between replicates (Table 1) of each sample indicates that the protocol established for this study was highly reproducible. The distribution of spots showed that the most strongly secreted proteins had molecular weights (MW) between 15.18 and 81.10 kDa. Most proteins showed different MW and pI values from those predicted after identification, suggesting protein modifications such as glycosylation and proteolytic cleavage, among others.

A total of 105 spots, from all conditions were excised, digested with trypsin and the resulting peptides analyzed by MALDI-TOF/TOF mass spectrometry. Among the 105 spots, 97 spots showed MS spectra with good ion intensity. The peptides *m/z* were selected and reanalyzed by means of LIFT fragmentation. A total of 300 MS/MS spectra were obtained and the primary amino acid sequence of each one was manually interpreted using PepSeq (Waters) and FlexAnalysis 3.0 (Bruker Daltonics). The resulting peptides were then compared to the NCBI-nr Database and to the *T. harzianum* CBS226.95 genome v1.0 in order to identify the proteins present in the excised spots (Table 2). A total of 94 proteins, from 37 different genes (Table 2 and Supporting Information Material 4) were identified in this study. Identified proteins were numbered from 1 to 67. Some proteins with the same parameters (MW and pI) in different conditions were excised and identified, yielding the same identifications and, consequently the same ID numbers. Some proteins were identified as the same but were given different ID numbers due to their different locations on the 2DE maps.

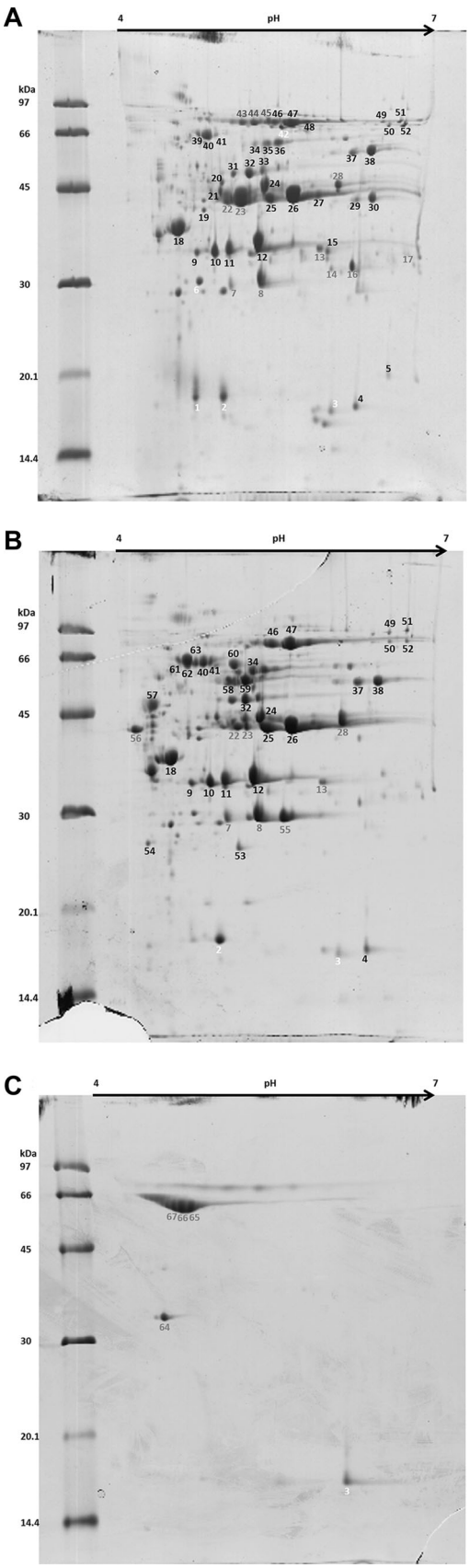
An automatic interpretation of MS spectra via MASCOT Peptide Mass Fingerprint and MS/MS spectra via MS/MS Ion search were also performed to identify protein spots. Both methods resulted in a lower protein identification when compared to manual de novo interpretation. MASCOT resulted in 41 identified proteins from 11 different genes as MS/MS Ion Search resulted in 76 identified proteins from 29 different genes (Supporting Information Material 5). MS/MS Ion Search tool failed to assign homology to proteins from database on correct sequences, lowering overall protein identification and protein coverage. Only 50% of the manually interpreted peptides sequences presented on Supporting Information Material 5 were confidently assigned to sequences from NCBInr database by MS/MS Ion Search ($p>0.05$) (Supporting Information 5 and 6).

To avoid these issues, we opted for a time-consuming but more reliable approach. Through manually interpreting MS/MS spectra, we were able to annotate a higher number of MS/MS spectra peptides sequences (Supporting Information Material 6) and avoid misinterpretation or interpretation failure due to glycosylation or di- or tripeptides, among other problems that could decrease the total level of identification [30]. This approach therefore led to a high level of protein/gene identification. This is the first secretome study of *Trichoderma* spp. to show such a high diversity of different proteins/genes identified on mycoparasitic-related studies, identifying over 89% of the analyzed protein spots. Studies to date were able to identify only three to ten different proteins [8–10,28], most of them known enzymes such as 42 kDa chitinases, B-1,3-glucanases and trypsin-like proteases. Through de novo interpretation, we were able to explore a new set of proteins, many of them not related to date with *T. harzianum* mycoparasitic mechanisms.

This high diversity of different proteins/genes identified in this study is also closely related to the study of genes related to mycoparasitism over several years and, more recently, facilitated by widespread analysis approaches such as EST analysis [16], subtractive library hybridization [17], RNA-seq [31] and the public accessibility of *Trichoderma* genomes.

3.2 Identified proteins, functions and gene expression analysis

The highest diversity of proteins was identified for TLE+FSCW and MM+FSCW (Table 2 and Fig. 2). Among



all of the differently identified proteins/genes, only one was observed in all conditions, and 24 were observed both in TLE+FSCW and MM+FSCW, while three were exclusive to TLE+FSCW, 7 to MM+FSCW and two to TLE+GLU (Table 2 and Fig. 2A). All 37 proteins/genes were separated in three classes: CAZYmes, Proteases and other functions (Fig. 2B) based on the NCBI-nr Blast search.

Protein domains were searched using Pfam database to assign putative functions for the identified proteins (Fig. 3). Five proteins (cbd13, cbd9, hypot, lyso and cys, gene name references present in Table 2) did not show any Pfam matches. All matches for TLE+FSCW and MM+FSCW are shown in Fig. 3, except for the glutaminase-like protein (Gene ID: glut), which showed a domain of unknown function (DUF), and it was not considered for further discussion. TLE+GLU exclusive proteins were assigned to Proteases classes (aspartate Protease, Asp; and tripeptidyl protease, S8 peptidase) and one in Other functions (cerato-platanin class, epl1).

