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Research Article

Plant growth promotion and induced defense response in safflower (*Carthamus tinctorius* L.) by *Trichoderma*

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ABSTRACT: Nine potential *Trichoderma* strains were tested for mycoparasitic, defence enzyme activity and root colonizing behaviour against *Macrophomina phaseolina* and *Fusarium oxysporum f. sp. carthami* in safflower (*Carthmus tinctorius* L). Among them three strains *viz*., *T. harzianum* Th4d, *T. asperellum* TaDOR7316 and *T. asperellum* Tv5 were found to be most effective showing superior antagonistic activity. Hyphal interaction studies revealed that the inhibition was caused by an interaction that took place in close contact with the host hypha, causing lysis, swelling and coiling of mycelia resulting potentially reduced mycelial growth of *M. phaseolina* and showed lytic enzymes activity to various extent in Th4d, Tv5 and TaDOR 7316. These strains were also able to solubilize inorganic (P). Increased activity of defense related enzymes *viz*., peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activity in these three potential strains pre-treated safflower plants challenged with *M. phaseolina* was observed. PO, PPO and PAL activity was also increased two-three folds more in all these bioagents. Interaction between the bioagents and the safflower root system showed profuse adhesion of hyphae to the plant roots as well as colonization of the root epidermis and cortex cells but not the vessels at early stages of safflower root system. Levels of hydrogen peroxide (H_2O_2) in the bioagents treated leaves and untreated (control) were determined microscopically. Application of these bioagents under field conditions reduced the incidence of root rot and Fusarium wilt, increased growth and plant biomass to a reasonable extent with better root colonization, which is directly correlated with the resistance of the plant against infection and high seed yield, was observed with bioagents treatment. Thus, it is evident that the hyphal interaction and enzymes play a key role to stimulate the defense mechanism which aid in disease management as well as plant growth promotion of the host plant against pathogen attack.

KEY WORDS: Antagonistic activity, defense enzymes, plant-fungus interaction, safflower, *Trichoderma* spp

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INTRODUCTION

Among the various biological control agents (BCA's), members of the fungal genus *Trichoderma* are considered as a potent bioprotectants against many plant pathogens and attracted considerable scientific attention. *Trichoderma* possess ability to promote plant growth and soil remediation activity compared to other microbial agents (Gamal *et al.*, 2007). *Trichoderma* can promote plant growth and crop yield by increasing plant nutrient uptake that help plants to grow more rapidly and reducing disease incidence by their biocontrol activity (Altomare *et al.*, 1999; Yedidia *et al.*, 2001). Role of mycolytic enzymes and antibiotic substances of *Trichoderma* spp. in fungal pathogen inhibition is well established. However, efficiency in their activity is variable among the strains. These fungi have shown to be plant symbionts, in this symbiotic process, they infect plant roots, but through chemical communication factors they induce the plant to wall the invading *Trichoderma* hyphae so that the organism is restricted to the outer layers of the root (Yedidia *et al.*, 1999). In so doing, they would induce systemic resistance (ISR) responses to a wide spectrum of pathogens and adverse environmental conditions (Lorito *et al.*, 2010). Thus even though the *Trichoderma* spp. are restricted to roots, the foliage becomes resistant to plant diseases.

To understand the mechanism of the effect of bioagents against *Fusarium oxysporum* f. sp. *carthami* (*Foc*) and *Macrophomina phaseolina (Mp)* in safflower, histochemical and biochemical studies have been conducted and also antagonistic potential of promising *Trichoderma* isolates and their role in host root colonization has been assessed.

