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Research Article

Induction of defense enzymes in *Trichoderma harzianum* treated groundnut plants against *Macrophomina phaseolina*

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ABSTRACT: Antagonistic activity of *Trichoderma harzianum* isolated from the rhizosphere of groundnut was determined *in vitro*. *T. harzianum* decreased the root rot incidence *in vivo*. Potential of *T. harzianum* to induce systemic resistance was tested in groundnut against *Macrophomina phaseolina*. Biochemical changes in *T. harzianum* treated plants, *M. phaseolina* inoculated plants and healthy plants were assayed at different stages of infection. Treatment with *T. harzianum* and challenge inoculation of *M. phaseolina* enhanced induction of defense enzymes such as peroxidase (PO) and polyphenol oxidase (PPO) and defense compounds like total phenol and ortho-dihydric phenol. Total phenols, ortho-dihydric phenols, peroxidase and polyphenol oxidase activities increased at different stages of infection. *T. harizanum* treatment along with challenge inoculation of the pathogen significantly increased the activity of peroxidase and polyphenol oxidase by about 28.2 % and 95.5%, respectively, in roots at stage 2 compared to untreated plants. Increased levels of peroxidase and polyphenol oxidase were induced in root and shoot of treated plants indicating the systemic protection offered to groundnut by *T. harzianum*. The observations revealed that *T. harzianum* was capable of inducing systemic resistance against *M. phaseolina* by eliciting the production of defense enzymes.

KEY WORDS: *Trichoderma harzianum*, *Macrophomina phaseolina*, total phenols, ortho-dihydric phenols, peroxidase, polyphenol oxidase, induced systemic resistance (ISR)

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INTRODUCTION

Root rot of groundnut caused by *M. phaseolina* remains a challenge in terms of management. *Trichoderma* spp., which are common saprophytic filamentous fungi in almost any soil and rhizosphere microflora, are well recognized as biocontrol agents against various plant pathogenic fungi that cause a lot of soil-borne diseases and post-harvest diseases in several crops (Hajieghrari *et al*., 2008; Howell, 2003). In addition to biocontrol ability, some *Trichoderma* species are able to promote plant growth (Hoyos-Carvajal *et al*., 2009; Shanmugaiah *et al*., 2009; Harman *et al*., 2004; Ousley *et al*., 1994; Baker, 1988, 1989). Biological control has become a promising source of control in the management of root rot disease. *Trichoderma* spp. are effective in control of soil/seed-borne fungal diseases in several crop plants (Kubicek *et al*., 2001), including groundnut (Podile and Kishore, 2002). Several isolates of *T. viride, T. harzianum* and *T. pseudokoningi* suppress soil-borne pathogens by diversified mechanisms, *viz.,* production of a wide range of broad spectrum antifungal metabolites, mycoparasitism, competition with the pathogen for nutrients and for occupation of the infection court, induced resistance, production of protease and fungal cell wall degrading enzymes (Perello *et al*., 2003). *Trichoderma* spp. induce localized and systemic resistance to a variety of plant pathogens (Hoitink *et al*., 2006; Honson and Howell, 2004). *Trichoderma* induces systemic resistance mechanism in plants against pathogens (Abd-El-Kareem, 2007, Haggag and Amin, 2001; Hibar *et al*., 2007; Prasad *et al*., 2002; Brunner *et al*., 2005). Peroxidase catalyses several reactions including those involved in the mechanism of phenols and indoles. Peroxidase (PO) and polyphenol oxidase (PPO) catalyze the formation of defense gene products like lignin. Phenylalanine ammonia lyase (PAL) is involved in the synthesis of phytoalexins and phenolics (Karthikeyan *et al*., 2005). Selected strains of *Trichoderma* are potent inducers of plant defense responses. These responses are systemic and are termed as induced systemic

resistance (ISR). However, studies on induction of defense mechanisms in groundnut upon treatment with biocontrol agents are limited. The present study was carried out to assess the induction of phenolics and defense enzymes in *M. phaseolina* infected groundnut plants in response to application of biocontrol agents.

MATERIALS AND METHODS

Isolation of *Trichoderma* spp. from soil by serial dilution method

Trichoderma harzianum was isolated from healthy groundnut plants rhizosphere collected from the groundnut growing areas in and around Tirupati using serial dilution technique on *Trichoderma* specific medium (Elad and Chet, 1983) and isolated *T. harzianum* was maintained on potato dextrose agar medium.

