Study of proteome pattern of *Pseudomonas fluorescens* strain UTPF68 in interaction with *Trichoderma atroviride* strain P1 and tomato

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Abstract

Saprophitic *Pseudomonas* species are root-colonizing bacteria that can improve plant health. Efficient exploitation of these bacteria in agriculture requires knowledge of traits that enhance ecological performance in the rhizosphere. Some *Pseudomonas fluorescens* strains present biocontrol properties, protecting the roots of some plant species against plant pathogens. These bacteria induce systemic resistance in the host plant, so it can better resist attack by a true pathogen. The bacteria outcompete other (pathogenic) soil microbes, e.g., by siderophores, giving a competitive advantage at scavenging for iron. The bacteria produce compounds antagonistic to other soil microbes, such as phenazine - type antibiotics or hydrogen cyanide. In this study the changes in the protein profile of *P. fluorescens* strain UTPF68, involved in the multiple interactions between plant (tomato) and an antagonistic agent (*Trichoderma atroviride* strain P1) investigated. Two-dimensional electrophoresis was used to analyze separately collected proteins from each one, two or three partner interactions. The results about differential produced spots in *Pseudomonas* proteome in each collation, showed that 18 differential spots became visible as new, 16 spots were absent, 17 spots were up-regulated and 1 spot was down-regulated, when Tomato-*Pseudomonas* (TP) condition was compared with control *Pseudomonas* alone (P). Also more than 84 differential spots were accumulated in proteome of *Pseuodomonas* due to the presence of *Trichoderma*, as new, absent, increased and decreased spots. By comparison of conditions revealed 2 protein spots that detected by MS, have newly expressed in present of Plant and *Trichoderma*. These proteins corresponded to arginine deiminase of *P. putida* GB-1 and Chaperonin GroEL protein of *P. putida* S16 that their expressions associated to stress condition.The results indicated that the presence of Plant and *Trichoderma* induces major changes in the protein profile of *Pseudomonas*.

Keywords: 2-DE; Interaction; Antagonist; *Pseudomonas fluorescens* strain UTPF68; *Trichoderma atroviride* strain P1

INTRODUCTION

The use of traditional methods to protect crops from disease have been largely based on the use of chemical pesticides. But chemical methods are not cost effective in the long run because they damage the environment, leave harmful residues, pollute the atmosphere and can lead to the development of resistant strains among the target organisms with repeated use [22].

One of the most promising means to reduce doses of chemicals is by the use of biocontrol agents (BCAs) in the control of plant pathogens [6]. BCAs are available as commercial products such as *Agrobacterium*, *Streptomyces*, *Bacillus*, *Pseudomonas* and fungal genera such as *Gliocladium*, *Trichoderma*, *Amplomyces*, *Candida* and *Coniothyrium* [2]. These agents have developed the ability to interact simultaneously both with themselves and plants. These microorganisms have separated by biocontrol mechanisms and distinct regulation of key biocontrol genes. *Pseudomonas* spp. produce

different secondary metabolites with antimicrobial activity, including hydrogen cyanide and metal-chelating (siderophores). Also produce salicylic acid and the polyketide antimicrobial compound, pyoluteorin [2]. *Trichoderma* biocontrol activity is distinctively due to mycoparasitism facilitated by the production of cell wall-degrading enzymes (CWDE) [9]. The CWDEs act synergistically to advance mycoparasitism of phytopathogenic fungi. The most important are the ECH42 endochitinase encoded by *ech42* and an *N*-acetyl-β-d-glucosaminidase encoded by *nag1* [4].

The use of bacterial and fungal strain mixtures is a promising way to improve the efficacy of biocontrol treatments. Recent studies suggested to *Pseudomonas* and *Trichoderma* strains in using of biocontrol agents. The introduction of two or more antagonists in a strain mixture has been proposed as an approach to improve the level and reliability of biocontrol treatments across an expanded broader range of environments [18].

Our aim in this research was to determine impact of one of the most studied biocontrol organisms, *P. fluorescens*, on the changes of proteome profile. To achieve this goal the three way interaction between plant-*Trichoderma*–*Pseudomonas* investigated by using proteomeic technique. This technique clearly represents an effective tool to analyze such biocontrol processes. By using of this method, we studied the current interactions of biocontrol agent *P. fluorescens* strain UTPf68 with a host plant and *T. atroviride* strain P1, in order to identify and analyze differentially produced proteins by the three players. A method has been developed and optimized to obtain 2-D gel maps of separately collected proteins from each partner, as well as all the possible combinations of the components. In each experiment was assessed changes in the proteome profile of *P. fluorescens* UTPF68. Also, differently produced proteins were obtained from the analysis of 2-D gel containing the proteins of the antagonists during the interaction with tomato plant.

MATERIALS AND METHODS

Antagonistic compatibility test

In vitro compatibility test between *P. fluorescens* strain UTPF68 and *T. atroviride* strain P1 was conducted in order to determine whether they can be used in combination. Dual culture plate method described by seddiqui and Shaukat [27] was employed. Accordingly, an overnight culture of *P. fluorescens* which was grown in King's broth was streaked on one side of the plate (9 cm diameter) containing Kings' B agar (KBA). The other side of the plate was inoculated with 1 cm disc of *T. atroviride* (9 days old). The plates were incubated at 25 °C and zone of inhibition (if any) was measured. The test was performed in triplicates.

Growth and interaction conditions for proteomeics experiments

Tomato seeds (*Lycopersicon sculentum*) were sterilized for 1 min with a 1% hypochlorite solution, rinsed in sterile water and placed in water agar (WA) plates 15 cm in diameter which covered with a sterile cellophane membrane (CM).

P. fluorescens strain UTPF68 were cultivated at 27˚C on King's B agar which were covered with a sterile CM. After 16-24 h of incubation the CM with the bacterial cells was transferred on top of the WA plate containing the plant tissue.

A spore suspension of *T. atroviride* strain P1 (ATCC74058) obtained from a colony grown on potato dextrose agar (PDA) was used to inoculate CM-covered PDA plates (100 µl of a 5×10^5 spores /ml suspension). After 4 days of incubation at $25^{oc,}$ the CM with the antagonist mycelia was transferred on top of the WA plate containing the 2 weeks seedling plant tissue.

The CM was used to separate the antagonists between themselves and from the tomato plant allowed separation and transfer fungal mycelia and bacterial cells, but still permitted micro- and macro-molecules diffusion [15].

The plates hosting three-player (plant-fungal antagonist- bacterial antagonist) and two players (plant – bacterial antagonist) interactions and the relative control (bacterial antagonist alone) were maintained at room temperature for 3 days and then the plant/antagonists samples were separately collected for protein extraction.