MATERIALS AND METHODS

Bioagents and Pathogens

Trichoderma strains used are isolated from rhizosphere soils of various crops obtained from different locations in India plating on *Trichoderma* selective medium (TSM) and pure cultures were maintained on PDA. The isolates were identified morphologically by using www.isth.info.com (Trichokey 2.0) http://nt.ars-grin.gov/taxadescriptions/keys/*Trichoderma* Index.cfm is a link that provides an interactive key, images, descriptions, distributions and nomenclature for the genus *Trichoderma.* To further characterize the potential *Trichoderma* isolates both ITS sequencing using primer ITS1 (5'TCCGTAGGTGAACCTGCGG 3') for forward and the primer ITS2 (5'GCTGCGTTCTTCTTCATCGATGC 3') for reverse (White *et al.*, 1990) and for further confirmation section of elongation factor 1 alpha gene was amplified using the primers EF1-728 F: 5′-CATCGAGAAGTTCGAGAAGG-3′ (Carbone and Kohn 1999) and TEF1 R: 5′-GCCATCCTTGGGAGATACCAGC-3′ (Samuels *et al.*, 2002). Template DNA for sequencing was prepared directly from PCR products with the QIA quick PCR purification kit (Qiagen, Valencia, California). The sequences were subjected to BLAST in NCBI and also analyzed using TrichoKey and TrichoBlast tools (Druzhinina *et al.*, 2005) (http://www.isth.info/tools/blast/) to obtain the identification of the isolates. After confirmation the sequences were submitted to NCBI GenBank. Two pathogens *Macrophomina phaseolina (Mp)* and *Fusarium oxysporum f. sp. carthami* (*Foc*) was isolated from infected safflower plants at Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad and maintained on PDA at 4°C. Nine *Trichoderma* strains were deposited at National Agriculturally Important Microbial Culture Collection (NAIMCC), National Bureau of Agriculturally Important Microorganisms (NBAIM), Maunath Bhanjan, U.P., India.

Phosphate solubilisation

Inorganic P solubilization was estimated by inoculating the bioagents into Pikovskaya's broth. The supernatant containing released P was extracted on 7th day and the available P content was estimated by phosphomolybdic blue color method.

Hydrolytic enzymes production

Chitinase production by bioagents was studied by inoculating in minimal medium supplied with 1% colloidal chitin as the sole source of carbon. Supernatant was extracted on 7th day as the source of crude enzyme and the chitinase activity assayed as explained by Xu-fen *et al.*, (2007). β-1, 3-glucanase and β-1, 4-glucanase activities were estimated as explained by Paul et al., (2005).

In vitro and *in vivo* screening of bioagents against pathogens of safflower *viz.*, *Macrophomina phaseolina* (Mp) and *Fusarium oxysporum* f. sp. *carthami* (Foc)

In vitro screening

In vitro antagonistic potential of the biocontrol agent against *M. phaseolina* and *F. oxysporum carthami* was tested by dual culture technique (Dennis and Webster, 1971) using potato dextrose agar (PDA). The radial growth of the pathogens in dual culture and control plates was measured after contact of the both test antagonist and the pathogen and repeated till complete colonization of plate by one organism. The per cent inhibition of the pathogen in dual culture plates was calculated as per formula proposed by Vincent (1927).

Hyphal interactions

Hyphal interaction between *M. phaseolina* and *Trichoderma* spp. was studied by scanning electron microscope (SEM). Mycelial samples from interaction zone of 7 days old culture plate were cut by a cork borer and were processed for SEM as per Pham *et al.* (2005). Samples were fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.2) for 24 h at 4°C and post fixed 2% in aqueous osmium tetroxide for 4 h. Dehydrated in series of graded alcohols and dried to critical point drying with CPD unit. The processed samples were mounted over the stubs with double-sided carbon conductivity tape and a thin layer of gold coat over the samples were done by using an automated sputter coater (Model-JEOL JFC-1600) for 3 minutes and scanned under Scanning Electron Microscope (SEM-Model: JOEL-JSM 5600) at required magnifications as per the standard procedures at Ruska Laboratories, College of Veterinary Science, S.V. Veterinary University, Rajendranagar, Hyderabad, India.

In vivo screening

Three isolates *viz., T. harzianum* Th4d, *T. asperellum* TaDOR 7316 and *T. asperellum* Tv5 which have shown maximum antagonism against *F. oxysporum carthami* and *M. phaseolina in vitro* were used for further studies. To test the efficacy of bioagents against *M. phaseolina* safflower seeds (var. A1) were surface disinfected with 2% sodium hypochlorite and treated with TaDOR7316, Th4d and Tv5 conidia suspension (2 x 10^7 conidia/ml) and seeds were immersed in the suspension and incubated overnight in incubator shaker and spread onto plastic trays for air drying. Carbendazim at 2 g/kg seed treatment serves as fungicide check. Surface sterilized plastic pots (15 cm diameter) filled with sterilized soil, sand and manure in the ratio of 1:1:1 (2 kg/pot) and inoculated with 15-day-old inoculum of *M.*

