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RESEARCH ARTICLE

Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach

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Abstract The main molecular factors involved in the complex interactions occurring between plants (bean), two different fungal pathogens (*Botrytis cinerea*, *Rhizoctonia solani*) and an antagonistic strain of the genus *Trichoderma* were investigated. Two-dimensional (2-D) electrophoresis was used to analyze separately collected proteomes from each single, two- or three-partner interaction (i.e., plant, pathogenic and antagonistic fungus alone and in all possible combinations). Differential proteins were subjected to mass spectrometry and in silico analysis to search for homologies with known proteins. In the plant proteome, specific pathogenesis-related proteins and other disease-related factors (i.e., potential resistance genes) seem to be associated with the interaction with either one of the two pathogens and/or *T. atroviride*. This finding is in agreement with the demonstrated ability of *Trichoderma* spp. to induce systemic resistance against various microbial pathogens. On the other side, many differential proteins obtained from the *T. atroviride* interaction proteome showed interesting homologies with a fungal hydrophobin, ABC transporters, etc. Virulence factors, like cyclophilins, were up-regulated in the pathogen proteome during the interaction with the plant alone or with the antagonist too. We isolated and confidently identified a large number of protein factors associated to the multi-player interactions examined.

Keywords *Trichoderma* · Proteomics · Three-way interactions · Plant · Fungal pathogens · Differential proteins

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Introduction

Studies published so far on plant–pathogen interactions have been mainly focused on the molecular changes related to pathogen attack and/or plant response (Baker et al. 1997; Dangl and Jones 2001; Hammond-Kosack and Parker 2003; Martin et al. 1993; Ronald 1997; Suzuki et al. 2004). Several signal molecules and defence factors have been identified in plant (Cánovas et al. 2004; Ramonell and Somerville 2002; Rep et al. 2002), as well as virulence and avirulence factors in microbes (Kazemi-Pour et al. 2004; Smolka et al. 2003). Nevertheless, the molecular bases of multiple-player systems that may produce beneficial effects on plant health are largely unknown. Moreover, the influence that a biocontrol agent may have on the interactions

between a plant and a pathogen has not yet been investigated by using proteomics, while this technique clearly represents an effective tool to analyze such biological processes (Woo et al. 2006).

Since biocontrol fungi of the genus *Trichoderma* have developed the ability to interact simultaneously both with plants and fungal pathogens, they can be used as model microorganisms to study complex and multiple-player plant–microbe interactions. Antagonistic *Trichoderma* spp. use numerous mechanisms against pathogens, including production of antifungal compounds, direct parasitism or inhibition of pathogen growth and, as determined more recently, induction of plant systemic and localized resistance (ISR and LAR) (Benítez et al. 2004; Brunner et al. 2005; Chet 1987; Handelsman and Stabb 1996; Harman and Kubicek 1998; Harman et al. 2004a; Lorito and Woo 1998; Sivasithamparam and Ghisalberti 1998). The plant interaction with *Trichoderma*, which often involves an ISR effect, normally increases fitness and ability to withstand both biotic and abiotic stresses (Bigirimana et al. 1997; De Meyer et al. 1998; Howell 2003; Miller and Jastrow 1990). De facto, the activity of these fungal biocontrol agents commonly determines the level of plant susceptibility or resistance to pathogens (Bigirimana et al. 1997; De Meyer et al. 1998; Lo et al. 2000; Lu et al. 2004).

Structural genomic studies have provided vast information on the identity and structure also of genes expressed in plant–microbe interactions (Baker et al. 1997; Singh et al. 2004; Talbot 2003). Functional genomics combined with bioinformatics provides an overall picture of the metabolic status of an organism in a given moment or condition (Pandey and Mann 2000; Zhu et al. 2003). In particular, proteomics permits a large-scale analysis of protein production, and in recent years has been widely used to investigate protein profiles produced from diverse interaction conditions and physiological states of cells and tissues (Pandey and Mann 2000; Zhu et al. 2003), especially in pathology-related research (Lim and Elenitoba-Johnson 2004).

By using a proteomic approach, we studied the concurrent interactions of the biocontrol agent *T. atroviride* strain P1 with a host plant and different fungal pathogens, in order to identify and analyze the proteins differentially produced by the three players. 2-D maps of protein extracts were obtained from plant and fungi singly and in any possible combination. Differential proteins in the gels were confidently identified and characterized by using tryptic digestion, mass spectrometry (MS) and in silico analysis. Proteins putatively important for plant–pathogen–antagonist interaction were analyzed in order to determine their accumulation pattern, gather hints on their role and eventually improve methods for disease control.

Materials and methods

Growth and interaction conditions

Bean plants (*Phaseolus vulgaris* L. cv. Cannellino) were grown in sterile soil. After 3 weeks bean leaves were collected, washed and placed on the top of 1.5% water agar (WA) plates 15 cm in diameter (Fig. 1). For root interaction, bean seeds were sterilized for 1 min with a 1% hypochlorite solution, rinsed with sterile water and placed in sterilized magenta filled with a sterile inert support (perlite). After seed germination, the magenta were opened and the seedlings were rinsed with water two times per week. Three-week-old plants were collected, their roots washed extensively, and entirely transferred to WA plates by laying them on the medium (Fig. 1).

The fungal pathogens *Botrytis cinerea* (strain 309, isolated from tobacco) and *Rhizoctonia solani* (strain 1556, isolated from tomato) were maintained on malt extract (SIGMA, St. Louis, MO, USA) and potato dextrose agar (PDA) (SIGMA) plates, respectively. One hundred microliters of a 5×10^5 spores/ml suspension

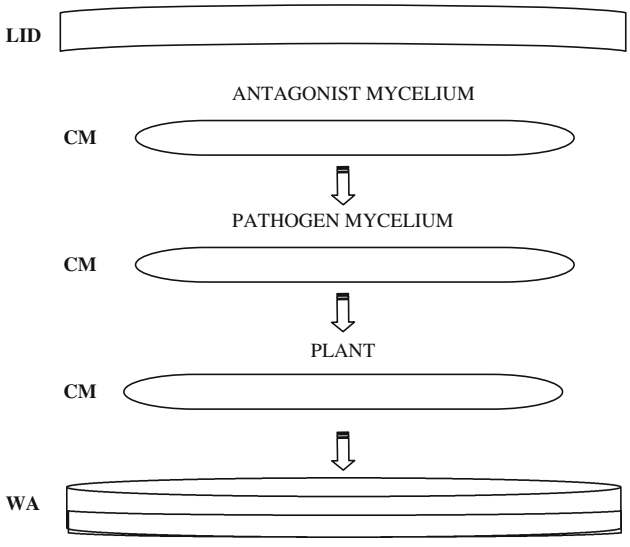


Fig. 1 Scheme of the plate system used to arrange three- and two-way interactions between plant, pathogens and the antagonist *Trichoderma atroviride* strain P1. Plant material (bean leaves or roots) was placed on the water agar (*WA*) plate of a large Petri dish covered with a sterile cellophane membrane (*CM*). Fungal mycelia of the antagonistic or pathogenic microbes, previously grown on PDA plates on a sterile CM, were layered on the top of the plant tissues

of *B. cinerea*, and 1 cm^2 of a *R. solani* colony were used to separately inoculate PDA plates covered with a sterile cellophane membrane (CM).

