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Differential expression of defense related enzymes and protease inhibitors in two different genotypes of chickpea by *Trichoderma harzianum* L1

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Abstract

Induction of some defense related enzymes and protease inhibitors by *Trichoderma harzianum* L1 was investigated in two different genotypes of chickpea cvs. JG62 and A1 which were susceptible and resistant to wilt/root rot diseases caused by *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomina phaseolina*, respectively. Phenylalanine ammonia-lyase activities were increased by two fold in resistant cv. A1 than that of susceptible cv. JG62 upon treatment with salicylic acid and *T. harzianum* L1. Induction of the multiple isoforms of catalases was observed in roots and shoots of cv. JG62 and they were insensitive to salicylic acid (SA) or *T. harzianum* L1. However, no new isoforms of catalases were observed in either roots or shoots of cv. A1 and the native CAT-2 was inhibited. Induction of new isoforms of peroxidase, chitinase (PR-3) and β-1, 3-glucanse (PR-2) was observed in the resistant cv. A1. New protease inhibitors (PIs) were elicited in both the cultivars; however PIs of cv. A1 were highly effective in inhibiting the fungal growth as well as proteases secreted by *F. oxysporum* f. sp. *ciceri* and *M. phaseolina*.

Keywords: Chickpea, *F. oxysporum*, *M. phaseolina*, protease inhibitors, *Trichoderma harzianum*.
Abbreviations: cv: cultivar; Foc: *Fusarium oxysporum*; DAG Days after growth; ISR: Induced systemic acquired resistance; PI: Protease inhibitors; PR: Pathogen related; SA: Salicylic acid; SAR: Systemic acquired resistance.

Introduction

Trichoderma spp. members represent widespread soilborne saprophytic organisms. These fungi are attractive candidates for various biological control applications as they are cultivable on inexpensive substrates (Harman, 2006) and thus are being used in bio-control of diseases in many different crops, like cotton, grapes, sweet corn, lettuce, onion, peas, plum apples carrots, caused by pathogens such as *Pythium, Phytophthora, Rhizoctonia, Sclerotinia, Botrytis* and *Fusarium* spp. (Sariah, 2005). Various isolates have been used with success in greenhouse and field applications in soil and in the phyllosphere, as well as in cold storage (Chet, 1987). The ability of *Trichoderma* strains to protect plants against root pathogens has long been attributed to an antagonistic effect against the invasive pathogen (Monte, 2001 Vinale et al., 2008). Strains of *Trichoderma* added to the rhizosphere protect plants against numerous classes of pathogens by SAR and ISR in plants (Harman et al., 2004). The activation of SAR correlates with the expression of pathogenesis-related (PR) genes, including acidic and basic β–glucanase and chitinase, which supposedly act against the cell walls of the pathogen (Ferreira et al., 2007). At the molecular level, resistance results in an increased activity of some enzymes related to defensive mechanisms, such as phenylalanine ammonia lyase and chalcone synthase which are involved in the biosynsthesis of phytoalexins (Lorito et al., 2010). Activation of defense responses using elicitors could be a valuable strategy as an alternative to the use of conventional fungicide and insecticide to protect plants against pathogens (Harman, 2006). A variety of strains of *T. virens, T. asperellum, T. atroviride and T. harzianum* induce metabolic changes in plants that increase resistance of broad range plants to a wide range of plant-pathogenic microorganisms and viruses (Harman, 2006; Lorito et al., 2010). Salicylic acid is a key signal molecule involved in plant's defense response to pathogen invasion and controls development of systemic acquired resistance SAR (Vlot, 2009; Hayat et al., 2010). The role of SA in disease resistance was elucidated (Chen and Klessig, 1991; Chen et al., 1993). Plants respond to SA by increasing the production of reactive oxygen species such as O_2^- that are dismutated by superoxide dismutase to H_2O_2 (Chen and Klessig, 1991; Chen et al., 1993). Increased production of H_2O_2 initiates cell wall cross-liking (Tenhaken et al., 1995) and enhances the enzymes involved in the biosynthesis of lignin and SA (Leon et al., 1995). SA was a known inducer of SAR in chickpea (Raju et al., 2007, 2008). Chickpea (*Cicer arietinum* L.) is the third most important pulse crop in the world but its production suffers from various biotic and abiotic stresses (Singh and Ocampo, 1997). Amongst the diseases of chickpea, wilt caused by *Fusarium oxysporum* f. sp. *ciceri* and root rot caused by *Macrophomina phaseolina* are important pathogens causing loss to a tune of 10-100 percent depending upon sickness of soil (Singh et al., 1998). *F. oxysporum* f. sp. *ciceri* is soil and internally seedborne and can also survive

Table 1. Effect of different doses of *T. harziamum* L1 on chickpea wilt complex 20 at DAG

Treatments*	Percent mortality of plants			
	A1 cv.		JG62 cv.	
	Foc (Wilt)	*M. phaseolina* (Root rot)	Foc (Wilt)	*M. phaseolina* (Root rot)
T1	23.21 (28.79)	22.00 (27.97)	80.52 (63.79)	83.65 (66.19)
T2	20.22 (26.71)	20.38 (26.85)	53.00 (45.00)	60.73 (51.18)
T3	19.93 (26.49)	18.83 (25.70)	50.13 (46.72)	64.00 (53.13)
Control	29.96 (32.04)	30.00 (33.21)	1000.00 (90.00)	100.00 (90.00)
SE±	0.503	0.634	0.712	1.021
CD (p=0.05)	1.50	2.00	2.10	3.00