phaseolina multiplied on sorghum grains at 7.5 g/kg soil 5 days before sowing and untreated soil served as check. In parallel, the seeds coated with pathogen *M. phaseolina* were sown and maintained for comparison. Each pot consisted of three plants with five replicates for each treatment was maintained. Defence enzymes were assayed and plant growth parameters were noted. The root rot incidence was calculated by counting the total number of diseased plants in each treatment which was then divided by the total number of plants per treatment and expressed as a percentage.

Assay of induced enzymes

Inoculum preparation

For inoculation purposes, *M. phaseolina* was cultured on 9 cm diameter petri plates containing PDA (potato dextrose agar medium) at 25°C for 7 days. Mycelia suspension was prepared in sterilized distilled water and inoculum load was adjusted to 18 x 10^6 infective propagules (mycelial fragments) per ml by using a haemocytometer

Tissue collection

The *M. phaseolina* inoculated leaf portions of safflower plants treated with biocontrol agents, Carbendazim and untreated were collected at various time intervals (0, 24, 48, 72, 96 and 164 h) after pathogen inoculation and quickly frozen in liquid nitrogen and stored at -20°C.

Determination of peroxidase, polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activity

Fresh plant leaves 1 g homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) were with prechilled mortar and pestle. The homogenate was centrifuged at 18,000 rpm at 58°C for 15 minutes and used within 2-4 hours. Supernatant served as an enzyme source. Peroxidase, PAL and PPO activity was estimated as per the protocols followed by Hammer Schimdt *et al.*, 1982; Singh and Prthiviraj (1997) and Mayer *et al.,* 1965.

Histo-chemical analysis of hydrogen peroxidase (H_2O_2)

For histochemical detection of H_2O_2, Leaves were unfiltered with 0.1% 3, 3-diaminobenzidine (DAB) in 10mM Tris buffer (pH 7.8). Samples were incubated under day light for two hours after the vaccum infiltration. After the staining, DAB treated samples were incubated under day light for 20 min and subsequently cleared in 0.15% trichloroacetic acid (wt/vol) in ethanol:chloroform 4:1 (vol/vol). The solution was exchanged once during the next 48 h of incubation (Huckel-Hoven *et al.*, 1999). Subsequently leaves were stored in 50% glycerol prior to microscopic evaluation. Samples were harvested 24, 48, 72 and 96 hours after spray. Discoloration of leaf discs resulted by DAB staining was observed using Leica microscopy.

Plant-fungus interactions in hydroponic culture

Trichoderma isolates germlings were obtained by inoculating 10^6 conidia/ml in 200 ml of potato dextrose broth (PDB). To study *Trichoderma* interaction with host plant roots in hydroponic culture, bright field microscopy was performed as per protocol described by Mariola *et al.*, (2007). For intercellular assay transmission electron microscopy (TEM) analysis was performed as per the procedures given by RUSKA Lab, Hyderabad and the growth parameters on 60 days old plant *viz.,* (the shoot length, root length, vigour index, dry biomass weight of root and shoot). In addition, number of heads per plant, seed weight per treatment was determined.

Production and development of WP formulations of bioagents

The *Trichoderma* spp. were grown on molasses-soy medium (Prasad and Rangeshwaran, 2000) by inoculation of 5 ml of spore suspension from sporulating cultures on potato dextrose agar slants to 250 ml of medium in 500 ml Erlenmeyer flasks. Inoculated flasks were kept in shaking incubator for 3 days at 200 rpm and culture flasks were removed and kept in incubation room for 2 days for profuse conidia production on the surface. The five-day-old inoculum was homogenized in a blender and mixed with sterilized talc powder (1:2 v/w). The powder formulation was air-dried by spreading as thin layer in a clean room for 2 days to reduce moisture to 8-10 % and carboxymethyl cellulose (CMC) as sticker was added @ 5 g/kg formulation at the end. The formulation was packed in milky white bag.