3.3 CAZymes

The CAZymes identified in this study were only observed on the 2DE maps when *T. harzianum* was induced by FSCW compounds. We observed by RT-qPCR that all of them were overexpressed in the presence of FSCW confirming the gel results; the exception was the α-galactosidase (agalac) gene (Table 2). Specific enzyme activities were performed to validate the gel results for some classes (Table 3), but correlation to gene expression was not further discussed. *Trichoderma harzianum* possess several genes from the same class, like GH18 family which has 32 genes on *T. harzianum* CBS226.95 genome (Supporting Information Material 7). We only identified the product of four of these genes. There are evidences that other enzymes from GH18 family may be present in *T. harzianum* ALL42 secretome [16], and contribute to overall chitinase activity, although not identified in our study.

The mycoparasitic capacity of *Trichoderma* spp. is related to its ability to control the host fungus and to assimilate the carbon sources from its cell wall and cytosol [32]. A comparative analysis of genomes reveals the abundance of genes encoding chitin- and β-glucan- degrading enzymes, with respect to which *Trichoderma* (particularly the vigorous mycoparasites *T. virens* and *T. atroviride*) outperform other fungi [11].

◀ **Figure 1.** Reference bidimensional gels of *T. harzianum* ALL42 secreted proteins when cultivated in different conditions. (A) TLE+FSCW; (B) MM+FSCW and; (C) TLE+GLU. Numbers indicate the proteins that were identified after mass spectrometric analysis (Table 2 and Supporting Information Material 4) and colors indicate the putative classes of each identified spot. Black – CAZymes; Gray – Proteases; White – Other functions. As showed in Table 1, the difference in protein quantification profiles when *T. harzianum* was cultivated in the presence or absence of FSCW showed a very different protein profile between these conditions. Between the conditions supplemented with FSCW, most of the differences were identified (Table 2 and Supporting Information Material 4).

Table 2. Identified proteins, gene expression and Pfam classes analysis from *T. harzianum* ALL42 when cultivated in different conditions

Protein class	Spot ID[a)]	Gene ID[b)]	Description (protein name/species)[c)]	NCBI identity	JGI protein ID[d)]	Present in	Pfam classes confirmation[e)]	Gene expression analysis[f),g)]	
								TLE+FSCW	MM+FSCW
CAZYmes (22 different proteins)	4	cdb13	Carbohydrate-binding module family 13 protein (*T. virens*)	EHK19601.1	511345	TLE+FSCW & MM+FSCW	ND	ns	+
	5,34,58	abf	Alfa-L-arabinofuranosidase (*Trichoderma virens*)	EHK20391.1	503269	TLE+FSCW & MM+FSCW	GH54-CBM42	++	++
	9,10	b134gluc	Glycoside hydrolase family 16 protein - Endo-1,3(4)-β-glucanase (*T. virens*)	EHK27028.1	150678	TLE+FSCW & MM+FSCW	GH16	++++	++++
	11,12	chit33	Endochitinase 33 kDa (*T. harzianum)*	CAA56315.1	529621	TLE+FSCW & MM+FSCW	GH18	+++++	+++++
	15	cdb9	Hypothetical protein - CDB9-like domain - carbohydrate binding (*T. virens*)	EHK26772.1	511848	TLE+FSCW	ND	+++	+++
	18	chit37	Endochitinase 37 kDa (*T. harzianum*)	ABG46358.1	505895	TLE+FSCW & MM+FSCW	GH18	+++++	++++
	19	hypot	Hypothetical protein -β-1-6-glucan synthase (*T. virens)*	EHK17331.1	487382	TLE+FSCW & MM+FSCW	ND	++	++
	24	agluc42	Glycoside hydrolase family 71 protein (α-1,3-glucanase)	EHK22586.1	71044	TLE+FSCW & MM+FSCW	GH71	+++	+++
	25–27	chit42	Endochitinase 42 kDa (*T. harzianum*)	AAA98644	101028	TLE+FSCW & MM+FSCW	GH18	+++++	+++++
	35,36	agalac	Glycoside hydrolase family 27 protein - Alpha-Galactosidase (*T. reesei*)	EGR51161.1	509041	TLE+FSCW & MM+FSCW	GH27	ns	ns
	31–33	aman	Glycoside hydrolase family 47 protein - α-1,2D-mannosidase (*T. virens*)	EHK21505.1	487999	TLE+FSCW & MM+FSCW	GH47	+	ns
	29,30,37,38	b16gluc	Glycoside hydrolase family 30 protein - β-1,6-glucanase (*T. harzianum*)	CAC80490.1	485240	TLE+FSCW & MM+FSCW	GH30	++	++
	20,21,39-41,61, 62,63	gamy	Glycoside hydrolase family 15 protein - Glucoamylase (*T. harzianum*)	CAI67598.1	81392	TLE+FSCW & MM+FSCW	GH15-CBM20	+	+
	45–48	agluc75	Glycoside hydrolase family 71 protein - Alpha-1,3-glucanase (*T. harzianum*)	CAC80493.1	525334	TLE+FSCW & MM+FSCW	GH71-CBM24	+++	++++
	49,51	bgluc	Glycoside hydrolase family 3 protein - β-D-glucosidase (*T. virens*)	EHK22982.1	71613	TLE+FSCW & MM+FSCW	GH3	++++	+++++
	50,52	b13gluc	β-endo-1,3-glucanase (*T. harzianum*)	P53626.1	84648	TLE+FSCW & MM+FSCW	GH55	+++	+++
	54	endogluc	Endo-beta-1,4-glucanase (*T. harzianum*)	AFK32784.1	91916	MM+FSCW	GH12	++	+++++
	53	lyso	Glycoside hydrolase - lysozyme-like (*T. reesei*)	EGR45706.1	503484	MM+FSCW	ND	++++	++++
	57	gel	Glycoside hydrolase family 72 protein - β-1,3-glucanosiltransferase (*T. virens*)	EHK19699.1	150179	MM+FSCW	GH72-CBM43	+	++
	57	chit48	Glycoside hydrolase family 18 protein - chitinase (*T. atroviride*)	EHK50815.1	101981	MM+FSCW	GH18-CBM1	+++	+++
	59	cbh2	Cellobiohydrolase II (*T. virens*)	EHK21827.1	508869	MM+FSCW	GH7-CBM1	ns	+++++
	60	cbh1	Exoglucanase I - Cellobiohidrolase I (*T. harzianum*)	Q9P8P3.1	7497	MM+FSCW	GH6	+	+++++

