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Production of Pathogenesis-Related proteins during the induction of resistance to *Phytophthora capsici* in pepper plants treated with *Burkholderia cepacia* and *Trichoderma harzianum* in combination compatible

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Abstract

We evaluate the defensive reaction of pepper plants after inoculating the stems with *Phytophthora capsici* using a decapitation method and treating the roots with the antagonists *Burkholderia cepacia* and *Trichoderma harzianum* jointly. Infection of the stems with the pathogen produces a hypersensitivity reaction but necrosis is slowed down in plants treated with the two antagonists, as a result of induced resistance. The addition of the antagonists to plants inoculated with *P. capsici* produces a defensive reaction involving the production of proteins showing β-1,3-glucanase activity. The increase in activity affects both constitutive enzymes and those synthesised "de novo", although the increase in activity detected in control plants is due to the effect of the wound rather than to the treatment since it does not increase with time. Treatment with the antagonists induces a systemic reaction in the roots and stems, which is resolved by the production of PR proteins with β-1,3-glucanase activity, even though the stems are not affected by the antagonist or by the pathogen. These PR-glucanases are basic (pI 9.0 and pI 7.8) and PR-glucanase acid (pI 4.5) are detected in stems and roots treated with antagonistic's 3 and 9 days after inoculation, common in stems and roots and in the different treatments of time but that differ in their concentration. Increased β-1,3-glucanase activity is also observed in stems and roots and the production of a PR protein is observed both at 9 days. This isoenzyme is acidic (RF 0.21) and seems to be produced as a result of treatment with the antagonistic's, but does not appear in the control or in the stems and roots treated two water-peptone. The production of PR-proteins with β-1,3-glucanase activity presumably forms part of the hypersensitive defense mechanism of pepper plants and would be responsible for the induction of the resistance developed by the plant after treatment with the antagonists and infection by decapitation by *P. capsici*.

1. Introduction

Root rot in pepper (*Capsicum annuum*) caused by the oomycete *Phytophthora capsici* results in substantial losses worldwide. The disease is fatal; by the time the first symptoms appear, the tissues are already totally invaded and the plant dies within a few days. *P. capsici* produces different types of propagules that are involved in infection and dispersal. Zoospores are short-lived propagules that survive for short periods, generally from days to weeks. In contrast, sporangia and hyphae (vegetative stages of the pathogen) survive in soil for between 4 to 8 weeks. Oospores are the primary overwintering propagules and persist for longer [1]. Pepper (*Capsicum annum* L.), one of the most widely grown vegetables, is susceptible to root rot caused by *P. capsici*, and this disease can cause substantial crop losses [2]. Since pepper varieties with only intermediate resistance are known, the disease was controlled by soil fumigation with methyl bromide up to 2005, when this substance was banned [3]. Several of the chemicals used to manage this pathogen usually fail as a result of the development of fungicide resistance [4] or variable efficacy against the diverse propagules of the pathogen [5,6]. "Pathogenesis-

related" (PR) proteins have been described in plants infected with various types of potential pathogens: fungi, bacteria, viruses, and viroids [7]. The most widely used operational definition of PR proteins is that of polypeptides with relatively low molecular weights (M_r, 10,000- 40,000) that accumulate extracellularly in infected plant tissue, exhibit high resistance to proteolytic degradation, and often, but not always, possess extreme isoelectric points [7]. PR proteins have been studied in several systems with respect to physical properties, relationship to the corresponding mRNAs and cDNAs, and gene activation following pathogen infection or elicitor treatment [7-15]. However, the biochemical functions of PR proteins have not been reported. Plants possess innate defensive mechanisms which ensure their resistance or tolerance to certain pathogens. It was found possible to trigger these mechanisms to provide protection against pathogens to which the plants are normally susceptible. Systemic acquired resistance (SAR) is a plant resistant response to a microbial challenge as a result of induced signal transduction pathway. SAR results in broadspectrum resistance [16] and can be induced by chemicals like acibenzolar-S-methyl (ABM) [17], by the use of plant-associated bacteria [18] or by inoculation of avirulent strain of the same species [19]. Several studies have established the role of selected strains of nonpathogenic plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF) in enhancing plant resistance [20-21]. An example of PGPF are *Trichoderma* spp., which have recently been shown to induce local and systemic defense responses in cucumber [22-23] and other agricultural crops, such as cotton, tobacco, lettuce, and bell pepper [24-26]. Induced systemic resistance (ISR) [27-29], mediated by such nonpathogenic rhizospheric microorganisms, has been demonstrated in several plant species and shown to be effective against bacterial, viral, and fungal disease [30]. Plants have developed an arsenal of defense mechanisms to protect themselves against pathogen attacks. These include synthesis of pathogenesis-related (PR) proteins and phytoalexins, accumulation of reactive oxygen species (ROS), rapid alterations in cell walls and enhanced activity of various defense-related enzymes [31-35]. Plant peroxidases (POs) have been implicated in a variety of defense-related processes, including the hypersensitive response, lignification, cross-linking of phenolics and glycoprote in s, suber iz ation and phytoalexin production [36]. Close relationships have been found between enhanced levels of POs and resistance of plants upon infection with pathogens [37-39]. Differences in PO levels have been used as a biochemical marker for preliminary selection of different plant species resistant to different pathogens [40-41]. Furthermore, Lebeda and Dolezal [42] reported that PO zymograms should offer a quick and reliable method for discriminating specific cucumber genotypes with high levels of field resistance against cucumber downy mildew *Pseudoperonospora cubensis*. β-1,3-glucanases are one group of pathogenesis-related (PR) enzymes and the enhancement of such enzymes in different plant species in response to microbial pathogens infection has been estimated [43-45]. β-1,3-glucanases have been proposed to serve as antifungal properties by their ability to degrade isolated fungal cell walls and to inhibit growth of fungi *in vitro* [46]. A correlation between resistance and accumulation of β-1,3-glucanases in response to pathogen attacks has been recorded [47-49]. Karasuda *et al.*[50] have used the partially purified β-1,3-glucanase from sweet potato as a biocontrol agent instead of chemical fungicides for controlling the powdery mildew infecting strawberries and leaves.

