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Biocontrol mechanisms of *Trichoderma harzianum* against soybean charcoal rot caused by *Macrophomina phaseolina*

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Abstract: Throughout the world, charcoal rot, caused by *Macrophomina phaseolina*, is one of the most destructive and widespread diseases of crop plants such as soybean. In this study, the biological control capability of 11 *Trichoderma* spp. isolates against *M. phaseolina* was investigated using screening tests. Among all the tested *Trichoderma* spp. isolates, inhibition varied from 20.22 to 58.67% in dual culture tests. Dual culture, volatile and non-volatile tests revealed that two isolates of *Trichoderma harzianum* (including the isolates T7 and T14) best inhibited the growth of *M. phaseolina in vitro*. Therefore, these isolates were selected for biocontrol of *M. phaseolina in vivo*. The results of greenhouse experiments revealed that disease severity in the seed treatment with *T. harzianum* isolates was significantly lower than that of the soil treatment. In most of the cases, though, soil treatment with *T. harzianum* resulted in higher plant growth parameters, such as root and shoot weight. The effects of *T. harzianum* isolates on the activity of peroxidase enzyme and phenolic contents of the soybean root in the presence and absence of *M. phaseolina* were determined in greenhouse conditions. Our results suggested that a part of the inhibitory effect of *T. harzianum* isolates on soybean charcoal rot might be related to the indirect influence on *M. phaseolina*. Plant defense responses were activated as an elicitor in addition to the direct effect on the pathogen growth.

Key words: biocontrol, *Macrophomina phaseolina*, peroxidase, total phenol, *Trichoderma harzianum*

Introduction

Soybean (*Glycine max* L.) is one of the most important crops. It is a source of vegetable protein and oil (Herridge *et al*. 2008; Prévost *et al*. 2010). Soybean, though, suffers from various diseases. Soybean charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goidanich is one of the most important diseases of soybean in tropical and subtropical regions of the world resulting in reduced yields and seed quality. In Iran, charcoal rot is an economically important disease of various oilseed plants in northern regions, especially in the provinces of Golestan and Mazandaran (Rayatpanah *et al*. 2011). *Macrophomina phaseolina* is a necrotrophic phytopathogen with a wide host range including more than 500 cultivated and wild plant species belonging to more than 75 families (Khan 2007; Salik 2007). Many of the species are economically important crops, such as corn, sorghum, bean, cotton, sesame, sunflower, melon, tobacco, and safflower, in addition to soybean (Su *et al*. 2001; Purkayastha *et al*. 2006; Singh *et al*. 2008). Although only one species is recognised within the genus *Macrophomina*, great variability in morphology and pathogenicity was recognised among isolates from different hosts (Fernandez *et al*. 2006). It mainly produces either microsclerotia or pycnidia. The fungus is not only soil-borne, but also seed-borne and infects plants from seedling stage to maturity (Purkayastha *et al*. 2006). *Macrophomina phaseolina* is considered a high-temperature pathogen. Disease severity increases as the air and soil temperatures increase from 28 to 35°C, and when soil moisture is limited (Pearson *et al*. 1984; Gary *et al*. 1991). Under favorable conditions, the fungus causes many diseases like damping off, seedling blight, collar rot, stem rot, charcoal rot, and root rot, in various economically important crops (Babu *et al*. 2007). Estimates of yield losses due to charcoal rot in the United States were 2.5 × 10, 3.0 × × 10, 2.2 × 10 and 0.9 × 10 million metric tons in 2006, 2007, 2008, and 2009, respectively. This disease ranked in the top six of diseases among economically important soybean diseases in the United States from 2006–2009 (Koenning and Wrather 2010).

Charcoal root rot may be a difficult disease to control because of the nature of causal pathogen. *Macrophomina phaseolina* invades the roots of a host at an early stage, 1–2 weeks after planting, but the symptoms appear only in mature plants (Pearson *et al*. 1984). Efforts to control soybean charcoal rot by adjusting planting dates, crop rotation, planting densities, and irrigation have all been suggested as important ways of disease management (Bowen and Schapaugh 1989; Todd 1993). Host resistance may be a practicable method to manage this destructive disease. However, soybean genotypes with high levels of resistance have not been identified (Mengistu *et al*. 2007). Other management options are needed. Chemical fungicides are being replaced with biocontrol agents because of the emergence of fungicide-resistant fungal isolates and public concerns regarding the health and environ-

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mental impacts of these chemicals. During the past few decades, several potential biocontrol organisms have been isolated, characterised, and commercialised. Thus, biocontrol of plant diseases has received more consideration in disease management strategies (Shali *et al*. 2010).

Trichoderma spp. are considered as potential biocontrol agents and growth promoting fungi for many crop plants (Verma *et al*. 2007; Bai *et al*. 2008; Savazzini *et al*. 2009). The genus *Trichoderma* comprises a great number of fungal species. The antagonistic properties of these species are based on the activation of multiple mechanisms. *Trichoderma* spp. exert biocontrol activity against fungal phytopathogens either indirectly, by competing for nutrients and space, which modify mechanisms and antibiosis, or directly, by mechanisms such as mycoparasitism (Benitez *et al*. 2004). These biocontrol agents are effective against soil and/or seed borne fungal diseases of various plants (Kubicek *et al*. 2001). It is relatively easy for *Trichoderma* populations to be established in different types of soil and the populations can continue to persist at detectable levels for several months (Verma *et al*. 2007). Control of several soil borne fungal pathogens using *Trichoderma harzianum* has been reported by many investigators (Zeidan *et al*. 2005). Yet there is little information available on the use of *T. viride* (Kehri and Chandra 1991) and *T. harzianum* (Elad *et al*. 1986; Adekunle *et al*. 2001) as biocontrol agents against *M. phaseolina* in different plants. Aly and associates (2007) reviewed differential antagonism of *Trichoderma* sp. against *M. phaseolina*. The percentage of the melon plant in the commercial *T. harzianum* (Bi) + *M. phaseolina* treatment (96.7%) was greater than that of *M. phaseolina* inoculation alone (46.7%) and demonstrated the best results among various *Trichoderma* spp. in the control of melon charcoal stem rot (Etebarian 2006). Singh and associates (2004) suggested that cell surface hydrophobicity and the cell surface electrostatic charge of mycoparasitic *Trichoderma* spp. may contribute to non-specific adhesion onto the sclerotial surfaces of *M. phaseolina* that may be influenced by growth and environmental conditions. Studies of Sreedevi and associates (2011) revealed the antifungal activity of *T. harzianum* and *T. viride* isolates against *M. phaseolina*, the causal agent of groundnut root rot.