SE ± standard error mean; CD- critical difference mean. *Mean of four replicates.
Figure in parenthesis are arcsine-transformed values. T1-T3: 2, 4, and 6 g of *T. harzianum* L1 respectively applied to per kg of seeds

as chlamydospores in plant debris in the field (Stevenson et al., 1995). In soil *M. phaseolina* survives primarily as sclerotia which are formed in the host tissues and released in to the soil as tissues decay (Cook et al., 1973; Iliyas et al., 1974). In recent times, there has been a worldwide swing to the use of eco-friendly methods for protecting the crop from pests and diseases (Vinale et al., 2008). Chemical methods are not economical in the long run because they pollute the atmosphere, damage the environment, leave harmful residues, and can lead to the development of resistant strains among the target organisms with repeated use (Naseby et al., 2000). The use of biocontrol agents for the control of plant diseases is one of the most promising means to achieve the minimal usage of chemicals on the environment (Vinale et al., 2008). The first requirement of biological control is the identification and deployment of highly effective strains. The filamentous fungi, *Trichoderma* have attracted the attention because their multiprong action against various plant pathogens (Harman et al., 2004). Earlier, *Trichoderma* strain L1 has been employed as a potent antagonistic bioagent against the wilt causing pathogens (Jayalakshmi et al., 2003, 2008) and induced defense responses in chickpea against wilt disease caused by *F. oxysporum* f. sp. *ciceri* (Jayalaksmi et al., 2009). In view of the importance of *Trichoderma* spp. and SA in the elicitation of disease resistance in plants against various pathogens, in the present study, *T. harzianum* strain L1, a local isolated was screened and compared with SA for the induction of some potential defense enzymes and protease inhibitors in two different genotypes of chickpea plants.

Results

Phenylalanine ammonia-lyase activity

The PAL activities increased by 1.5, and 1.4 fold in roots and by about 1.7 to 2 fold in shoots of susceptible cultivar (Fig.1A) whereas 2.0 and 1.9 fold in roots 2.3 and 2.0 fold in shoots of cv. A1 respectively, treated with SA and *T. harzianum* L1 compared with their untreated controls at 240 h (Fig.1B).

Influence of T. harzianum L1, SA, Foc and M. phaseolina on the isoform composition of catalase, POD, PPO and chitinase

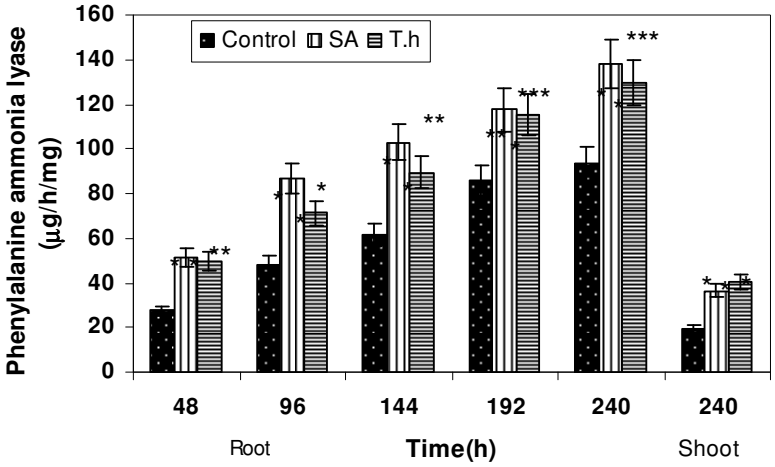
In plant cells CAT and POD exhibit as multiple isoforms and their isoform composition was analysed by native PAGE. Gels stained for CAT activity revealed two isoforms (CAT-1 and CAT-2) in control roots of susceptible cultivar while a third isoform CAT-3 was observed in roots treated with SA and bioagent (Fig.2A). On the other hand, shoots showed three isoforms (CAT-2 CAT-3 and CAT-4) in both control as well as the seeds treated with SA and *T. harzianum* L1 (Fig. 2B). In contrast, control root extract of the resistant cultivar revealed three CAT isoforms, in which two isoforms (CAT-1 and CAT-2) were completely inhibited and isoform CAT-3 was partially inhibited (Fig. 2C) in the roots treated with SA and *T. harzianum* L1 whereas, control shoots expressed three isoforms of CAT in which CAT-1 and 2 were inhibited upon treatments with SA and *T. harzianum* L1 (Fig. 2D). No inductions of CAT isoforms were observed in resistant cultivar A1 upon treatment with SA. No major changes were observed for POD isoforms in shoots. Whereas, induction of new POD isoform was observed in susceptible cultivar roots upon treatements (Fig 3A and B). In contrast, there was an induction of a new isoforms of POD in roots and shoots of resistant cultivar treated with SA and *T. harzianum* L1. Further the intensity of band pattern in roots increased with the treatments (Figure 3C and D). Native gel stained for PPO activity revealed two isoforms (PPO-1 and PPO-2) in control as well as in treated roots and shoots of susceptible cultivar (Fig. 4C and D). However, an induction of a newly synthesized PPO-2 isoform was observed in roots and shoots of resistant cultivar treated with *T. harzianum* L1 and SA (Fig. 4A and B).