Protein extraction from plant tissue and fungi mycelia

The protein extraction protocol described by Jacobs et al. 2001 was applied with some

modifications for plant tissue and fungal mycelia [14]. Approximately 1 g of fungal mycelium (wet weight) from the antagonist, or plant tissue (leaves and roots) were 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 (T25basic, IKA Labortechnik, Germany) by keeping the tube in an ice bath. Samples were maintained at -20˚C for at least 3h allowing protein precipitation, and then centrifuged at 30,000g for 20 min 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 [9M 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 2h to obtain complete protein solubilization. Samples were centrifuged at 30,000g for 60 min 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 -80˚C.

Protein extraction from bacterial cells

Free cells of *P. fluorescens* strain UTPF68 harvested from top of the CM by adding 15 ml phosphate buffer enriched with 1g/L glucose (PBG) pH 7.9. Then centrifuged at 8000g for 20 min. The pellet was washed with PBG again and resuspended in 20 ml of isoelectrofocusing (IEF) buffer of the following composition (8M urea, 65mM CHAPS, 10mM DTT and 0.8% (w/v) carrier ampholytes (pH 4-7, SIGMA). Cells in IEF buffer were maintained in -80˚C for at least 1h. Then cells in IEF buffer were disrupted by thermal shock (from -24˚C to 20˚C) followed by ultrasonication at 4˚C (30W, 15pulses of 2s separated by 2s breaks). Protein extracts were centrifuged at 10,000g for 20 min to eliminate cell debris. The protein content of the supernatant was evaluated by using the Bio-Rad protein assay. Supernatants were stored at -24˚C.

Two-dimensional electrophoresis (2-DE):

Isoelecric focusing (IEF) was conducted by using 7 cm immobilized-pH-gradient (IPG) strips (Bio-Rad) (with a pH range from 3 to 10 for plant and fungi and 4 to 7 for bacteria) 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 into the focusing tray and absorbed into the gel strip (1h passively at room temperature and 12h actively with a 50 V current applied). IEF was carried out by a PROTEAN IEF CELL system (Bio- Rad). IPG strips were focused up to a total of 14 kv for 3h and until 19kv were reached). The strips were equilibrated by placing them in a solution of 6M urea, 0.05M Tris/HCL pH 8.8, 20% glycerol, 2% SDS, 2%DTT for 10 min, and then in 6M urea, 0.05M Tris/HCL pH8.8, 20% glycerol, 2% SDS, 2.5% iodoacetamide 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). After equilibration, strips were located 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 8h. Gels were stained for at least 3h with SimplyBlue SafeStain G-250 (Invitrogen, California, USA) according to the manufacturer's instructions. Each protein extract was run on triplicate gels for 7 cm strips. Gel images were acquired by a GS-800 Imaging Densitometer (Bio-Rad) [19].

Bioinformatics analysis

2DE gels were scanned and gels were analyzed by non linear progenesis same spot software to compare gels together and compare the spots in one statement in gels and get the density of same spot in each of gel.

In-gel digestion, mass spectrometry and in silico analysis

Some differential spots, such as those produced by *P. fluorescens* in the interaction with Tomato plants and *T. atroviride* were subjected to MALDI-TOF/TOF analysis by using a Proteomeics Analyzer 4700 (Applied Biosystems) that was calibrated prior to each experiment. The samples were desalted and concentrated using ZipTips C18 (Millipore, Bedford, MA, U.S.A.), as described by Marra et al. (2006) [19]. The acquired MS/MS data were submitted to the National Center Biotechnology Information nonredundant (NCBInr) database for protein

identification by using the GPS Explorer Software with integrated Mascot search engine.

RESULTS

Data on compatibility showed that both *P. fluorescens* and *T. atroviride* were compatible to each other in dual culture. Either of microorganisms formed no inhibition zone in any of the plates. The proteome of *P. fluorescens* grown alone (P) used as a control specimen for comparisons with two-way and three-way interactions. In figure 1 show 2-DE gel of *P. fluorescens* in condition P(a), TP (b) and TTP (c). Two experiments group gels were analyzed by using non linear progenesis same spot software. 18 differential spots produced new, 16 spots were absent, 17 were up-regulated and 8 was down-regulated when TP condition was compared with control P (Table_1). These results indicated that the presence of Plant (tomato) induces major changes in proteome of the *Pseudomonas* while the *Pseudomonas* is interacting with the tomato plant (Table 1).

More than 39 differential spots were noted as new, absent, increased or decreased if *Trichoderma* was exposed to the plant and *Pseudomonas* together (Table 1). When TTP was compared to the TP, 14 spots appeared was produced new, 39 spots were absent, 6 spots were up-regulated and 29 spots were down-regulated, indicating that the presence of *Trichoderma* induces important variations in the protein of *Pseudomonas* interacting with the plant (Table 1). A statistical analysis by progenesis same spot software obtained protein clustering that divides protein profile of each group to subdivisions of same expression of proteins. In Fig. 1, illustrate protein dendrogram of P condition compared to TP condition. In fig. 1a, exist dendrogram, indicate lower protein expression in P than TP. While in fig. 2b, dendrogram release higher protein expression in P than TP. Fig. 2a, illustrate protein dendrogram of TP compared to TTP with lower protein expression in TP, and in fig. 3b, dendrogram shows the higher protein expression in TP than TTP.

Other results from progenesis same spot software were shown of different comparable protein spots exist in each group, that details of some spots were presented in figures 4 and 5. Fig. 4a, shows differential intensity levels of 2-D gel spots produced in condition P which were lower than condition TP. Spots 192, 204, 369, 364, 306, 284 and 155, were absent in the control (P) and were induced by the presence of tomato. Fig. 4b, shows differential intensity levels of 2-D gel spots produced in condition P which were more than condition TP. Spots 350, 349 and 133 were present in the control and were absent by the presence of tomato. Fig.5a. shows differential intensity levels of 2-D gel spots produced in condition TP which were lower than condition TTP**.** For example, in comparison to the control (TP), spots 397 and 244 showed increased intensity when *Trichoderma* exposed to the interaction. Fig. 5b. Shows differential intensity levels of 2-D gel spots produced in condition TP which were more than condition TTP. Spots 409, 404, 261 and 408 showed particularly high decrease in intensity when *Trichoderma* entered in the interaction.

About 10 of the most strongly modified *P. fluorescens* protein spots (examples shown in Fig. 6) were further characterized by MALDI-TOF MS followed by in silico analysis, but only two identification cases are reported here (Table 2). These include an arginine deiminase and a chaperonin GroEL proteins. These proteins in the interaction between Tomato and *Pseudomonas* (TP) were expressed significantly higher up-regulated in comparison with Tomato-*Trichoderma-Pseudomonas* (TTP) interactions, while they are not produced in the control specimen (*Pseudomonas* alone), as indicated by the increased spot intensities (Fig 6).