Induction of plant defense responses is believed to be one of the most important mechanisms for the biocontrol effects of *Trichoderma* spp. (Harman 2006). In addition to pre-formed physical and chemical barriers, plants have an inducible immune system. This system is capable of detecting motifs or domains with conserved structural traits typical of entire classes of microbes but not present in their host plants, namely the pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively). MAMP-triggered plant defense responses are elicited rapidly and transiently. Early MAMP responses involve ion fluxes across the plasma membrane, the generation of reactive oxygen species (ROS), nitric oxide, various phytohormones, priming several defense-related genes, activation of corresponding proteins and also, but later, the deposition of callose and biosynthesis of antimicrobial compounds such as phenolics. Effective *Trichoderma* spp. isolates produce a variety of MAMPs which are the most widely described among plant-beneficial fungi so far (reviewed by Lorito *et al*. 2010; Hermosa *et al*. 2012). Various species of this powerful biocontrol agent induce cellular and molecular changes in plants that increase resistance to a variety of biotic and abiotic stresses (Brotman *et al*. 2013; Kumar 2013). The activity of defense-related enzymes such as peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase were reported to be significantly increased in greengram plants treated with a *T. viride* alone or in combination with *Pseudomonas fluorescens* as biocontrol agents of *M. phaseolina* (Thilagavathi *et al*. 2007). Treating the tomato plants with the use of *T. arundinaceum* resulted in priming the expression of defense-related genes belonging to the salicylic acid (SA) and jasmonic acid (JA) pathways against *Botrytis cinerea* and *Rhizoctonia solani* (Malmierca *et al*. 2012).

There are a few investigations on the biological control capability of *T. harzianum* against soybean charcoal rot (Taliei *et al*. 2012; Vasebi *et al*. 2013). So far, to our knowledge, there has not been any report on the involvement of priming plant defense responses by *Trichoderma* spp. as a mechanism of their biocontrol against *M. phaseolina* in soybean plants. Therefore, the objectives of the present investigation were (i) to screen and select effective *Trichoderma* spp. isolates against the soybean charcoal rot pathogen *M. phaseolina*, and (ii) to determine the role of selected *Trichoderma* spp. isolates, which were capable of controlling the pathogen *in vitro*, in activating soybean defense responses such as peroxidase activity and total phenolics as a part of mechanisms involved in *Trichoderma*-induced resistance in our pathosystem.

Materials and Methods

Antagonistic and phytopathogenic fungal isolates

Five isolates of *T. harzianum* and six *T. viride* isolates (obtained from the culture collection of plant pathology laboratory, Abureihan Campus, University of Tehran, Iran) were used in this study (Table 1). The phytopathogenic fungus, *M. phaseolina,* which was isolated from infested soybean Golestan Province, Iran, was also obtained from the same culture collection. All fungi were maintained on Potato Dextrose Agar (PDA) medium and sub-cultured monthly.

Table 1. List of *Trichoderma* spp. isolates used in this study

Genus and species	Isolate
Trichoderma harzianum	T7
T. harzianum	T8
T. harzianum	T14
T. harzianum	BI
T. harzianum	T5
Trichoderma viride	T6
T. viride	T10
T. viride	T17
T. viride	T20
T. viride	T23
T. viride	T24

Evaluation of antagonistic activity of *Trichoderma* spp. isolates on mycelial growth of *Macrophomina phaseolina in vitro*

Dual culture technique

All of the *T. harzianum* and *T. viride* isolates were screened individually against *M. phaseolina* by employing the dual culture technique described by Asran-Amal *et al*. (2010) with a few modifications. The *Trichoderma* spp. isolates and *M. phaseolina* were cultured, separately, on PDA medium for 7 days at 25±1°C. Four-day old *Trichoderma* spp. cultures were inoculated on one side of a PDA plate and *M. phaseolina* cultures were inoculated at the opposite side of the Petri dish and the plates were incubated at 25±1°C for 6 days. The distance between discs was approximately 5 cm. In the control treatment, a sterile agar disc (5 mm diameter) was placed in the dish instead of *Trichoderma* spp. isolates. There were four replicates for each treatment and the experiment was repeated three times. At the end of the incubation period, the radial growth of the pathogen was measured. The efficiency of *Trichoderma* spp. isolates to suppress radial growth was calculated as described by Asran-Amal *et al*. (2010) using the formula:

$$L = \frac{C - T}{C} \times 100,$$

where: L – inhibition of radial mycelial growth; C – the radial growth measurement of the pathogen in the control; T – the radial growth measurement of the pathogen in the presence of antagonists.

Production of volatile metabolites

Two selected *T. harzianum* isolates based on the mycelial growth inhibition assay against *M. phaseolina* were evaluated for the possibility of producing volatile inhibitory substances *in vitro* following the modified methods of Dennis and Webster (1971a). Briefly, a 5 mm diameter disc from the margin of the *T. harzianum* colony was placed in the centre of the Petri dishes containing PDA medium and each treatment had four replications. The Petri plates were sealed at the edges and incubated at 25±1°C. After 5 days, *M. phaseolina* was inoculated on fresh PDA and the lids of the Petri plates inoculated with antagonist were replaced by *M. phaseolina* on PDA. The plates were fixed with cellophane-tape and incubated at 25±2°C. The control plates were only inoculated with *M. phaseolina*. The radial growth of *M. phaseolina* was recorded after 7 days of incubation. The percentage inhibition was calculated in relation to the control, by the above mentioned formula.

Production of non-volatile metabolites

The production of non-volatile substances by *T. harzianum* isolates against *M. phaseolina* was studied using the modified methods described by Dennis and Webster (1971b). The *T. harzianum* isolates were inoculated in 100 ml sterilised Potato Dextrose Broth (PDB, Merck, Germany) in 250 ml conical flasks, and incubated at 25±1°C on a rotatory shaker set at 100 rpm for 15 days. The control flasks were not inoculated with any of the antagonistic isolates. The culture was filtered through Whatman filter paper for removing mycelial mats. Then, the culture was sterilised by passing through a 0.2 μm pore biological membrane filter. The filtrate was added to molten PDA medium (at 40±3°C) to obtain a final concentration of 10% (v/v). The PDA containing Petri dishes were inoculated with mycelial plugs (5 mm diameter) of *M. phaseolina* at the centres. The dishes were incubated at 25±2°C until the colony reached the plate edge. There were four replicates for each treatment and the experiment was repeated three times. Then, colony diameters and percentage inhibition were calculated in relation to the control by the above-mentioned formula.

Preparation of *Macrophomina phaseolina* inoculum

Inoculum preparation was carried out using the method described by Nerey *et al*. (2010) with a few modifications. Briefly, disks which were 5 mm in diameter were taken from a 4-day old colony of *M. phaseolina* grown on PDA at 25±1°C, and placed into flasks containing millet grains, previously autoclaved at 121°C for 21 min on 2 successive days. The flasks were incubated for 10 days at 25±1°C in the dark and shaken every 2–3 days.

Greenhouse experiments

Experiments were conducted using soil and seed treatments. The soil used in this experiment was a combination of clay, sand, and farmyard manure with the ratio of 1 : 1 : 1 (v/v). For treatments with autoclaved soil, the soil was autoclaved at 121°C for a minimum of 30 min at 100 kPa (15 psi) on 2 successive days.

To obtain a conidial suspension of *T. harzianum*, the fungus was grown on PDA for 7 days at 25±2°C. Conidia were harvested from the surface of the plates and washed in sterile distilled water and suspended in 0.05% tween 20. The fungal spore concentration was adjusted to 10^5 spores · ml^{-1}. Seeds of soybean cv. DPX were surface disinfected by soaking the seeds in 1% sodium hypochlorite for 3 min, then rinsing the seeds three times in sterile distilled water. The seeds were soaked in 10 ml of Trichoderma spore suspension (with a concentration of 10^5 spores · ml^{-1}) and shaken using an electric shaker for 0.5 h at room temperature, then air dried in a laminar flow hood. For adhering the conidia to the seed surface, methyl cellulose was used. For methyl cellulose coatings, conidial suspensions were added (1/1) to 2% methyl cellulose.