Isolation of *Macrophomina phaseolina* from infected groundnut plants

Macrophomina phaseolina was isolated from naturally infected groundnut plants showing root rot symptoms cultivated in and around Tirupati and isolation of the pathogen was performed on potato dextrose agar (PDA) medium.

Pot culture experiment

Oatmeal sand medium was prepared in 250 ml conical flasks. Each flask inoculated with *T. harzianum* and *M. phaseolina* separately and incubated at 28±2°C for 10 days. One kg of soil and one kg of sand was taken into polythene bags and sterilized at 121°C for 30 min at 15 lbs pressure for two successive days. 9¨ earthenware pots were taken; sterilized sandy soil was added into the pots. Surface sterilized groundnut seeds were sown in pots filled with sandy soil containing *M. phaseolina, T. harzianum* separately and in combinations. Three replicates were maintained for each treatment. There were four treatments, viz., *T. harzianum, T. harzianum*+ *M. phaseolina, M. phaseolina* and control. For analysis of total phenols, ortho-dihydric phenols, peroxidase and polyphenol oxidase, samples were randomly collected from pots at different stages of infection. The progress of the disease in groundnut could be differentiated into the following three stages, on the basis of lesion development (Mehan, 1997).

Stage 1 (S1): Characterized by water-soaked lesions on the hypocotyl near the soil surface.

Stage 2 (S2): Infected tissues eventually have a dull, light-brown appearance. Later, affected areas become covered with sclerotia.

Stage 3 (S3): Roots become rotten and blackened with shredding of the taproot. The dead tissues rot and turn black, as sclerotia of the fungus develop profusely. Infected pegs and pods also rot and become covered with sclerotia.

Estimation of total phenols

Total phenol content was estimated by Folin-Ciocalteu reagent method (Bray and Thorpe, 1954). To one ml of ethanol extract in a test tube, one ml of Folin-Ciocalteu reagent and two ml of 20% sodium carbonate were added. The mixture was heated on a boiling water bath for exactly 1min and cooled and diluted to 25ml with distilled water. The absorbance of the blue colour developed was determined in Spectronic–20 colorimeter at 725çm. A reagent blank was maintained with one ml of distilled water in the place of ethanol extract. Total phenols were calculated from the standard curve plotted for catechol.

Estimation of ortho-dihydric phenols

Ortho-dihydric phenols (OD phenols) were estimated by employing Arnow's reagent, which is specific to ortho-groups (Johnson and Schaal, 1957). The reagent was prepared by dissolving 10 g of sodium nitrite and 10 g sodium molybdate in 100ml of distilled water. To one ml of the ethanol extract in a test tube, one ml of 0.5N HCl, one ml Arnow's reagent and two ml of 1N NaOH were added. The volume was raised to 12.5ml with distilled water and the light pink colour developed immediately was read in Spectronic–20 colorimeter at 522çm. A reagent blank contained one ml of distilled water instead of ethanol extract. The quantity of OD phenols in the sample was calculated from a standard curve prepared for an authentic sample of catechol.

Quantification of peroxidase (PO) activity

The procedure adopted for determining the activity of peroxidase was essentially as that of Fehrmann and Diamond (1967). The peroxidase enzyme activity was determined from both leaves and roots of uninoculated and inoculated cultivars of groundnut after inoculation. About 0.5 g of freshly harvested material was ground in a prechilled mortar with 20 ml of 0.1M ice cold phosphate buffer (pH 7.1) and centrifuged at 2000 rpm for 10min. The supernatant was made up to 25 ml and used for assay. Freshly prepared pyrogallol (0.2 M) reagent (0.1 ml) and 1.0 ml of the enzyme extract, 1.4 ml of 0.1M phosphate buffer (pH–7.1) were mixed in a cuvette tube and the mixture was immediately adjusted to zero absorbance of a spectrophotometer. H_2O_2 solution (0.5 ml

of 0.01M) was added to it and the content was mixed by inverting the cuvette. Enzyme activity was recorded as the change in absorbance per minute (ÄA / min/ä) at 430 çm.

Quantification of polyphenol oxidase (PPO) activity

The reaction mixture consisted of 0.5 ml of enzyme extract and 2.3 ml of 0.1M phosphate buffer (pH–6.1) that were mixed together in a cuvette and adjusted to zero absorbance of a spectrophotometer (Mahadevan and Sridhar, 1982). 0.2ml of 0.1 M catechol solution was added to the above mixture and the reactants were quickly mixed. The enzyme activity was measured as the change in absorbance per minute (ÄA/min) at 400 çm immediately after the addition of 0.2 ml of 0.1M catechol solution which initiated the reaction.