Table 2. Continued

Protein class	Spot ID[a)]	Gene ID[b)]	Description (protein name/species)[c)]	NCBI identity	JGI protein ID[d)]	Present in	Pfam classes confirmation[e)]	Gene expression analysis[f),g)]	
								TLE+FSCW	MM+FSCW
Proteases (11 different proteins)	7,8,55	pra1	Trypsin-like protease (*T.harzianum*)	CAC80694.2	526221	TLE+FSCW & MM+FSCW	Trypsin	++++	++++
	13	sprt	Serine protease - SprT (*T. koningii*)	ABN04079.1	511032	TLE+FSCW & MM+FSCW	S8 Peptidase	+++	+++
	14,16	m28	Hypothetical protein - Peptidase M28 (*T. reesei*)	EGR50305.1	501003	TLE+FSCW	M28 Peptidase	++++	++++
	17	serin33	Serin endopeptidase (*T. harzianum*)	CAL25580.1	110777	TLE+FSCW & MM+FSCW	S8 Peptidase	+++++	+++++
	22,23	m36	Hypothetical protein - Peptidase M36, Fungalysin (*T. virens*)	EHK26892.1	511763	TLE+FSCW & MM+FSCW	M36 Peptidase	+++	++
	28	m14-442	Hypothetical protein - Peptidase m14 (*T. virens*)	EHK25112.1	514267	TLE+FSCW & MM+FSCW	M14 Peptidase	+	++
	28	m14-422	Hypothetical protein - Peptidase m14 (*T. virens*)	EHK23409.1	494007	TLE+FSCW & MM+FSCW	M14 Peptidase	++++	++++
	43–45	serin75	Serin endopeptidase (*T. harzianum*)	CAL25578.1	477752	TLE+FSCW & MM+FSCW	S8 Peptidase	+++	+++
	56	asp1	Putative aspartate protease (*T. harzianum*)	CAC17811.1	493562	MM+FSCW	Asp	ns	+
	64	asp2	Putative aspartic protease (*T. harzianum*)	CAI91181.1	86893	TLE+GLU	Asp	0	–
	65–67	tripep	Hypothetical proteins - Propeptidase S53 (tripeptidyl peptidase) (*T. virens*)	EHK16285.1	98670	TLE+GLU	Peptidase S8	–	–
Other function (4 different proteins)	3	epl1	Epl1 (*Trichoderma harzianum*)	AER09349.1	508110	All	Cerato-platanin	-	-
	6	npp1	Hypothetical protein - Necrosis inducing protein (NPP1) (*T. atroviride*)	EHK39942.1	507042	TLE+FSCW & MM+FSCW	NPP1	+++	+++
	1,2	cys	Predicted small secreted cysteine-rich protein (*T. virens*)	EHK19462.1	511478	TLE+FSCW & MM+FSCW	ND	+++++	+++++
	42	glut	Hypothetical protein - glutaminase-like (*T. virens*)	EHK25695.1	84515	TLE+FSCW	DUF1793	++	++

a) Protein ID refers to numbers on Fig. 1.
b) Gene ID is the name used to identify the gene for RT-qPCR analysis.
c) Protein Identification is listed as CAZymes, proteases and other functions.
d) JGI Protein ID was obtained from *Trichoderma harzianum* CBS226.95 genome v1.0 (http://genome.jgi.doe.gov/Triha1/Triha1.home.htmL).
e) Pfam analysis and confirmation of the putative classes obtained from protein description. ND, Not determined.
f) *T. harzianum* gene expression analysis after growth on TLE+FSCW and MM+FSCW. TLE+GLU was set as reference. Ns, Not statisticaly significant from TLE+GLU; 0 – no expression was detected; + 1–10 fold overexpressed than TLE+GLU; ++ 10.1–100 fold; +++ 100.1–1000 fold; ++++ 1000.1–10000;+++++ > 10000.1 fold. The minus (-) symbol was used for underexpressed when compared to TLE+GLU, following the same scale for overexpression.
g) Gene expression values are available at Supporting Information Material 8.

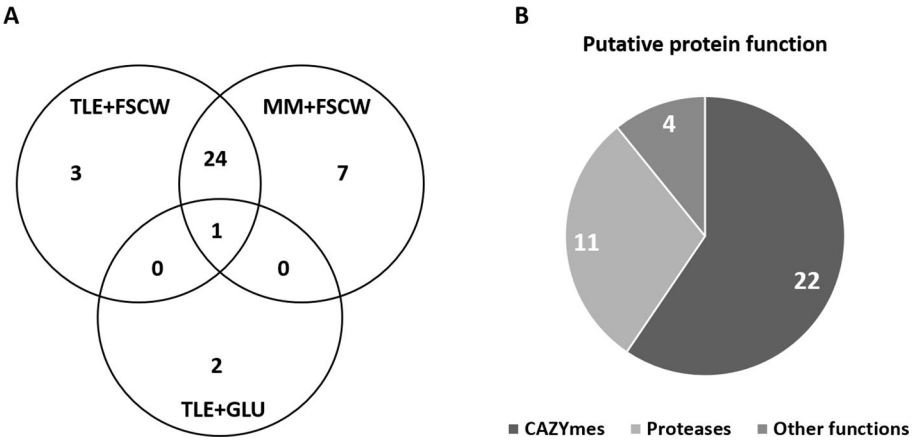


Figure 2. Distribution and putative functions of the different identified proteins. (A) Venn diagram of the distribution of proteins/genes per condition on 2DE maps. Out of all the identified proteins/genes, only one was observed in all conditions (epl1), 24 were observed both in TLE+FSCW and MM+FSCW, three were exclusive to TLE+FSCW (cbd9, m28 and glut), 7 of MM+FSCW and two to TLE+GLU (aps2 and tripep). (B) Putative function of the different identified proteins from all growth conditions based on NCBInr Blast search.

Although cell wall composition varies among fungal species, the structural scaffold is composed of chitin and β-1,3-glucan, which are together embedded in the amorphous fraction of α-glucans, galactomannans and other carbohydrate polymers, that account for > 90% of the cell wall [33, 34].