Detection of chitinase and β-1, 3 glucanse activities

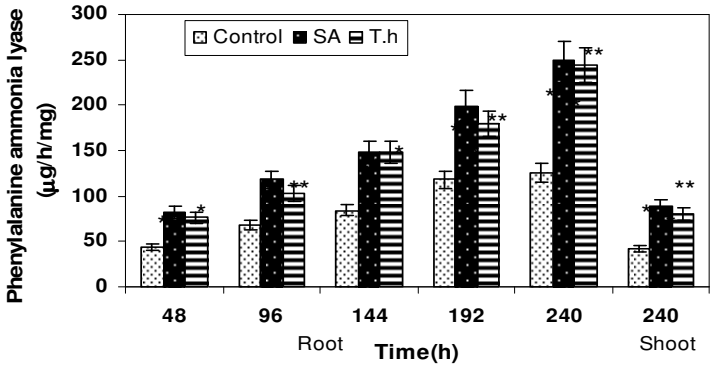
Chitinase activity was observed in the resistant cultivar of roots and shoots treated with SA, *T. harzianum* L1 and *T. harzianum* L1 + SA (Fig. 5A) whereas, no chitinase activity was detected in susceptible cultivar (data not shown). Western blot showed constitutive expression of β-1, 3-glucanase in roots of resistant cultivar (Fig. 5B). Expression of β-1, 3-glucanase was at a low level in control and induced upon treatments with the SA and *T. harzianum* L1 (Fig. 5B). In contrast, no β-1, 3-glucanase activity was detected in either roots or shoots of susceptible cultivar before and after treatments.

Electrophoretic visualization of proteases and proteases inhibitors

Four isoforms of proteases were found in roots of the cv. A1 treated with water (control). However, in roots treated with *T. harzianum* L1 seedlings, isoform 3 and 4 were inhibited (Fig. 6a). In contrast, the banding pattern of proteases in roots of



(A)



(B)

Fig 1. Effect of SA and *T. harzianum* L1 on phenylalanine ammonia-lyase activities of (A) cv JG62 and (B) cv A1. Differences from control values were significant at. $^{*}_{P<}0.05$; $^{**}_{P<}0.01$ $^{***}_{P<}0.001$, according to ANOVA variance for regression.

cv. JG62 revealed two isoforms in control and induction of new isoform of protease was observed in seedlings treated with *T. harzianum* L1 (Fig. 6b). Earlier Jayalakshmi et al. (2009) have shown that the cv. JG62 possess two isoforms of trypsin inhibitors (TI-4 and 5) and chymotrypsin inhibitors (CTI-1 and 3) in control roots. Upon treatment with *T. harzianum* L1, induction of three new isoforms TIs (TI-1, TI-2 and TI-3), and two new isoforms CTIs (CTI-2 and CTI-4) was observed. The isoform TI-4 was further induced in roots inoculated with strain *T. harzianum* L1. Since TI-3 was also detected in *T. harzianum* L1 extract and its presence in root extract could be of fungal origin. The banding pattern of TI reveals four TI (TI-2, TI-3, TI-5, TI-6) and three CTI (CTI-3, CTI-4, CTI-5) in roots of water treated control plants of resistant cv. A1. Further, induction of two TI (TI-1 and TI-4) and two CTI (CTI-1 and CTI-3) isoforms in root was observed after treatment with *T. harzianum* L1 (Fig. 7A and B).

Visualization of in-vitro effect of PI extracts on protease of Foc and M. phaseolina

Four proteases were detected in the extracellular crude extract of *M. phaseolina* (Fig. 8A), but no inhibition was observed by control root extract. However protease-4 was inhibited by the root extract of JG62 treated with *T. harzianum* L1 (Fig 8A) whereas, proteases 2, 3 and 4 of *M. phaseolina* were partially inhibited by the control root extract and complete inhibition was observed by roots extract of cv. A1 treated with *T. harzianum* L1 (Fig 8B). Two proteases were detected in the extracellular crude extract of *F. oxysporum* (Jayalakshmi et al. 2009), of which protease-2 was completely inhibited by the roots extract of A1 inoculated with *T. harzianum* L1 and partially by the control roots extract (Fig 8C). Similar results were reported to cv JG62 (Jayalakshmi et al., 2009).