RESULTS AND DISCUSSION

In the present study *T. harzianum* was isolated from the rhizosphere of healthy groundnut plants and exploited for the control of *M. phaseolina,* the causative agent of root rot in groundnut plants through induced systemic resistance. The results revealed that the contents of total phenols, ortho-dihydricphenols and the phenol oxidizing enzymes, peroxidase and polyphenol oxidase activities were higher in *T. harzianum* + *M. phaseolina* treated groundnut plants than *M. phaseolina* inoculated plants as well as control.

Total phenols and ortho-dihydric phenols assay

Total phenol content increased in *T. harzianum* treated groundnut plants at different stages of infection. The results in Table 1, 2 and 3 explain that total phenol content markedly increased in *T. harzianum*+ *M. phaseolina* (1.42 mg g^{-1} fresh weight.) treated groundnut plants than *M. phaseolina* inoculated plants at stage 2 in roots. Total phenol content increased in infected plants (0.73 mg g^{-1} fr.wt.) than control (0.51 mg g^{-1} fr.wt.) at stage 2 in roots. Increase in total phenol content was observed in *T. harzianum* + *M. phaseolina* (1.45 mg g^{-1} fr.wt.) treated groundnut plants than *M. phaseolina* alone inoculated plants (0.95 mg g^{-1} fr.wt.) and control (0.75 mg g^{-1} fr.wt.) at stage 2 in shoot. Significant differences were found among three stages.

The maximum accumulation of ortho-dihydric phenols was observed in all the treatments. The maximum accumulation of ortho-dihydric phenols was observed in *T. harzianum*+*M. phaseolina* treated groundnut plants (0.59 mg g^{-1} fr.wt.) followed by *M. phaseolina* infected plant (0.47 mg g^{-1} fr.wt.) than control (0.19 mg g^{-1} fr.wt.) at stage 3 in roots. The maximum accumulation of ortho-dihydric phenols was observed in *T. harzianum*+*M. phaseolina* treated groundnut plants (0.73 mg g^{-1} fr.wt.). Ortho-dihydric phenols content increased in infected plant (0.63 mg g^{-1} fr.wt.) than control (0.5 mg g^{-1} fr.wt.) at stage 3 in shoot. The total phenols, ortho-dihydric phenol activities increased in different stages of infection and reached a maximum level at stage 3. At later stages of disease development, when the rotting developed fully, the extent of increase in ortho-dihydric phenols was significant (Table 1, 2 and 3). In the present study accumulation of phenolics was observed in all the treatments. Sivakumar and Sharma (2003) also expressed the view that there was an increase in phenolic content in maize leaf sheaths inoculated with *R. solani* or plants raised from *P. fluorescens* treated seeds.

Table 1. Effect of *Trichoderma harzianum* and *Macrophomina phaseolina* applied either alone or in combination on total phenols and ortho-dihydric phenols content of groundnut plants at stage – 1

Treatments	Total phenol (mg g^{-1} fresh weight)		Ortho-dihydric phenol (mg g^{-1} fresh weight)	
	Root	Shoot	Root	Shoot
T. harzianum	0.48^{c} ±0.007	0.76^{c} ±0.02	0.29^{c} ±0.005	0.41^{c} ±0.01
T. harzianum + *M. phaseolina*	0.81^{a} ±0.01	1.24^{a} ±0.03	0.39^{a} ±0.007	0.5^{a} ±0.004
M. phaseolina	0.53^{b} ±0.005	0.85^{b} ±0.01	0.33^{b} ±0.005	0.47^{b} ±0.006
Control	0.26^{d} ±0.008	0.59^{d} ±0.002	0.12^{d} ±0.002	0.38^{d} ±0.01

Each value is an average of 3 replicate samples. ± Standard error; in a column, means followed by a common letter are not significantly differ at 5% level by DMRT (Duncan's Multiple Range Test)

Table 2. Effect of *Trichoderma harzianum* and *Macrophomina phaseolina* applied either alone or in combination on total phenols and ortho-dihydric phenols content of groundnut plants at stage – 2