Effect of biocontrol agents under field conditions

T. harzianum Th4d, *T. asperellum* TaDOR 7316 and *T. asperellum* Tv5 were selected based on their superior performance in laboratory and greenhouse conditions for field evaluation. Wettable Powder (WP) formulation of potential isolates (Th4d, TaDOR 7316 and Tv5) was evaluated against root rot and wilt of safflower under field conditions at ICAR-IIOR, Hyderabad. The soil in the experimental field area is sandy loam with pH: 7.8, Electrical conductivity (ds/m): 0.45; total N, P and K of 225, 14.5 and 446 kg/ha, respectively). Field trial was performed in a randomized block design (RBD) with 8 treatments and each treatment replicated thrice. The treatments are T1: Carbendazim + Mancozeb (saff) @ 0.2%, T2: Carbendazim @ 0.2%, T3: Captan @ 0.2% T4: Cymoxanil 8% + Mancozeb 64% @ 0.2%, T5: *T. harzianum* Th4d WP @ 10g/ kg, T6: *T. asperellum* TaDOR 7316 @ 10 g/kg, T7: *T. asperellum* Tv5

Table 1. Gene bank and NAIMCC accessions of *Trichoderma* isolates

S. No.	Organism	NCBI GeneBank accession numbers		NAIMCC accession No.	Place of collection
		ITS1 and ITS2	*tef1*		
1	*Trichoderma asperellum* - Tv5	JQ976275	KR002594	NAIMCC-F-02225	Guntur, A.P.
2	*Trichoderma asperellum* - T12	JQ976276	KR002605	NAIMCC-F-02060	Narayankhed, Telangana
3	*Trichoderma asperellum* - Ts12	JQ976277	KR002602	NAIMCC-F-02044	Sadashivpet, Telangana
4	*Trichoderma asperellum* - T33	JQ976278	KR002603	NAIMCC-F-02067	Guntur, A.P.
5	*Hypocrea lixii* - Tv3	JQ976279	KR002604	NAIMCC-F-02052	Gulbarga, Karnataka
6	*Trichoderma asperellum* - Tv2	JQ976282	KR002600	NAIMCC-F-02059	Nalgonda, Telangana
7	*Trichoderm aasperellum* - TN13	JQ976285	KR002601	NAIMCC-F-02051	Mau, Uttar Pradesh
8	*Trichoderma asperellum* - Tv16	JQ976286	-	NAIMCC-F-02053	Gulbarga, Karnataka
9	*Trichoderma harzianum* - Th4d	F471117	KR002606	NAIMCC-F-02188	Gurajala, A.P.

WP @ 10 g/kg and T8: Pathogen checks. The plot size was 45 x 20 cm and surface sterilized safflower seeds (var.A1) were treated with WP formulation of bioagents and chemical treatments accordingly. Disease incidence variations in plant growth were also recorded from emergence until seed setting. Ultimately the yield was assessed and tabulated.

Statistical analysis

Percentage data were transformed to arcsine values and the analysis of variance (ANOVA) was carried out. In disease severity and enzyme activity data were transformed with logarithmic transformation.

