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Trichoderma harzianum L_1 as a potential source for lytic enzymes and elicitor of defense responses in chickpea (*Cicer arietinum* L.) against wilt disease caused by *Fusarium oxysporum* f. sp. *ciceri*.

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Abstract

The effect of some natural lignocellulosic substances on the production of β-glucanase, chitinase, protease and xylanase from *Trichoderma harzianum* L_1 has been studied under solid state fermentation conditions. Maximum activities of all these enzymes were observed in the fermentation medium containing the mixture of 1% rice bran, neem cake and 0.1% crab shell powder. The induction of plant defense response was investigated by inoculating the roots of chickpea cv JG62 with the biocontrol agent, *T. harzianum* L_1. A root extract of chickpea inoculated with *T. harzianum* L_1 showed increased activities of phenylalanine ammonia lyase and polyphenol oxidase, as well as induction of new trypsin and chymotrypsin inhibitors. The *Fusarium oxysporum* protease-2 was inhibited completely by root extract of chickpea inoculated with *T. harzianum* L_1 and showed maximum resistance to rotting of roots caused by wilt disease.

Key words: *Trichoderma harzianum* L_1, biocontrol, *Fusarium oxysporum*, chickpea, protease inhibitors, polyphenol oxidase.

Abbreviations*:* PAL_Phenyl ammonia lyase; PPO_ Polyphenol oxidase; FOX_ *Fusarium oxysporum* f.sp. *ciceri*; PIs_ protease inhibitors.

Introduction

The genus *Trichoderma* is widespread in soil and on decaying wood and vegetable matter. As saprophytic organisms, *Trichoderma* spp. are able to use a wide range of compounds as carbon and nitrogen sources and secrete a variety of enzymes to break down recalcitrant plant polymers into simple sugars for energy and growth. The high degree of ecological adaptability shown by strains within the genus *Trichoderma* is reflected its world wide distribution, under different environmental conditions, and its

survival on various substrates. This considerable variation, coupled with their amenability of cultivation on inexpensive substrates, makes *Trichoderma* isolates attractive candidates for a variety of biological control applications (Harman, 2006). Several modes of action have been proposed to explain the biocontrol of plant pathogens by *Trichoderma*; these include production of antibiotics and cell wall degrading enzymes, competition for key nutrients, parasitism, stimulation of plant defense mechanisms and combination of these possibilities. *Trichoderma* spp. generally grows in its natural habitat on plant root surfaces and therefore it controls root diseases in particular. The dual role of antagonistic activity against plant pathogens and plant growth promoter make *Trichoderma* strains appealing alternatives to hazardous fumigants and fungicides. *Trichoderma* spp. were shown to be very efficient producers of extracellular enzymes, and some of these have been implicated in the biological control of plant diseases (Monte, 2001; Harman, 2006).

Plants are capable of producing an immune response after primary pathogen infection, which is known as systemic acquired resistance (SAR) (van Loon et al., 1998). The activation of SAR correlates with the expression of pathogenesis-related (PR) genes, including acidic and basic β-1,3-glucanase and chitinase, which supposedly act against the cell walls of the pathogen (Ferreira et al., 2007). Non-pathogenic rhizobacteria and fungi, such as *Trichoderma* spp. can induce systemic resistance in plants that is phenotypically similar to SAR (Yedidia, 2000). No single biocontrol strain is known to possess all of the mechanisms discussed above, and the genetic and biochemical bases for their efficacy are still explored. Therefore, although potential biocontrol agents with suitable antagonistic characteristics may be readily found, they must be screened carefully for other traits relevant to their use in a given application. In the present study we have screened a local isolate of *Trichoderma harzianum* strain L_1 for secretion of some lytic enzymes, and report its exploitation as a biocontrol agent against chickpea wilt, a soil-borne fungal disease caused by *Fusarium oxysporum* f. sp. *ciceri*.