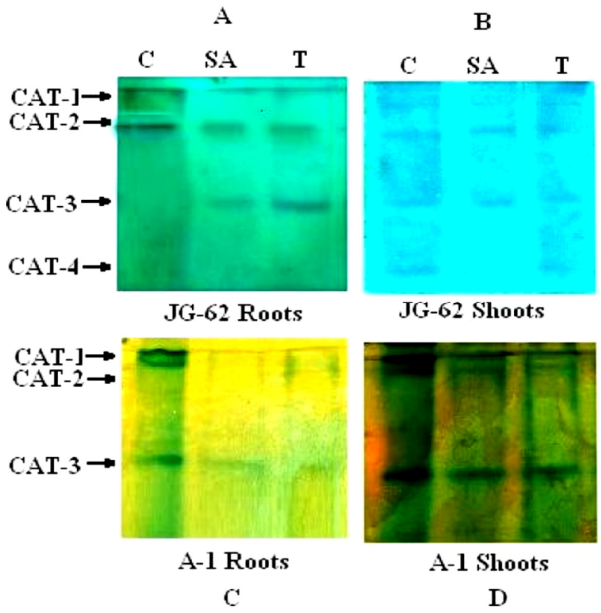


Fig 2. Native gel stained for catalase activity at 15 DAG. A: roots and B: shoots of susceptible cv. JG62; C: roots and D: shoots of resistant cv. A1. Equal amounts of protein loaded on gels (200 µg). Lane C: control; SA: salicylic acid; T: *T. harzianum*

Pot experiment

Results of pot experiments (Table-1) revealed that the seeds treated with strain *T. harzianum* L1 at 2, 4 and 6 g/kg seeds significantly reduced the wilt and root rot in both the cultivars compared with control. Maximum percent reduction of wilt and root rot was observed in the seeds treated with the *T. harzianum* L1 at the rate of 6g/kg seeds. The wilt and root rot incidence recorded in T2 and T3 were statistically significant.

Bioassay of for the inhibition of Foc and M. phaseolina

Crude PI extract of both the cultivars was checked for their ability to inhibit fungal growth. Foc and *M. phaseolina* mycelial growth was inhibted more effectively by *T. harzianum* L1 treated root extracts of resistant cv. A1 than the susceptible cv. JG62. The inhibition of fungal growth is negligible in control roots of both the varieties. The activity of PI may be a result of inhibition of Foc and *M. phaseolina* proteases which are responsible for growth and development of both the pathogens (Fig. 9 A and B).

Discussion

In the present study, the differential induction of isozymes and PR proteins was observed in both the cultivars upon treatments with the *T. harzianum* L1 and SA. This could be a partially responsible for the resistance or susceptibility to various pathogens. In this study, PAL activity increased in response to *T. harzianum* L1/ SA treatments. PAL is a key enzyme in the biosynthesis of phenyl propane unit via phenylpropaoid pathway (Hahlbrock and Schell, 1989) which is a component of phenolic acids, flavonoids and lignins. Increased PAL activity level in response to pathogen or elicitor spray has been reported (Song et al., 1993). Similarly, chickpea seedlings treated with *Rhizobium* Pch43 followed by *Fusarium oxysporum* f. sp. *ciceri* race 0, resulted high accumulation of phenolics and PAL activity (Arfaoui et al., 2007). Peroxidases are critical in the biosynthesis of plant cell walls as they provide the peroxidative polymerization of monolignols coniferyl, p-coumoryl and sinapyl alcohols to lignin (Greisbach et al., 1991). Lignification serves to strengthen and reinforce cell walls and overall result is a toughening of plant tissue. The induction of multiple isozymes of CAT and POD are responsible for elevated levels of antioxidant enzyme activities in susceptible cultivar JG62. Production of ROS at elevated levels is a common feature of defense response in plants, when they are challenged by pathogens and elicitors and H_2O_2 produced as the end product functions as a second messenger, mediating the systemic expression of various defense related genes in tomato plants (Orozco-Cardenas et al., 2001) and also serves to strengthen the cell wall by cross-linking of lignin polymers. The co-ordinated induction of antioxidant enzymes in response to various biotic and abiotic stresses has been reported in tomato (Mittler et al., 2002) and chickpea (Raju et al., 2007). This study clearly demonstrates that the cv. A1 expressed catalases which were sensitive to SA and *T. harzianum* L1 treatments whereas, the susceptible cv. JG62 expressed catalases which were insensitive to SA/ *T. harzianum* L1 treatments. As a result cv. A1 could produce high levels of H_2O_2 that lead to induce SAR when the seedlings were treated with *T. harzianum* L1 or SA and became "primed" to resist future pathogen invasion, whereas the susceptible cv. JG62 could not produce any H_2O_2 as it is unable to establish SAR. Raju et al., (2007) have screened different genotypes of chickpea for the induction of SAR and found that the susceptible genotypes (for wilt disease) expressed multiple catalases and were insensitive to SA, did not express SAR while the resistant genotypes did not express multiple catalases and their native catalase isoforms were sensitive to SA, followed by expression SAR. The PPO over expressing transgenic tomato plants exhibited high resistance to *Pseudomonas syringae*, the causative agent of speck disease compared with control plants (Li and Stiffens, 2002). The induced PPO-2 isoform in roots and shoots treated with *T. harzianum* L1, SA and with pathogen might have also been implicated in induced defense responses against the pathogen invasion.