Numerous bacteria and fungi are known to possess an antagonistic capacity against other microorganisms. Such antagonism is manifested in many different ways, including inhibiting the development of the pathogens responsible for causing disease. The antagonists can be exploited for biocontrol as long as their lack of pathogenicity towards plants or even animals and man can be demonstrated. Among the genera classified as antagonists are the bacteria *Pseudomonas* and *Bacillus* [51-56].

There are many studies reporting that biological control with genus *Trichoderma* is found to be effective in control of *R. solani* promoting plant growth as well as stimulating plant defense responses [57]. *Trichoderma* spp., are typically anaerobic, facultative, and cosmopolitan filamentous fungi that can be found in large numbers in agricultural soils and in other substrates such as decaying wood. The genus *Trichoderma* display a remarkable range of lifestyles and interactions with *R. solani* and can be used as biological control of plant diseases [58-59]

The main objectives of this study are to demonstrate the biochemical changes induced in pepper, *Capsicum annuum,* in response to *P. capsici* infection and purification and characterization of an isoenzyme of β-1,3-glucanase and the evaluation of the two antagonists against the pathogen and between themselves was carried out by dual *in vitro* interactions in several cultural media, at different pH and temperature conditions.

2. Material and Methods

2.1. Plant material

Pepper plants (*Capsicum annuum* cv. California wonder) were grown from seed previously disinfected with 5% sodium hypochlorite for 8 min and sown in alveolar trays containing a 2:1 mixture (v/v) of peat and sand that had been sterilized twice at 121°C for one hour on each of two consecutive days. The trays were placed in a

Fisons growth chamber with a 16 h photoperiod at 25°C and a relative humidity of 75–80%. They were watered with running water every three days until the plants had five leaves, at which stage they were used for experiments

2.2. Pathogenic fungi culture and plant inoculations

Phytophthora capsici isolate 17*,* isolated in our laboratory from diseased pepper plant, were cultivated in potato dextrose agar (PDA) *(Difco,* Detroit, USA) medium at 25°C and maintained in a freezer at 4°C. The tops of the stems of 90 plants (approximately with 5–6 true leaves) were cut off and inoculated with plugs of actively growing mycelium of *P. capsici*. The stems of control plants were inoculated in the same way, but only with culture medium (without mycelium) described previously by to Egea-Gilabert *et al*., [60].

2.3. Culturing antagonists

As antagonist, we used the fungus *Trichoderma harzianum*, strain 2413 from the Spanish type collection (CECT) in Valencia (Spain), was grown on potato dextrose agar (PDA) (Difco, Detroit). Synthetic medium for *T. harzianum* was prepared according to Ezziyyani *et al*., [3]. The inoculum consisted of 1 ml (109 spores, counted by hemocytometer) of 7-day-old *T. harzianum* cultured on PDA added to a 250-ml flask containing 100 ml of synthetic medium. The flask was shaken at 150 rpm for 24 h at 30°C to allow spore germination. The inoculum was then separated from the growth medium by centrifugation at 10,000 × g at 4°C and washed twice in 100 ml of distilled water. *T. harzianum* mycelial inoculum was added under aseptic conditions to the PGM of 7-day-old seedlings to a final concentration of about 105 germinated spores/ml of PGM. The bacterial strain *Burkholderia cepacia*, isolate 322 was also obtained from the Spanish type collection (CECT), Valencia (Spain) and kept on NA medium (Nutritive Agar, standard II, Merck). Depending on the type of assay to be carried out, the inocula and bacterial suspensions were prepared in solid or liquid medium to treat the seeds and plants by three different procedures described previously by to Ezziyyani *et al*., [51].