Materials and methods

The isolate of *T. harzianum* L_1 used in the present study was obtained from the rhizosphere of healthy chickpea plants from a plot contaminated with wilt disease at the Agriculture Research Station, Gulbarga, using a standard procedure. It was identified on the basis of its morphological characteristics as *T. harzianum* (L_1). This local isolate has been shown to possess the highest efficacy against pigeon pea wilt caused by *F.* udum Butler, when compared to other *Trichoderma* spp. (Jayalakshmi et al., 2003).

Cultivation of T. harzianum L_1 for the production of lytic enzymes by solid state fermentation method

T. harzianum L_1 was maintained on slants of PDA media with glucose (2%) as carbon source for 7 days to generate spores. These spores were scraped aseptically from the surface of the agar plate and suspended in 0.1% sterile Tween 80 in physiological saline; this solution was used as inoculum. *T. harzianum* L_1 was cultured in 250 mL Erlenmeyer flasks on medium containing 10 g of rice bran and 10 mL of mineral salts solution (g/l; KH_2PO_4, 2.5; KNO_3, 5.0; $MgSO_4$. $7H_2O$, 1.0; Na_2SO_4, 1.0; $FeCl_2$, 0.02; $ZnSO_4$. $7H_2O$, 0.0015; $CuSO_4$. $5H_2O$, 0.003 and $MnSO_4$, 0.001) in distilled water, which was sterilized twice, cooled and inoculated with 1 mL of conidia suspension (4×10^7 conidia/mL). The fungal culture was incubated at 37 °C for 5 days. The production of different lytic extracellular enzymes (viz: protease, xylanase, β-1,3-glucanase, and chitinase) was tested replacing the rice bran by one of the various natural lignocellulosic substrates (Table 1) that were moistened with mineral salt solution (10 g/10 mL) in the presence and absence of 0.1% crab shell powder. Enzymes produced by *T. harzianum* L_1 grown in the above media were extracted twice with 100 mL of 50 mM sodium acetate buffer pH 6.5 and squeezed through a wet muslin cloth.

The pooled enzyme extract was centrifuged at 15000 × g for 10 min at 4 °C. The clear supernatant was used as the enzyme source for determination of protease (Kunitz, 1947), xylanase (Miller, 1959), β-1,3-glucanase (Somogyi, 1957; Nelson, 1957), and chitinase (Boller and Mauch, 1988) activities.

Table 1. Effect of different lignocellulosic substrates on the production of lytic enzymes by *T. harzianum* L_1

Substrate	β -1, 3-glucanase[1]	chitinase[2]	protease[3]	xylanase[4]
1% peptone	15.23 ± 0.12	0.22 ± 0.02	120.56 ± 1.3	52.76 ± 0.75
Rice bran	45.78 ± 0.34	11.86 ± 1.2	125.34 ± 1.7	172.35 ± 0.49
Jowar bran	30.12 ± 0.56	10.57 ± 0.99	122.38 ± 1.1	79.33 ± 0.58
Saw dust	29.86 ±0.96	9.35 ± 0.65	130.89 ± 1.4	51.85 ± 0.91
Neem cake	46.22 ± 0.68	8.92 ± 0.85	132.34 ± 1.5	32.46 ± 0.85
Wheat bran	43.61 ± 0.42	11.60 ± 0.22	140.81 ± 1.5	52.33 ± 0.69
Gram bran	42.98 ± 0.87	12.10 ± 0.12	145.98 ± 1.9	72.78 ± 0.76
Sugar bagasse	48.78 ± 0.59	10.54 ± 0.45	117.97 ± 1.78	45.89 ± 0.36
Rice bran+neem cake	48.87 ± 0.45	11.98 ± 0.98	133.98 ± 1.09	172.45 ± 0.98
Rice bran+ Crab shell+ Neem cake	105.25 ± 0.55	26.86 ± 0.72	175.25 ± 1.67	225.33 ± 0.67

Each value in the table represents the mean ± S.E from three independent experiments. [1]µM of glucose equivalent/min; [2]nmol N-acetylglucosamine min^{-1} $.g^{-1}$ protein; [3]µg of tyrosine/min; [4]µg of xylose /mL/ min.