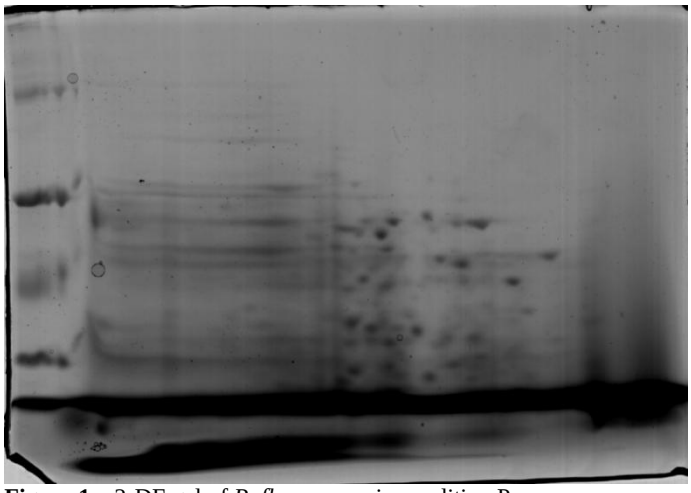


Figure 1a. 2-DE gel of *P. fluorescens* in condition P

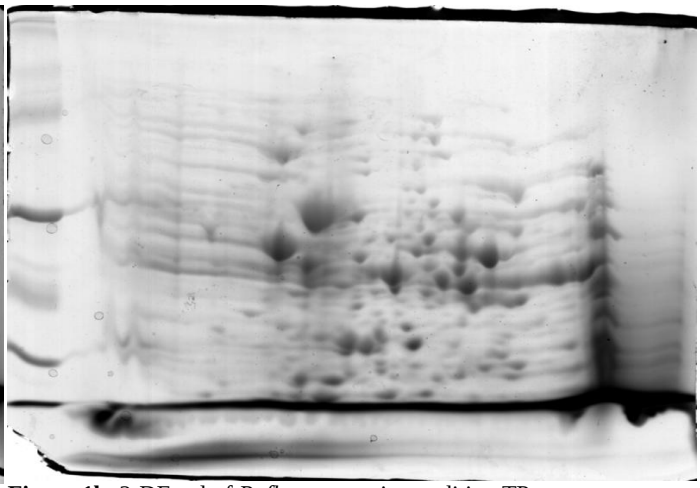


Figure 1b. 2-DE gel of *P. fluorescens* in condition TP

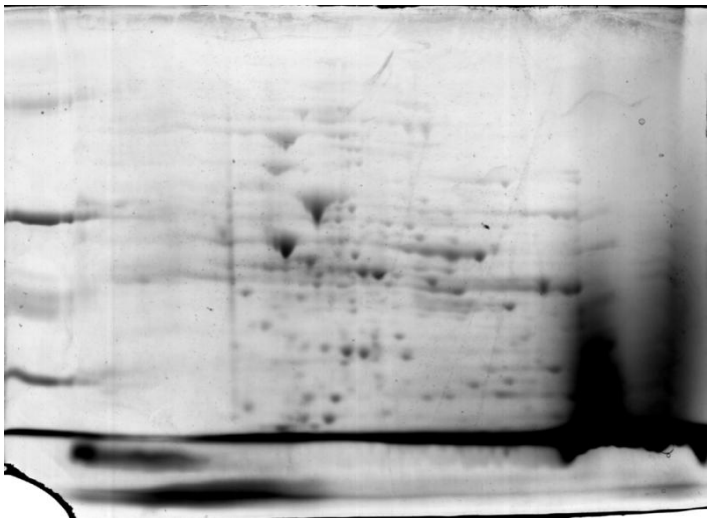


Figure 1c. 2-DE gel of *P. fluorescens* in condition TTP

Table1. Comparison of differential spots produced in *P. fluorescens* proteome in each gel.

Vs P (condition A)

Spot	Increased[c]	Decreased[c]	ON[b]	OFF[b]
condition B:				
TP	17	8	18	16

Vs TP (condition A)

Spot	Increased[c]	Decreased[c]	ON[b]	OFF[b]
condition B:				
TTP	6	29	14	39

[b]Number of spots present in the first condition compared to the second (ON) or vice versa (OFF)

[c]Number of spots whose intensity in the first condition increased or decreased at least twofold compared to the second one.

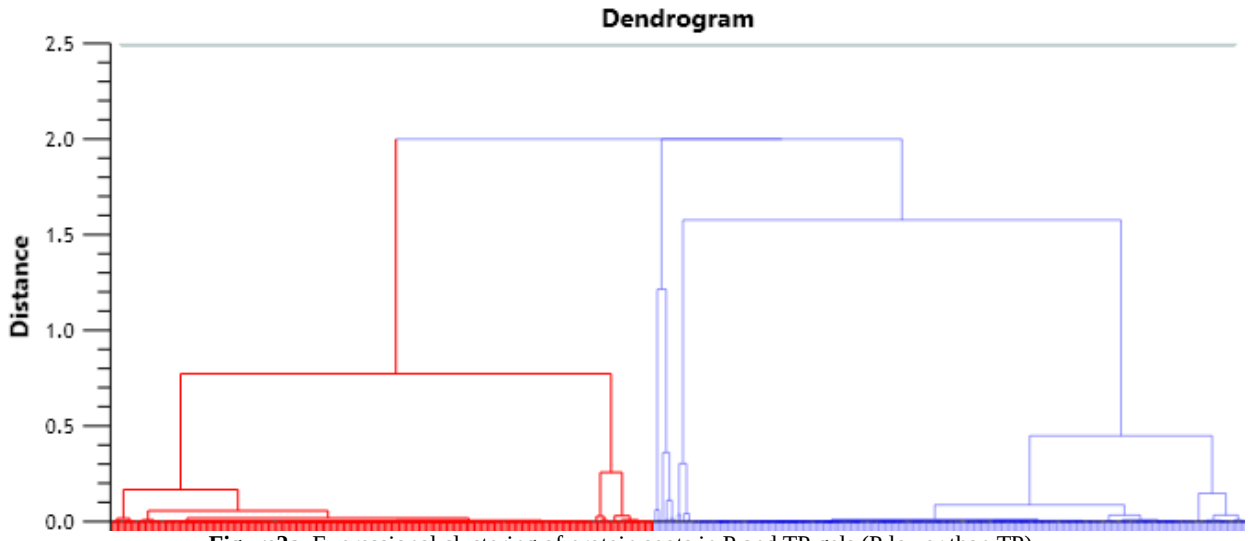


Figure2a. Expressional clustering of protein spots in P and TP gels (P lower than TP)