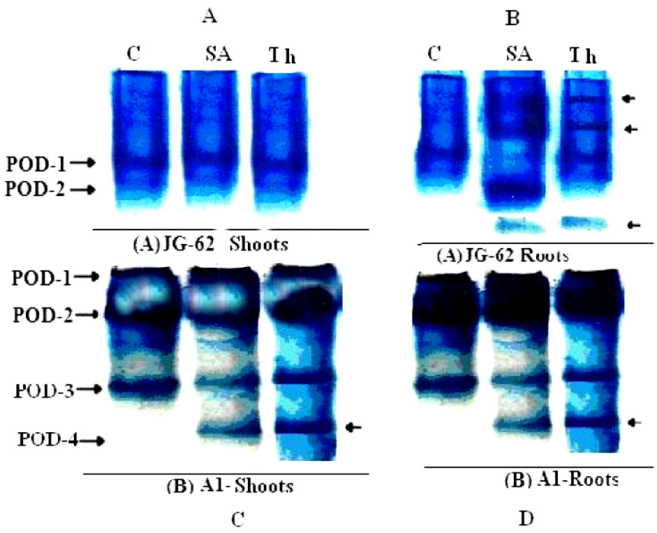


Fig 3. Native gel stained for peroxidase activity at 15 DAG. (A): shoots and (B) roots of susceptible cv. JG62; (C) shots and (D) roots of resistant cv. A1. Lane C: control; Lane SA: salicylic acid; Lane T.h: *T. harzianum* L1. Arrows indicate newly synthesized isoforms

However, no induction of PPO was observed in susceptible cv. JG62. Raju et al., (2008) have screened different genotypes of chickpea for the induction of PPO isoforms and found that the resistant genotypes (ICCV10, GBS-963, GBC-2 and A-1) for wilt disease expressed multiple isoforms of PPO, while the susceptible genotypes (L550, GBC-6 GCP-101, and GCP-107) did not. Earlier, they also have reported that the induction of PO isoforms, chitinase (PR-3), and β-1, 3-glucanase (PR-2) were observed in the resistant cv. ICCV10 but not in the susceptible cultivar L-550 on treatment with SA, spermin and SA+spermin. Ferraris et al., (1987) reported that infection with *F. oxysporum* f. sp. *lycopersici* caused several fold increase in chitinase and β-1, 3-glucanase activity in susceptible and resistant cultivars of chickpea. However, our results suggest that the expression of chitinase (PR-3) (Fig. 5A), β-1, 3-glucanase (PR-2) (Fig. 5B) and peroxidase (PR-9) activities occurs only in the resistant cultivar A1 but not in susceptible cv. JG62 after treatment with SA and bioagents. Chitinase along with β-1, 3-glucanase imparts resistance against a wide array of plant pathogens (Lee and Hwang, 2005). A direct role for chitinase in defense of plants against pathogens has been proposed because the substrate for those enzymes is a major component of the cell walls of many fungi. Expression of defense related genes such as those encoding pathogenesis-related proteins are used as markers for the establishment of SAR (Du and Klessig, 1997). Exogenous SA application induced PR-1 proteins (Yalpani et al., 1991). In the present study it is interesting to note that the induction of antioxidant enzyme activities, isozymes and PR proteins by *T. harzianum* L1 were similar as that of SA which mediates the SAR in plants. In the present investigation, it was also observed that strain L1 elicited trypsin and chymotrypsin inhibitors in the roots of chickpea. Proteinase inhibitors (PR 6) in plants are able to suppress enzymatic activities of phytopathogenic microorganisms (Ferreira et al., 2007). 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). Our results indicate that protease inhibitors of chickpea cv A1 roots inhibited the protease-2 of *F. oxysporum*. Earlier, Jayalakshmi et al., (2009) have reported that seeds of cv. JG62 treated with *T. harzianum* L1 shown to produce trypsin and chymotrypsin inhibitors and inhibited protease-2 of Foc. Raju et al., (2009) showed that new trypsin and chymotrypsin inhibitors were synthesized in response to SA and spermine treated roots and shoots of chickpea cv. ICCV10 and shown to inhibit the growth of *F. oxysporum*. Protease inhibitors isolated from healthy bean and tomato plants reduced the activities of proteinases from *Fusarium solani* and *Colletotrichum lindemuthianum* (Mosolov et al., 1979). In the present study, it was observed that the fungal growth was inhibited by the root extract of the cvs. A1 and JG62 following treatment of the seedlings with *T. harzianum* L1. Protease inhibitors of potato were shown to inhibit the pathogenicity and growth of *Botrytis cineaare* (Hermosa et al., 2006). *Trichoderma* spp. have been shown to decrease wilt incidence in chickpea plants (Dubey et al., 2007) and increase root development in numerous other plants (Harman, 2006). *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. *Trichoderma* spp. are known to induce ISR and SAR against several pathogens (Herman, 2004; Yedidia et al, 1999; 2003; Brunner et al., 2005) The application of *Trichoderma* or its metabolites for crop protection, such as the host defense inducers may become a reality in the near future as they can be produced cheaply in large quantities on an industrial scale and formulated for spray or drenching process. In conclusion, the differential expression of isozymes and PR proteins in moderately resistant and susceptible cvs of chickpea in response to treatments with *T. harzianum* L1 and SA was observed. However the induction of defense related enzymes/PR proteins in moderately resistant cv A1 was further induced and more effective compared with JG62. The defense mechamisms induced by *T. harzianum* L1 in both the cvs are comparable to SA mediated changes indicating that plant protecting mechanism of *T. harzianum* L1 is probably mediated by SA pathway.