Treatments	Total phenol (mg g^{-1} fresh weight)		Ortho-dihydric phenol (mg g^{-1} fresh weight)	
	Root	Shoot	Root	Shoot
T. harzianum	0.73^{b} ±0.04	0.84^{c} ±0.03	0.36^{c} ±0.005	0.48^{c} ±0.01
T.harzianum + M. phaseolina	1.42^{a} ±0.04	1.45^{a} ±0.05	0.55^{a} ±0.03	0.62^{a} ±0.02
M. phaseolina	0.73^{b} ±0.005	0.95^{b} ±0.01	0.41^{b} ±0.01	0.51^{b} ±0.02
Control	0.51^{c} ±0.04	0.75^{d} ±0.01	0.16^{d} ±0.004	0.45^{d} ±0.01

Each value is an average of 3 replicate samples. In a column, means followed by a common letter are not significantly differ at P = 0.05 by DMRT (Duncan's Multiple Range Test)

Table 3. Effect of *Trichoderma harzianum* and *Macrophomina phaseolina* applied either alone or in combination on total phenols and ortho-dihydric phenols content of groundnut plants at stage – 3

Treatments	Total phenol (mg g^{-1} fresh weight)		Ortho-dihydric phenol (mg g^{-1} fresh weight)	
	Root	Shoot	Root	Shoot
T. harzianum	0.61^{c} ±0.03	0.80^{c} ±0.05	0.41^{c} ±0.01	0.55^{c} ±0.03
T.harzianum + M. phaseolina	1.3^{a} ±0.08	1.35^{a} ±0.08	0.59^{a} ±0.03	0.73^{a} ±0.005
M. phaseolina	0.68^{b} ±0.02	0.91^{b} ±0.04	0.47^{b} ±0.02	0.63^{b} ±0.02
Control	0.45^{d} ±0.04	0.70^{d} ±0.01	0.19^{d} ±0.01	0.5^{cd} ±0.04

Each value is an average of 3 replicate samples. In a column, means followed by a common letter are not significantly differ at P = 0.05 level by DMRT (Duncan's Multiple Range Test)

According to Singh *et al.* (1998) soil application of *T. viride* enhanced the concentration of phenols in chickpea plants that led to induced resistance to *M. phaseolina.* The phenolic compounds as constituents of lignin may contribute to enhance the mechanical strength of the host cell wall and may also inhibit fungal growth as they are fungi toxic in nature. M'Piga *et al.* (1997) found that the hyphae of the pathogen surrounded by phenolic substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. Observation of the current study pertaining to suppression of *M. phaseolina* infection in groundnut supports this view.

Peroxidase and polyphenol oxidase activity

The induction of defense enzymes, PO and PPO in groundnut was studied at different stages of infection after challenge inoculation with *M. phaseolina* and *T. harzianum*. The enzyme activity was increased up to 25 days after challenge inoculation and the maximum induction was observed during this period. The enzyme activity declined at 35 days after challenge inoculation in *T. harzianum* treated plants. In control plants, the enzyme activity started declining drastically from 25 days. Peroxidase and polyphenol oxidase activities increased at different stages of infection in all the treatments. In *T. harzianum + M. phaseolina* treated plants there is an increase in peroxidase (0.359 OD min^{-1} g^{-1} of sample) and polyphenol oxidase activities (0.391 OD min^{-1} g^{-1} of sample) at stage 2 in roots. Peroxidase activity increased in infected plant (0.342 OD min^{-1} g^{-1} of sample) than control (0.28 OD min^{-1} g^{-1} of sample) at stage 2 in roots. In case of plants infected with *T. harzianum* alone (0.321 OD min^{-1} g^{-1} of sample), the enzyme activities remained slightly lower compared to *M. phaseolina* treated plants. Polyphenol oxidase activity increased in infected plant (0.37 OD min^{-1} g^{-1} of sample) than control (0.2 OD min^{-1} g^{-1} of sample) at stage 2 in roots. In *T. harzianum + M. phaseolina* treated plants increase in peroxidase

Table 4. Peroxidase activities in groundnut root and shoot due to the pathogen and *Trichoderma harzianum* at various stages of disease development