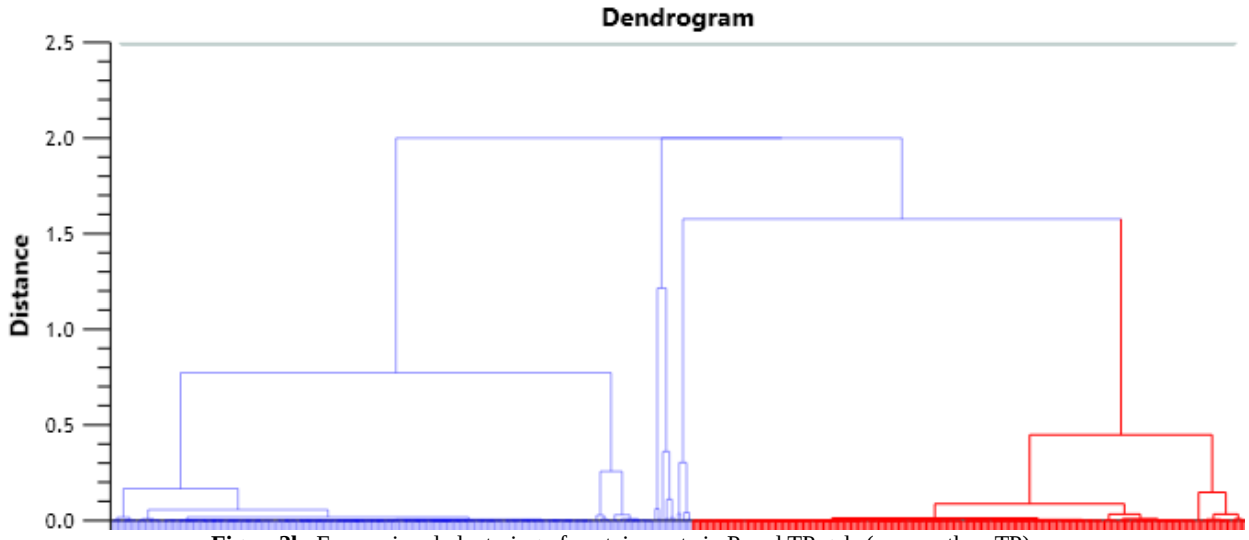


Figure2b. Expressional clustering of protein spots in P and TP gels (p more than TP)

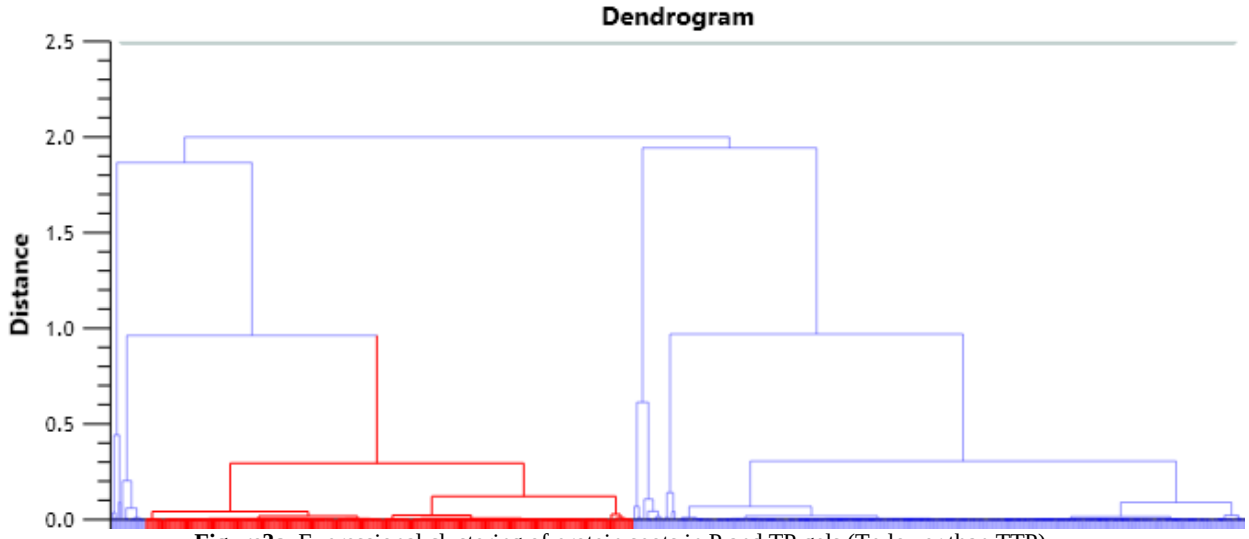


Figure3a. Expressional clustering of protein spots in P and TP gels (Tp lower than TTP)

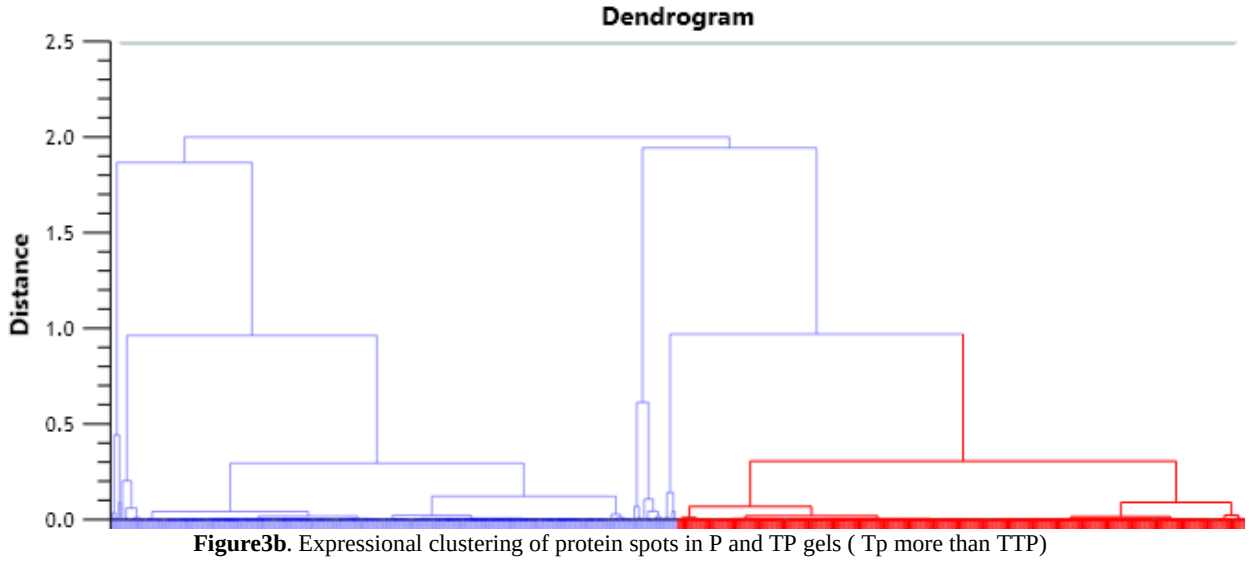


Figure3b. Expressional clustering of protein spots in P and TP gels (Tp more than TTP)