Four different endochitinases (GH18) were identified, and among these chit48 was only observed in MM+FSCW. Chit33, 37 and 42 have been purified, characterized and their roles in mycoparasitism studied [35–37]. Chit33 and 42 were previously observed to be overexpressed in the presence of FSCW [16, 17]. To date there is no further information available for the endochitinase designated here as chit48. Another protein that can hydrolyze β-1,4-N-acetylglucosamine and *N*-acetylmuramic acid linkages, usually found in peptidoglycans [38], is a lysozyme-like protein (lyso) identified only on MM+FSCW supernatant. Although all endochitinase genes, except for chit48 were overexpressed in TLE+FSCW (Table 2), endochitinase specific activity was

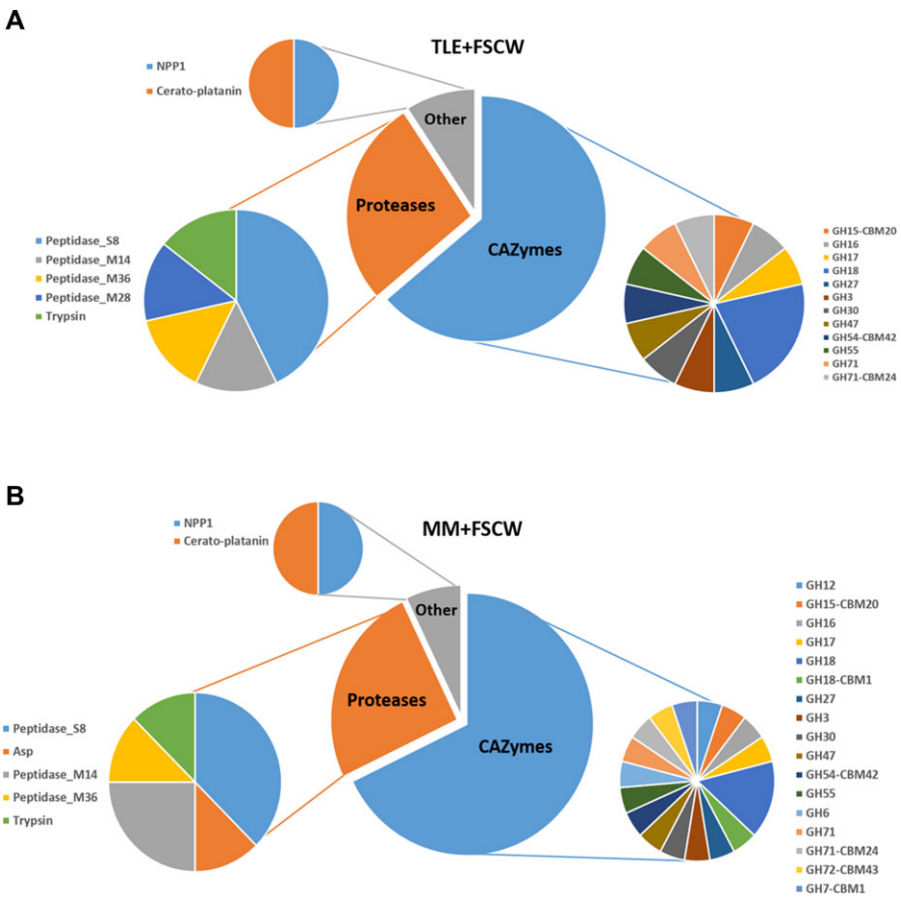


Figure 3. Pfam analysis of the identified proteins from *T. harzianum* ALL42 when grown in different conditions. (A) TLE+FSCW; B. MM+FSCW. Four proteins (cys, cbd13, cbd9 and glut) did not show any Pfam match. TLE+FSCW showed 12 CAZymes and five proteases matches as MM+FSCW showed 17 TLE+GLU and 5 proteases matches as well, but M28 was only present in TLE+FSCW and Asp only present in MM+FSCW. TLE+GLU showed three Pfam matches (cerato-platanin, S8 Peptidase and Asp), but are not represented in this figure.

Table 3. Enzymatic activity of the supernatant of *T. harzianum* ALL42 cultivated in different conditions

Enzymes (CAZYmes classes)	TLE+GLU	TLE + FSCW	MM + FSCW
	Specific activity ± SD (U/mg)		
Endochitinase (GH18,GH18-CBM1)	0	1.35 ± 0.04	1.51 ± 0.01
β-1,3-glucanase (GH55, GH16)	0	10.54 ± 0.75	20.84 ± 1.06
Glucoamylase (GH15-CBM20)	0	2.99 ± 0.03	4.09 ± 0.06
α-mannosidase (GH47)	0	0.006 ± 0.00	0.04 ± 0.00
α-L-arabinofuranosidase (GH54-CBM42)	0	0.02 ± 0.00	0.02 ± 0.01
β-glucosidase (GH3)	0	0.63 ± 0.03	1.51 ± 0.00
Endoglucanase (GH12, GH16)	0	0.42 ± 0.02	1.94 ± 0.32
Exoglucanase (GH6, GH7-CBM1)	0	0	0.33 ± 0.00
Acid protease	12.50±2.52	6.27 ± 0.157	3.92 ± 0.12
Neutral protease	3.93±0.50	7.80 ± 0.754	4.37 ± 0.09
Basic protease	2.50±0.51	6.29 ± 1.010	3.78 ± 0.07

higher in MM+FSCW, suggesting that lyso and other chitinases not visualized/identified in our study might be contributing for this activity.

An endo-β-1,3-glucanase (GH55) [39] and an endo-β-1,3(4)-glucanase (GH16) were also identified. These enzymes play important roles in mycoparasitism and in morphogenetic-morpholitic processes during development [40], and in the mobilization of β-glucans under conditions of carbon and energy starvation [41]. The endo-β-1,3(4)-glucanase MW is similar to an endo-β-1,3-glucanase purified from *T. harzianum* [42] and from *T. asperellum* [43] and its gene was also identified on an EST library when *T. harzianum* ALL42 was grown in the presence of FSCW [16].

Four different spots, with different molecular weights (42.5 and 57 kDa) were identified as β-1,6-glucanases (GH30), products of the same gene, and were also identified in an EST library [16] and a subtractive library hybridization [17] of *T. harzianum* ALL42 grown in the presence of FSCW. These differences could be due to proteolytic activity involved in activation, since *T. harzianum* secretes a large set of proteases (as discussed later) or alternatively may be due to alternative splicing. De la Cruz et al. [44] observed that at least two endo-β-1,6-glucanase were secreted by *T. harzianum* and a 43 kDa enzyme (BGN16.2) has been purified. This enzyme acted synergistically with β-1,3-glucanase and chitinases in the degradation of fungal cell walls. A 51 kDa β-1,6-glucanase (BGN16.1) purified and characterized by de la Cruz & Llobel [45], was also identified and the authors ruled out the possibility that BGN16.2 was a proteolytic major product of BGN16.1 after observing that an antibody did not bind to both proteins, suggesting that they were products of different genes. However, almost all of the peptides obtained in our study were observed in all of the protein spots, and matched the same gene in the database. Further analysis of this gene is required in order to determine whether these β-1,6-glucanases are products of different genes, or the same gene, or whether this is a strain-specific finding.