Treatments	Peroxidase (changes in OD min^{-1} g^{-1} of sample)					
	Root			Shoot		
	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
T. harzianum	0.254^{c} ±0.006	0.321^{c} ±0.004	0.272^{c} ±0.007	0.325^{c} ±0.002	0.369^{c} ±0.007	0.35^{c} ±0.005
T. harzianum+ *M. phaseolina*	0.287^{a} ±0.007	0.359^{a} ±0.007	0.323^{a} ±0.004	0.38^{a} ±0.01	0.459^{a} ±0.03	0.4^{a} ±0.02
M. phaseolina	0.27^{ab} ±0.003	0.342^{b} ±0.004	0.297^{b} ±0.006	0.36^{ab} ±0.005	0.4^{b} ±0.006	0.371^{b} ±0.07
Control	0.187^{d} ±0.003	0.28^{d} ±0.003	0.270^{c} ±0.003	0.27^{d} ±0.006	0.3^{d} ±0.005	0.287^{d} ±0.004

Each value is an average of 3 replicate samples; ± standard error; in a column, means followed by a common letter are not significantly differ at 5% level by DMRT (Duncan's Multiple Range Test)

Table 5. Polyphenol oxidase activities in groundnut root and shoot due to the pathogen and *Trichoderma harzianum* at various stages of disease development

Treatments	Polyphenol oxidase (changes in OD min^{-1} g^{-1} of sample)					
	Root			Shoot		
	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
T. harzianum	0.264^{c} ±0.003	0.3^{c} ±0.006	0.27^{c} ±0.006	0.314^{c} ±0.003	0.349^{c} ±0.006	0.329^{c} ±0.004
T.harzianum + *M. phaseolina*	0.333^{a} ±0.007	0.391^{a} ±0.008	0.359^{a} ±0.008	0.36^{a} ±0.006	0.414^{a} ±0.004	0.372^{a} ±0.005
M. phaseolina	0.288^{b} ±0.004	0.37^{b} ±0.007	0.3^{b} ±0.005	0.324^{b} ±0.008	0.362^{b} ±0.007	0.338^{b} ±0.008
Control	0.162^{d} ±0.002	0.2^{d} ±0.004	0.179^{d} ±0.002	0.26^{d} ±0.004	0.285^{d} ±0.006	0.272^{d} ±0.002

Each value is an average of 3 replicate samples; ± standard error; in a column, means followed by a common letter are not significantly differ at 5% level by DMRT (Duncan's Multiple Range Test)

(0.459 OD min^{-1} g^{-1} of sample) and polyphenol oxidase activities (0.414 OD min^{-1} g^{-1} of sample) at stage 2 in shoot was observed. Peroxidase activity increased in infected plant (0.4 OD min^{-1} g^{-1} of sample) than control (0.3 OD min^{-1} g^{-1} of sample) at stage 2 in shoot. Polyphenol oxidase activity increased in infected plant (0.37 OD min^{-1} g^{-1} of sample) than control (0.2 OD min^{-1} g^{-1} of sample) at stage 2 in roots. The peroxidase, polyphenol oxidase activities increased at different stages of infection and reached a maximum level at stage 2 (Table 4 and 5). The results show that *T. harzianum* induced the accumulation of enzymes such as peroxidase and polyphenol oxidase which play an important role in plant defense mechanism against the pathogen. The induction of defense related enzymes by *Trichoderma* was correlated with the percentage of root rot suppression in biocontrol treated plants upon challenge inoculation with the pathogen. Peroxidase activity was significantly more in plants treated with *T. harzianum* as compared to other treatments. Increase in the activity of peroxidase and polyphenol oxidase was observed in all the treatments. High level of expression of peroxidase was reported in *P. fluorescens* treated tomato plants challenged with *F. oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al*., 2002). There are some evidences indicating that the activation of peroxidase, polyphenol oxidase plays a crucial role in the biological control and resistance of plant to pathogenic attack (Chérif *et al*., 2007; Mohammadi and Karr, 2002; She-ze *et al*., 2008). Polyphenol oxidase activity was significantly higher in plants treated with *T. harzianum*. The greater activity of PO and PPO, along with higher amount of total phenols enhanced the host resistance. Nawar and Kuti (2003) reported that there were positive relationships between peroxidase and resistance development in plants. Elad (2000) also demonstrated the role of induced systemic resistance in the control of the foliar pathogen *Botrytis cinerea* in cucumber using *Trichoderma*. From this study it is concluded that groundnut plants treated with *T. harzianum* followed by

inoculation with *M. phaseolina* exhibit induction of defense related enzymes such as peroxidase and polyphenol oxidase which could be very effective in the control of root rot of groundnut.

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