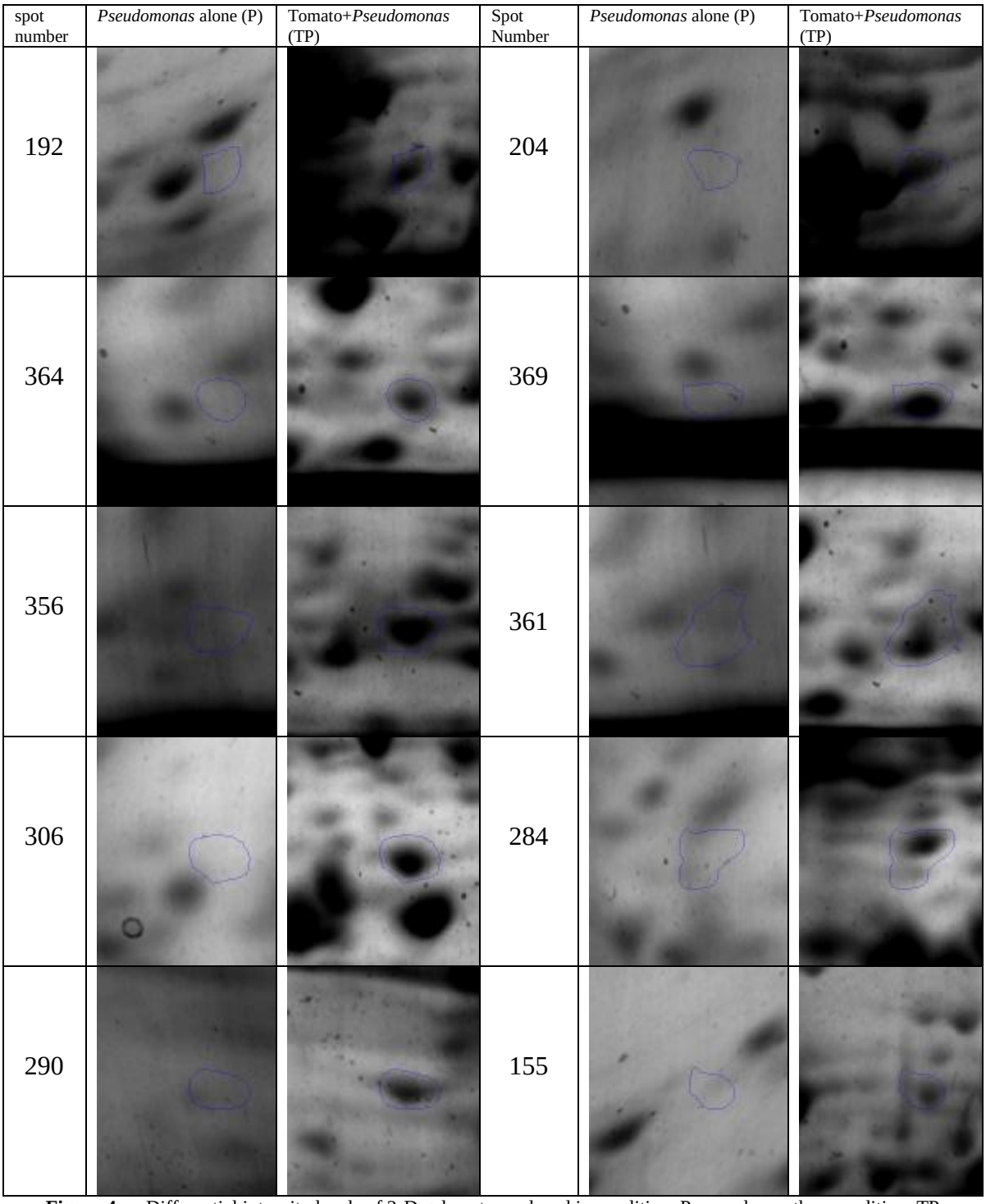


Figure 4a. Differential intensity levels of 2-D gel spots produced in condition P were lower than condition TP

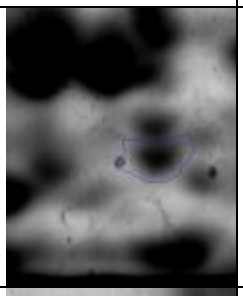
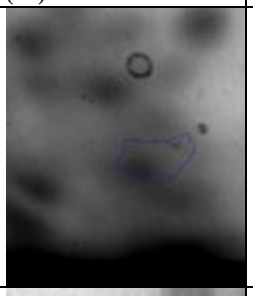
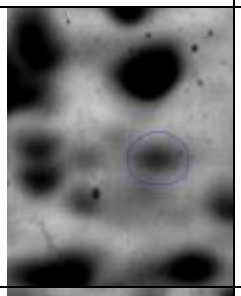
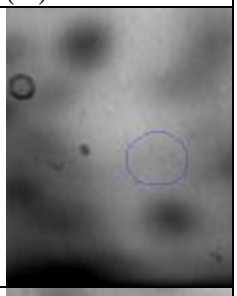
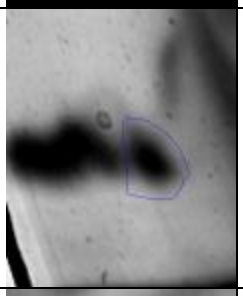
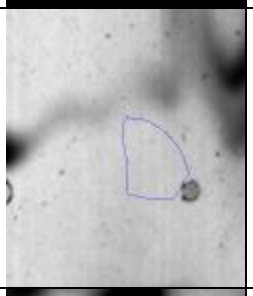
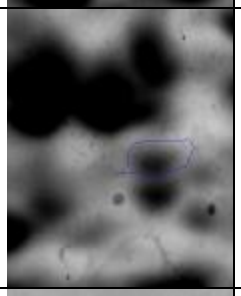
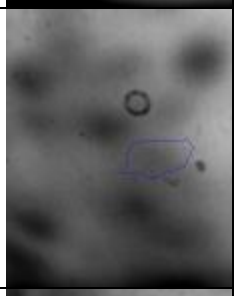
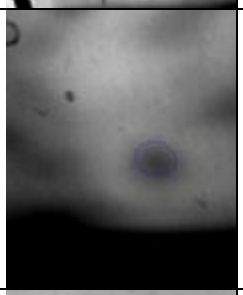
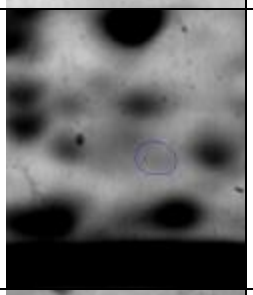
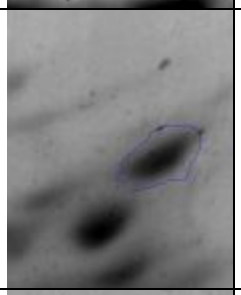
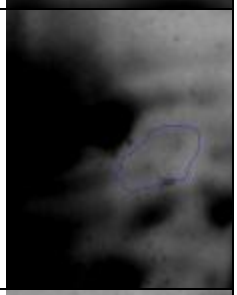
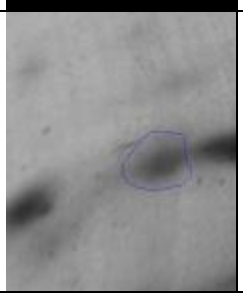
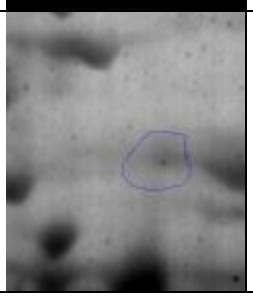
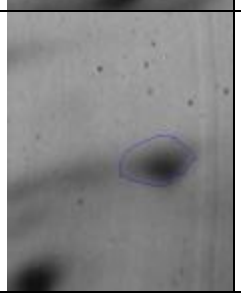
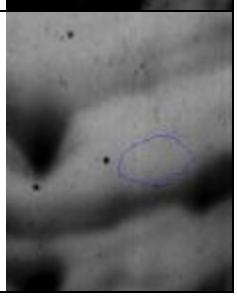
Spot Number	*Pseudomonas* alone (P)	Tomato+*Pseudomonas* (TP)	Spot Number	*Pseudomonas* alone (P)	Tomato+*Pseudomonas* (TP)
358			350		
349			347		
366			173		
109			133		