For soil treatment, the surface-sterilised seeds were incubated for 3 days on a wet sterile filter paper in Petri dishes at 28±1°C on distilled water-soaked filter papers. Then, the seeds were each sown in 15 cm-diameter plastic pots. Inoculation of soybean plants by the pathogen was carried out on the stem near the soil-line, using seven-day-old seedlings. Colonised millet grains were filled 1 cm above the soil surface, which contained 3 g of millet grain inoculums per pot. Sterilised millet grains which were placed on PDA without the fungus served as the control. Inoculated plants were grown at 28±1°C for 4 weeks in a greenhouse. In all of the cases in which

lesions developed, the pathogen was re-isolated from the infected plants. Four replicate plants were inoculated in a completely randomised design and the experiment was repeated twice.

Five weeks after planting, the plants were removed from their pots and washed with running tap water. Then, disease severity was estimated using the disease scale (0 to 100) as described by Persson *et al*. (1997). Disease severity (*DS*) was estimated using the formula:

$$DS = \sum (XiNi)/N,$$

where: *X* – the disease grade (0, 5, 10, 25, 50, 75, and 100), *Ni* – represented the number of diseased plants on the *i*-th grade of the disease scale, and *N* – the total number of plants evaluated.

In addition, the average stem height and the wet and dry weights of soybean roots and shoots, were recorded 5 weeks after planting.

Effect of *Trichoderma harzianum* isolates on phenolic accumulations and peroxidase (POX) activity

For elucidating the mode of action of two *T. harzianum* isolates (T7 and T14) *in vivo*, we examined the phenolic accumulations and POX activity as part of the plant defense responses. The effect of treating the soybean seeds with *T. harzianum* isolates on the accumulation of total phenolics and POX activity, were evaluated using six treatments:

1. Treatment H – healthy plants;
2. Treatment M – plants treated with *M. phaseolina*;
3. Treatment T7 – plants treated with *T. harzianum* T7;
4. Treatment T14 – plants treated with *T. harzianum* T14;
5. Treatment MT7 – plants treated with *T. harzianum* T7 since infected with *M. phaseolina*;
6. Treatment MT14 – plants treated with *T. harzianum* T14 since infected with *M. phaseolina*.

In all the above mentioned treatments, the seeds were sown in autoclaved soil. Plants were carefully uprooted without causing any damage to root tissues. The uprooting was done at different time points (0, 1, 2, 3, 4, 5, 6, 7, and 8 days after the pathogen inoculation). Three plants were sampled for each time point and these plants were kept separate for biochemical analysis. Fresh roots were washed in running tap water and homogenised with liquid nitrogen in a pre-chilled mortar and pestle. The homogenised root tissues were stored at –80°C until used.

Enzyme extraction and POX activity assay

Root tissues collected from the plants were immediately homogenised with liquid nitrogen. One gram of powdered sample was transferred to 1 ml of extraction buffer (0.1 M sodium phosphate, pH 6.0). The extracts were centrifuged at 14,000 g for 20 min at 4°C. The supernatant (enzyme extract) was transferred to a new tube and freeze at –80°C until assayed. The protein concentration was determined as described by Bradford (1976) using bovine serum albumin (Sigma) as a standard. For determining POX activity, enzyme extracts (containing 30 µg of total protein) were added to 30 µl of 200 mM guaiacol and 25 mM citrate phosphate (pH 5.4). To each sample, 30 µl of 30% H_2O_2 was added. The absorbance was measured at 470 nm using a spectrophotometer. Enzyme activity was expressed as the change in the absorbance of the reaction mixture $min^{-1} \cdot g^{-1}$ on a fresh weight basis.

Quantification of phenolics accumulation

The total phenolic contents of the methanol extracts were determined colorimetrically using Folin & Ciocalteu's phenol reagent (Merck, Germany) as described by Seevers and Daly (1970). A standard curve was prepared using different concentrations of gallic acid. The results were expressed as mg gallic acid equivalent (GAE) $\cdot$ g^{-1} fresh weight basis. Briefly, portions of 1 g of the root tissues were ground with a pestle and mortar in 16 ml of 80% methanol for 10 min at 4°C in an N-saturated atmosphere. The extract was filtered through cloth and centrifuged for 10 min at 4,000 ×g and the liquid phase collected. The procedure was repeated three times. The pooled supernatants were evaporated at 40°C to a volume of 1–2 ml and used for the analysis. Then, 0.5 ml of the methanol extracts were dissolved in 7 ml of distilled water and 0.5 ml of Folin & Ciocalteu's phenol reagent were added to them in a 10 ml volumetric flask. The contents were mixed and allowed to stand for 5–8 min at room temperature. Next, one ml of a sodium carbonate solution (75 g $\cdot$ l^{-1}; Na_2CO_3) was added, followed by bringing the volume to 10 ml with the addition of distilled water. Solutions were mixed and allowed to stand at room temperature for 1 h prior to the determination of total phenolic contents using a spectrophotometer at 725 nm. The experiment was conducted in triplicates and repeated three times.

Statistical analysis

The greenhouse experiments were arranged as a factorial in a randomised complete block design with four replications. Statistical analysis of the data was carried out by analysis of variance (ANOVA) using SAS software (version 9.1). The means were separated using Tukey's multiple range tests at $p < 0.05$, where the F-value was significant.

Results

Effect of *Trichoderma* isolates on mycelial growth of *Macrophomina phaseolina in vitro*

The comparison of the data obtained from the dual culture revealed that all 11 isolates of *Trichoderma* spp. inhibited the mycelial growth of *M. phaseolina* from 58.67% to 20.22%. The highest level of inhibition belonged to *T. harzianum* T7 and the lowest level was observed for *T. viride* T24 (Table 2).

Two isolates of *T. harzianum* (T7 and T14), which showed the highest levels of inhibiting the pathogen growth in the dual culture test, were used for determining the capability of producing volatile and non-volatile

Table 2. *In vitro* screening of *Trichoderma* spp. isolates against *Macrophomina phaseolina* by Dual culture test (7 days post inoculation)

Isolates *Trichoderma*	Dual culture	
	mean radial growth [mm]	inhibition[a] [%]
T. harzianum T7	18.6±0.3	58.7 a
T. harzianum T14	19.2±0.5	57.3 a
T. viride T20	21.7±0.1	51.8 b
T. harzianum BI	23.9±0.2	46.9 c
T. harzianum T8	25.9±0.2	42.4 d
T. viride T17	28.3±0.3	37.1 e
T. viride T10	29.5±0.4	34.4 ef
T. viride T23	30.1±0.5	33.1 f
T. viride T6	33.5±0.0	25.3 g
T. harzianum T5	35.9±0.2	20.2 h
T. viride T24	35.9±0.2	20.2 h
Control	45.0±0.1	0 i

Four replicates were used for each treatment and the experiment was repeated three times.
[a]the means with the same letter do not have significant difference according to Tukey's multiple range test at $p < 0.05$

metabolites. The results indicated that *T. harzianum* isolates apparently produced volatile and non-volatile substances that suppressed the pathogen growth. Data presented in table 3 clearly indicate that *T. harzianum*-T7-derived volatile substances caused maximum inhibition of the mycelial growth of *M. phaseolina*. Mycelial growth of *M. phaseolina* was reduced to 56.72% and 49.52% by volatile metabolites of *T. harzianum* T7 and T14 isolates, respectively. A significant difference was not observed among the two *T. harzianum* isolates tested for the effect of non-volatile metabolites against *M. phaseolina* (Table 3).