Protein content was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard.

Treatment of seeds with T. harzianum L_1

Chickpea seeds cv JG62 (susceptible to wilt disease) were obtained from the Agriculture Research Station, Gulbarga, India. The seeds were treated with *T. harzianum* L_1 solution containing 10^6 conidia mL^{-1}, at a ratio of 1 mL per 10 gram of seeds. Control seedlings were treated with sterile distilled water. The roots of seedlings were collected at different time intervals (1-5 d), and grinded them in a prechilled mortar with pestle in a ice cold 0.1 M Tris-HCl buffer (pH 6.0) containing $5x10^{-3}$ M 2-mercaptoethanol for extraction of enzymes. The enzymes extract was centrifuged at 10,000 x g for 25 min at 4 °C. The supernatant thus obtained was used as an enzymes source for the determination of phenylalanine ammonia-lyase (PAL) (Burrell and Rees 1974) and polyphenol oxidase (PPO) (Mayer et al., 1965) activities (PPO activity was expressed as the change in absorbance at 495 $nm.min^{-1}$ $.mg^{-1}$ protein).

Detection of protease inhibitors (PIs) by X-ray film contact print method

Extraction of plant protease inhibitors (PIs) was performed using the method described by Plunkett (1982). Briefly, roots of 5 day-old chickpea plants were ground in a pre-chilled pestle and mortar with 50 mM Tris-HCl buffer, pH 8.0 (3 $mL.g^{-1}$ tissue) containing 10 mM 2-mercaptoethanol and 5% (w/v) PVP-40. The extract was further subjected to continuous stirring on a magnetic stirrer at 4 °C for 4 h. The resulting suspension was centrifuged at 4 °C for 20 min at 10,000 × g, and the resulting supernatant was used as crude extract of PI to measure inhibitory activity. Root extracts of chickpea seedlings (200 µg) treated with water, and *T. harzianum* L_1 or extract of *T. harzianum* L_1 (200 µg) were separated by PAGE on a vertical slab gel using the discontinuous buffer system of Davis (1964). After electrophoresis, the gel was processed for activity staining of PIs, using the gel X-ray film contact print method (Pichare and Kachole 1994). Native gels were equilibrated in 0.1 M Tris-HCl buffer, pH 7.8, for 10-15 min, followed by incubation

in trypsin solution (0.1 mg/mL, Sigma) or chymotrypsin (20 µg/mL) for 15 min at 37 °C in a shaker at 45 rpm. The gels were washed with the same buffer and placed on a piece of undeveloped X-ray film for 3-5 min. The films were then washed with warm water and bands displaying inhibitor activity were visualized as unhydrolyzed gelatin on X-ray films.

Visualizastion of In-vitro effect of PI extract on proteases of Fusarium oxysporum

A culture of *Fusarium oxysporum* f. sp. *ciceri* (race 1) causing wilt disease in chickpea was obtained from the Agriculture Research Station Gulbarga, India. *F. oxysporum* was grown in a 250 mL Erlenmeyer flask containing 50 mL of Sabouraud`s dextrose broth for 5 days at 28 °C. The fungal cells were separated by filtration and the filtrate was used to determine extracellular protease activity in polyacrylamide gels. Crude protease (100 µg) from *F. oxysporum* was resolved using electrophoresis on a native polyacrylamide gel (8%) and inhibitory activity was checked using a crude PI extract. After electrophoresis the gel was equilibrated with 0.2 M glycine-NaOH buffer (pH 10) for 5-10 min. After equilibration, the *F. oxysporum* resolved gel strips were incubated in PI extracts (100 mg/mL) obtained from roots treated with water and *T. harzianum* L_1 or *T. harzianum* L_1 extract alone, for 30 min at 37 °C. The gel strips were washed in buffer and overlaid on X-ray film for 45 min. The films were then washed with warm water to reveal hydrolyzed gelatin as protease activity bands on X-ray film.