Figure 4b. Differential intensity levels of 2-D gel spots produced in condition P were more than condition TP

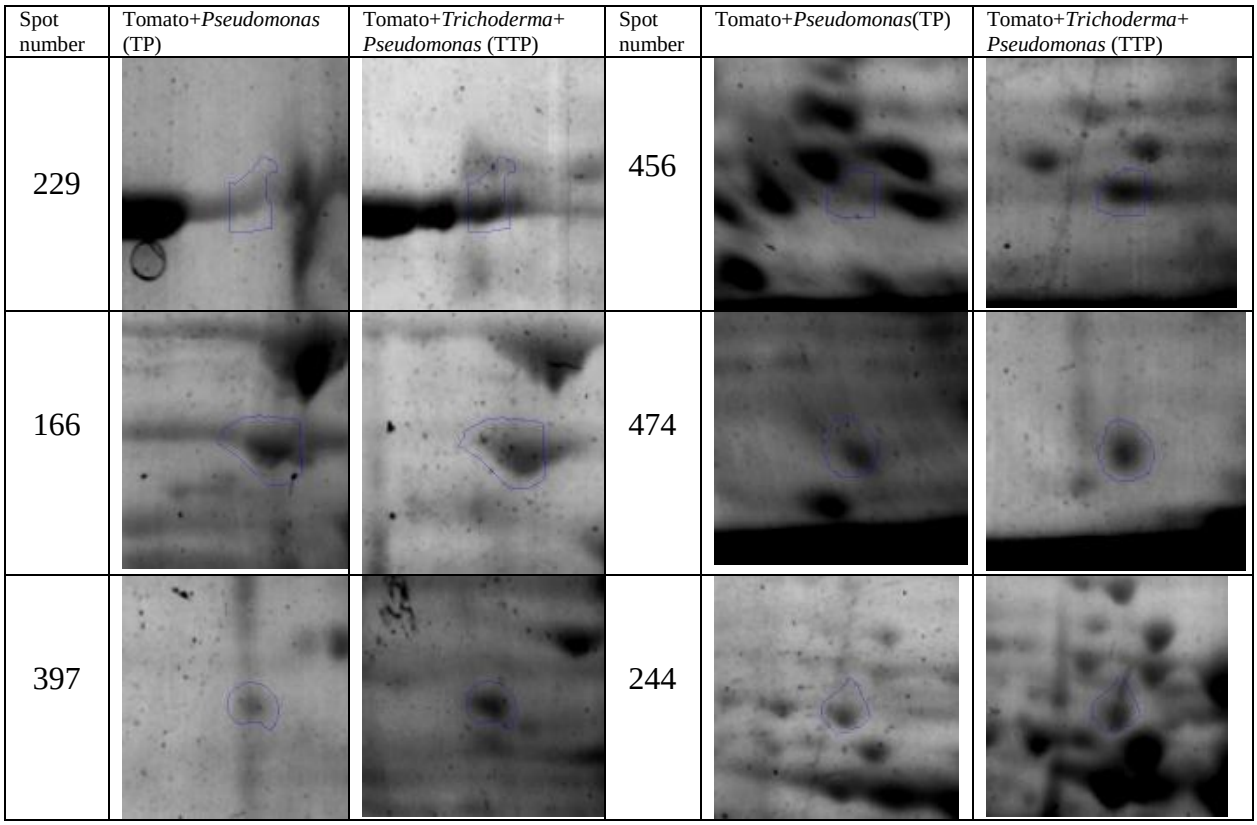


Figure 5a. Differential intensity levels of 2-D gel spots produced in condition TP were lower than condition TTP