A spore suspension of *T. atroviride* strain P1 (ATCC 74058) obtained from a colony grown on PDA was used to inoculate CM-covered PDA plates (100 µl of a 5×10^5 spores/ml suspension). After 3–4 days of incubation at 25°C, the CM with the pathogen/antagonist mycelia was transferred on top of the WA plate containing the plant tissue (bean leaves or roots), as shown in Fig. 1. The CM used to separate the pathogens and the antagonist between themselves and from the plant allowed separation and transfer of fungal mycelia, but still permitted micro- and macro-molecules diffusion (Kullnig et al. 2000).

The plates hosting the three-player (plant–pathogen–antagonist) and the two-player (plant–pathogen, plant-antagonist) interactions and the relative controls (plant/fungi alone) were maintained at room temperature for 3 days and then the plant/fungi samples were separately collected for protein extraction.

Protein extraction

The protein extraction protocol described by Jacobs et al. (2001) was applied with some modifications. Approximately 1 g of fungal mycelium (wet weight) from the pathogen, the antagonist, or of a plant tissue (leaves or roots) was suspended in 10 ml of a cold (−20°C) acetone solution [20% trichloroacetic acid (TCA) and 0.2% dithiothreitol (DTT)] and ground with an ultraturrax (T25 basic, IKA Labortechnik, Germany) by keeping the tube in an ice bath. Samples were maintained at −20°C for at least 3 h to allow protein precipitation, then centrifuged (20 min, 30,000*g* at 4°C). The pellet was washed three times with cold (−20°C) acetone solution containing 0.2% DTT, then resuspended in a rehydration buffer [9 M urea, 2% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), 1% DTT, 10 mM phenylmethylsulfonyl fluoride (PMSF)], vortexed and kept on an orbital shaker for 2 h to obtain complete protein solubilization. The samples were centrifuged (60 min, 30,000*g* at 20°C) and the supernatants were recovered. Protein concentration was determined by a Bradford Dc protein assay (Bio-Rad, Richmond, CA, USA) and samples were stored at −40°C until use.

Two-dimensional electrophoresis (2-DE)

Isoelectric focusing (IEF) was conducted by using 7 cm immobilized-pH-gradient (IPG) strips (Bio-Rad) with a pH range from 3 to 10, rehydrated in a solution of 9 M urea, 2% CHAPS, 1% DTT, 2% carrier ampholyte and 10 mM PMSF proteinase inhibitor (SIGMA). Two hundred microliters of the total protein solution (equivalent to 200 µg) were loaded in the focusing tray and absorbed into the gel strip (1 h passively at room temperature and 12 h actively with a 50 V current applied). IEF was carried out with a PROTEAN IEF Cell system (Bio-Rad). IPG strips were focused up to a total of 14 kVh by using a three-step program (250 V for 1 h, 4 kV for 3 h and until 10 kVh were reached). The strips were equilibrated by placing them in a solution of 6 M urea, 0.05 M Tris/HCl pH 8.8, 20% glycerol, 2% SDS, 2% DTT for 10 min, and then in 6 M urea, 0.05 M Tris/HCl pH 8.8, 20% glycerol, 2% SDS, 2.5% iodocetamide for 10 min more. IPG strips were finally loaded on a 15% polyacrylamide gel in a Mini-Protean 3 Cell (Bio-Rad), and run with a constant current of 150 V for 75 min in 1X tris-glycine-SDS (TGS) buffer (Bio-Rad). The same rehydration protocol was used for the 17 cm IPG strips. The IEF program was 300 V for 2 h, 10 kV for 4 h and until 40 kVh were reached. After equilibration, strips were loaded onto 8–16% polyacrylamide gradient gels for SDS-PAGE in a Protean plus Dodeca Cell (Bio-Rad) which was run at 10°C, with a constant current of 200 V for about 8 h. Gels were stained for at least 3 h with SimplyBlue SafeStain G-250 (Invitrogen, California, USA) according to the manufacturer instructions. Each protein extract was run on triplicate or duplicate gels for the 7 cm and 17 cm IPG strips, respectively.

Gel images were acquired by a GS-800 Imaging Densitometer (Bio-Rad) and analyzed with the PD-Quest software. Image files were recorded by using a red filter (wavelength 595–750 nm) and a resolution of 36.3×36.3 µm. The signal intensity of each spot was determined in pixel units (Optical Density, OD) and normalized to the sum of the intensities of all the spots included in the standard gel. Protein spots were considered to be differentially produced if at least a twofold intensity variation was detected when responses to different interaction conditions were compared.

In-gel digestion, mass spectrometry and in silico analysis

Protein spots were excised from gels and digested with trypsin (SIGMA), as described by Ha et al. (2002). Tryptic peptides were resuspended in 10 µl of a 1% acetic acid solution. The samples were mixed 1:1 with a matrix of a saturated α-cyano-4-hydroxycinnaminic acid solution [10 mg/ml acetonitrile (ACN)/0.2% trifluoroacetic acid (TFA), 70/30] (SIGMA), and 1 µl aliquots were applied to the MALDI (matrix-assisted laser

desorption/ionization) sample plate and dried. Peptide mass spectra were obtained on a Voyager-DE Pro MALDI-TOF (time of flight) mass spectrometer (Applied Biosystem, Foster City, CA, U.S.A.) equipped with a 337 nm laser and delay extraction, operated in positive-ion reflector mode for the mass range between 890 and 3,500 Da. Mass calibration was performed with the ions from human adrenocorticotropic hormone—ACTH (fragments 18–39) (SIGMA) at 2,465.1989 Da, and Angiotensin III human (MP Biomedicals, Irvine, CA, USA) at 931.5154 Da as internal standards.

Peptide mass fingerprint (PMF) data were matched to the National Centre Biotechnology Information non-redundant (NCBInr) database entries against proteins from fungal, plant or all species, using the Mascot software (Matrix Science, London, UK). The following search parameters were applied. One incomplete cleavage was allowed and alkylation of cysteine by carbamidomethylation was set as possible modification. The Mascot program compares theoretical and experimental peptide values derived by virtual hydrolysis of proteins present in the database with a specific proteolytic agent, then supplies a list of hypothetical candidates with the probability that the peptides found belong to that entries. A modified Mascot analysis was also performed by using a *Trichoderma* Expressed Sequence Tags (EST) database, built as described by Suárez et al. (2005) and supported by TRICHOEST European Union project (http://www.trichoderma.org; Rey et al. 2004). The database contains more than 14,000 cDNA clones obtained from libraries of mRNAs isolated from the mycelia of different *Trichoderma* species, including *T. atroviride* strain P1, grown also in the presence of plant and/or pathogens. Similarities between the peptide fragmentation of some *Trichoderma* spp. clones and known proteins were determined.

Moreover, the Motif program (http://www.motif.genome.jp) was applied to Pfam and Pfam_fs databases to determine if the known proteins share a significant degree of homology with the analyzed protein spots or contained conserved domains in the areas that matched the peptide deduced sequences.