Pot experiment

A pot experiment was conducted during 2006 and 2007 using a completely randomized block design with 5 treatments and 4 replicates to evaluate the performance of *T. harzianum* L_1 as a biocontrol agent against wilt. Ten seeds of the susceptible chickpea cultivar JG62 were sown in each 15 cm diameter surface-sterilized (1% mercuric chloride) plastic pot, which was filled with 1 kg of sterilized soil (3 subsequent sterilizations at 1.1 kg .cm for 1 h for 3 days). Each pot was then mixed with 20- day-old culture of the pathogen, which was mass-multiplied on sand maize meal water medium (90 g sand, 10 g maize meal, 20 mL distilled water) at 50 g/kg soil one week before sowing (Dubey et al., 2007). Before sowing the chickpea seeds , *T. harzianum* L_1 was applied to them at a ratio of 2 g of 7- to 10-day-old spores with mycelial culture mass per kg of seed, by shaking in a conical flask to ensure uniform distribution of the biocontrol agent on the seed surface. The biomass production of *T. harzianum* L_1 was carried out in a 250 mL conical flask containing (all sterilized) 10 g rice bran, 1 g neem cake, 0.1 g crab shell powder, and 10 mL of water inoculated with the mycelial plugs of a 3-day-old culture of *T. harzianum* L_1 grown on PDA medium. The flask was incubated at room temperature for 5 days and mixed with the pot soil containing wilt sick pathogen. The pots were observed for wilt incidence after 20 days of sowing and compared with the control pots.

Results

Screening of lytic enzymes on different substrates

The secretion of lytic enzymes by *T. harzianum* strain L_1 was tested in solid-state fermentation using various lignocellulosic substrates as raw materials. The strain L_1 was shown to secrete some of the lytic enzymes constitutively (Table 1). The maximal enzyme activities were reached during its cultivation on rice bran. Upon addition of crab shell powder (0.1%) to the rice bran, chitinase and β -glucanase activities were increased by more than 50%. Xylanase and protease activities were also increased by 30% (Table 1). During the early stages of incubation, a low level of enzyme activity was detected (data not shown) which steadily increased, reaching a maximum level at 120 h of incubation. Beyond 120 h, a steep loss in production was observed, which could be due to loss of moisture as a consequence of prolonged incubation at 40 °C. Various divalent cations (Zn^{+2}, Mn^{+2}, Ca^{+2}, Pb^{+2}, Mg^{+2}, Fe^{+3}) and the chelating agent EDTA at concentrations in the 0.1-1 mM range did not have any noticeable effect on enzyme production (data not shown).

PAL and PPO activities

Application of strain L_1 led to significant increase in PAL (150.22 ± 0.62 µg of cinnamic acid min/ mg) and PPO (39.40 ± 0.23 Units. min^{-1} .mg^{-1}) activities by 50% in roots was noticed, after 5

Table 2. Effect of *T. harzianum* L_1 on chickpea wilt incidence caused by *F. oxysporum* f. sp. *ciceri* during cropping seasons of 2005-06 and 2006-07.