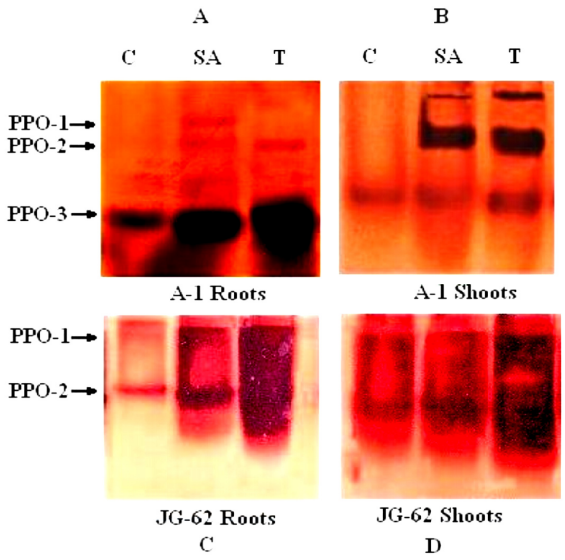


Fig 4. Native gel stained for polyphenol oxidase activity at 15 DAG: (A) roots and (B) shoots of resistant cv. A1 (C) roots and (D) shoots of susceptible cv. JG62. Lane C: control; SA: salicylic acid; Lane T: *T. harzianum* L1.

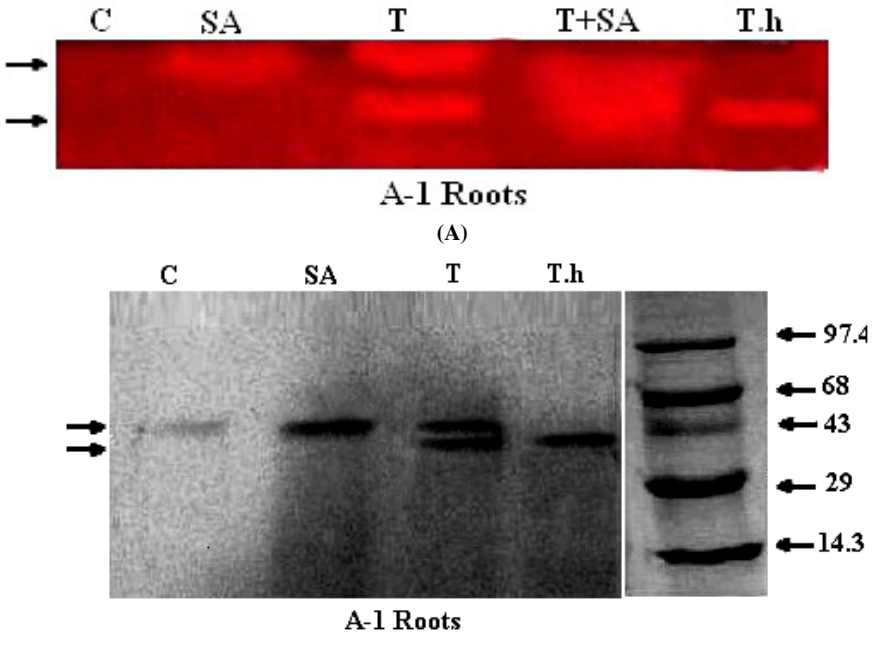


Fig 5. (A) Native gels stained for chitinase activity in roots of resistant cv. A1 at 15 DAG. (B) Immunoblot for β-1, 3-glucanase in roots of resistant cv. A1. Lane C: control, lane SA: salicylic acid; Lane T: *T. harzianum* L1 treated root extract and T.h: *T. harzianum* L1 extract alone.

Materials and methods

Plant material and fungal cultures

Chickpea seeds of the susceptible cv. JG62, moderately resistant cv. A1 to wilt and root rot diseases and local isolates of the wilt causing pathogens, *Fusarium oxysporum* f. sp. *ciceri* (Foc) (race 1) and *Macrophomina phaseolina* were obtained from the Agricultural Research Station, Gulbarga. *T. harzianum* L1 used in this study was isolated from the rhizosphere soil of healthy chickpea plants by using standard mycology procedures from wilt sick plot at the Agricultural Research Station, Gulbarga, India (Jayalakshmi et al., 2003, 2008).