2.4. In vitro experiments

To check the feasibility of using the two microorganisms jointly, they were grown together and also along with *P. capsici*. To measure the degree of antagonism, two discs, each 5 mm in diameter, one covered with actively growing mycelium of *P. capsici* and one with either of the two organisms antagonistic to it, were placed 6 cm apart on either side of a Petri dish. The interactions were studied in Petri dishes (85 mm diameter) containing three different media: V8c, Czapek and water agar (WA), all adjusted to pH 5.6. A disc (5 mm diameter) from the edge of an actively growing *P. capsici* colony was transferred to each dish and a similar sized disc of *T. harzianum*, cut in the same manner, was placed at a distance of 4 cm. The dishes were incubated at 25ºC in darkness. As controls, discs of agar were added to similar plates inoculated with *P. capsici*. Any interactions were observed daily for six days using an optical microscope, noting any morphological changes and recording the inhibition according to the following formula : $I = 100-(100R_2/R_1)$

where I ¼ inhibition of vegetative growth of the fungi, R1¼ radius of the control colony in mm, and R2¼ the distance in mm travelled by the *P. capsici* colony over the *T. harzianum* colony. Such confrontations were prepared in triplicate and the experiment was repeated three times.

2.5. Protein analysis

After inoculation with *P. capsici* (3 and 9 days) assays were performed in the intercellular fluid (IF) and in the intracellular fraction (INTRA-F) of an area 0.5 cm below the necrotic zone of the stems. IF extraction as described by Alcazar et al., [58]. The INTRA-F extraction sections of IF-free stems were ground using a mortar and pestle in liquid nitrogen and the fine powder was extracted with 5 ml of 0.05 M acetate buffer, pH 5.0. The suspension was vigorously stirred in a tube mixer at maximum velocity for 2.5 min at 4°C. The resultant homogenate was filtered through two layers of cheesecloth and centrifuged at 2600 x g for 30 min at 4°C. The supernatants were stored at -20°C and used for INTRA-F protein analysis. Finaly the total protein was measured according to the method of Lowry [59]. Fifty plants were used for each treatment and the trials were repeated three times.

2.6. β-1,3-Glucanase Assay

β-1,3-glucanase Assay: Total β-1,3-glucanase activity was assayed colorimetrically using the laminarin-dinitrosalicylic method according to Abeles and Forrence [61] and modified by Ji and Kuc [62]. The reaction mixtures contained in 1.0 ml: 5 mg laminarin, 100 mM sodium acetate buffer, pH 5.5 and appropriate concentration of enzyme crude extract. The reaction mixtures were incubated for 1 h at 37^{o}C, 0.5 ml of dinitrosalicylic reagent that is prepared according to Fischer and Kohtes [63] was added followed by heating.

2.7. Isoelectric focusing and electrophoresis

The proteins from IF and INTRA-F were analysed by isoelectric focusing (IEF) and PAGE analysis of acidic and basic proteins under native conditions using the Phast System separation equipment (Pharmacia, Uppsala, Sweden). IEF was performed on pH 3–9 gradient gels, prefoc using was run at 5 W, 2000 V and 2.5 mA at 15°C for 75 V h. Native PAGE was performed on Phast Gel Gradient 8–25 and the buffer strips for native PAGE of acidic proteins were from Pharma-cia. For PAGE of basic proteins under native conditions, buffer strips were made according to Phast System Application File No. 300 (Pharmacia) [58]. A total of 5 µg protein was applied per lane. After IEF or native PAGE, the gels were washed with water, incubated with 0.05M sodium acetate (pH 5.0) for 5 min, and then incubated at 40°C for 30 min in a mixture containing 75ml of 0.05 M sodium acetate (pH 5.0) and 1 g of laminarin dissolved in 75 ml of water by heating in a boiling water bath

2.8. Densitometry and quantification of β-1,3-glucanase isoenzymes

The stained gels were scanned by transmittance at 540 nm using a PhastImage densitometer (Pharmacia).The different β-1,3-glucanase isoenzymes appearing in the gels were quantified by determining the percentage of the area of each band with respect to total peak area, which referred to the enzymatic activity deposited in each lane.

2.9. Data analysis

To compare the results, all data were subjected to analysis of variance (ANOVA) using Statgraphics Plus for Windows. Means were separated with the LSD multiple range test ($P < 0.05$).

3. Results and discussion

Vegetative growth of the mycelium of the pathogen was inhibited *in vitro*. *B. cepacia* produced a zone of inhibition with the *P. capsici* (Fig. 1).

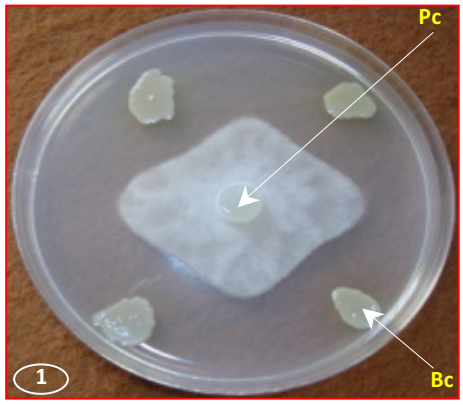

Figure 1: Inhibition of the vegetative growth of *P. capsici* by *B. cepacia* (Bc) on PDA.
Data calculated using the Samaniego formula, where PICR: $I = 100-(100R_2/R_1)$

T. harzianum was capable to invading the whole surface of the pathogen colony. *T. harzianum* grows rapidly at the outset and then invades the colony of *P. capsici* by a marked process of hyperparasitism. The arrows indicate the interaction zones (hyperparasitism) (Fig. 2).