The same situation was observed for the protein spots identified as glucoamylase (GH15), but there were also differences in pI. The major product was of approximately 63.89 kDa, a similar MW to that of the protein encoded by the 2.1 kb transcript of the *Gla66* gene [46]. As observed in our study, this gene is repressed by the presence of high glucose concentration, and its minor transcript does not seem to encode splicing variants, suggesting that the other spots were the product of proteolytic breakdown.

Other glycosyl hydrolases, such as α-1,3-glucanases (GH71), α-galactosidase (GH27), α-1,2-mannosidase (GH47) and α-L-arabinofuranosidase (GH54) were also identified. These enzymes might be related to the degradation of the amorphous fraction of the fungal cell wall, but the α-mannosidase may be also involved in the deglycosylation of glycoproteins present in the fungal cell wall, since *F. oxysporum* has a high mannose concentration among its cell wall glycoproteins [47]. It may also be involved in the deglycosylation of proteins secreted by other microorganisms, rendering these proteins more susceptible to proteolytic cleavage [48].

Two α-1,3-glucanases (agluc42 and agluc75) were identified in this study. The gene encoding the protein here defined as agluc75 was upregulated when *T. harzianum* was cultivated under mycoparasytic conditions [49]. *F. oxysporum* cell wall is rich in α-1,3-glucan [47], suggesting that *F. solani* might have a similar cell wall composition.

The identification of α-L-arabinofuranosidase and α-galactosidase was unexpected since these enzymes, together with xylanases are involved in the degradation of hemicellulose [50], a polymer not found in fungi. There is evidence that a 22 kDa xylanase secreted by *Trichoderma* spp. leads to ethylene production and induces local defense mechanisms in plants [51]. α-L-arabinofuranosidase and α-galactosidase can be involved in inducing defense responses in plants in the presence of phytopathogens as discussed later for npp1. Studies regarding these enzymes so far have been focused on *T. reesei* due to its efficient cellulolytic enzyme system, and its applicability in the biotechnology industry. However, α-galactosidase can be also involved in the cleavage of galactomannans from fungal cell wall, together with α-mannosidase produced by *T. harzianum*.

The hypothetical protein defined here as a putative β-1,6-glucan synthase requires further study to identify its real function. BLAST results indicate that this protein is similar to a β-1,6-glucan synthase from *Metarhizium anisopliae*, and Interpro scan results (IPR017853) indicate that this protein possesses a domain that belongs to several glycosyl hydrolase families. The CAZymes discussed so far were observed in TLE+FSCW and MM+FSCW.

We observed that five CAZymes were only identified in MM+FSCW, and gene expression analysis confirmed these findings (Table 2 and Supporting Information Material 8). β-1,3-glucanosyltransferase (GH72) is a GPI-anchored protein that participates in the biogenesis and elongation of β-1,3-glucan chains, and is important for fungal growth [52]. As a membrane-anchored protein, we did not expect to observe such a high concentration in the extracellular medium, although it has a signaling peptide indicating secretion.

At first, we believed that this was a response to nitrogen starvation, but we identified the same protein as well as a glycosylphosphatydilinositol phospholipase in the supernatant of *T. harzianum* ALL42 when cultivated in TLE medium supplemented with *F. oxysporum* cell walls (Ramada et al., unpublished data), though in a lower concentration than in MM+FSCW. This may reflect a process of protein cleavage for protein turnover, or may be related to cell-morphogenesis processes, or alternatively may be important for another physiological process as yet undetermined, since one β-1,3-glucanosyltransferase from *Saccharomyces cerevisiae* has been shown to be involved in transcriptional silencing [53].

The proteins endoglucanase (GH12), cellobiohydrolase I (GH7) and cellobiohydrolase II (GH6), involved in the degradation of cellulose polymers, were only observed in MM+FSCW, while β-glucosidase, was also observed in TLE+FSCW. Endoglucanase and β-glucosidase-specific enzymatic activities were higher in MM+FSCW than in TLE+FSCW, while exoglucanase activity was only observed in this condition (Table 3). Gene expression analysis confirmed that these genes were overexpressed on MM+FSCW (Table 2) when compared to the other two, suggesting nitrogen regulation, although Ilmen et al. [54] reported that carbon and nitrogen starvation is not sufficient to trigger significant expression of cellulolytic genes on *Trichoderma reesei*.

3.4 Proteases

Although there was a greater number of protein spots identified as CAZymes, a large set of different proteases were also identified in the three different conditions. Proteases may play important roles in host cell lysis by attacking lipids and proteins, which are also a part of the cell-wall, and in the inactivation of enzymes secreted by phytopathogens during the infection process in plants, as well as in self-protein recycling [55, 56]. *Trichoderma* may have one of the largest sets of proteases among fungi [57]. Around 20% of the predicted *Trichoderma* proteases possess a signal peptide and are therefore destined for the secretory pathway. Druzhinina et al. [26] observed that the dominant groups were aspartyl proteases, serine proteases, subtilisin-like proteases, dipeptidyl peptidases and tripeptidyl peptidases, although the larger set of proteases identified in our study included members of the serine protease, subtilisin-like protease and metalloprotease classifications.

An aspartic protease (asp2), which was previously believed to be only expressed when induced with fungal cell walls [28], was identified only on TLE+GLU. One other protease was only observed in TLE+GLU, and was identified as a propeptidase from the family s53, a serine tripeptidyl peptidase (tripep). Gene expression analysis showed that these genes are overexpressed on TLE+GLU (Table 2), and a higher specific activity was also observed for acid proteases in this supernatant (Table 3). The pH in TLE+GLU was around 2.3, suggesting that these proteases are pH-regulated.

Another aspartic protease (asp1) was observed only on MM+FSCW, and gene expression analysis confirmed its overexpression in this condition. Aspartic proteases have been mostly associated with proteolytic degradation. In *T. reesei*, an aspartic protease with the same molecular weight as the one observed in our study was isolated under a cellulose-inducing condition and was involved in the degradation of this GH [58]. Asp1 and the cellulase proteins were only identified in MM+FSCW, indicating that this enzyme expression might act in a similar manner to the one isolated from *T. reesei*.