Some differential spots, such as those produced by *B. cinerea* in the interaction with bean leaves and *Trichoderma* or by bean leaves in the interaction with *Trichoderma* and *B. cinerea*, were subjected to MALDI-TOF/TOF analysis by using a Proteomics Analyzer 4700 (Applied Biosystems) that was calibrated immediately prior to each experiment. The samples were desalted and concentrated using ZipTips C18 (Millipore, Bedford, MA, USA), washed with 0.1% TFA solution (10 µl × 2), and eluted with 0.1% TFA in a 1:1 water : ACN solution (10 µl). They were applied onto the MALDI sample plate and treated with a matrix solution of saturated α-cyano-4-hydroxycinamic acid in a 1:1 water : ACN solution + 0.1% TFA. The analyte and matrix were air-dried at room temperature. The acquired MS/MS data were then submitted to the NCBInr database for protein identification by using the GPS Explorer Software with the integrated Mascot search engine.

Results

Multi-player interactions and proteome separations

The use of large water agar Petri dishes to support the interaction and cellophane membranes to separate the three players, permitted extensive contact and selective recovery of the different fungal and plant tissues (Fig. 1). Instead, co-cultures in liquid medium by separating the three partners with dialysis membranes did not allow a good interaction between the plant and the two microbes (data not shown).

Experimental procedures have been optimized by changing the length of the interaction time, the quantity of the microbial inoculum, the age of the plantlets transferred in the Petri plates, etc. in order to achieve the best separation and recovery of proteins from the live material. Generally, good yield and quality of proteins from both leaves and mycelia were obtained. For instance, 5–10 mg of total proteins were obtained from 1 g of fresh material (both from plant and fungal mycelia). On the other hand, the proteins from root samples were not well resolved on 2-D gels, even when a specific protocol for root protein extraction was applied (Saravanan and Rose 2004). Although the experiments were performed with different plants (i.e., tobacco, potato and tomato), only the results obtained on bean are included in the present report.

First, proteins were separated on 7 cm long IPG strips across a gradient of pH from 3 to 10, and results were successively confirmed by using a larger gel format (17 cm strip size). The gel analysis performed with the PD-Quest software (Bio-Rad) was found to be useful to generate the 2-D maps from the various conditions of interaction, and perform quantitative and qualitative analysis of the differential protein spots. In fact, the gels obtained from the interaction plates allowed the separation of hundreds of proteins and provided a representative picture of each proteome. For some spots, the homology found was confirmed by the presence in the known proteins of conserved domains matching the deduced sequences of the peptides obtained from 2-D gels. An example is provided

by the matching of spot 3703 with a *Brassica oleracea* SGT1-like protein (gi|40974917) containing an SGS conserved domain (Fig. 2).

Preliminary experiments with protein extracts from liquid cultures of *T. harzianum* grown in the presence of various fungal pathogen sources (i.e., cell walls or heat killed mycelia of *Pythium ultimum, R. solani* or *B. cinerea*) or plant tissues, produced more differential spots (present, absent, increased, decreased) than cultures supplemented with different simple sugars as the only carbon source (data not shown). As expected, the presence of the fungal host or plant extensively modified the proteome of the antagonist and activated a variety of interaction-related genes in comparison to a non-induced phase. These results were confirmed by using the WA plate-based method, when *Trichoderma* mycelia were left to interact either with the plant, the pathogen or both.

Differential proteins from the interaction between *T. atroviride*, bean leaves and *B. cinerea*

The proteome of *T. atroviride* grown alone was used as a control for comparisons with the two-way (*Trichoderma*–bean leaves) and the three-way interactions (*Trichoderma*–bean leaves–*Botrytis*). More than 220 differential spots were noted as ex novo, absent, increased or decreased if *Trichoderma* was exposed to the plant or to the plant and the pathogen together (Table 1). When the *Trichoderma*–plant–pathogen profile was compared to the *Trichoderma*–plant interaction, 57 spots appeared to be produced ex novo, 93 were absent, 25 were up-regulated and 62 down-regulated, indicating that the presence of *B. cinerea* induces major changes in the proteome of the antagonist interacting with the plant (Table 1). About 50 of the most strongly modified *T. atroviride* protein spots (examples shown in Fig. 3) were further characterized by MALDI-TOF MS followed by in silico analysis, but only a few identification cases are reported here (Table 2). These include a kinase containing a DnaJ conserved domain, a cyclophilin A-like protein and a chitin synthase.

In the bean proteome, the presence of *Trichoderma* or *Botrytis* determined a similar accumulation of differential proteins (about 140), as observed by comparing the three- versus two-way interactions

A)

Spot 3703 Matched to: **gi|40974917** Score: **40** Expect: **22**
SGT1-like protein [Brassica oleracea]

Nominal mass (M_r): **39017**; Calculated pI value: **4.95**
Variable modifications: Carbamidomethyl (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **15**
Number of mass values matched: **6**
Sequence Coverage: **20%**
Matched peptides shown in **Bold**

1 MASELAEKAK EAFLDDDFDV AADLYSKAID LDPSCASFFA DRAQANIKLL
51 NFTEAVADAN KAIELEPTLA KAYLRKGAAC MKLEEYATAK AALE**KGASVA**
101 **PNESKFEK**MI DECNLLIAEE EKDLVQQVPP TLPSSSTTPL AIAADAPPAA
151 PAKPMF**RHEF YQKPEEVGVA IFAKGIPK**QN VNVEFGDQIL SVVIDVAGEE
201 AYHFQP**RLFG KIIPEKCRYE VLSTK**VEIRL AKAEIVTWAS LEYGKGQALL
251 PKPNVASAVS QRPVYPSSKP G**KDWDKLEAE VKK**QEKDEKL DGDAAMNKFF
301 SDIYQSADED MRRAMNKSFA ESNGTVLSTN WKEVGTK**KVE STPPDGMELK**
351 KWEY

B)

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Deduced sequence
96 - 108	1364.00	1362.99	1362.68	0.31	1	**KGASVAPNESKFEK**
158 – 178	2387.00	2385.99	2386.27	-0.27	1	**RHEFYQKPEEVGVAIFAKGIPK**
208 – 216	1045.00	1043.99	1043.63	0.35	1	**RLFGKIIPEK**
217 – 225	1098.00	1096.99	1097.55	-0.56	1	**KCRYEVLSTK**
273 – 282	1232.00	1230.99	1231.61	-0.61	1	**KDWDKLEAEVKK**
339 – 350	1302.00	1300.99	1301.62	-0.62	0	**KVESTPPDGMELK**

Fig. 2 Peptide mass fingerprint of spot 3703 from bean leaves, in the presence of *T. atroviride* and *B. cinerea*, and identification of conserved domains. **a** Sequence of the *Brassica oleracea* SGT1-like protein (gi|40974917) found to be homologous to spot 3703. Matched peptides are shown in *bold*. The sequence of the SGS conserved domain is *underlined*. **b** Sequences of the digested peptides obtained. *Start–End* = amino acid position indicating the portion of the known protein matching the peptide deduced sequence, *Observed* = experimental m/z value, *Mr* (*expt*) = experimental m/z value transformed to a relative molecular mass, *Mr* (*calc*) = calculated relative molecular mass of the matched peptide, *Delta* = difference (error) between the experimental and calculated masses, *Miss* = number of missed enzyme cleavage sites, *Deduced sequence* = sequence of the peptide in one-letter code