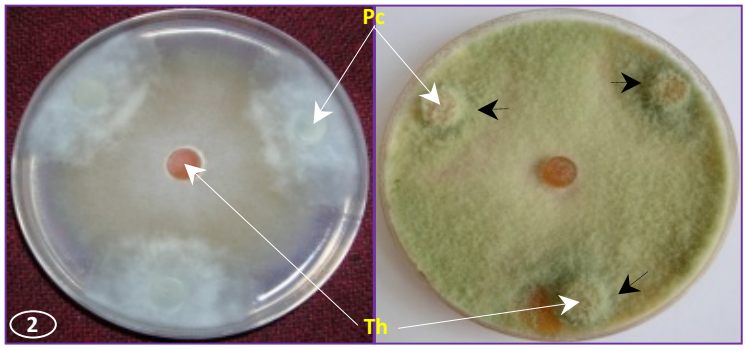

Figure 2: Inhibition of the vegetative growth of *P. capsici* by *T. harzianum* (Th) on PDA.
Data calculated using the Samaniego formula, where PICR: $I = 100-(100R_2/R_1)$

In plates seeded simultaneously with *T. harzianum* and *B. cepacia* the fungal mycelium surrounded the bacterial colony, demonstrating their compatibility. The interaction between the antagonist's *B. cepacia* and *T. harzianum* indicating good compatibility between them. The arrows indicate the interaction zones of the microorganisms.

B. cepacia and *T. harzianum* despite belonging can be added together to produce a greater effect on the pathogen *P. capsici*. (Figs. 3-4).

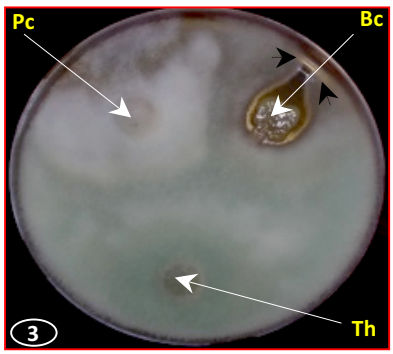

Figure 3: Inhibition of the vegetative growth of *P. capsici* by *B. cepacia* and *T. harzianum*. Data calculated using the Samaniego formula, where PICR: $I = 100-(100R_2/R_1)$

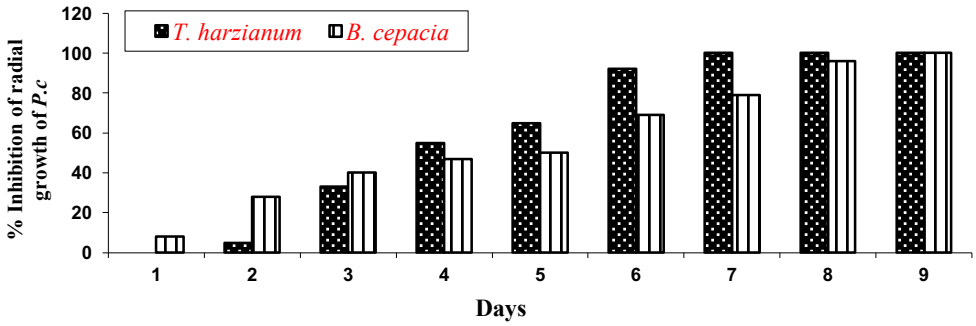

Figure 4. The percentage of inhibition of radial growth of *P.capcisi* versus the antagonists. Data calculated using the Samaniego formula, where PICR = (R1-R2) / R1x100

Pseudomonas fluorescens and *Trichoderma* spp. are among the most commonly used biocontrol agents (BCAs) against plant diseases [64]. In addition to the suppressive action against target pathogens, the application of these BCAs triggers or activates latent defense mechanisms in plants [65]. Induced resistance may be defined as a physiological state of enhanced defensive capacity elicited in response to specific environmental stimuli and consequently the plant's innate defenses are potentiated against subsequent biotic challenges [66-67]. Biopriming plants with some plant growth promoting rhizobacteria can also provide systemic resistance against a broad spectrum of plant pathogens. Total β-1,3-glucanase activity was assayed colorimetrically in the intercellular fraction from stems and roots of both pepper cultivars at 0, 3 and 9 days after infection with *P. capsici* in pepper plants and treated with *B. cepacia* and *T. harzianum* in combination (Table 1).