The majority of extracellular proteases related to biocontrol processes in *Trichoderma* spp. have been characterized as serine proteases. In our study we identified two subtilisin-like serine proteases (serin33 and serin75), but serin75 was only observed on TLE+FSCW, and gene expression confirmed its overexpression in this condition. However, *Trichoderma* proteases are not only efficient against fungi, but are also important for the control of other phytophatogens. The trypsin-like protease identified was isolated from *T. harzianum*, characterized and displayed nematicidal activity [59]. This nematicidal activity was also observed for the serine protease sprt, (matched in our study to a SprT from *T. koningii*) characterized from *T. pseudokoningii*, that showed closer similarity to nematicidial serine proteases from nematode parasistic fungi than to serine proteases from *Trichoderma* [60].

Four metalloproteases (M36, M28 and two M14) were also induced by FSCW, indicating that these enzymes may play roles in this process, as is the case for the serine proteases and some aspartyl proteases [26]. The two M14 proteins were identified in the same spot, showing similar predicted MW and pI, while the genes encode for predicted 442 and 422 amino acid proteins. Both were overexpressed in MM+FSCW, while M36 and M28 were overexpressed in TLE+FSCW. The high diversity of proteases secreted by *T. harzianum* in the mycoparasitic-related conditions may reflect a synergistic system for the efficient use of proteins by this mycotrophic fungus.

3.5 Other functions

One of the largest groups of proteins secreted by *Trichoderma* is the small secreted cysteine-rich proteins (SSCPs). They were identified under the criteria that they should be ≤ 300 amino acids long and contain four or more cysteine residues [61]. This group is subdivided into four groups [13]. The only protein that was observed in all three conditions was epl1. Seidl et al. [62] suggests that this protein is constitutively expressed by *Trichoderma atroviride*. Epl1 is a protein that belongs to the second SSCP group, the cerato-platanin protein family, associated with the induction of defense responses in plants [62, 63]. Gene expression analysis showed that epl1 is overexpressed on TLE+GLU (Table 2). Another SSCP protein was identified in our study (cys). This protein is 143 amino acid residues long and has 18 cysteine residues. Gene expression was similar for the mycoparasitic-related conditions, and was overexpressed when compared to TLE+GLU. Apparently, this protein belongs to the fourth group of SSCPs, the largest and most unique group, but with no function assigned so far.

The protein identified as npp1 is an interesting protein that might act as another inducer of plant resistance. Infiltration of npp1 from *Phytophthora* sp. into leaves of *Arabidopsis thaliana* plants results in transcript accumulation of pathogenesis-related (PR) genes, production of reactive oxygen species (ROS) and ethylene, callose apposition and hypersensitive response-like cell death [64]. The npp1 gene might be induced by some elicitor from the fungal phytopathogen or by some mechanism other than the presence of the plant, since npp1 was overexpressed in the presence of FSCW.

The remaining identified proteins/genes (cdb13, cdb19 and glut) did not show any match with Pfam database search or with a class with known function. They were overexpressed in FSCW supplemented media, but their role in mycoparasitic-related condition is still unknown.

In this study, we were able to identify a larger number of different proteins, when compared to previous studies, which are potentially involved in the response of *T. harzianum* ALL42 to *F. solani*. From the 37 different proteins identified from the secretome, just four genes (chit42, chit33, b16gluc and b134gluc) were also identified on the transcriptome analysis of *T. harzianum* ALL42 in response to FSCW by EST library [16] and subtractive library hybridization [17], highlighting the importance of proteomics as a powerful approach to analyze and obtain novel information from mycoparasitic-related conditions.

4 Concluding remarks

The nature of fungal secretomes is closely dependent on their biotic and abiotic environment, a property that depends on the ecological spectrum of fungal species. The structure of these secretomes consists of hydrolases necessary to the fungal food supply. Different strata of effectors can increase the fungal secretome "strike force," as manifested by the production of isoforms/multiforms of hydrolases functionally required in different physicochemical environments, and/or synthesis of specific protective proteins [65].

Trichoderma harzianum secretes a complex protein profile according to the environment in which the fungus finds itself. Several proteomic, ESTs, RNA-seq or prediction studies have been or are being performed in an attempt to obtain novel information concerning the important and characteristic process from *Trichoderma* spp, known as mycoparasitism. The number of genes/proteins related to mycoparasitism is growing fast, showing a high level of diversity of proteins that does not include only cell wall degrading enzymes, such as chitinases, β-1,3-glucanases and proteases. Proteins that display functions related to the symbiotic interaction with plants are also secreted, probably including proteins that interact with other microorganisms from the environment, as well as a host of other genes/proteins without known functions. The synergism between enzymes and metabolism is reported in the literature and represents an efficient strategy for *Trichoderma´s* opportunistic success. Many studies have been carried out using cell wall, a complex structure, as an inducer of *Trichoderma´s* arsenal in order to visualize its potency in these mycoparasitic-related conditions.

A wider view of the process allows the identification of many proteins that might be involved at a given moment or in a given mechanism, but the lack of information in the databases is a rising concern in the era of data generation. Further gene or protein studies are necessary in order to validate the prediction studies and the assigned functions for many genes/proteins identified by large-scale analyses such as EST analysis, RNA-seq and proteomics. We believe that this is the next step for a better understanding of the mycoparasitism of *Trichoderma* along with the study of the complex *Trichoderma*–phytopathogen interaction.

This work was supported by a biotechnology research grant to C.J.U (CNPq and FUNAPE/UFG). M.H.S.R was supported by FINEP/CAPES/CNPq.

The authors have declared no conflict of interest.

5 References

[1] Tolêdo-Souza, E. D., Lobo-Júnior, M., Silveira, P. M., Café-Filho, A. C., Interações entre *Fusarium solani* f. sp. *phaseoli* e *Rhizoctonia solani* na severidade da podridão radicular do feijoeiro. *Pesq. Agropec. Trop.* 2009, *39*, 13–17.

[2] Carvalho, D. D. C., Mello, S. C. M., Lobo-Júnior, M., Silva, M. C., Controle de *Fusarium oxysporum* f. sp. *phaseoli* in vitro e em sementes, e promoção do crescimento inicial do feijoeiro comum por *Trichoderma harzianum*. *Trop. Plant. Pathol.* 2011, *36*, 28–34.

[3] Zaccardelli, M., Vitale, S., Luongo, L., Merighi, M., Corazza, L., Morphological and molecular characterization of *Fusarium solani* isolates. *J. Phytopathol.* 2008, *156*, 534–41.

[4] Druzhinina, I. S., Kopchinskiy, A. G., Kubicek, C. P., The first one hundred of Tricrhoderma species is characterized by molecular data. *Mycoscience* 2006, *47*, 55–64.