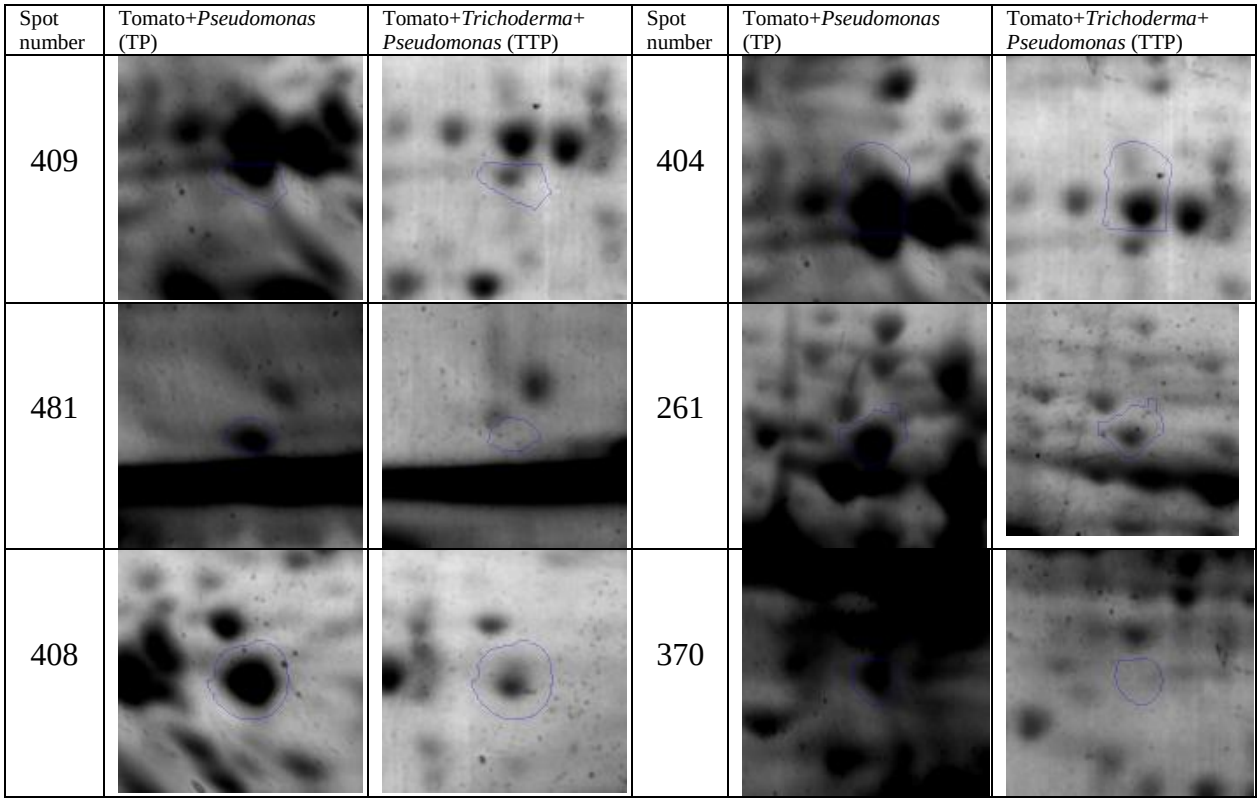


Figure 5b.Differential intensity levels of 2-D gel spots produced in condition TP were more than condition TTP

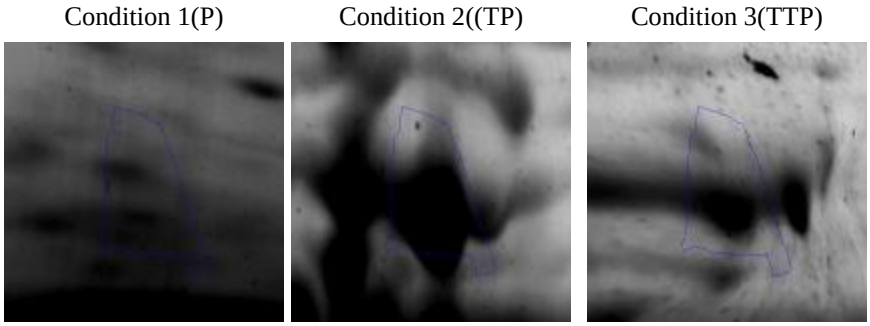


Fig 6a. Differentially level of Spot 395 in *Pseudomonas* proteome: condition 1 *Pseudomonas* alone (no interaction), condition 2 Tomato+ *Pseudomonas*, condition3 Tomato+ *Trichoderma*+ *Pseudomonas*

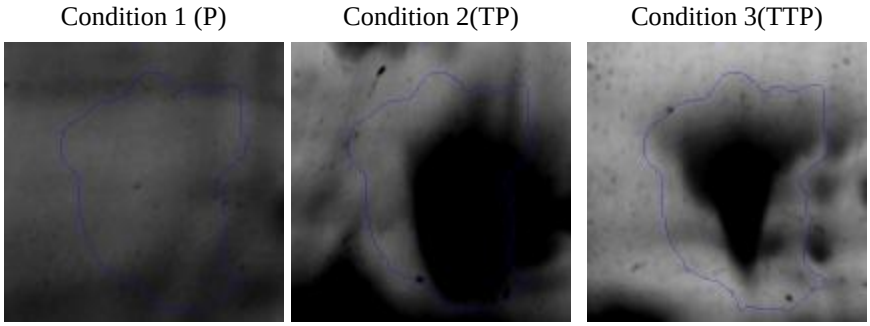


Fig 6b. Differentially level of Spot 195 in *Pseudomonas* proteome: condition 1: *Pseudomonas* alone (no interaction), condition 2: Tomato+ *Pseudomonas*, condition3: Tomato+ *Trichoderma*+ *Pseudomonas*

Table 2. Homologies of differential proteins in the *P. fluorescens* UTPF68 proteome during interaction with tomato and *T. atroviride*. MW (KDa): molecular weight and PI: isoelectric point of defferential proteins determined experimentally (from gels) and theoretically (from in silico analysis)

Spot number	MW(KDa)/PI	Protein name (source)	Accession number	No. of mass values searched	No. of mass values matched	Sequence coverage(%)	Calculated PI value	Score
395	46	Arginine deiminase (*Pseudomonas putida* GB-1)	gi|167032015	56	18	36	5.66	127
195	56	Chaperonin GroEL (*Pseudomonas putida* S16)	Gi|339489217	336	49	66	5.00	114

DISCUSSION

In this study, we conducted compatibility tests between *T. atroviride* and *P. fluorescens* to understand their compatibility to each others before studying their interaction by proteomeics technique. Data on compatibility showed that both *P. fluorescens* and *T. atroviride* were compatible to each other in dual culture. Either of the organisms formed no inhibition zone in any of the plates. Similar observations have been reported previously also. For example *T. viride*, *T. harzianum* and *P. fluorescens* were reported to be compatible when these were applied together [25]. Lutz et al. showed that the compatibility of antagonists applied as strain mixtures is not only dependant on compatible utilization of nutrients, minerals or space but can also be influenced by molecular signaling between the individual

antagonists. In addition the benefit of enhancing the biocontrol mechanisms of individual strains in the mixture, there is potential for synergistic activity [18]. Fogliano et al. reported that *Pseudomonas* lipodepsipeptides primed pathogen cells such that CWDE penetration and activity was increased [9].

We used tomato plants and two antagonistic agents (*T. atroviride* and *P. fluorescens)* to analyze the changes in the proteome of the *P. fluorescens* caused by three player interaction. 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 the individual proteomes. Placing of such type of membranes between fungal spores, bacterial cells and seed surface did not affect the improvement of seed germination. Kulling et al. 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 *Rhizoctonia solani* [15]. Figure1 show the 2DE gels of *P. fluorescens* in 3 conditions. During the interaction between *Pseudomonas* and tomato versus control specimen (*Pseudomonas* alone) induced more new protein spots (18 spots) and intensity level of 17 spots were increased. As expected these data indicated that the presence of plant induced important variations in the antagonist proteome, while it is interacting with tomato plant, which maybe is related to increase expression of *Pseudomonas* genes which are involved in a direct way to promote the growth of plants such nitrogen fixation, increasing the availability of iron and phosphorus from the soil or their capture and delivery of these minerals to the plant form in which it has used, and the synthesis of plant hormones that stimulate the proliferation of plant cells. Also some molecules, which are found to be small oligosaccharides consisting of two monomers with or without an amino acid residue, act as inducers of genes associated with biocontrol, and in some cases also function as elicitors of plant defense mechanisms [12,31]. Antagonistic microorganisms can interact with the plant through the processes of colonization, increased bioavailability of nutrients, production of useful substances in plant metabolism, induction of resistance mechanisms (ISR) and activation of the hypersensitive response (HR).