Table 1: Time course of total β-1,3-glucanase activity (nkat mg^{-1} protein) in the INTRA-F of stems and roots at 0, 3 and 9 days after inoculation with *P. capsici* and treatments with *B. cepacia* and *T. harzianum* in combination. 0 days: non-inoculated and non-treated plants

Time after inoculation (days)	Roots	Stems
0	0	0
3+-	22+-	5+-
9+-	41+-	12+-

The total β-1,3-glucanase activity increases with inoculation time although in a different way, although the initial values of both stem and root activity are practically the same. At three days after treatment with the combination of the antagonists, the activity increases exponentially. At nine days of treatment, the activity

begins to grow slightly in the stems and continues to increase in roots of the treated plants, but stabilizes in stems and roots of control plants (Figure 5).

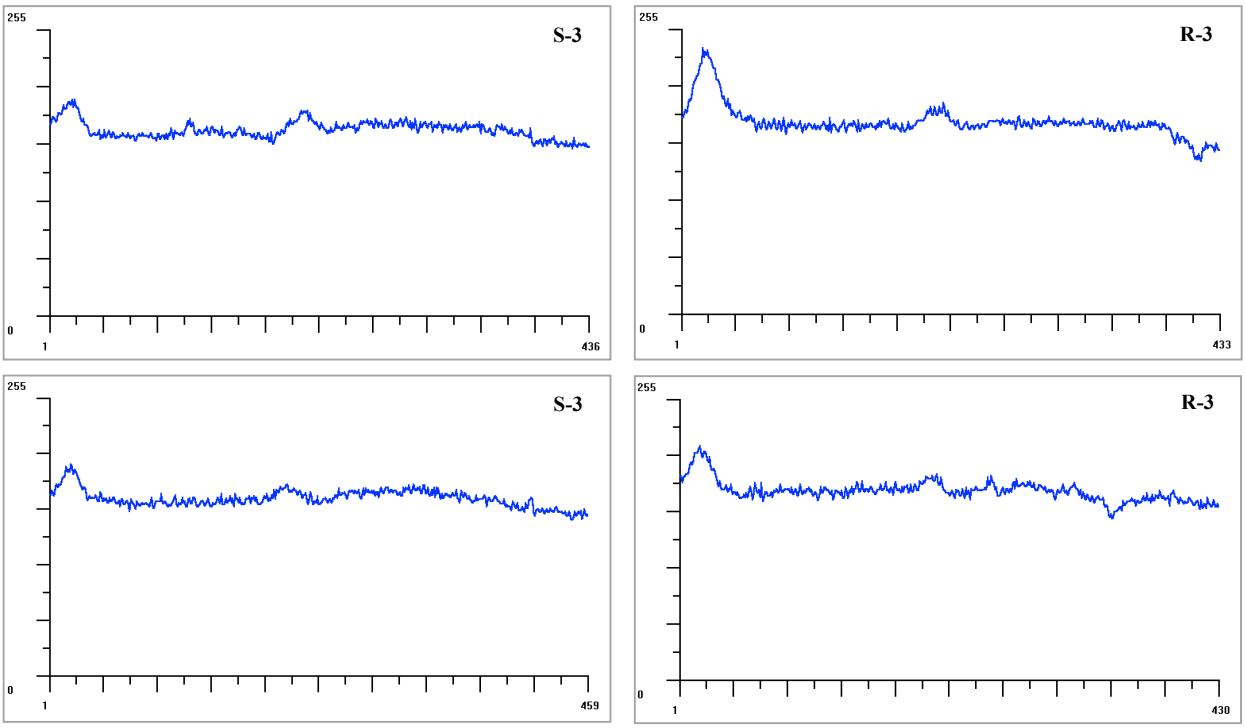

Figure 5 : Densitometry and quantification of β-1,3-glucanase isoenzymes. Changes in β-1,3-Glucanase activity with time for each isozyme in INTRA-F of pepper stems (S) and roots (R); Three (3) days after infection with *P. capsici* and treated with *B. cepacia* and *T. harzianum* in combination

Total β-1,3-glucanase activity in the INTRA-F of stems and roots at 3 and 9 days after inoculation with *P. capsici* and treatments with *B. cepacia* and *T. harzianum* in combination. After separation by IEF, the induction of three β-1,3-glucanases, two basic of (pI 9.0 and pI 7.8) and one acidic of pI 4.5, was detected in the intercellular fraction in stems and roots treated with antagonistic's 3 and 9 days after inoculation but which differ in their concentration. Subsequent separationby native PAGE again revealed revealed the presence of a common isoenzyme at the two treatment times (Rf 0.68) and an additional exclusive, of the stems of the plants treated with the combination 9 days, Rf 0.21. The isoenzymes separated by Native-Page acids have both the same pI of 4.5. Native-Page for basic proteins revealed the presence of two isoenzymes (Rf 0.23 and 0.53) in stems from Plants treated with the combination at both 3 and 9 days. These isoenzymes have, one of them a pI 9 and the other a pI 7.8. The main relevance is that although it is detected in all the stems (treated and control) its concentration increases, mainly with the time of treatment. That is, the plant detects the existence of the antagonist combination and maintains the enzyme synthesis while the interaction continues. Most significant is the higher concentration of the β-1,3-glucanases extracted in roots compared to those obtained in stems and especially at 9 days of treatment (Fig. 6).