[5] Lorito, M., Woo, S. L., Harman, G. E., Monte, E., Translational research on *Trichoderma*: from 'Omics to the field. *Annu. Rev. Phytopathol.* 2010, *48*, 395–417.

[6] Hermosa R., Viterbo A., Chet I., Monte E., Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 2012, *158*, 17–25.

[7] Shoresh, M., Mastouri, F., Harman, G. E., Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 2010, *48*, 21–43.

[8] Grinyer, J., Hunt, S., Mckay, M., Herbert, B. R., Nevalainen, H., Proteomic response of the biological control fungus *Trichoderma atroviride* to growth on the cell walls of *Rhizoctonia solani*. *Curr. Genet.* 2005, *47*, 381–388.

[9] Yang, H. H., Yang, S. L., Peng, K. C., Lo, C. T., Liu, S. Y., Induced proteome of *Trichoderma harzianum* by *Botrytis cinerea*. *Mycol. Res.* 2009, *113*, 924–932.

[10] Monteiro, V. N., Silva, R. N., Steindorff, A. S., Costa, F. T. et al., New insights in *Trichoderma harzianum* antagonism of fungal plant pathogens by secreted protein analysis. *Curr. Microbiol.* 2010, *61*, 298–305.

[11] Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A. et al., *Trichoderma*: the genomics of opportunistic success. *Nat. Rev. Microbiol.* 2011, *9*, 749–759.

[12] Hermosa, R., Woo, S. L., Lorito, M., Monte, E., Proteomic approaches to understand *Trichoderma* biocontrol mechanisms and plant interactions. *Curr. Proteomics* 2010, *7*, 298–305.

[13] Kubicek, C. P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D. A. et al., Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome Biol.* 2011, *12*, R40.

[14] Almeida, F. B., Cerqueira, F. M., Silva, R. N., Ulhoa, C. J., Lima, A. L., Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Lett.* 2007, *29*, 1189–1193.

[15] Lopes, F. A. C., Steindorff, A. S., Geraldine, A. M., Brandão, R. S. et al., Biochemical and metabolic profiles of *Trichoderma* strains isolated from common bean crops in the Brazilian Cerrado; and potential antagonism against *Sclerotinia sclerotiorum*. *Fungal Biol.* 2012, *116*, 815–824.

[16] Steindorff, A. S., Silva, R. N., Coelho, A. S. G., Nagata, T. et al., *Trichoderma harzianum* expressed sequence tags for identification of genes with putative roles in mycoparasitism against *Fusarium solani*. *Biol. Control.* 2012, *61*, 134–140.

[17] Vieira P. M., Coelho A. S. G., Steindorff A. S., Siqueira S. J. L. et al., Identification of differentially expressed genes from *Trichoderma harzianum* during growth on cell wall of *Fusarium solani* as a tool for biotechnological application. *BMC Genomics* 2013, *14*, 177.

[18] Bradford, M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, *72*, 248–254.

[19] Laemmli, U. K., Cleavage of structural proteins assembly of the head of bacteriophage T_4. *Nature* 1970, *227*, 680–685.

[20] Shevchenko, A., Thomas, H., Havlis, J., Olsen, J. V., Mann, M., In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 2007, *1*, 2856–2860.

[21] Horton, P., Park, K-J., Obayashi, T., Fujita, N. et al., WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* 2007, W585–W587.

[22] Xiao, Z., Storms, R., Tsang, A., Microplate-based filter paper assay to measure total cellulose activity. *Biotechnol. Bioeng.* 2004, *88*, 832–837.

[23] Xiao, Z., Storms, R., Tsang, A., Microplate-based carboxymethylcellulose assay for endoglucanase activity. *Anal. Biochem.* 2005, *342*, 176–178.

[24] Ramada, M. H. S., Lopes, F. A. C., Ulhoa, C. J., Silva, R. N., Optimized microplate β-1,3-glucanase assay system for *Trichoderma* spp. screening. *J. Microbiol. Meth.* 2010, *8*, 6–10.

[25] Livak K. J., Schmittgen T. D., Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 2001, *25*, 402–408.

[26] Druzhinina, I. S., Shelest, E., Kubicek, C. P., Novel traits of *Trichoderma* predicted through the analysis of its secretome. *FEMS Microbiol. Lett.* 2012, *337*, 01–09.

[27] Donzelli, B. G. G., Harman, G. E., Interaction of ammonium; glucose; and chitin; and chitin regulates the expression of cell-wall degrading enzymes in *Trichoderma atroviride* strain P1. *Appl. Environ. Microb.* 2001, *67*, 5643–5647.

[28] Suárez, M. B., Sanz, L., Chamorro, M. I., Rey, M. et al., Proteomic analysis of secreted proteins from *Trichoderma harzianum* Identification of a fungal cell wall-induced aspartic protease. *Fungal Genet. Biol.* 2005, *42*, 924–934.

[29] Adav, S.S., Ravindran, A., Chao, L. T., Tan, L. et al., Proteomic analysis of pH and strains dependent protein secretion of *Trichoderma reesei*. *J. Proteome. Res.* 2011, *10*, 4579–4596.

[30] Fryksdale, B. G., Jedrzejewski, P.T., Wong, D. L., Gaertner, A. L., Miller, B. S., Impact of deglycosylation methods on two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-time of flight-mass spectrometry for proteomic analysis. *Electrophoresis* 2002, *2*, 2184–2193.

[31] Steindorff, A. S., Ramada, M. H. S., Coelho, A. S. G., Miller, R. N. G. et al., Identification of mycoparasitism-related genes against the phytopathogen *Sclerotinia sclerotiorum* through transcriptome and expressions profile analysis in *Trichoderma harzianum*. *BMC Genomics* 2014, *15*, 204.

[32] Benítez, T., Rincón, A. M., Limón, M. C., Codón, A. C., Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* 2004, *7*, 249–260.

[33] Latgé, J. P., The Cell Wall: a carbohydrate armour of the fungal cell. *Mol. Microbiol.* 2007, *66*, 279–290.

[34] Feofilova, E. P., The fungal cell wall: modern concepts of its composition and biological function. *Microbiology* 2010, *79*, 711–720.

[35] de la Cruz, J., Hidalgo-Gallego, A., Lora, J. M., Benitez, T. et al., Isolation and characterization of three chitinases from *Trichoderma harzianum*. *Eur. J. Biochem.* 1992, *206*, 859–867.

[36] Carsolio, C., Gutierrez, A., Jimenez, B., Montagu, M., Herrera-Estrela, A., Characterization of *ech42*; a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proc. Natl. Acad. Sci. USA* 1994, *91*, 10903–10907.