RESULTS AND DISCUSSION

Nine *Trichoderma* isolates used in this study were collected from soil samples of various agroclimatic regions of Andhra Pradesh, Karnataka and one strain was obtained from Uttar Pradesh and identified morphologically, these strains were characterized by sequencing the ITS region using ITS1 and ITS2 primers and further confirmed using translational elongation factor *(Tef1)* 1 alpha gene was amplified using EF1-728F and TEF1R primers. According to NCBI blast search against the GenBank sequence database and comparison using TrichoBLAST and TrichoKey tools, seven strains were identified as *T. asperellum,* one *T. harzianum* Th4d and one strain was found to be *H. lixii* (Tv3) and their corresponding accession numbers were obtained from the NCBI. The geographical location of these isolates was also listed. Further these strains were deposited in National Agriculturally Important Microbial Culture Collection (NAIMCC) and accessions are listed for reference (Table 1). Based on *in vitro* dual culture studies with *Trichoderma* spp. against *M. phaseolina* and *F. carthami* and microscopic observations indicated that the inhibitory effect was caused by an interaction that took place in close contact with the host hypha, causing lyses, swelling and coiling mycelia resulting reduced mycelial growth of *M.phaseolina* over control treatment (Table 2; Fig. 1). Lytic enzymes of mycoparasitic fungi of the genus *Trichoderma* is capable of suppressing several fungal phytopathogens (De Marco *et al.*, 2003). *Trichoderma* spp. were found efficient *in vivo* in suppressing many soil borne plant pathogenic fungi, were tested *in vitro* for their efficacy in solubilizing the inorganic (P) and to produce the mycolytic enzymes in liquid cultures. All the isolates were found to be tricalcium phosphate solubilizers (TCP) in the liquid culture and mycolytic enzyme activity by *Trichoderma* spp. (Table 3). Chitinase (17.7 U/ml), β-1,3-glucanase (49.3 U/ml) and β-1,4-glucanase (0.9 U/ml) activities were found highest in *T. harzianum* Th4d over other isolates. Acidification may not the major mechanism of solubilization in *Trichoderma* spp., since the pH of cultures never fell below 5.0 except *T. harzianum* Th4d and *Aspergillus niger*. This observation is in agree with the findings of Altomare *et al.* (1999) where they could not detect any organic acids in the culture filtrates which is thought to be having major role in solubilization of phosphate. Instead they reported the role of chelating metabolites complexing with Ca, Fe and Al ions. Similarly, all the bioagents produced significant quantity of extracellular mycolytic enzymes. Production of lytic enzymes and the factors which influence their production are therefore the aspects which will determine the potential of any *Trichoderma* isolate selected for the biological control of plant pathogenic fungi (Karasuda *et al.*, 2003). Inducing the plants for their own defense mechanisms by prior application of bioagents is a novel technique for plant protection. Increased activity of defense related enzymes *viz.,* peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activity in three potential *Trichoderma* spp. *viz., T. harzianum* (Th4d), *T. asperellum* (Tv5) and *T. asperellum* (TaDOR7316) pre-treated safflower plants challenged with *M. phaseolina* was observed. Significant accumulation of PO, PPO and PAL activity at 30 days interval was high compared to 60 days in all these bioagents. The activity of PO reached the highest level in plants at 72 hr after treatment with *T. harzianum* (Th4d) and then slowly decreased (Fig. 2). Similarly PPO activity increased

significantly within 48hr after treatment and reached the highest level at 72 hr of inoculation there after reduction in the activities of polyphenol oxidizing enzymes was recorded when plants treated with both *M. phaseolina* and antagonist compared to control alone (Fig. 3). PAL activity reached maximum at 48 hr of inoculation (Fig. 4). Biocontrol agents induced defense in safflower at varied levels of efficiency in suppression of pathogen establishment or multiplication depending upon the ability of the pathogen to avoid activated host defenses. High peroxidase activity was observed by treatment with *P. fluorescens* and *T. harzianum* followed by *B. subtlis* over control are associated with stages of the infection process and are involved in generation of hydrogen peroxides it inhibit the pathogen directly by producing free radicals with antimicrobial effects and lignifications (Hammer Schmidt *et al.*, 1982). Accumulation of PAL was high at 24hr after challenge inoculation and it is a product of cinammic acid; it is directly linked with lignifications. Our results confirm the findings of Silva *et al.*, (2004). Activity of PPO is also enhanced due to treatment with biocontrol agents and it catalyses the last step of biosynthesis of lignin and other oxidative phenols. Apart from these application of the Th4d reduced the incidence of root rot and enhanced the plant growth promotion to a reasonable extent against *M. phaseolina,* which is directly correlated with the resistance of the plant against infection.

Table 2. *In vitro* screening of *Trichoderma* isolates against *Fusarium oxysporum* f.sp. *carthami* and *Macrophomina phaseolina*

Isolate	*F. carthami vs. Trichoderma* isolates		*M. phaseolina vs. Trichoderma* isolates	
	Colony diameter (mm)	Percent Inhibition of pathogen (%)	Colony diameter (mm)	Percent Inhibition of pathogen (%)
T. asperellum Tv5	28.5	68.3	29.0	67.7
T. asperellum T12	30.5	66.1	38.0	57.7
T. harzianum Ts12	34.0	62.2	36.5	59.4
T. asperellum T33	36.5	59.4	40.0	55.5
T. asperellum Tv3	30.5	66.1	31.0	65.5
T. asperellum Tv2	30.5	66.1	32.5	63.8
T. asperellum TvN13	31.5	65	30.5	66.1
T. harzianum Th4d	26.5	70.5	27.5	69.4
T. asperellum TaDOR 7316	27.0	70	28.0	68.8
Pathogen	90	-	90	-

Mean - denotes mean value of 3 replications.