Table 1 Changes occurring in the proteome of plant (Pln), pathogens (Bot = *Botrytis cinerea*; Rhi = *Rhizoctonia solani*) and *Trichoderma atroviride* strain P1 (Trc) when different interaction conditions (three-way, two-way and no interaction) were compared

Condition	TOT[a]	ON[b]	OFF[b]	INCR[c]	DECR[c]
T. atroviride proteome interacting with bean leaves and *B. cinerea*					
(Trc + Pln) versus (Trc)	248	82	64	77	25
(Trc + Pln + Bot) versus (Trc)	222	88	54	45	35
(Trc + Pln + Bot) versus (Trc + Pln)	237	57	93	25	62
Bean leaves proteome interacting with *T. atroviride* and *B. cinerea*					
(Pln + Trc) versus (Pln)	191	39	87	27	38
(Pln + Bot) versus (Pln)	267	87	70	39	71
(Pln + Trc + Bot) versus (Pln)	194	34	78	45	37
(Pln + Trc + Bot) versus (Pln + Trc)	141	57	53	22	9
(Pln + Trc + Bot) versus (Pln + Bot)	144	51	49	31	13
(Pln + Trc) versus (Pln + Bot)	125	43	34	29	19
B. cinerea proteome interacting with bean leaves and *T. atroviride*					
(Bot + Pln) versus (Bot)	157	23	44	44	46
(Bot + Pln + Trc) versus (Bot)	226	39	38	106	43
(Bot + Pln + Trc) versus (Bot + Pln)	204	64	15	82	43
T. atroviride proteome interacting with bean roots and *R. solani*					
(Trc + Pln) versus (Trc)	215	91	42	65	17
(Trc + Pln + Rhi) versus (Trc)	192	84	66	30	12
(Trc + Pln + Rhi) versus (Trc + Pln)	235	63	116	27	29
Bean leaves proteome when roots interact with *T. atroviride* and *R. solani*					
(Pln + Trc) versus (Pln)	149	37	51	35	26
(Pln + Rhi) versus (Pln)	300	151	23	107	19
(Pln + Trc + Rhi) versus (Pln)	155	45	47	37	26
(Pln + Trc + Rhi) versus (Pln + Trc)	127	40	29	28	30
(Pln + Trc + Rhi) versus (Pln + Rhi)	312	28	168	23	93
(Pln + Trc) versus (Pln + Rhi)	306	19	176	20	91
R. solani proteome interacting with bean roots and *T. atroviride*					
(Rhi + Pln) versus (Rhi)	193	37	69	39	48
(Rhi + Pln + Trc) versus (Rhi)	234	89	32	43	70
(Rhi + Pln + Trc) versus (Rhi + Pln)	200	81	12	51	56

[a] Total number of differential spots

[b] Number of spots present in the first condition compared to the second ("ON") (i.e., present in Trc + Pln and not present in Trc) or viceversa ("OFF")

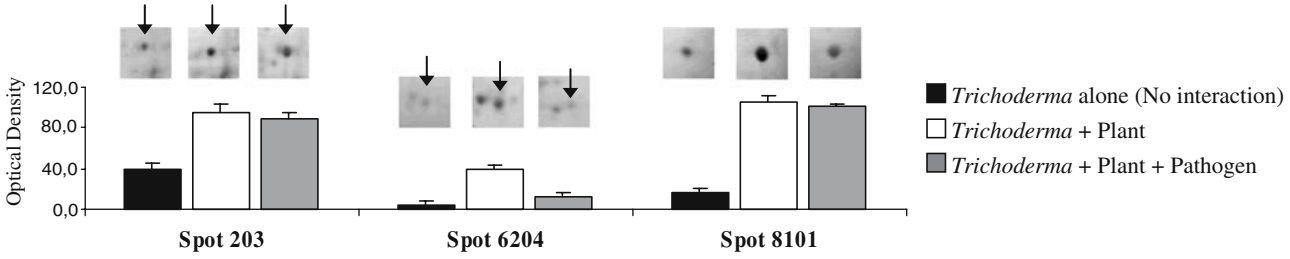
[c] Number of spots whose intensity in the first condition increased ("INCR") or decreased ("DECR") at least twofold compared to the second one

(Table 1). However, the comparison between the two two-way interactions (plant–*Trichoderma* vs. plant–*Botrytis*) indicated a different proteomic response in the plant to the antagonist and the pathogen. Bean accumulated 43 novel, 34 absent, 29 increased and 19 decreased spots in the presence of the antagonist instead of the pathogen. Four of the about 30 selected spots for PMF analysis are presented here (Fig. 3). All corresponded to proteins involved in defence response against pathogens, and in particular the MS data matched with specific domains related to disease resistance (i.e., leucine rich repeats, SGS and Barwin domains, thaumatin family sequences) (Table 3).

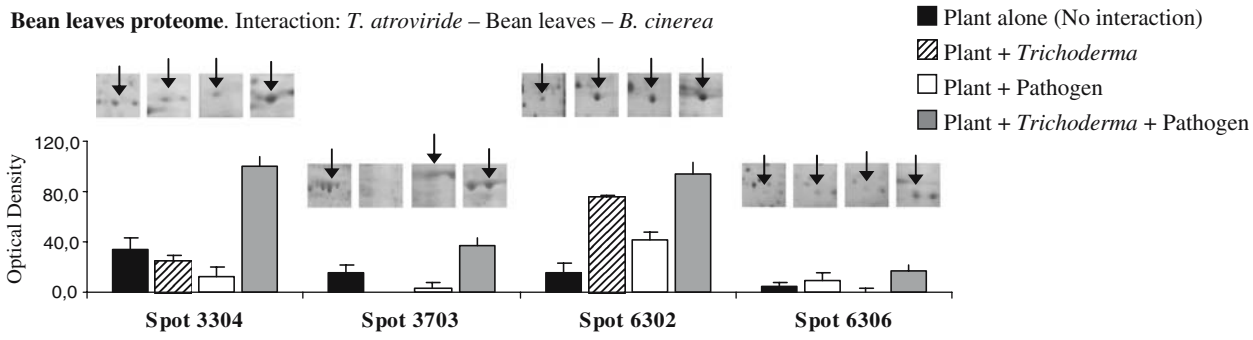
Differential proteins were also produced by the pathogen *B. cinerea* in the presence of the plant alone or in combination with *Trichoderma* (i.e., 157 and 204 differential spots, respectively, in the interaction with plant vs. *Botrytis* alone and in the three-player vs. the *Botrytis*–plant interaction) (Table 1). These results also indicated that the presence of *Trichoderma* induces major changes in the *Botrytis* proteome while the fungus is interacting with bean plant. Of the spots further analyzed by 4700 MALDI-TOF/TOF, four gave the homologies reported in Table 4. The proteins involved in the infection process of *Botrytis*–plant and *Botrytis*–plant–*Trichoderma* inter-

Fig. 3 Differential intensity levels of two-dimensional (2-D) gel spots produced by the three-way, two-way and no-interaction conditions of bean plants, *B. cinerea*, *R. solani* and *T. atroviride*. Spot intensity changes are shown by the enlarged gel regions (*picture insets*) placed over the corresponding relative intensity (*histogram*). 2-D gels of intracellular proteins were separated in the first dimension on IPG strip (7 cm, pH 3–10) and in the second dimension on 15% polyacrylamide SDS gel. Proteins were visualized by SimplyBlue SafeStain G-250 (Invitrogen). Spot intensity was quantified by using the PD-QUEST software (Bio-Rad). The relative intensity of a spot is the sum of the signal intensities (expressed as optical density units) of all the pixels that make up the object