[37] Dana, M. M., Limón, M. C., Mejías, R., Mach, R. L. et al., Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*. *Curr. Genet*. 2001, *38*, 335–342.

[38] Dziarski, R., Gupta, D., Peptidoglycan recognition in innate immunity. *J. Endotoxin Res.* 2005, *11*, 304–310.

[39] de la Cruz, J., Pintor-Toro, J. A., Benítez, T., Llobel, A., Romero, L. C., Novel endo-β-1,3-glucanase; BGN13.1; involved in the mycoparasitism of *Trichoderma harzianum*. *J. Bacteriol.* 1995, *177*, 6937–6945.

[40] Adams, D. J., Fungal cell wall chitinases and glucanases. *Microbiology* 2004, *150*, 2029–2035.

[41] Pitson, S. M., Seviour, R. J., Mcdougall, B. M., Noncellulolytic fungal β-glucanases: their physiology and regulation. *Enzyme Microb. Techn.* 1993, *15*, 178–192.

[42] Noronha, E. F., Kipnis A., Junqueira-Kipnis, A. P., Ulhoa, C. J., Regulation of a 36-KDa β-1,3-glucanase synthesis in *Trichoderma harzianum*. *FEMS Microbiol. Lett*. 2000, *188*, 19–22.

[43] Aires, R. S., Steindorff, A. S., Ramada, M. H. S., de Siqueiram S. J. L., Ulhoa, C. J., Biochemical characterization of a 27 kDa 1,3-β-D-glucanase from *Trichoderma asperellum* induced by cell wall of *Rhizoctonia solani*. *Carbohyd. Polym*. 2012, *87*, 1219–1223.

[44] de la Cruz, J., Pintor-Toro, J. A., Benítez, T., Llobell, A., Purification and characterization of an endo-β-1;6-glucanase from *Trichoderma harzianum* related to its mycoparasitism. *J. Bacteriol*. 1995, *177*, 1864–1871.

[45] de la Cruz, J., Llobell, A., Purification and properties of a basic endo- β -1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum*. *European J. Biochem*. 1999, *265*, 145–151.

[46] Dana, M. M., Pintor-Toro, J. A., Post-transcriptional control of a glucoamylase gene from *Trichoderma harzianum* under stress conditions. *Mol. Microbiol.* 2005, *57*, 250–260.

[47] Schoffelmeer, E. A. M., Klis, F. M. J., Sietsma, H., Cornelissen, B. J. C., The cell wall of *Fusarium oxysporum*. *Fungal Genet. Biol.* 1999, *27*, 275–282.

[48] Barros, M. C., Silva, R. N., Ramada, M. H. S., Galdino, A. S. et al., The influence of N-glycosylation on biochemical properties of Amy1; an α-amylase from the yeast *Cryptococcus flavus*. *Carbohyd. Res*. 2009, *344*, 1682–1686.

[49] Ait-Lahsen, H., Soler, A., Rey, M., de La Cruz, J. et al., An antifungal Exo-α-1;3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. *Appl. Environ. Microbiol*. 2001, *67*, 5833–5839.

[50] Saha, B. C., α-L-Arabinofuranosidases: biochemistry; molecular biology and application in biotechnology. *Biotechnol. Adv.* 2000, *18*, 403–423.

[51] Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., Lorito, M., *Trichoderma* species- Opportunistic; avirulent plant symbionts. *Nat. Rev. Microbiol.* 2004, *02*, 43–56.

[52] Hurtado-Guerrero, R., Schüttelkopf, A. W., Mouyna, I., Ibrahim, A. F. M. et al., Molecular mechanisms of yeast cell wall glucan remodeling. *J. Biol. Chem.* 2009, *284*, 8461–8469.

[53] Koch, M. R., Pillus, L., The glucanosyltransferase Gas1 functions in transcriptional silencing. *Proc. Natl. Acad. Sci. USA* 2009, *106*, 11224–11229.

[54] Ilmen, M., Saloheimo, A., Onnela, M. L., Penttilä, M. E., Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Appl. Environ. Microbiol*. 1997, *63*, 1298–1306.

[55] Viterbo, A., Ramot, O., Chernin, L., Chet, I., Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Anton. Leeuw*. 2002, *81*, 549–556.

[56] Suárez, M. B., Vizcaíno, J. A., Llobell, A., Monte, E., Characterization of genes encoding novel peptidases in biocontrol fungus *Trichoderma harzianum* CECT 2413 using the TrichoEST funcional genomics approach. *Curr. Genet.* 2007, *51*, 331–342.

[57] Rawlings, N. D., Barrett, A. J., Bateman, A., MEROPS: the database of proteolytic enzymes; their substrates and inhibitors. *Nucleic Acids Res*. 2012, *40*, D343–D350.

[58] Haab, D., Hagspiel, K., Szakmary, K., Kubicek, C. P., Formation of the extracellular protease from *T. reesei* QM 9414 involved in cellulose degradation. *J. Biotechnol*. 1990, *16*, 187–198.

[59] Suárez, B., Rey, M., Castillo, P., Monte, E., Llobell, A., Isolation and characterization of PRA1; a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. *Appl. Microbiol. Biot.* 2004, *65*, 46–55.

[60] Chen, L. L., Liu, L. J., Shi, M., Song, X. Y. et al., Characterization and gene cloning of a novel serine protease with nematicidal activity from *Trichoderma pseudokoningii* SMF2. *FEMS Microbiol. Lett.* 2009, *299*, 135–142.

[61] Martin, F., Aerts, A., Ahrén, D., Brun, A. et al., The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature* 2008, *452*, 88–92.

[62] Seidl, V., Marchetti, M., Schandl, R., Allmaier, G., Kubicek, C. P., Epl1; the major secreted protein of *Hypocrea atroviridis* on glucose; is a member of a strongly conserved protein family comprising plant defense response elicitors. *FEBS J.* 2006, *273*, 4346–4359.

[63] Frischmann, A., Neudl, S., Gaderer, R., Bonazza, K. et al., Self-assembly at air/water interfaces and carbohydrate binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. *J. Biol. Chem.* 2012, *288*, 4278–4287.

[64] Fellbrich, G., Romanski, A., Varet, A., Blume, B. et al., NPP1; a phytophthora-associated trigger of plant defense in parsley and *Arabidopsis*. *Plant J.* 2002, *32*, 375–390.

[65] Girard, V., Dieryckx, C., Job, C., Job, D., Secretomes: The fungal strike force. *Proteomics* 2013, *13*, 597–608.