Effect of *Trichoderma harzianum* isolates on plant growth parameters and *T. harzianum* isolates' control of soybean charcoal rot in the greenhouse

The results of greenhouse experiments showed that charcoal rot of soybean was significantly reduced by two *T. harzianum* isolates (T7 and T14) tested in both the autoclaved and non-autoclaved soils (Table 4). Average stem height, fresh and dry weights of the roots and shoots in the treatment in which the soil or soybean seeds were inoculated with *T. harzianum* isolates, were greater than those of the controls (Table 5). So, these isolates were effective in promoting plant growth parameters. *Macrophomina phaseolina* was isolated from all inoculated plants showing symptoms with different disease-severity levels. In most of the treatments investigated, the isolate T7 was more effective in promoting plant growth and reducing charcoal rot compared to isolate T14. Seed treatment with *T. harzianum* isolates promoted plant growth. But in most of the treatments, the amount of this promotion was less than that of the soil treatment with these isolates. Whereas, significantly more reduction of disease severity was observed by seed treatment with *T. harzianum* isolates in both autoclaved and non-autoclaved soils (Table 5).

Table 3. Effect of volatile metabolites and non-volatile metabolites of *Trichoderma harzianum* isolates on mycelial growth of *Macrophomina phaseolina*

Isolates of *T. harzianum*	Non-volatile metabolites		Volatile metabolites	
	colony area [mm^2]	inhibition[a] [%]	colony area [mm^2]	inhibition[a] [%]
T7	615.4±34.1	87.7 a	1,962.5±30.8	56.7 a
T14	706.5±32.7	85.9 a	2,289.1±6.9	49.5 b
Control	5024±39.8	0 b	4,534.2±19.5	0 c

Each treatment was replicated four times and the experiment was repeated three times with similar results.
[a]the means with the same letter do not have significant difference according to Tukey's multiple range test at $p < 0.05$

Table 4. Mean square of combined ANOVA on disease severity and several growth parameters of plants for biological-control treatments against *Macrophomina phaseolina*

Source of variation	df	Stem height [cm]	Roots weight [g · plant^{-1}]		Shoots weight [g · plant^{-1}]		Disease severity
			fresh	dry	fresh	dry	
Experiment (E)	1	31.48*	38.47*	74.99**	20.62*	6.86 ns	19.29**
Rep within experiment	6	11.95	32.16	18.08	23.97	8.63	0.0
Soil (S)	1	28.88**	18.60**	0.49 ns	11.09**	0.06 ns	100.19**
Trichoderma harzianum (T)	1	50.60*	80.75**	233.91**	23.46**	8.53*	0.09**
E × S	1	4.86 ns	31.67 ns	94.73*	13.78 ns	3.07 ns	15.34*
E × T	1	0.0 ns	21.95*	67.04*	14.56*	3.68 ns	0.19*
S × T	1	0.0 ns	4.42*	0.0 ns	3.04*	0.0 ns	0.13*
E × S × T	1	0.0 ns	0.0 ns	0.0 ns	0.0 ns	0.28 ns	0.06*
Pooled error	25	15.91	58.94	0.99	70.30	0.47	0.03
CV [%]	28	3.05	14.73	8.86	7.85	36.64	7.42

*, ** and ns – significant at $p = 0.05$, $p = 0.01$ and non-significant probability levels, respectively; CV – coefficient of variation

Table 5. Effect of *Trichoderma* isolates on several growth parameters of soybean plants

Treatments	Stem height [cm]	Roots weight [g · plant^{-1}]		Shoots weight [g · plant^{-1}]		Disease severity
		fresh	dry	fresh	dry	
A	33.48 a	4.61 ab	0.60 b	13.47 a	0.45 a	0 j
N	30.72 b	4.65 ab	0.61 b	11.39 b	0.39 ab	0 j
AM	19.03 h	1.02 e	0.05 d	5.21 e	0.05 d	0.55 a
NM	21.69 g	1.11d e	0.06 d	7.88 d	0.08 cd	0.45 b
AMOT14	27.95 cd	4.86 a	0.78 a	9.45 cd	0.29 abc	0.21 g
AMOT7	29.47 bc	4.93 a	0.79 a	11.06 bc	0.37 ab	0.19 h
AMET14	26.07 de	3.07 c	0.48 bc	8.36 d	0.22 bcd	0.19 h
AMET7	25.64 ef	3.22 bc	0.51 bc	8.49 d	0.20 bcd	0.17 i
NMOT14	23.77 f	2.49 cde	0.39 c	8.00 d	0.17 bcd	0.28 c
NMOT7	24.82 ef	2.66 cd	0.42 c	8.30 d	0.20 bcd	0.24 e
NMET14	25.61 ef	2.33 cde	0.36 c	8.21 d	0.19 bcd	0.25 d
NMET7	26.45 de	2.50 cde	0.40 c	8.14 d	0.19 bcd	0.22 f

Each treatment was replicated four times. The experiment was repeated three times with similar results: A – autoclaved soil; N – non-autoclaved soil; AM – autoclaved soil + *M. phaseolina;* NM – non-autoclaved soil + *M. phaseolina;* AMOT14 – autoclaved soil + *M. phaseolina* + soil treatment with *T. harzianum* T14; AMOT7 – autoclaved soil + *M. phaseolina* + soil treatment with *T. harzianum* T7; AMET14 – autoclaved soil + *M. phaseolina* + seed treatment with *T. harzianum* T14; AMET7 – autoclaved soil + *M. phaseolina* + seed treatment with *T. harzianum* T7; NMOT14 – non-autoclaved soil + *M. phaseolina* + soil treatment with *T. harzianum* T14; NMOT7 – non-autoclaved soil + *M. phaseolina* + soil treatment with *T. harzianum* T7; NMET14 – non-autoclaved soil + *M. phaseolina* + seed treatment with *T. harzianum* T14; NMET7 – non-autoclaved soil + *M. phaseolina* + seed treatment with *T. harzianum* T7.
The means with the same letter do not have significant difference according to Tukey's multiple range tests at $p < 0.05$

POX activity

At all time points investigated except 1 day post-inoculation (dpi), the POX activity increased in soybean roots (compared to healthy plants as the control) due to the *T. harzianum* treatments. Also, inoculating the pathogen leads to an increase in the enzyme activity at these time points (Fig. 1). Analysis of variance revealed significant differences among the POX activity of plants with various treatments. Enzyme activity in all treatments reached a maximum level at 3 dpi, and decreased afterward. The highest levels of POX activity were observed in MT7, MT14, T7, and T14 treatments at 3 dpi, which were not significantly different from each other (Fig. 1).