The increase in glucanase activity is therefore one of the components of the mechanisms involved in the biocontrol of *P. capsici*. Developed by adding the combination of the antagonists, to the growing soil of pepper plants.In short, nine days after treatment, β-1,3-Glucanase activity increases about three times as much in stems and about five times more in roots than plants without treatment. These data confirm the strong implication of β-1,3-glucanase activity as part of the plant reaction to treatment with the combination. This reaction should form part of the defense mechanism against the interaction of microorganisms added to their rhizosphere and that will eventually increase their resistance capacity against pathogens such as *P. capsici* that have glucan in their cell walls. The increase in glucanase activity could be directly related to the defensive response of plants as we observed an increase in total activity, both in stems and in plant roots treated with the combination of the antagonists. It is significant however those only new glucanase isoenzymes are detected in roots and stems only to increase the activity of the constitutive ones. This may mean that the plant develops a specific reaction in the direct interaction in the roots, and nonspecific and systemic in stems. Most notable is the induction of the pI 4.5 isoenzyme only in roots treated with the combination and absent in control roots. This increase in glucanase activity favors the resistance of the plant to subsequent infection by *P. capsici* telluric fungus and that its main area of infection is the rhizosphere of the plant. Most noteworthy and the fortunate finding is that when the

plants are treated with the combination of the antagonists, they produce a direct and systemic reaction that increases the concentration of proteins related to the pathogenesis and, specifically, of enzymes with glucanase activity. This reaction is very favorable for the increase of resistance against the attack of oomycetes that do not contain chitin but glucan in its walls like *P. capsici* than the fungus object of our study.

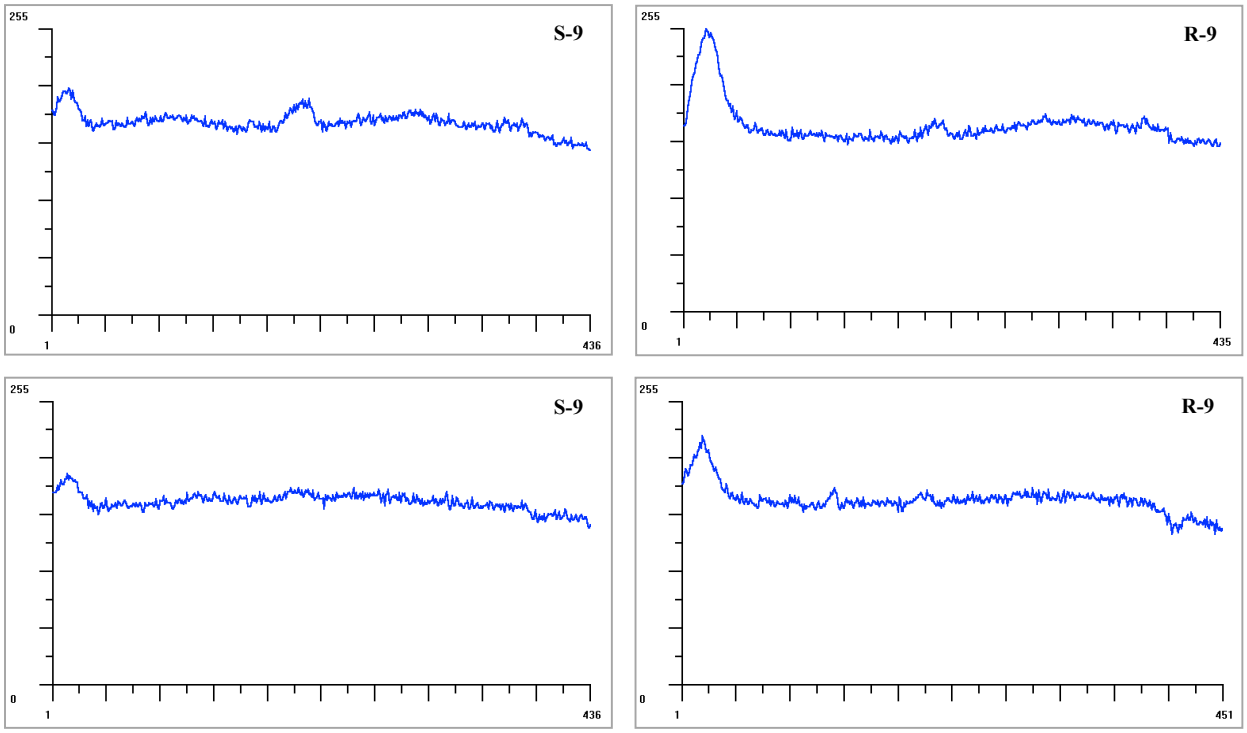

Figure 6 : Densitometry and quantification of β-1,3-glucanase isoenzymes. Changes in β-1,3-Glucanase activity with time for each isozyme in INTRA-F of pepper stems (S) and roots (R); Nine (9) days after infection with *P. capsici* and treated with *B. cepacia* and *T. harzianum* in combination