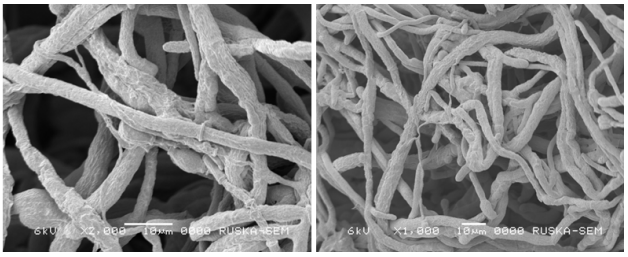

Fig. 1. Scanning electron micrographs of hyphal interaction between *Macrophomina phaseolina* and *Trichoderma harzianum* Th4d, on 5 days old PDA dual culture plate.

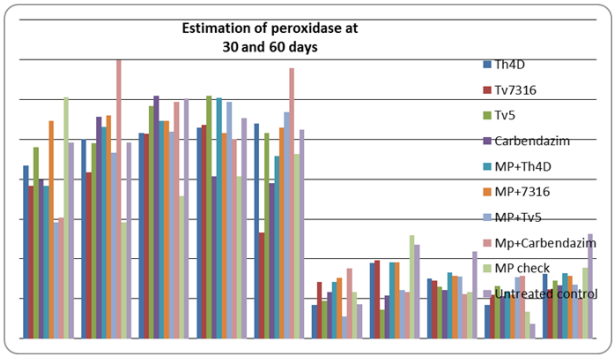

Fig.2.Inductionofperoxidaseinsafflowerresponsetobiocontrolagents (Th4d - *Trichoderma harzianum*,TaDOR7316 - *T.asperellum*,Tv5 - *T. asperellum*) against *Macrophomina phaseolina* (Mp).

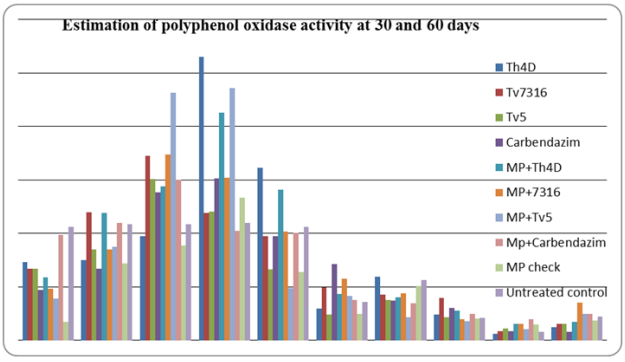

Fig. 3. Induction of PPO activity in safflower treated with biocontrol agents (Th4d - *Trichoderma harzianum*, Tv7316 - *T. asperellum*, Tv5 - *T. asperellum*) against *Macrophomina phaseolina* (Mp).

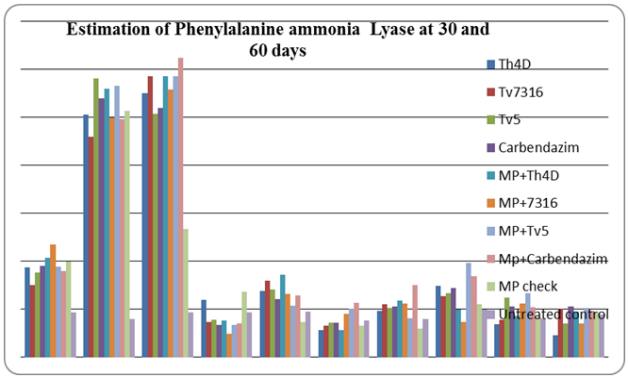

Fig. 4. PAL activity in safflower leaf in response to biocontrol agents (Th4d - *Trichoderma harzianum*, TaDOR7316 - *T. asperellum*, Tv5 - T. *asperellum*) against *Macrophomina phaseolina* (Mp).