Total phenolic contents

Plants have an almost unlimited ability to synthesise aromatic substances, most of which are phenolics or their oxygen-substituted derivatives. In many cases, these substances are involved in plant immunity against pathogens. Our data revealed significant difference in the phenolic contents of various treatments used in this study (Fig. 2). The accumulation of phenolics considerably increased after the soybean seedlings were inoculated with the pathogen, when compared to non-inoculated controls. Also, significantly higher phenolic contents were observed in the treatments with *T. harzianum* isolates.

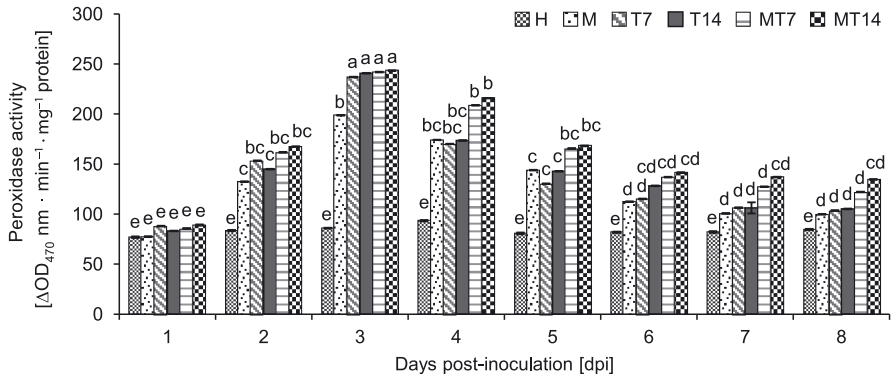

Fig. 1. Peroxidase activity of soybean roots in the presence or absence of *Trichoderma harzianum* isolates and *Macrophomina phaseolina* inoculation: H – healthy plants; M – plants inoculated with *M. phaseolina;* T14 – plants inoculated with *T. harzianum* T14; T7 – plants inoculated with *T. harzianum* T7; MT14 – plants inoculated with *T. harzianum* T14 and *M. phaseolina;* MT7 – plants inoculated with *T. harzianum* T7 and *M. phaseolina*. The activity of peroxidase was presented as ΔOD_{470} nm · min^{-1} · mg^{-1} protein. The means with the same letter do not have significant difference according to Tukey's multiple range tests at $p < 0.05$

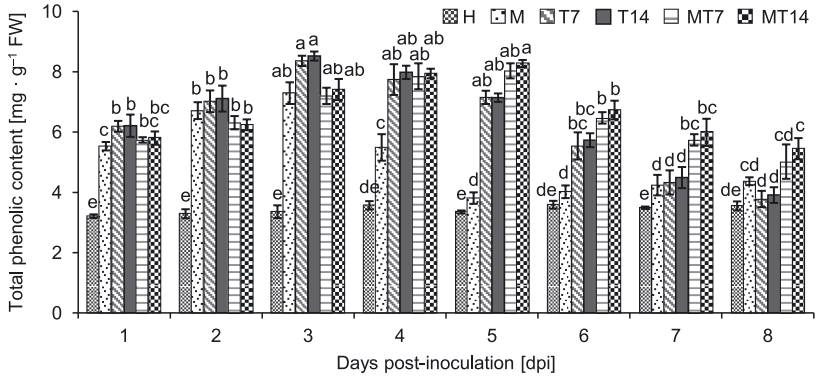

Fig. 2. Total phenolics of soybean root tissues in the presence or absence of *Trichoderma harzianum* isolates and *Macrophomina phaseolina*: H – healthy plants; M – plants inoculated with *M. phaseolina;* T14 – plants inoculated with *T. harzianum* T14; T7 – plants inoculated with *T. harzianum* T7; MT14 – plants inoculated with *T. harzianum* T14 and *M. phaseolina;* MT7 – plants inoculated with *T. harzianum* T7 and *M. phaseolina*. Total phenolics of the roots were expressed as mg caffeic acid · g^{-1} of root fresh weight (FW). The means with the same letter do not have significant difference according to Tukey's multiple range tests at $p < 0.05$

At 1, 2, 3, and 4 dpi, in the treatments with *T. harzianum* and *M. phaseolina* (i.e. MT7 and MT14), the phenolics levels slightly decreased, when compared to the phenolics detected in treatments with *T. harzianum* isolates alone. Whereas, at the next time points investigated (5, 6, 7, and 8 dpi) a slight increase in the phenolics of the MT7 and MT14 treatments was observed, when compared to that of the treatments with *T. harzianum* isolates alone. At 4, 5, and 6 dpi, phenolics levels were significantly higher in all treatments in which *T. harzianum* was isolated, than that of the plants only inoculated by the pathogen (Fig. 2). At all the investigated time points, the lowest level of phenolics was detected in the control which consisted of healthy plants.

Discussion

In the present study, the biocontrol capability of various isolates of *T. harzianum* and *T. viride* against *M. phaseolina*, the causual agent of soybean charcoal rot, was investigated using *in vitro* and *in vivo* assays. Furthermore, the effect of the most antagonistic isolates (*T. harzianum* isolates T7 and T14) in priming POX activity and phenolic accumulations in soybean roots were determined. The obtained data revealed that all *T. harzianum* and *T. viride* isolates used in this study had a considerable antagonistic effect on mycelial growth of *M. phaseolina* in dual cultures, when compared to the controls. Out of all the tested *Trichoderma* isolates, the isolates T7 and T14 showed a maximum inhibition against mycelial growth of the pathogen. Numerous reports indicated that *Trichoderma* spp. isolates (Ehteshamul *et al*. 1990; Ehteshamul and Ghaffar 1992) are effective biocontrol agents, and these indications support our results. According to Hermosa *et al*. (2000), *T. harzianum* had potential biocontrol activity in dual culture studies against various phytopathogenic fungi such as *Phoma betae*, *Rosellinia necatrix*, *Botrytis cinerea*, and *Fusarium oxyporum* f. sp. *dianthi*. It was reported by Asran-Amal *et al*. (2010), that *in vitro* screening of five isolates of *T. harzianum*, one isolate of *Chaetomium globosum*, and one isolate of *Conetherium mentance*, revealed that all of them significantly reduced the growth area of *M. phaseolina*, *Fusarium solani*, and *Rhizoctonia solani*. The higher antagonistic effect of *T. harzianum* against *M. phaseolina* compared to *T. viride* was previously observed by Sreedevi *et al*. (2011). This finding is in agreement with our results on the potent antagonistic activity of *T. harzianum* isolates T7 and T 14 against *M. phaseolina,* (from among all *Trichoderma* isolates tested). This antagonistic nature might be due to antibiosis, nutrient competition, and/or cell wall degrading enzymes (Kumar 2013).

To determine the mechanisms involved in microbial antagonism, we tested *T. harzianum* isolates T7 and T 14 for their capability to produce volatile and non-volatile compounds that inhibit the pathogen growth *in vitro*. From our results, it is evident that volatile compounds of both *T. harzianum* isolates decreased the mycelial growth of *M. phaseolina*. A large variety of volatile secondary metabolites could be produced by *Trichoderma* spp. such as ethylene, hydrogen cyanide, aldehydes, and ketones, which play an important role in controlling various plant pathogens (Vey *et al*. 2001). The non-volatile secondary metabolites of *T. harzianum* isolates used in this assay were found to be more effective in suppressing the mycelial growth of *M. phaseolina* when compared to volatile compounds. Similar to this finding, Tapwal and associates (2011) reported that non-volatile metabolites of *T. viride* were more effective against *R. solani*, *Curvularia lunata*, and *Alternaria solani*. Whereas, volatile compounds of this biocontrol agent were found to be more effective against *F. oxysporum* (Tapwal *et al*. 2011). The potential of *Trichoderma* spp. to produce many volatile (e.g. pyrones, sesquiterpenes) and non-volatile secondary metabolites (e.g. terpenes) has been reviewed by Reino *et al*. (2008). However, our research in the first to compare the effect of volatile and non-volatile compounds produced by *T. harzianum* against *M. phaseolina*.