Treatments*	Mean wilt incidence (%)	
	2007-06	2006-07
T1	100.00 (90.00)	100.00 (90.00)
T2	00.00 (00.00)	00.00(00.00)
T3	87.82 (69.62)	86.15 (68.19)
T4	47.03 (43.30)	42.67 (40.86)
T5	12.90 (21.03)	13.12 (21.22)
SEM±	0.714 (0.517)	0.692(0.492)
CV	4.88 (4.31)	3.90 (3.46)
CD (P=005)	2.199(2.31)	1.82 (2.17)

SEM ± standard error mean. CV - critical value mean; CD - critical difference mean *Mean of four replicates. Figure in parentheses are arcsine-transformed values. T1- seeds sown in wilt sick pot. T2 (control)-seeds sown in a pot containing natural (un-inoculated pathogen) soil. T3- Seeds treated with *T.* harzianum L_1 before sown in wilt sick pot. T4-Seeds treated with *T. harzianum* L_1 before sown in wilt sick pot containing *T. harzianum* L_1 biomass. T5-Seeds treated with *T. harzianum* L_1 before sown in wilt sick pot containing *T. harzianum* L_1*bio*mass with crab shell powder.

days of treatment with *T. harzianum* L_1 compared with their respective controls roots of chickpea. However, low levels of PAL (15 ± 0.12 µg of cinnamic acid min/ mg) and PPO (8.0 ± 0.11 Units. min^{-1} .mg^{-1}) activities were also found in the extract of strain L_1.

Visualization of trypsin inhibitors and chymotrypsin inhibitors

Figs. 1A and B show that the cultivar JG62 possess two native isoforms of trypsin inhibitors (TI-4 and 5) and chymotrypsin inhibitors (CTI-1 and 3) in control roots. Upon treatment with *T. harzianum* L_1, we observed induction of three new isoforms TI-1, 2 and 3, and two new isoforms CTI-2 and 4. The native isoform TI-4 was further induced in roots inoculated with strain L_1. Since TI-3 was also detected in *T. harzianum* L_1 extract (Fig. 1A: Lane-L_1) and its presence in root extract could be originated from the fungi.

Inhibition of FOX proteases by PIs extract

Two proteases were detected in the extracellular crude extract of *F. oxysporum* (Fig. 1C), in which protease-2 was completely inhibited by the root extract inoculated with *T. harzianum* L_1 and partially by the extract of strain L_1.

Pot experiment

In the pot culture assay under glasshouse conditions, the biomass formulation of *T. harzianum* L_1 in rice bran neem cake medium caused a significant ($p = 0.05$) wilt reduction as compared to control (Table 2).

Discussion

Trichoderma strains with biocontrol potential are applied in agricultural soils with certain characteristics. Therefore, it is also of great importance to collect information about the effects of different substrates on the growth and enzyme activities of the biocontrol strains. The maximum activities of lytic enzymes were detected when the strain L_1 was grown on rice bran containing neem cake and crab shell powder. A number of *Trichoderma* isolates produce a wide variety of fungal cell wall-degrading enzymes, such as chitinase, β-1,3-glucanase β-1,6-glucanases and proteases, when grown on polysaccharides, fungal cell walls, or autoclaved mycelium as a carbon source (Lorito 1998; Kucuk 2007; Kuck & Kivanc