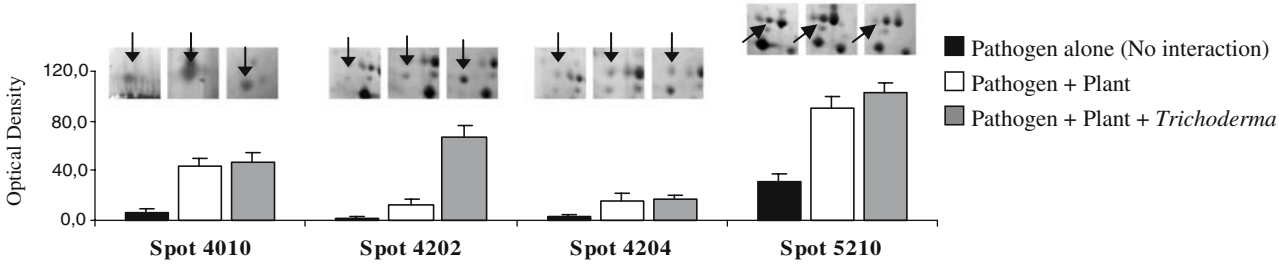
***Trichoderma* proteome**. Interaction: *T. atroviride* – Bean leaves – *B. cinerea*



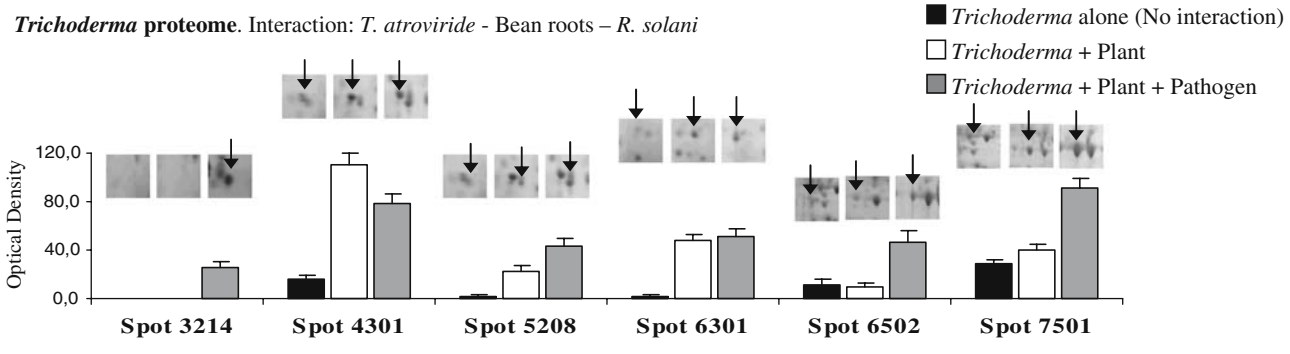
Bean leaves proteome. Interaction: *T. atroviride* – Bean leaves – *B. cinerea*



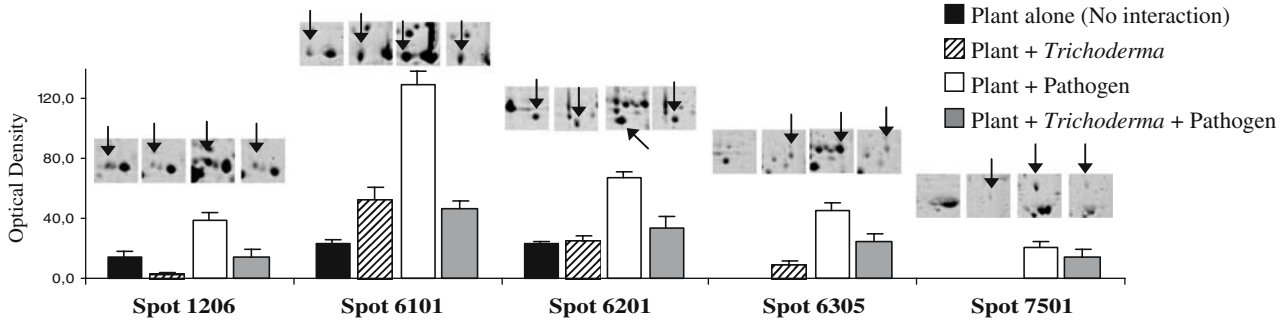
***Botrytis* proteome**. Interaction: *T. atroviride* – Bean leaves – *B. cinerea*



***Trichoderma* proteome**. Interaction: *T. atroviride* - Bean roots – *R. solani*



Bean leaves proteome. Interaction: *T. atroviride* - Bean roots – *R. solani*



actions were significantly higher than in the control, as indicated by the increased spot intensities (Fig. 3).

Differential proteins from the interaction between *T. atroviride*, bean plants and *R. solani*

When the *Trichoderma*–bean roots–*Rhizoctonia* was compared to the *Trichoderma*–plant interaction, more than 230 differential spots were accumulated in the antagonist proteome. Sixty-three spots appeared ex novo, 116 were absent, 27 up-regulated and 29 down-regulated (Table 1), indicating that the presence of *R. solani* induces major changes in the proteome of *Trichoderma* interacting with the plant. In addition, about 200 differential spots present ex novo, absent, increased or decreased were produced by *Trichoderma* during the interaction with the plant alone or in combination with *Rhizoctonia*, when compared to the control (*Trichoderma* grown alone). Figure 3 shows the selected spots that were further subjected to in silico analysis and the main results are presented in Table 5. Spot 4301 revealed a strong similarity to cyclophilin A, a protein containing a peptidyl-prolyl-*cis–trans* isomerase (PPIase) domain involved in protein folding and possibly in other cellular functions (Arévalo-Rodriguez et al. 2000; Gothel and Marahiel 1999). Spot 6301 was confidently identified as a fungal hydrophobin, while different members of the ABC transporter family were found to be differentially accumulated in the antagonist proteome (spots 6502 and 7501). Finally, spot 5208 was found to be a homologue of *T. reesei* Hex1 protein by searching the *Trichoderma* EST database.