The evaluation of the interaction among soybean plants, *M. phaseolina* and *T. harzianum* isolates revealed

that both *T. harzianum* isolates reduced charcoal rot disease and promoted plant growth in the greenhouse. Disease severity was lower in the seed treatment in autoclaved soil compared to other treatments. Similar to our results, seed treatment with *T. harzianum* and *T. viride* was more effective in controlling *R. solani* in *Phaseolus vulgaris* under both greenhouse and field conditions (Prashanthi *et al*. 1997). These results indicated that introducing *Trichoderma* spp. was effective for the success of these isolates in plant growth promotion and for reducing disease severity.

Soil treatment with *T. harzianum* seems to be more effective for promoting plant growth parameters. Whereas, the seed treatment was more effective for suppressing disease progress compared to the soil treatment in both autoclaved and non-autoclaved soils. The stem height and shoot weight were higher in plants cultivated in autoclaved soil when compared to plants cultivated in non-autoclaved soil. This is in accordance with the observations of Al-Khaliel (2010) on peanut plants which showed reduced growth in non-autoclaved soil despite the fact that they were healthy. Reduced growth in non-autoclaved soil may be due to the presence of lower levels of nutrients available or indigenous microbes that may have negative effects on the plant growth.

The effect that *T. harzianum* isolates have on enhancing growth parameters in the plants inoculated by the pathogen and the effect the isolates have on the reduction of disease severity were higher in autoclaved compared to non-autoclaved soil. This phenomenon might be due to the better ability of *T. harzianum* isolates in colonising rhizosphere with the consequent better effect on plant growth and disease control in autoclaved soil because of the absence of other microorganisms (Harman and Kubicek 1998).

The current study revealed that antagonistic *T. harzianum* isolates were capable of inducing defense responses in soybean plants against *M. phaseolina*. Total phenolics and POX activity increased in response to inoculation with *M. phaseolina*, or soybean seeds treated with *T. harzianum* T7 or T14 isolates alone. Furthermore, seed treatment using *T. harzianum* isolates together with inoculation of the plants by *M. phaseolina* primed these defense responses in soybean. It is known that the presence of phenolic compounds in plants and their synthesis in response to infection, is associated with resistance (Taheri and Tarighi 2011; Nikraftar *et al*. 2013). Induced accumulation of phenolics into the cell wall in a SA-dependent way (Alonso-Ramirez *et al*. 2014) and also by releasing anti-microbial compounds, which leads to the resistance response (Taheri and Tarighi 2010). Phenolic compounds and flavonoids are two kinds of metabolites of phenylpropanoid pathway in plants (Cao *et al*. 2005). Phenolics are oxidised by peroxidases to form more toxic compounds, known as quinines. It is the quinines which are directly toxic to fungal pathogens (Gogoi *et al*. 2001).

The present data also revealed a significant increase in POX activity of all treatments having *T. harzianum* isolates compared to the healthy and the diseased (*M. phaseolina* infected) control plants. Peroxidase is a useful marker of plant development, physiology, infection and defense (Zhang and Kirkham 1994). The induction and accumulation of peroxidases is often correlated with the onset of induced resistance, suggesting an active role for these enzymes in defense against pathogenic fungi (Sticher *et al*. 1997; Van Loon *et al*. 1998). Similar to our results, enhanced peroxidase activity during plant-fungus interactions have been previously reported by several researchers (Heath 1996). Vidhyasekaran (1997) demonstrated that the POX activity was more in the plants over infection by the pathogens in some plants and it has great role in inhibit the pathogen development. On the other hand, POX catalyses the condensation of phenolic compounds into lignin which is associated with disease resistance in plants and increases in host plants following pathogen infection (Scott-Craig *et al*. 1995). Peroxidase may be rapidly involved in the peroxidation of substrate molecules, leading to the accumulation of highly toxic compounds (i.e. phenolics), which may contribute to plant defense via their antifungal potential (Ward 1986). However, these compounds may, to some extent, be toxic to the plant itself, and it seems reasonable to assume that mechanisms designed to limit POX expression are activated during the resistance process in order to keep phenolic compounds below the phytotoxic levels. So, the decrease in POX activity observed at 4 dpi, and afterward observed in this research, may reflect a process by the plant to protect itself until such activity is needed, such as upon pathogenic attack. Similar findings about the increase in POX activity after the application of biocontrol agents have been reported in different crops (Oostendorp *et al*. 2001).

Guleria and Kumar (2006) reported a higher activity of POX and increased levels of phenolics in sesame (*S. indicum*) leaves treated with a leaf extract of neem (*Azadirachta indica* A. Juss.) and inoculated with *Alternaria*, the casual agent of leaf spot. It is suggested that neem leaf extract induced POX activity in sesame leaves, which resulted in increased biosynthesis and metabolism of phenolics. Increased phenolic accumulations have been demonstrated to be involved in protecting sesame plants against from *Alternaria sesame* (Guleria and Kumar 2006). We obtained similar results on priming POX activity upstream of phenolic accumulations by *T. harzianum* isolates in the soybean-*M. phaseolina* pathosystem which might be involved in disease suppression by the biocontrol agents.

Conclusions

The present study demonstrated that two isolates of *T. harzianum* (T7 and T14) had the potential, as biocontrol agents, to control soybean charcoal root rot caused by *M. phaseolina.* This is the first report on the higher effect of non-volatile metabolites produced by *T. harzianum* isolates compared to their volatiles against this necrotrophic pathogen. Also, we demonstrated for the first time, the capability of these biocontrol agents in priming plant defense responses such as POX activity and phenolics accumulation. Future cellular and molecular investigations on resistance responses activated by *Trichoderma* spp. or other plant growth promoting fungi against various pathogens, will not only improve our knowledge on the mechanisms involved in biological induction of immu-

nity but would also be useful in developing novel and environmentally safe strategies to control economically important soybean diseases.

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References

Adekunle A.T., Cardwell K.F., Florini D.A., Ikotun T. 2001. Seed treatment with *Trichoderma* species for control of damping-off of cowpea caused by *Macrophomina phaseolina*. Biocontrol Science and Technology 11 (4): 449–457.

Alonso-Ramírez A., Poveda J., Martín J.I., Hermosa R., Monte E., Nicolás C. 2014. Salicylic acid prevents *Trichoderma harzianum* from entering the vascular system of the roots. Molecular Plant Pathology 15 (8): 823–831.

Al-Khaliel A.S. 2010. Effect of salinity stress on mycorrhizal association and growth response of peanut infected by *Glomus mosseae*. Plant, Soil and Environment 56: 318–324.