Seed treatments and germinations

Chickpea seed were surface sterilized with 0.01% aqueous $HgCl_2$ for 1-2 min and then thoroughly washed with double distilled water. There were three replications with 60 seeds for each treatment. The first set was supplied with distilled water to serve as a control, while set two was supplied with 0.8 mM salicylic acid (SA), (at higher concentration seed germination was delayed, data not shown), set three was

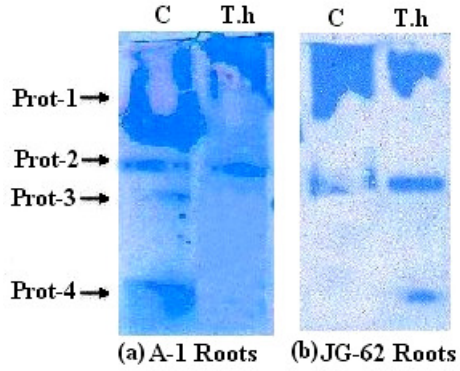


Fig 6. Detection of protease activity by X-ray gel film contact method. (a) roots of cv. A1 and (b) cv. JG62 at 15 DAG. For protease activity, the gel was equilibrated with 0.2 M Tris-HCl buffer pH 7.8 for 10 min, and overlaid on an undeveloped X-ray film (Kodak) for 1 h at 37 ^{o}C. The film was then washed with warm water and protease activity bands were visualized as hydrolyzed gelatin.

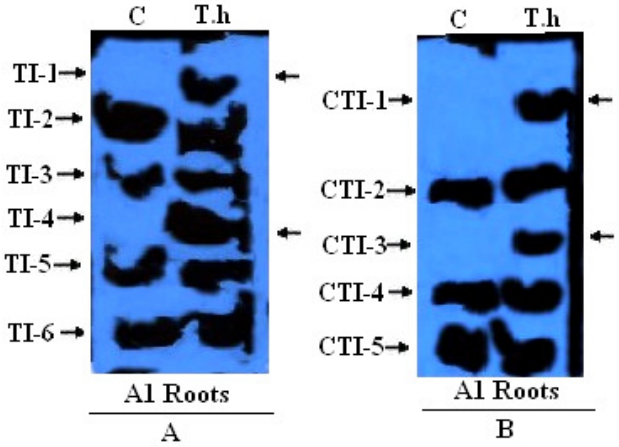


Fig 7. Detection of trypsin inhibitors (A) and chymotrypsin (B) by gel X-ray film contact print method in roots of resistant cv. A1 at 15 DAG. Arrows indicate newly synthesized inhibitors. C: control; T.h: *T. harzianum* L1 treated root extract.

supplied with *Trichoderma* solution containing 10^6 conidia mL^{-1} at the rate of 1 ml 10 g^{-1} seeds. All the Petri-plates lined with double layer of filter paper were kept wet by supplying respective solutions and allowed to germinate at 26 ^{0}C for 10 day period..

Enzymes extraction and assays

Weighed samples (g) (roots or shoots) were ground in a pre-chilled mortar with pestle in 5 mL of ice cold 0.1 M Tris-HCl buffer (pH 7.5) containing 5×10^{-3} M 2-mercaptoethanol. The extract was centrifuged at 10,000g for 25 min at 4 ^{0}C. The supernatant obtained was assayed for phenylalanine ammonina-lyase (PAL) (Burrell and Rees (1974). PAL Enzyme activity is expressed as µg of trans-cinnamic acid h^{-1} mg^{-1} protein. The isozyme patterns of catalase (CAT) (Woodbury et al., 1971), peroxidase (POD) (Vallejos, 1983) and polyphenol oxidases (PPO) (Jayaraman et al., 1987) were analyzed by native PAGE. Chitinase activity was determined by the modified method of Trudel and Asselin (1989). β 1,3 glucanase activity was detected in the gel as described earlier (Raju et al., 2008). The protein concentration of supernatant was determined according to Lowry et al., (1951) using bovine serum albumin as a standard.

Extraction of protease and protease inhibitors (PIs) from chickpea plants

The roots and shoots were ground in a pre-chilled pestle and mortar in an ice-cold 50 mM phosphate buffer pH (7.8). The extract was centrifuged at 4 ^{o}C for 25 min at 10,000 g. The resulting supernatant was used as an enzyme source for further determination of protease activities. Trypsin and chymotrypsin inhibitors were extracted by the modified method of Casaretto et al., (2004).

Extraction of proteases from F. oxysporum and M. phaseolina

F. oxysporum and *M. phaseolina* were grown for 4–5 days at 28 ^{o}C in 250-ml Erlenmeyer flasks containing 50 mL of Sabouraud`s dextrose broth. The fungal mycelia were separated by filtration and the filtrate was used as an enzyme source (Raju et al., 2009).

Electrophoretic visualization of proteases and PI

Root and shoot protein extracts of chickpea seedlings treated with *T. harzianum* L1 were electrophoresed on a vertical slab gel using a discontinuous buffer system by Davis (1964). For protease activity, the gel was equilibrated with 0.2 M Tris-

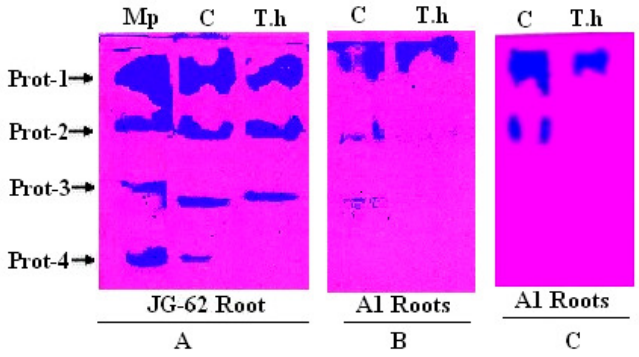


Fig 8. Electrophoretic separation and inhibition of *M. phaseolina* (A and B) and C-inhibition of Foc proteases. Proteases resolved gel was incubated with C (water- control) and T.h: *T. harzianum* L1 treated root extract. Native Foc proteases and their inhibition by cv. JG62 was reported earlier (Jayalakshmi *et al*. 2009).