Proteins were extracted from bean leaves and analyzed in order to characterize the systemic defence response in plants whose roots were in contact with pathogen and/or antagonist mycelia. The protein profiles from the bean roots-*Trichoderma–Rhizoctonia* (three-way) as compared to the bean roots-*Trichoderma* (two-way) interaction indicated 127 differential spots caused by the presence of the pathogen, of which 40 were novel, 29 absent, 28 of increased and 30 of

Table 2 Homologies of differential proteins in the *T. atroviride* strain P1 proteome during interaction with bean leaves and *B. cinerea*. MW (kDa)/pI$^{exp/th}$: molecular weight and isoelectric point of differential proteins determined experimentally (from gels) and theoretically (from in silico analysis)

Spot number	MW (kDa)/pI^{exp}	Protein name (source)	Accession number	Conserved domains	No. of matched peptides	MW (kDa)/pIth	Sequence coverage (%)	Score
203[a]	20.0; 4.7	Protein kinase HSP40 homologue (*Bos taurus)*	gi|27807457	DnaJ domain	6/6	57.5; 5.60	10	48
6204[a,b]	22.0; 6.7	Cyclophilin A (*Beauveria bassiana*)	gi|23664288	Peptidyl-prolyl cis-trans isomerase	5/10	17.5; 8.65	28	47
8101[a]	16.0; 7.0	Chitin synthase *(Malbranchea gypsea)*	gi|452090	Chitin synthase	4/8	21.3; 9.32	25	31

[a] Search in NCBInr database

[b] Search in *Trichoderma* EST database

Table 3 Homologies of differential proteins in the bean leaves proteome during interaction with *T. atroviride* strain P1 and *B. cinerea*. MW (kDa)/pI$^{exp/th}$: molecular weight and isoelectric point of differential proteins determined experimentally (from gels) and theoretically (from in silico analysis)

Spot number	MW (kDa)/ pI^{exp}	Protein name (source)	Accession number	Conserved domains	No. of matched peptides	MW (kDa)/ pIth	Sequence coverage (%)	Score
3304	19.4; 5.1	Resistance candidate RPP8-like protein (*Arabidopsis arenosa*)	gi|37783161	Leucine rich repeats (LRR)	5/6	59.6; 6.57	8	32
3703	54.6; 5.2	SGT1-like protein (*Brassica oleracea*)	gi|40974917	SGS domain	6/15	39.0; 4.95	20	40
6302[a]	25.4; 6.9	Thaumatin-like protein PR-5b (*Cicer arietinum*)	gi|3549691	Thaumatin family	-	25.9; 5.26	C.I.%: 100	130
6306	19.6; 6.8	Pathogenesis-related protein 4B (*Nicotiana tabacum*)	gi|19966	Barwin domain	2/2	15.2; 6.06	16	32

[a] Identification method: 4700 MALDI-TOF/TOF Mass Spectrometer

Table 4 Homologies of differential proteins in the *B. cinerea* proteome during interaction with bean leaves and *T. atroviride* strain P1. Data obtained by using the 4700 MALDI-TOF/TOF apparatus. MW (kDa)/pI$^{exp/th}$: molecular weight and isoelectric point of differential proteins determined experimentally (from gels) and theoretically (from in silico analysis)

Spot number	MW (kDa)/pI^{exp}	Protein name (source)	Accession number	Protein score	MW (kDa)/pIth	Protein C.I. (%)
4010	12.0; 6.5	Cutinase (*Botryotinia fuckeliana*)	gi|1177244	182	16.1; 9.20	100
4202	20.1; 6.3	Superoxide dismutase (*Botryotinia fuckeliana*)	gi|40642968	121	15.9; 5.85	100
4204	21.1; 6.3	Cytosolic cyclophilin 1 (*Botryotinia fuckeliana*)	gi|33357682	259	19.6; 6.30	100
5210	21.0; 6.9	Putative mitochondrial cyclophilin 1 (*Botryotinia fuckeliana*)	gi|33357681	490	24.2; 9.14	100

Table 5 Homologies of differential proteins in the *T. atroviride* strain P1 proteome during interaction with bean roots and *R. solani*. MW (kDa)/pI$^{exp/th}$: molecular weight and isoelectric point of differential proteins determined experimentally (from gels) and theoretically (from in silico analysis)

Spot number	MW (kDa)/pI^{exp}	Protein name (source)	Accession number	Conserved domains	No. of matched peptides	MW (kDa)/pIth	Sequence coverage (%)	Score
3214[a]	14.9; 4.9	Brefeldin A Resistance protein (*Schizosaccharomyces pombe*)	gi|6473569	RanBP1 domain	6/16	22.3; 8.95	35	32
4301[a]	19.8; 5.9	Cyclophilin A (*Bos taurus*)	gi|7767529	Peptidyl-prolyl cis-trans isomerase	3/4	10.3; 6.26	38	46
4307[a]	24.8; 5.9	Mitochondrial ribosomal protein (*Saccharomyces cerevisiae*)	gi|6323344	RNase 3 domain	5/13	25.8; 9.21	24	40
5208[b]	19.2; 6.1	Hex 1- Woronin body major protein (*Trichoderma reesei*)	gi|34014958	Eukaryotic initiation factor 5A	4/4	18.4; 9.13	29	63
5209[a]	15.9; 6.5	Hypothetical protein (*Magnaporthe grisea*)	gi|39964645	Acetyltransferase (GNAT) family	3/6	20.6; 5.46	21	33
5304[a]	20.1; 6.44	Conserved protein (*Pseudoalteromonas haloplanktis*)	gi|76876374	Unknown function	4/6	21.1; 4.78	30	51
5632[a]	36.2; 6.6	Lipoyl ligase (*Saccharomyces cerevisiae*)	gi|632368	Biotin/lipoate A/B protein ligase family	5/8	37.2; 9.28	14	40
6301[a]	20.9; 6.9	Hydrophobin 3 (*Pleurotus ostreatus*)	gi|17426760	Fungal hydrophobin	3/8	11.2; 7.48	34	30
6502[a]	35.2; 6.9	ABC transport protein (*Rhizobium leguminosarum* bv. *viciae*)	gi|17826825	ABC transporter	4/5	31.4; 7.14	14	42
7203[a]	15.6; 7.9	Unnamed protein (*Debaryomyces hansenii*)	gi|49656833	S25 ribosomal protein	5/13	11.6; 10.24	46	45
7239[a]	16.5; 8.0	Stress seventy family HSP70 (*S. pombe*)	gi|6473175	Hsp70 protein	5/22	19.6; 8.03	36	31
7501[a]	34.3; 7.3	ABC-type oligopeptide transport system ATPase component (*Ralstonia metallidurans*)	gi|22977505	ABC transporter	4/4	38.8; 8.70	8	42
8602[a]	44.4; 8.3	Unknown protein (*Xenopus laevis*)	gi|47939698	Mitochondrial carrier protein	6/7	33.1; 9.73	18	60

[a] Search in NCBInr database

[b] Search in *Trichoderma* EST database

Table 6 Homologies of differential proteins in the proteome of bean leaves whose roots were in contact with *T. atroviride* strain P1 and *R. solani*. MW (kDa)/pI$^{exp/th}$: molecular weight and isoelectric point of differential proteins determined experimentally (from gels) and theoretically (from in silico analysis)