Aly A.A., Abdel-Sattar M.A., Omar M.R., Abd-Elsalam K.A. 2007. Differential antagonism of *Trichoderma* sp. against *Macrophomina phaseolina*. Journal of Plant Protection Research 47 (2): 91–102.

Asran-Amal A., Moustafa-Mahmoud S.M., Sabet K.K., El Banna O.H. 2010. *In vitro* antagonism of cotton seedlings fungi and characterization of chitinase isozyme activities in *Trichoderma harzianum*. Saudi Journal of Biological Sciences 17 (2): 153–157.

Babu B.K., Saxena A.K., Srivastava A.K., Arora D.K. 2007. Identification and detection of *Macrophomina phaseolina* by using species-specific oligonucleotide primers and probe. Mycologia 99 (6): 797–803.

Bai Z., Jin B., Li Y., Chen J., Li Z. 2008. Utilization of winery wastes for *Trichoderma viride* biocontrol agent production by solid state fermentation. Journal of Environmental Sciences 20: 353–358.

Benitez T., Rincon A.M., Limon M.C., Condon A.C. 2004. Biocontrol mechanisms of *Trichoderma* strains. International Microbiology 7 (4): 249–260.

Bowen C.R., Schapaugh Jr, W.T. 1989. Relationships among charcoal rot infection, yield, and stability estimates in soybean blends. Crop Science 29 (1): 42–46.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72 (1–2): 248–254.

Brotman Y., Landau U., Cuadros-Inostroza A., Takayuki T., Fernie A.R., Chet I., Viterbo A., Willmitzer L. 2013. *Trichoderma*-plant root colonization: escaping early plant defense responses and activation of the antioxidant machinery for saline stress tolerance. PLoS Pathogens 9 (4), DOI: 10.1371/journal.ppat.1003221

Cao J., Jiang W., He H. 2005. Induced resistance in Yali pear (*Pyrus bretschneideri* Rehd.) fruit against infection by *Penicillium expansum* by postharvest infiltration of acibenzolar–S-methyl. Journal of Phytopathology 153 (11–12): 640–646.

Dennis C., Webster J. 1971a. Antagonistic properties of species groups of *Trichoderma* II. Production of volatile antibiotics. Transactions of the British Mycological Society 57 (1): 41–48.

Dennis C., Webster J. 1971b. Antagonistic properties of species groups of *Trichoderma* I, production of non-volatile antibiotics. Transactions of the British Mycological Society 57 (1): 25–39.

Ehteshamul H.S., Ghaffar A., Zaki M.J. 1990. Biological control of root rot diseases of okra, sunflower, soybean, and mungbean. Pakistan Journal of Botany 22 (2): 121–124.

Ehteshamul H.S., Ghaffar A. 1992. Efficacy of *Trichoderma* spp., and *Rhizobium meliloti* in the control of root rot of fenugreek. Pakistan Journal of Botany 24 (2): 217–221.

Elad Y., Zvieli Y., Chet I. 1986. Biological control of *Macrophomina phaseolina* (Tassi) Goid by *Trichoderma harzianum*. Crop Protection 5 (4): 288–292.

Etebarian H.R. 2006. Evaluation of *Trichoderma* isolates for biological control of charcoal stem rot in melon caused by *Macrophomina phaseolina*. Journal of Agricultural Science Technology 8 (3): 243–250.

Fernandez R.B., De Santiago A., Delgado S.H., Perez N.M. 2006. Characterization of Mexican and non-Mexican isolates of *Macrophomina phaseolina* based on morphological characteristics, pathogenicity on bean seeds and endoglucanase genes. Journal of Plant Pathology 88 (1): 1–8.

Gary F.A., Mihail J.D., Lavigne R.J., Porter P.M. 1991. Incidence of charcoal rot of sorghum and soil populations of *Macrophomina phaseolina* associated with sorghum and native vegetation in Somalia. Mycopathologia 114: 145–151.

Gogoi R., Singh D.V., Sivastava K.D. 2001. Phenols as a biochemical basis of resistance in wheat against Karnal bunt. Plant Pathology 50 (4): 470–476.

Guleria S., Kumar A. 2006. *Azadirachta indica* leaf extract induces resistance in sesame against Alternaria leaf spot diseases. Journal of Cell Molecular Biology 5 (2): 81–86.

Harman G.E. 2006. Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 96 (2): 190–194.

Harman G.E., Kubicek C.P. 1998. Trichoderma and Gliocladium, Enzymes, Biological Control and Commercial Applications. Vol. 2. Taylor & Francis, London, UK, 393 pp.

Heath M.C. 1996. Plant resistance to fungi. Canadian Journal of Plant Pathology 18: 469–475.

Hermosa R., Viterbo A., Chet I., Monte E. 2012. Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology 158 (1): 17–25.

Hermosa M.R., Grondona I., Iturriaga E.A., Diaz-Minguez J.M., Casytro C., Monte E. Garcia-Acha I. 2000. Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. Applied and Environmental Microbiology 66 (5): 1890–1898.

Herridge D.F., Peoples M.B., Boddey R.M. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311 (1): 1–18.

Kehri H.K., Chandra S. 1991. Antagonism of *Trichoderma viride* to *Macrophomina phaseolina* and its application in the control of dry root rot of mung. Indian Phytopathology 44 (1): 60–63.

Khan S.N. 2007. *Macrophomina phaseolina* as causal agent for charcoal rot of sunflower. Mycopathologia 5: 111–118.

Koenning S.R., Wrather J.A. 2010. Suppression of soybean yield potential in the continental United States by plant diseases

from 2006 to 2009. Online. Plant Health Progress. DOI: 10.1094/PHP-2010-1122-01-RS.

Kubicek C.P., Mach R.L., Peterbauer C.K., Lorito Kubicek M. 2001. *Trichoderma*: from genes to biocontrol. Journal of Plant Pathology 83 (2): 11–23.

Kumar S. 2013. *Trichoderma*: a biological control weapon for managing plant diseases and promoting sustainability. International Journal of Agricultural Sciences and Veterinary Medicine 1 (3): 106–121.

Lorito M., Woo S.L., Harman G.E., Monte E. 2010. Translational research on *Trichoderma*: from 'omics to the field. Annual Review of Phytopathology 48: 395–417.

Malmierca M.G., Cardoza R.E., Alexander N.J., McCormick S.P., Hermosa R., Monte E., Gutiérrez S. 2012. Involvement of *Trichoderma trichothecenes* in the biocontrol activity and induction of plant defense-related genes. Applied and Environmental Microbiology 78 (14): 4856–4868.

Mengistu A., Ray J.D., Smith J.R., Paris R.L. 2007. Charcoal rot disease assessment of soybean genotypes using a colony-forming unit index. Crop Science 47: 2453–2461.

Nerey Y., Van Beneden S., Franca S., Jimenez A. 2010. Influence of soil type and indigenous pathogenic fungi on bean hypocotyls rot caused by *Rhizoctonia solani* AG4 HGI in Cuba. Soil Biology and Biochemistry 42 (5): 797–803.

Nikraftar F., Taheri P., Falahati Rastegar M., Tarighi S. 2013. Tomato partial resistance to *Rhizoctonia solani* involves antioxidative defense mechanisms. Physiological and Molecular Plant Pathology 81 (1): 74–83.

Oostendorp M., Kunz W., Dietrich B., Staub T. 2001. Induced resistance in plants by chemicals. European Journal of Plant Pathology 107 (1): 19–28.