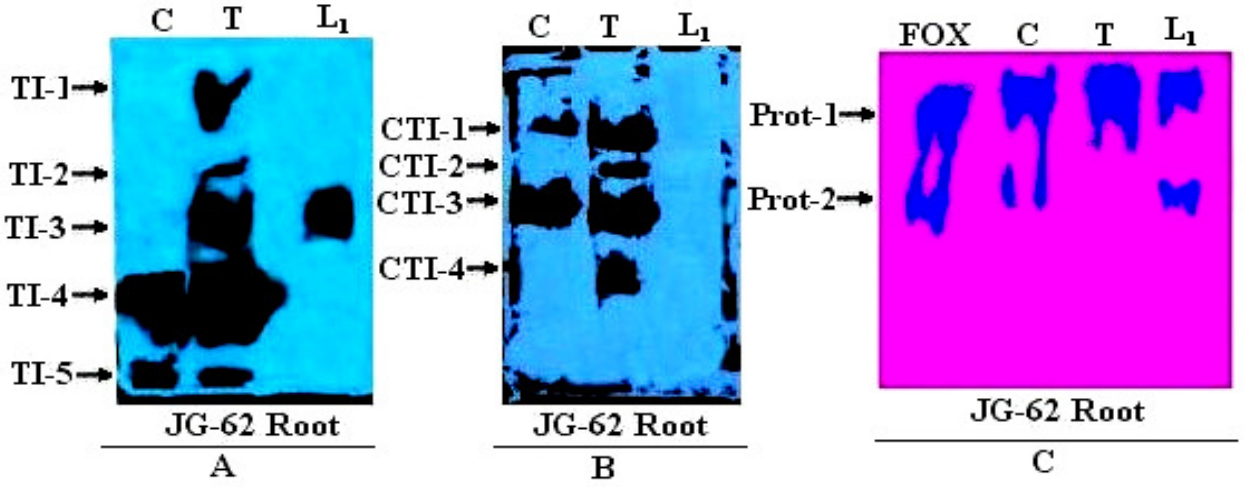

Fig 1. Detection of trypsin inhibitors (A) and chymotrypsin inhibitors (B) in roots of susceptible cultivar (JG-62) by the gel X-ray film contact print method. Lane C - control; Lane T - *T. harzianum* L_1 treated root extract; Lane L_1- extract of *T. harzianum* L_1. (C) In-vitro effect of protease inhibitor extract of roots on the extracellular proteases of *Fusarium oxysporum* using the gel X-ray film contact method. Lane FOX- *Fusarium* proteases; Lane C – control root extract; Lane T - *Trichoderma* strain L_1 treated root extract. Lane L_1 - *Trichoderma* extract; Other experimental details are given in methods and materials.

2008; Singh et al., 2008). These observations, together with the fact that chitin and β -1,3-glucan are the main skeletal polysaccharides of fungal cell walls (except from oomycetes and cellulose), suggest that chitinase and β -1,3-glucanases act as key enzymes in the lysis of phytopathogenic fungal cell walls during the antagonistic action of *Trichoderma*. Hence fungal cell wall-degrading enzymes of *Trichoderma* spp are of special importance in plant defense mechanisms. Rice bran, crab shell powder and neem cake combination was one of the suitable formulation for the induction of maximum levels of fungal hydrolytic enzymes.

PAL is a key enzyme in the biosynthesis of phenyl propane unit, which is a component of phenolic acids, flavonoids and lignins. Increased PAL activity in response to pathogen or elicitor spray has been reported (Song et al., 1993). De Meyer et al. (1999) reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated PAL in bean roots. In treated roots, the activity of PAL reached a maximum on the 5th day after treatment while the enzyme activity in control roots remained constant. Several studies have shown that phenolics and PAL activity are induced in chickpea upon treatment with pathogen (Karthikeyan et al., 2006; Arfaoui et al., 2007). PPO activity was increased by the treatment with strain L_1, implicating it in induced defense responses against root rot in chickpea. Induction of PPO activity and PPO isoforms by biological agents has been reported in several host-pathogen combinations (Karthikeyan et al., 2006). Meena et al. (2000) reported that *P. flurescence* induced the activities of PPO in response to infection by *Cercospora personnatum* in groundnut. Chen et al. (2000) reported that various rhizobacteria and *Pythium aphanidermatum* induced the PPO activity in cucumber root tissues. Induction of plant defense enzymes and phenolics by treatment with plant growth–promoting rhizobacteria *Serratia marcescens* NBRI1213 has been reported (Lavania et al., 2006). PPO overexpressing transgenic tomato plants exhibited high resistance to *Pseudomonas syringae*, the causative agent of speck disease, when compared with control plants (Li and Stiffens, 2002).