Spot number	MW (kDa)/pI^{exp}	Protein name (source)	Accession number	Conserved domains	No. of matched peptides	MW (kDa)/pIth	Sequence coverage (%)	Score
1206	20.2; 4.4	Disease resistance protein (*Capsicum annuum*)	gi|6648973	NB-ARC domain	6/11	23.4; 8.20	25	48
6101	15.7; 6.8	NBS-LRR type resistance protein (*Oryza sativa*)	gi|7489504	NB-ARC domain	7/18	18.7; 5.21	29	35
6201	26.8; 6.5	Pathogenesis-related protein 10c (*Sorghum bicolour*)	gi|58978001	PR-protein Bet v I family	5/14	16.8; 5.56	30	39
6305	29.4; 7.0	Ribonuclease-like PR-10a (*Malus x domestica*)	gi|15418744	PR-protein Bet v I family	4/11	17.5; 5.62	28	41
7501	40.0; 7.5	Resistance protein RPP8-like protein (*Arabidopsis thaliana*)	gi|32453361	NBS-LRR resistance protein domain	5/6	58.6; 6.28	12	40

decreased intensity (Table 1). By comparison with the bean roots–*Rhizoctonia* treatment, the three-way interaction revealed more than 300 differential spots: 28 novel, 168 absent, 23 of increased and 93 of decreased intensity. Interestingly, the presence of the antagonist resulted in a strong reduction in the number and the level of plant proteins produced as compared to the interaction of the plant with the pathogen, that produced the greatest number of novel and increased differential spots in comparison to the plant alone. In the case of plant–*Rhizoctonia* interaction compared to the control, 300 differential spots were obtained, of which 151 novel, 23 absent, 107 of increased and 19 of decreased intensity. Of the approximately 30 differential spots selected for PMF analysis, 5 are presented in Fig. 3. Spots 1206, 6101, 6201 and 6305 showed a particularly high increase in intensity when the plant was exposed to the pathogen and less in the three-way interaction with the fungal antagonist. These proteins were homologues to disease resistance or pathogenesis-related (PR) proteins (Table 6), and found to contain conserved motifs (NB-ARC, Bet v I family, NBS-LRR) known to be involved in pathogen- or stress-related responses. Spot 7501, which was similar to an *Arabidopsis thaliana* resistance protein, was absent in the control (plant grown alone) and was induced by the presence of *R. solani* (either alone or combined with *Trichoderma*).

The differential proteome of the soilborne pathogen *R. solani* was also studied. In comparison to the control, a total of about 200 spots, present ex novo, absent, increased or decreased, were obtained by exposing *Rhizoctonia* to plant roots, or to plant roots and the antagonist together (Table 1). In the interaction with the bean roots as compared to the pathogen alone, 37 novel spots, 69 absent, 39 of increased and 48 of decreased intensity were found. The differential spots of the three-player versus the *Rhizoctonia*–plant interaction were 81 novel, 12 absent, 51 of increased and 56 of decreased intensity. These data indicated that the presence of *Trichoderma* induced important variations in the pathogen proteome while the fungus is interacting with bean plant. Unfortunately, the MS results for the about 20 spots excised from gels and digested showed no significant homologies with proteins present in the NCBInr database.

Discussion

In this work we used bean plants, fungal pathogens and the antagonistic fungus *T. atroviride* strain P1 to analyze the changes in the proteome of the three organisms caused by multiple-player interactions. The use of cellophane membranes permitted both the exchange of compounds and thus a chemical interaction in situ, as well as the separated extraction and recover of the individual proteomes. Placing of such type of membranes between fungal spores and seed surface did not affect the improvement of seed germination or crop yield caused by treatment with *Trichoderma* (Benítez et al. 1998). Kullnig et al. (2000) reported that the cellophane membrane allowed the diffusion of proteins up to 90 kDa, as noted in confrontation assays between *T. atroviride* strain P1 and *R. solani*, whereas the dialysis membrane (12 kDa cut-off size) prevented macromolecule diffusion.

Comparison of results from different extraction methods demonstrated that the TCA/acetone-based precipitation was the best protocol in terms of quality and quantity of the proteins obtained, minimizing protein degradation and the presence of interfering

compounds (i.e., polysaccharides, salts, polyphenols, etc.) (Görg et al. 2004).

During the three-way interaction, major changes in the proteome of *T. atroviride*, as compared to single and double player (antagonist–plant) conditions, were observed. In particular, the presence of fungal pathogens (*B. cinerea* and *R. solani*) strongly modified the protein pattern of the antagonist during plant interaction. Many spots absent in the three-way (i.e., 93 spots with *B. cinerea* and 116 with *R. solani*, respectively) were present in the *Trichoderma*–plant condition (Table 1). Possibly, the activity of compounds released by either of the two pathogens may somehow interfere with the expression of *Trichoderma* genes used to interact with the plant. Alternatively, the increased extracellular protein production (i.e., cell wall degrading enzymes, antibiotics, etc.) that follows the activation of the antagonistic/mycoparasitic mechanisms may be associated with a reduced number of intracellular protein species.

In the bean proteome, the interaction with each pathogen induced more protein spots than the interaction with the antagonist alone or the combination of both fungi (Table 1). As expected, *Trichoderma* induced different sets of plant proteins than the pathogens, and the presence of the beneficial fungus clearly changed the expression pattern of plant genes responding to pathogen attack, which may be related to increased pathogen control. In fact, some of the spots analyzed by PMF corresponded to PR-proteins and were less up-regulated than by the pathogen alone when both *Trichoderma* and the pathogen were interacting with the plant (Fig. 3; Table 6). Both *B. cinerea* and *R. solani* produced the greatest number of novel and increased differential spots in comparison with the plant alone, thus confirming that the presence of the antagonist extensively modifies the proteome of the plant affected by a pathogen. These results underline the importance of conducting comparative analysis of the multiple interactions involved in biocontrol and pathogenesis processes.

The changes in the proteomes of each player during the complex three-way cross-talk were investigated and the most interesting differential spots were analyzed via PMF. These were selected by comparing proteomes obtained from three-way, two-way and no interaction conditions and selecting those spots showing the most evident and reproducible changes, either qualitatively or quantitatively. In addition to simple sequence matching, we also determined if the peptide fragments obtained by MS matched the sequence of conserved domains of known proteins. Both *Trichoderma* EST and all-species-entries databases were used, also to perform cross species identification (CSI) (Grinyer et al. 2004a, b, 2005; Wilkins and Williams 1997).

Several interesting proteins, among those differentially expressed, were found in the *T. atroviride* proteome during the three-way interaction with bean leaves and the foliar pathogen *B. cinerea* (Table 2) or with bean roots and the soilborne pathogen *R. solani* (Table 5). We confidently identified a homolog of a 40 kDa heat shock protein (HSP) (spot 203; Table 2) whose intensity increased in the presence of both the plant and the pathogen, including a conserved DnaJ domain typically related to environmental stress (Lindquist 1986; Morimoto et al. 1994) and possibly associated to a *Trichoderma* defence response. Spot 8101, whose intensity strongly increased in the presence of the plant (with or without the pathogen) (Fig. 3), was found to be a homologue of an enzyme (chitin synthase) involved in the synthesis of fungal cell wall components after chemical, physical or osmotic stress. This could also represent a stress-related protein, since increased expression of glucan and chitin synthase genes may be required to repair cell wall damages caused by the pathogen or plant enzymes (Valdivia et al. 2003). Similarly, spot 5208 (Table 5), which corresponded to a *T. reesei* Hex1 protein of comparable MW and pI (Lim et al. 2001), doubled in intensity when the pathogen was present (three-way vs. two-way interaction) (Fig. 3). Hex1 is one of the most abundant proteins in fungal cell walls, mainly contained in the Woronin body, and associated to repairing of damaged hyphae (Jedd and Chua 2000).