During three way interaction (Tomato-*Trichoderma-Pseudomonas*), important changes in the proteome of *P. fluorescens*, as compared to double player (*Pseudomonas*- Tomato) conditions were observed. In particular, the presence of fungal antagonist strongly modified the protein pattern of the bacterial antagonist during plant interaction. 39 spots which were absent in three ways condition, were present in the *Pseudomonas*- tomato condition (Table 1). (29 decreased spots, 6 increased spots). According to figures 2 and 3, in the profile of 2-D gels, we compared the changes of protein expression associated with increased or decreased expression in two conditions of P - TP and TP - TTP. Statistically, gene expression differences, were compared by using progenesis same spot software, and proteins with same expression were clustered in separate groups. Clustering based on expression, indicated the presence of the proteins that can be attached to each other, biologically. Thus, simultaneously increased expression of multiple proteins, must be compensated by decreased expression or absence of expression of other proteins. In fact, this is an establishment mechanism of cell homeostasis that silencing of a process lead to replacing new process and start to expression of new proteins. As depicted in fig. 4a, different intensity levels of 2-D gel spots produced in condition P which were lower than condition TP. Spots 192, 204, 369, 364, 306, 284 and 155, were absent in the control (P) and were induced by the presence of tomato plant. Fig. 4b, shows different intensity levels of 2-D gel spots produced in condition P which were more expressed than condition TP. Spots 350, 349 and 133 were present in the control and were absent from the presence of tomato. In other condition as shows in fig. 5a. differential intensity levels of 2-D gel spots were lower than condition TTP**.** Spots 397 and 244 showed increased intensity when *Trichoderma* exposed to the interaction. Fig. 5b. Shows differential intensity levels of 2-D gel spots produced in condition TP which were more than condition TTP. Spots 409, 404, 261 and 408 showed particularly high decrease in intensity when *Trichoderma* entered in the interaction.

As expected *Trichoderma* induced different sets of *Pseudomonas* proteins and clearly changed the expression pattern of *Pseudomonas* genes which may be related to increased synergistic control. There are several reports of biocontrol strain combinations that had more efficacy than single strain treatments against a variety of plant diseases. A study at the molecular level showed that when bacterial and fungal biocontrol agents were combined in a strain mixture, there were positive effects on the expression of key biocontrol genes in both antagonists [18]. They found that addition of *T. atroviride* P1 culture filtrates to grow cultures of *P. fluoescens* enhanced *phlA* expression transiently during growth. Shirzad et al. have analyzed the effects of two biocontrol agents, *T. atroviride* and *P. fluoescens* on the expression of their key biocontrol genes. They showed that when co-inoculated with *T. atroviride*, *phlD*-positive strains of *P. fluorescens* produced significantly higher levels of DAPG antibiotic [26]. In conclusion, the system, which needs more than one partner to act at the same time, indicated that a more integrated approach is necessary to profound a knowledge about the biology of biocontrol agents. In changes were shown of *P. fluorescens* UTPF68 proteome during the complex three-way and two-way interactions investigated and two of the most interesting differential spots were analyzed by PMF. These were selected by comparing proteomes obtained from three-way (TTP), two-way (TP) and no interaction conditions (P). We identified spot 395 as a protein similar to arginine deiminase from *Pseudomonas* proteome during a three-way and two-way conditions, with either tomato plant or *T. atroviride* and didn't find it in the control (Table 2, Fig 6a). This protein corresponded to an arginine deiminase of *P. putida* GB-1 and has a catalytic activity that catalyzes the degradation of arginine to citruline and ammonia.

Neubauer et al. (1996) and Pantel et al. (1998) have showed that in the absence of oxygen, *Staphylococcus aureus* can grow by fermentation of glucose or by using an altenative terminal electron acceptor such as nitrate. When neither glucose nor nitrate is available, arginine can serve as the sole energy source for growth [23, 24]. The main bacterial arginine catabolic pathway is the arginine deiminase (ADI) pathway, encoded by the *arc* operon [8]. ADI operons have been described for a wide variety of bacteria, including mycoplasma, *Pseudomonas* spp. and *Bacillus* spp. [3]. In *Streptococcus rattus*, the ADI pathway functions as a key defense mechanism against acidification [7]. Adaptation of many facultative anaerobic and some obligate aerobic bacteria to oxygen limitation is mediated by a family of transcriptional regulators related to the FNR (fumarate and nitrate reductase regulation) protein of *Escherichia coli* [28,30]. In *Pseudomonas aeruginosa* and other *Pseudomonas* species belonging to rRNA homology group I, the FNR homolog ANR (anaerobic regulation of arginine deiminase and nitrate reductase pathways) is a major regulator of genes involved in anaerobic metabolism, e.g., the genes required for anaerobic respiration [1,32] and the *arcDABC* (anaerobic arginine catabolism) operon of *P. aeruginosa* [10,11,33]. When oxygen levels decrease, the ANR protein binds to a conserved sequence, the ANR box, located about 40 bp upstream of the transcription initiation site and thereby activates transcription, as shown for the *arcDABC* operon [10, 11, 17, 20]. In our study, we can portray a new expression of arginine deiminase in the three way interaction. Interacting tomato plant and *T. atroviride* with *Pseudomonas*, probably causes oxygen-limiting condition in comparison with one way interaction (*Pseudomonas* alone). Blumer and Haas showed that *P. fluorescens* CHA0 produces hydrogen cyanide (HCN), a secondary metabolite that substantially contributes to this strain's biocontrol ability. Cyanogenesis is induced by oxygen-limiting conditions. In *P. fluorescens*, the anaerobic regulator ANR and the required for the maximal expression of the HCN biosynthetic genes *hcnABC* [5].

We also identified a homologue of a 56KDa heat shock protein (HSP) (spot 195, Table 2) whose intensity strongly increased in the presence of both tomato plant and *T. atroviride* (Fig 6b). This protein has a conserved domain DnaJ typically linked to environmental stresses [16,21] and probably associated with defence responses in *Pseudomonas*. This spot did not produced in the control (*Pseudomonas* alone). Spot 195 corresponded to a Chaperonin GroEL protein of *P. putida* S16. Chaperonins and typically bacterial

chaperonin (GroEL) are generally responsible for preventing damage to proteins in response to high levels of stress and for the transportation and refolding of newly synthesized proteins [29]. At this study, expression of chaperonin GroEL occurred while *Pseudomonas* was in interacton with tomato or *T. atroviride* (TP or TTP). Lake of chaperonin production in the control treatment, shows that when tomato or *T. atroviride* interacting with *P. flourescens*, induces new protein spots in *P. flourescens* proteome. These new produced proteins, need chaperonin molecules for refolding and transfering from cytoplasm and for preventing damage under stress conditions. Since, presence of tomato plant and *Trichoderma* in the interaction with *Pseudomonas* are as potential stresses, therefor *Pseudomonas* need defensive responses against them. In the control treatment (*Pseudomonas* alone) is not exist potential stress, which does not induce the production of chaperonins. In sum up, a proteomic approach indicated that differential proteins are involved in multiple players which normally are present in nature between plant and biocontrol agents and they extensively can induce important variations in the proteome of eachothers.

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