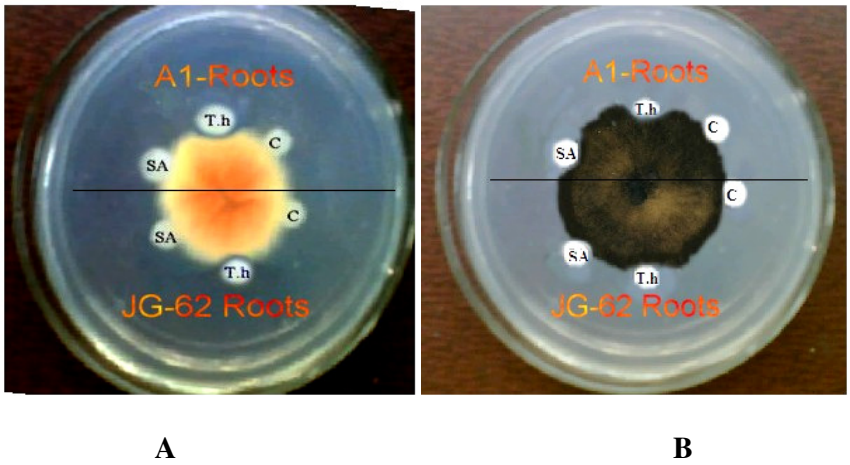


Fig 9. Bioassay of *F. oxysporum* f. sp. *ciceri* (**A**) and *M. phaseolina* (**B**). In vitro plate assay for antifungal activity of root extracts of both the cvs. towards pathogens. The plate containing potato dextrose agar were inoculated with hyphal mass of Foc or *M. phaseolina* in center and incubated at 37 ^{o}C for 2 days. The filter sterilized extracts were loaded using sterile paper discs at periphery of fungal growth and incubated for next 24 h. SA: salicylic acid; T.h: *T. harzianum* L1; C: control

HCl buffer pH 7.8 for 10 min, and overlaid on an undeveloped X-ray film (Kodak) for 1 h at 37 ^{o}C. The film was then washed with warm water and protease activity bands were visualized as hydrolyzed gelatin. For visualization of proteases of *F. oxysporum*, the gel was equilibrated in 0.2 M glycine/NaOH buffer, pH (10.0) for 10–15 min, and then overlaid on X-ray film for 40–45 min. Trypsin (TI) and chymotrypsin inhibitors (CTI), was visualized after native PAGE using the X-ray film contact print technique of Pichare and Kachole (1994). After electrophoresis of PIs, native gels were equilibrated in 0.1M Tris- HCL buffer, pH (7.8) for 10–15 min, followed by incubation in the trypsin (0.1 mg/mL) or chymotrypsin (20 µg/mL) solution or Foc protease (20 µg) for 15 min at 37 ^{o}C in a shaking water bath. The gels were then 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 water and inhibitor activity bands were visualized as unhydrolyzed gelatin on X-ray films. The X-ray films were developed using Kodak 163 DA developer.

Visualization of in-vitro effect of PI extracts on protease of F. oxysporum and M. phaseolina

F. oxysporum and *M. phaseolina* proteases were resolved in native PAGE as described above and the gel strips were incubated for 30 min at 37 ^{o}C in PI extracts (mg/ml of protein) obtained from roots and shoots upon treatment with *T. harzianum* L1. 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 remove hydrolyzed gelatin as proteinase activity bands on X-ray film.

Bioassay for the determination of antifungal activity

F. oxysporum and *M. phaseolina* growth inhibition was studied using the mycelial frowth inhibition assay of Roberts and Selitrennikoff (1986). For this, fungal mycelium from freshly grown culture was spot-inoculated at the center of a petri plate and incubated at 28 ^{o}C for 48 h. Sterile filter paper discs with filter-sterilized inhibitor extracts of roots and

shoots treated with water, *T. harzianum* L1, were placed on the periphery of the advancing fungal mycelium. The plates were further incubated at 28 ^{o}C for 24 h and observed for crescents of retarded mycelial growth.

Pot experiment

A pot experiment was conducted in completely randomized block design with four replications to evaluate the performance of *T. harzianum* L1 on the wilt and root rot diseases of chickpea. Ten seeds of chickpea cvs. each were sown in 15 cm diameter surface sterilized (1% $HgCl_2$) plastic pots, which were filled with 1 kg of sterilized soil and infested with 20 days old culture of the mass multiplied pathogens on sand maize meal water medium (90 g sand, 10 g maize meal, 20 ml distilled water) at 50 g/kg (Foc and *M. phaseolina*) one week before sowing. Seeds of both the cvs. were treated with *T. harzianum* L1 at a rate of 2, 4 or 6 g (talc formulation 1:1 w/w) per kg seed. The pots were observed for wilt and root rot incidence after 20 days of sowing and compared with the control pots.

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