We found proteins similar to different members of the cyclophilin family in the proteome of the antagonist during a three-player condition, with either *B. cinerea* or *R. solani* (Tables 2, 5). Cyclophilins have an enzymatic PPIase activity that has been demonstrated to have a role in protein folding (Marks 1996) and possibly in intracellular tracking, signal transduction, cell cycle regulation, differentiation and maintenance of multi-protein complex stability (Arévalo-Rodriguez et al. 2000; Gothel and Marahiel 1999). A protein with a predicted PPIase activity was previously identified by LC MS/MS in the proteome of *T. harzianum* grown in liquid culture (Grinyer et al. 2004b). These proteins could be used by *Trichoderma* in a wide range of processes, that may include the interaction with the plant since the relative spot intensity increased remarkably in the presence of bean leaves or roots (Fig. 3).

Both spots 6502 and 7501 were identified as membrane pumps of the ABC transporter family (Table 5), which could be related to the well-known resistance of *Trichoderma* spp. to natural toxins, antimicrobial com-

pounds, synthetic pesticides and chemical pollutants (Harman et al. 2004a, b). Recently, a few ABC transporter genes have been cloned from *T. atroviride* strain P1 (Lanzuise et al. 2002), and the culture filtrates of different pathogens (*B. cinerea*, *R. solani*, *P. ultimum*) were found to strongly induce their expression (Woo et al. 2006). Interestingly, the promoters of these genes have many putative regulation factor-binding sites corresponding to those of an endochitinase involved in biocontrol activity (Lorito et al. 1996). These results, including the fact that the relative intensity of spot 7501 increased remarkably in the presence of either the pathogen *R. solani* or the plant (Fig. 3), suggesting that several ABC transporters may support the antagonistic activity of *Trichoderma* and its ability to colonize the roots. Spot 3214 matched a protein that may confer resistance to the fungal toxin brefeldin A (Table 5) capable of interfering with protein secretion and transport (Fujiwara et al. 1988), and may be possibly involved in cell detoxification. The presence in *T. harzianum* of the RNA from a different brefeldin A resistance protein (accession no. P41820) has been recently reported by Liu and Yang (2005) who used an EST-based approach. These data support the hypothesis that *Trichoderma* possesses a variety of mechanisms to protect itself from microbial and plant toxins.

A fungal hydrophobin matched to spot 6301 (Table 5). This kind of protein helps fungi to penetrate barriers, has in some cases a structural function (Linder et al. 2005; Wösten 2001), and mediates the attachment of pathogenic fungi to the host plant surface (Kershaw and Talbot 1998). Several hydrophobins have been found in the *Trichoderma* genome, but their role has not been determined yet (Linder et al. 2005). Benítez et al. (2004) have recently suggested that hydrophobins are specifically up-regulated during colonization by *Trichoderma* of tomato roots, and similarly we have found in the culture filtrates of *T. harzianum* strain T22 a hydrophobin containing a chitin binding domain able to induce the hypersensitive response (HR) and systemic resistance in plant (Ruocco et al., unpublished data).

The differentially expressed proteins found in the proteome of the pathogen *B. cinerea* (Table 4) included the cutinase encoded by the *cutA* gene particularly important for pathogen infection (van der Vlugt-Bergmans et al. 1997), different isoforms of cyclophilin 1 has been suggested to act as virulence factors in pathogen penetration or *in planta* growth (Viaud et al. 2003), and a superoxide dismutase (SOD) that may support pathogenicity by removing reactive oxygen species (ROS) produced by the plant (Gil-ad et al. 2000). The over-expression of SOD in *B. cinerea* could be related to the induction of plant resistance mechanisms by *Trichoderma*, since the intensity of the relative spot increased considerably when the antagonist was added to the *B. cinerea*–bean interaction (Fig. 3).

A variety of differentially expressed proteins were identified in the plant proteome during the interaction with the pathogens, the antagonist and both fungi (Tables 3, 6). The in silico analysis of data from plant–*Botrytis* and plant–*Trichoderma* interactions revealed many homologues to PR-proteins. Conserved domains, such as leucine rich repeats (LRR), nucleotide binding sites (NBS) and SGS domains, as well as conserved sequences of Barwin and Bet v I PR-protein families, were found. For instance, a protein of the tobacco PR-4 family with a Barwin domain (spot 6306; Table 3) and a thaumatin-like protein (spot 6302; Table 3) involved in the defence response of rice to *Magnaporthe grisea* (Kim et al. 2004) were differentially accumulated in presence of *Trichoderma*, either alone or in combination with *B. cinerea* (Fig. 3). Some differential spots in the bean leaves proteome obtained from plants which had root interactions with *R. solani* and *T. atroviride* showed a high level of similarity to proteins associated with plant disease resistance and pathogen recognition (Table 6). Homologies with both PR-proteins or proteins involved in defence activation mechanisms were found, including a NBS-LRR type (spot 6101) (Table 6) that may recognize pathogen products and induce defence responses such as apoptosis and HR (Moffett et al. 2002). These proteins accumulated in the plant proteome particularly in the presence of *R. solani* (Fig. 3), which was expected considering the activation of plant defence mechanisms after a pathogen attack. Regardless, we noticed that in many cases spots corresponding to defence-related compounds showed a decreased intensity when *Trichoderma* was present as compared to plant–pathogen condition (Fig. 3), which is in agreement with the differences found in terms of spot number (see above). These results indicated that specific resistance genes may regulate the plant–*Trichoderma*–*R. solani* interaction, and that the presence of the antagonist may reduce quantitatively and qualitatively the protein-based response of the plant to the pathogens. However, the addition of *Trichoderma*, either alone or in combination with *B. cinerea*, induced an increased expression in bean leaves of at least two PR-proteins (spots 6302 and 6306) (Fig. 3), suggesting the activation of a specific response to the biocontrol agent (Yedidia et al. 2000, 2003). In fact, the simple comparison between the plant–*Trichoderma* and the plant alone conditions indicated up to 191 differential spots, of which 39 appeared ex novo, 87 were absent, 27 up-regulated and 38 down-regulated (Table 1). In addition, we found, both by proteome matching and

band hybridization, possible homologues in *T. harzianum* and *T. atroviride* of known avr proteins (avr4, avrE, NIP1) (Harman et al. 2004a), which is not surprising considering the "avirulent" nature of these plant-colonizing fungi. In particular, the homolog of NIP1 showed similarity to a *T. harzianum* β-1,3-exoglucanase with a conserved cysteine pattern typical of hydrophobins (Rohe et al. 1995). These findings, together with the absence of such homologies in *T. reesei* genome (data not shown), suggest a significant involvement of hydrophobins in the *Trichoderma* avirulence behaviour. Finally, we consider that there is a general similarity in the plant molecular interaction with pathogenic and beneficial saprophytic microbes like *Trichoderma* spp., including common gene-for-gene and avr-R gene mechanisms.

In conclusion, a proteomic approach allowed us to identify numerous differential proteins involved in multiple-player cross-talk normally occurring in nature between plant, pathogens and biocontrol agents. The majority of the studies reported so far have been focused on two-partner conditions (Baker et al. 1997; Dangl and Jones 2001; Hammond-Kosack and Parker 2003; Harman et al. 2004a; Suzuki et al. 2004), thus providing a relatively incomplete view of a pathogenicity/resistance processes as mediated by both beneficial and pathogenic microbes. Proteomic analysis can be very useful to provide both general and specific information on the "interaction proteomes" used by plants and microbes. However, the complexity of the system, which requires more than one player to act at the same time, indicated that a more integrated approach is necessary to deeply understand the biology of biocontrol agents such as *Trichoderma* spp.

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