Proteins with β-1,3-glucanase activity are considered to be part of the defense response of plants to fungal pathogens, presumably by inhibiting growth through their hydrolytic capacity [68]. The finding of proteins showing β-1,3-glucanase activity in both fractions agrees with the results obtained by otherauthors working with similar systems. For example, Sela-Buurlage et al., [69] detected several isoenzymes with glucanase activity in tobacco plants infected by *Fusarium solani*. Kim et al., [70] detected the accumulation of β-1,3-glucanases in pepper-*P. capsici* interaction and they implicated these hydrolases in the disease resistance, because they were found to accumulate earlier and to a higher extent in incompatible rather than compatible interactions. Of authors observed an inter- and intracellular β-1,3-glucanase activity increase in tobacco cells inoculated with *Phytophthora nicotianae*, strongly suggesting the defensive role of this type of hydrolase against pathogenic attack. The role of β-1,3-glucanases in defense in this interaction is supported by the evidence that their transcription is induced by pathogen attack. Although it has not been determined which of the isoforms correspond to the cloned β-1,3-glucanase, expression of this gene is clearly induced in the resistant cultivar. Interestingly, transcription is also induced in the susceptible cultivar, but the zone in which transcription is occurring corresponds to the zone into which the disease has progressed. The detection of time-dependent induction of β-1,3-glucanase in the stem and root of pepper, supports the hypothesis that this enzyme may be involved in the reaction against fungal infection and treated with *B. cepacia* and *T. harzianum* in combination. These results agree with the findings of Hyong et al., [71], who worked with the same plant-pathogen interaction, but using using only one pepper cultivar and two isolates of *P. capsici.* Induced resistance is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli [72]. In 1991, the research groups of B. Schippers in Baarn, The Netherlands, and J. W. Kloepper in Auburn, AL, discovered independently that induced systemic resistance (ISR) is a mode of action of plant growth-promoting rhizobacteria (PGPR), especially fluorescent *Pseudomonads*, in suppressing diseases[73-74]. The *Pseudomonas* bacteria were inoculated into the rhizosphere and remained spatially separated from the pathogen that was inoculated on the aboveground plant parts, either into the stem [75] or on the leaf surface [76]. By ensuring spatial separation between the *Pseudomonas* bacteria and the pathogen on the root system, for instance in a split root system, it was demonstrated that ISR is also effective against root-infecting pathogens [77-78]. A threshold