Pearson C.A.S., Schwenk F.W., Crowe F.J., Kelly K. 1984. Colonization of soybean roots by *Macrophomina phaseolina*. Plant Disease 68 (12): 1086–1088.

Persson L., Bodker L., Larsson-Wikstrom M. 1997. Prevalence and pathogenicity of foot and root rot pathogens of pea in Southern Scandinavia. Plant Disease 81 (2): 171–174.

Prashanthi S.K., Sreekanth K., Sreenivasa M.N., Kulkarni S. 1997. Integrated management of root rot disease of *Phaseolus vulgaris* caused by *Rhizoctonia solani*. Environmental Ecology 15: 800–802.

Prévost D., Bertrand A., Juge C., Chalifour F.P. 2010. Elevated CO_2 induces differences in nodulation of soybean depending on bradyrhizobial strain and method of inoculation. Plant Soil 331 (1): 115–127.

Rayatpanah S., Nanagulyan S.G., Alav S.V., Razavi M., Ghanbari-Malidarreh A. 2011. Pathogenic and genetic diversity among Iranian isolates of *Macrophomina phaseolina*. Chilean Journal of Agricultural Research 72: 40–44.

Reino J.L., Guerriero R.F., Hernàndez-Galà R., Collado I.G. 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. Phytochemistry Reviews 7 (1): 89–123.

Purkayastha S., Kaur B., Dilbaghi N., Chaudhury A. 2006. Characterization of *Macrophomina phaseolina*, the charcoal rot pathogen of cluster bean, using conventional techniques and PCR-based molecular markers. Plant Pathology 55 (1): 106–116.

Salik N.K. 2007. *Macrophomina phaseolina* as causal agent for charcoal rot of sunflower. Mycopath 5 (2): 111–118.

Savazzini F., Longa C.M.O., Pertot I. 2009. Impact of the biocontrol agent *Trichoderma atroviride* SC1 on soil microbial communities of a vineyard in northern Italy. Soil Biology and Biochemistry 41: 1457–1465.

Scott-Craig J.S., Kerby K.B., Stein B.D., Sommerville C.S. 1995. Expression of an extracellular peroxidase that is induced in barley (*Hordeum vulgare*) by the powdery mildew pathogen (*Erysiphe graminis* f. sp. *hordei*). Physiological and Molecular Plant Pathology 47 (6): 407–418.

Seevers P.M., Daly J.M. 1970. Studies on wheat stem rust resistance control at *sr6* locus, I. The role of phenolic compounds. Phytopathology 6: 1322–1328.

Shali A., Ghasemi S., Ahmadian G., Ranjbar G., Dehestani A., Khalesi N., Motallebi E., Vahed M. 2010. *Bacillus pumilus* SG2 chitinases induced and regulated by chitin, show inhibitory activity against *Fusarium graminearum* and *Bipolaris sorokiniana*. Phytoparasitica 38 (2): 141–147.

Singh T., Saikia R., Jana T., Arora D.K. 2004. Hydrophobicity and surface electrostatic charge of conidia of the mycoparasitic *Trichoderma* species. Mycological Progress 3 (3): 219–228.

Singh N., Pandey P., Dubey R.C., Maheshwari D.K. 2008. Biological control of root rot fungus *Macrophomina phaseolina* and growth enhancement of *Pinus roxburghii* (Sarg.) by rhizosphere competent *Bacillus subtilis* BN1. World Journal of Microbiology and Biotechnology 24: 1669–1679.

Sreedevi B., Charitha Devi M., Saigopal D.V.R. 2011. Isolation and screening of effective *Trichoderma* spp. against the root rot pathogen *Macrophomina phaseolina*. Journal of Agricultural Technology 7 (3): 623–635.

Sticher L., Mauch-Mani B., Metraux J.P. 1997. Systemic acquired resistance. Annual Review of Phytopathology 35: 235–270.

Su G., Suh S.O., Schneider R.W., Russin J.S. 2001. Host specialization in the charcoal rot fungus, *Macrophomina phaseolina*. Phytopathology 91 (2): 120–126.

Taheri P., Tarighi S. 2010. Riboflavin induces resistance in rice against *Rhizoctonia solani* via jasmonate-mediated priming of phenylpropanoid pathway. Journal of Plant Physiology 167 (3): 201–208.

Taheri P., Tarighi S. 2011. A survey on basal resistance and riboflavin-induced defense responses of sugar beet against *Rhizoctonia solani*. Journal of Plant Physiology 168 (10): 1114–1122.

Taliei T., Safaei N., Aghajani M.A. 2012. Survival of *Macrophomina phaseolina* and associated mycobiota on soybean residuals and the effect of *Trichoderma harzianum* on their population dynamics. Journal of Applied Researches in Plant Protection 1: 1–13.

Tapwal A., Singh U., Teixeira de Silva J.A., Singh G., Grag S., Kumar R. 2011. *In vitro* antagonism of *T. viride* against five phytopathogens. Pest Technology 5 (1): 59–62.

Thilagavathi R., Saravanakumar D., Ragupathi N., Samiyappan R. 2007. A combination of biocontrol agents improves the management of dry root rot (*Macrophomina phaseolina*) in greengram. Phytopathologia Mediterranea 46: 157–167.

Todd T.C. 1993. Soybean planting date and maturity effects on *Heterodera glycines* and *Macrophomina phaseolina* in southeastern Kansas. The Journal of Nematology 25 (4S): 731–737.

Van Loon L.C., Bakker P.A.H.M., Pieterse M.J. 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36: 453–483.

Vasebi Y., Safaie N., Alizadeh A. 2013. Biological control of soybean charcoal root rot disease using bacterial and fungal

antagonists *in vitro* and greenhouse condition. Journal of Crop Protection 2: 139–150.

Verma M., Brar S.K., Tyagi R.D., Sahai V., Prévost D., Valéro J.R., Surampalli R.Y. 2007. Bench-scale fermentation of *Trichoderma viride* on wastewater sludge: rheology, lytic enzymes and biocontrol activity. Enzyme and Microbial Technology 41 (6): 764–771.

Vey A., Hoagland R.E., Butt T.M. 2001. Toxic metabolites of fungal biocontrol agents. p. 311–346. In: "Fungi as Biocontrol Agents: Progress, Problems and Potential" (T.M. Butt, C. Jackson, N. Magan, eds.). CAB International, Bristol, UK, 390 pp.

Vidhyasekaran P., Rabindran R., Muthamilan M., Nayar K., Rajappan K., Subramanian N., Vasumathi M. 1997. Development of a powder formulation of *Pseudomonas fluorescens* for control of rice blast. Plant Pathology 46 (3): 291–297.

Ward E.W.B. 1986. Biochemical mechanisms involved in resistance of plants of fungi. p. 107–131. In: "Biology and Molecular Biology of Plant Pathogen Interactions" (J.A. Butt Baily, ed.). Spinger-Verlag KG., Berlin, Germany, 415 pp.

Zhang J., Kirkham M.B. 1994. Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant and Cell Physiology 35 (5): 785–791.

Ziedan E.H., Saad M., Farage S. 2005. Biological controls of grapevine root rot by antagonistic microorganism's. African Journal of Mycology and Biotechnology 13 (3): 19–36.