In the present investigation, it was observed that strain L_1 induced trypsin and chymotrypsin inhibitors in the roots of chickpea. In particular, protective plant proteins specifically induced in pathological or related situations have been

intensively studied from an agricultural perspective, and are called "pathogenesis-related proteins" (PR proteins). Proteinase inhibitors (PR 6) in plants are able to suppress enzymatic activities of phytopathogenic microorganisms (Ferreira et al., 2007). It is evident from Fig. 1C that protease inhibitors of chickpea roots inhibited the protease-2 of *F. oxysporum*. Several reports have shown that proteinase inhibitors are associated with resistance and that the inhibitors can block the proteinases of microbes including those of pathogens (Dunaevsky et al., 2005). Protease inhibitors isolated from healthy bean and tomato plants reduced the activities of proteinases from *Fusarium solani* and *Colletotrichum lindemuthianum* (Mosolov et al., 1979). Trypsin inhibitor from buckwheat seeds (*Fagopyrum esculentum* L. Moench) suppressed proteinase activity and spore germination of the fungus alternaria alternate and *F. oxysporum* (Ryan, 1996; Dunaevsky et al., 2005) The synthesis of proteinase inhibitors in response to infection by phytopathogenic microorganisms was first observed in tomatoes infected with the oomycete, *Pseudomonas infestans.* In that case, a correlation was observed between an increased content of trypsin inhibitors and chymotrypsin inhibitors and resistance to the pathogen. An increase in the activity of serine proteinase inhibitors was also observed in potato tubers infected with. *P. infestans*. (Valueva and Mosolov, 2004; Dunaevsky et al., 2005).

In the present study, the effect of treatment was found to be significant ($p = 0.05$) with respect of wilt incidence during the two years of the experiment, and the maximum wilt reduction was observed in pots with treatment T5 (Table 2). This could be due to a high degree of mycoparasitism and production of some lytic enzymes by *T. harzianum* L_1. *Trichoderma* spp have been shown to decrease wilt incidence in chickpea plants (Dubey, 2007) and increase root development in numerous other plants (Herman et al., 2006). This effect lasts through the entire life of annual plants, and can be induced by the addition of small amounts of the fungus (less than $1g.ha^{-1}$) applied as part of seed treatment. Such improvements in root development are frequently associated with increases in yield and biomass. *Trichoderma* treatments therefore have the potential to improve overall crop yield and might be particularly important in suboptimal field conditions. *Trichoderma* species produce many of the same or similar enzymes as plant species that are capable of digesting walls and membranes of plant pathogens as well as of other plants. It is possible that *Trichoderma* species release elicitor-active compounds from pathogenic fungi, plant cell walls, or organic matter in the soil or the root, thereby inducing resistance in associated plant tissues. Several Trichoderma spp can activate systemic induced resistance in plants (Brunner et al., 2005). The additionof sterilized or living *Rizoctonia solani* mycelia to the soil also increases resistance to *Botrytis cinerea* infection of bean leaves. The simultaneous presence of *R. soalni* in soil and *T. atrovirdie* on the seed or root surface produced the highest level of systemic resistance (Brunner et al., 2005). This result may have occurred as a result of a direct additive effect of the two fungi on the palnt or because the presence of *R. solani* stimulated *T. atroviride* to interact with the palnts by releasing resistance –inducing molecules. The mechanism of the *Trichoderma* spp–palnt interaction that activates the systemic plant resistance response has only recently been studied at molecular level, and our understanding of this response currently is limited to the identification of some of the palnt factors accumulated in response to root contact with this fungus.

From the present study, it can be postulated that there is a rapid high induction of enzymes involved in phenyl propanoid metabolism and of PR proteins in chickpea roots, in response to treatment with the biocontrol agent *T. harzianum* L_1. This species could be an excellent candidate for providing long-term induced resistance in chickpea. One of the emerging strategies for managing plant diseases is the use of microbial biocontrol agents with the aim of reducing pesticide residues. *T. harzianum* L_1 can be used as an efficient biocontrol agent for various soil-borne pathogens by formulation with neem cake, since neem cake acts as a biopesticide and does not interfere with enzyme secretion.

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