population density of 105 colony forming units per gram of root was required for effectiveness of the resistance-inducing *Pseudomonas* strain [79]. When used under commercial greenhouse conditions, the ISR triggering *P. fluorescens* strain WCS374r significantly protected radish from *Fusarium* wilt leading to average yield increases of 40% [80]. In the last decade it has become clear that elicitation of ISR is a widespread phenomenon, not only for fluorescent *Pseudomonads* but for a variety of nonpathogenic microorganisms and biological control agents. ISR is phenotypically similar to systemic acquired resistance (SAR) that is triggered by necrotizing pathogens in that disease caused by a challenging pathogen is reduced. Improving the effectiveness of biological control by fluorescent *Pseudomonas* spp. may be established by using combinations of strains that have different mechanisms of disease suppression, such as competition for iron and ISR [81]. Combining SA-dependent and SA-independent ISR is another possibility to increase effectiveness [82]. Several *Pseudomonas* strains produce SA under conditions of low iron availability and potentially are able to induce the SA-dependent signal transduction pathway. However, ISR by these SA-producing strains does not appear to depend on SA, and it is speculated that in most cases the SA is channeled into SA-containing siderophores [83-85]. Manipulating SA production in these bacteria by either uncoupling SA production from the biosynthesis of SA-containing siderophores [83] or by transfer of SA biosynthesis genes into non-SA-producers seems effective. Production of SA by strains that already possess determinants that effectively trigger SA-independent ISR may create strains that induce both signal transduction pathways simultaneously. Plant diseases caused by *Phytophthora spp.* are very crucial yield determinants in several horticultural crops. Use of antagonists such as *Trichoderma* and *Pseudomonas* is being explored for the management of many of the diseases. Selected strains of *Trichoderma* species are potent inducers of plant defense responses. These responses are systemic and are termed as induced systemic resistance (ISR). Unlike systemic acquired resistance (SAR) elicited by inducers of pathogen origin, ISR induced by biocontrol agent does not result in hypersensitive reaction, plant cell necrosis or phytotoxity [85] . Zong and Bing Sheng [86] demonstrated that application of *T. harzianum* T39 to soil instead of spraying resulted in a 75-90% reduction in *Sphaerotheca fusca* coverage on the leaves of green house cucumbers showing that the mode of action of *T. harzianum* T39 in powdery mildew control was induced resistance, not mycoparasitism or antibiotic action, and reported that photosynthesis and chlorophyll content in cotton seedlings increased with *T. koningii* treatment. Hanania *et al*., [87] observed that challenging tomato or tobacco varieties with ethylene inducing xylanase (EIX) from *T. viride* caused rapid induction of plant defense responses leading to programmed cell death. *Trichoderma* spp. are also suppressing *R. solani* by producing antifungal compounds. The antifungal compounds include antibiotics, mycotoxins and lower weight secondary compounds. *Trichoderma* spp. are also well knowing plant growth regulators. They proliferate root and increase the yield by uptake of nutrients [87]. As compared to fungicides the effect of *Trichoderma* spp. against *R. solani*is higher because it persists in soil for a longer period after application. The fungal cell wall degrading enzyme exo-β-1,3-glucanase was encoded by another gene and this enzyme showed strong parasitic activity against *R. solani*. This gene was isolated from T. aspererllum and characterized. The expression analysis of this gene was studied using real-time and reverse transcription-polymerase chain reaction (RT-PCR). Two various kinds of glucanases (β-1,3 and β-1,6 glucanase genes) isolated from *T. virens* found that these genes secrete cell wall degrading enzyme that helps in the biocontrol activity against *R. solani*. *T. virens* GV29.8 wild type and double over expression (DOE) transformant strains were used to detect the enzyme activity against pathogens like *R. solani* [88]. *Trichoderma* strains are well known for their ability to colonize roots, but *Trichoderma* conidia have also been applied to fruit, flowers and foliage, and plant diseases can be controlled by their application to any of these sites [89-90]. Crucial components of the associations that are considered in this review are microorganism–plant interactions. *Trichoderma* spp., and other beneficial root-colonizing microorganisms, also enhance plant growth and productivity. Intuitively, this might seem counterproductive, as most of these species also induce resistance in plants, and switching on resistance pathways must be energetically expensive to the plant. However, many resistance-inducing fungi and bacteria do increase both shoot and root growth. The specific examples that follow are from research on *Trichoderma*, but many other organisms also have similar effects; in fact, resistance-inducing rhizobacteria are widely known as plant-growth-promoting rhizobacteria [91]. At least some non-pathogenic root-colonizing fungi also have similar abilities [92]. Several studies have shown that root colonization by *Trichoderma* strains results in increased levels of defence-related plant enzymes, including various peroxidases, chitinases, β-1,3-glucanases, and the lipoxygenase-pathway hydroperoxide lyase [93]. In cucumber, the addition of *Trichoderma asperellum* T-203 led to a transient increase in the production of phenylalanine ammonia lyase in both shoots and roots, but within 2 days this effect decreased to background levels in both organs. However, if leaves were subsequently inoculated with the bacterial pathogen *Pseudomonas syringae* pv. *lachrymans*, the expression of many defence-related genes increased several times over. In the environment, secreted glucanases are positioned potentially to hydrolyze polysaccharides of other fungal cell walls, perhaps as a source of glucose which can serve metabolic needs. While we cannot exclude

such a role for *Histoplasma* Eng1 and/or Exg8, our data indicates that Eng1 and Exg8 have likely evolved to facilitate *Histoplasma* pathogenesis. First, Eng1and Exg8 are expressed at high levels by pathogenic yeasts but are only minimally expressed by the environmental mycelial form [94]suggesting Eng1 and Exg8 function specifically in yeast cell biology (pathogenesis as opposed to the saprobic mycelia). Second, during infection, *Histoplasma* yeasts are found almost exclusively within the phagosome of host phagocytes in which *Histoplasma* is the only fungal cell present, making it unlikely that Eng1 and Exg8 act on other fungi. Third, Eng1 and Exg8 have evolved to be compatible with conditions within the host phagosome, whereas glucanase activity is reduced in acidic conditions, common of *Histoplasma*-containing soils. Finally, Eng1 in particular has been shown to be important for pathogenesis by reducing surface exposure of yeast cell wall β-glucans [95].

Conclusion

In the present study, application of the saprophytic fungus *T. harzianum* and *B. cepacia* in combination compatible to the rhizosphere of young pepper seedlings initiated in the plants a series of morphological (slowing down of necrosis, whereas the stem of was only partially invaded) as well as biochemical changes (production of PR-proteins with β-1,3-glucanase activity) which are considered to be part of the plant defense response. Biochemical analyses revealed that inoculation with *T. harzianum* and *B. cepacia* in combination initiated increased β-1,3-glucanase activity with in 3 and 9 days, respectively. These results were observed for both the roots and the stems, providing evidence that *T. harzianum* and *B. cepacia* may induce systemic resistance mechanisms in pepper plants. The results presented here demonstrate that striking modifications of epidermal and cortical cell walls, as well as deposition of newly formed barriers, are triggered in pepper root tissues by colonization. To our knowledge, this study is the first to provide evidence that *T. harzianum* and *B. cepacia* in combination penetrates the root system without causing extensive damage and triggers the transient elaboration of host defense reactions. As with immunization, *T. harzianum* and *B. cepacia* in combination inoculated plants may be sensitized to respond faster and to a greater extent to potential pathogen attacks.

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