Table 3. Potential of *Trichoderma* spp. and bacterial bioagents in solubilization of Tricalcium Phosphate (TCP) and production of mycolytic enzymes in liquid culture

Isolates	Final pH of Pikovskaya's broth	(µg P ml^{-1})	Chitinase (U/ml)	β-1,3-glucanases (U/ml)	β-1,4-glucanases (U/ml)
T. asperellum Tv5	5.2	050.3	14.5	42.7	0.7
T. harzianum Th4d	5.8	079.0	17.7	49.3	0.9
T. asperellum TaDOR 7316	5.3	091.2	16.6	41.3	0.9
Aspergillus niger	1.9	146.7	15.1	29.5	1.1
Uninoculated control	7.0	012.0	-	-	-
SED	1.5	002.2	00.9	01.4	0.2
CD ($P = 0.05$)	3.5	005.2	02.1	03.4	0.5
CV%	7.3	003.6	09.0	05.6	3.2

Mean - denotes mean value of 3 replications.

Hydrogen peroxide accumulation were detected microscopically in safflower leaves treated with *T. asperellum* TaDOR7316, *T. asperellum* TaDOR673 and *T. harzianum* Th4d showed increased levels of Hydrogen Peroxide (H_2O_2) at 48h of inoculation as compared to control. However the level of H_2O_2 did not change significantly after 48 and 72 hours of treatment (Fig. 5). The increased levels of H_2O_2 and O_2^- (Reactive Oxygen Species = ROS) could inhibit the spore germination and the hyphal growth of powdery mildew pathogens (Hafez and Kiraly, 2003). These findings are correlated with our findings treatment with bioagents increased the levels of induced defense enzymes (PO, PPO and PAL), H_2O_2 after 48 hr treatment could have inhibited the growth of *M. phaseolina* leading to better resistance and plant growth promotion when compared to control in safflower.

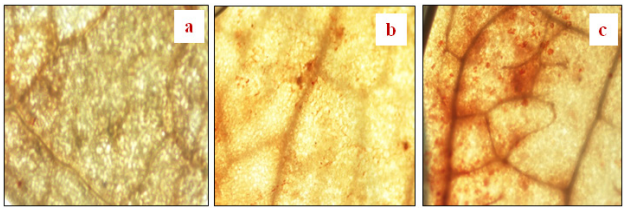

Fig. 5. H_2O_2 production (arrows) in safflower leaves as visualized by 3, 3'-diaminobenzidine (DAB) staining. H_2O_2 production (revealed by reddish-brown stain) was detected in (a) untreated control, (b) *Macrophomina phaseolina* and (c) *Trichoderma harzianum*, Th4d + *M. phaseolina* treated.

The results obtained from the present study indicate that the *Trichoderma* treated plants played a positive role in protecting against *M. phaseolina*-induced oxidative stress and enhance tolerance by partially increasing the H_2O_2 content and other defense enzymes.

An oxidative burst is one of the earliest and most universal resistance responses mounted by plant tissues against an invading microbe: a controlled release of O_2 and H_2O_2 at the point of pathogen challenge is believed to be required for pathogen defense (Bestwick 1997). H_2O_2 not only directly inhibits and reduces the growth of plant pathogens, directly inhibits and reduces the growth of plant pathogens, but in the early plant-pathogen interaction it contributes to cell wall strengthening by papillae formation, lignifications, cross-linking of hydroxyproline/proline-rich proteins and other cell wall polymers (Kuzniak and Urbanek, 2000) In the present investigation, it was found that H_2O_2 content initially increased significantly in the BCA- challenged plants, indicating that plants treated with microbes were better equipped to mitigate stress generated by *M. phaseolina*, which is known to suppress H_2O_2 production. However, a down ward trend appeared thereafter in the BCA-treated plants after a temporary increase, with a maximum decrease in plants treated with a *Trichoderma.* In uninoculated plants, although an increase in H_2O_2 content was observed, it was insignificantly in comparison to plants treated with BCAs.

Based on the microscopic observations *T. harzianum,* Th4d, *T. asperellum,* TV5 and *T. asperllum,* TaDOR7316 conidia inoculated into the liquid medium of hydroponically grown safflower (*Carthamus tinctorius* L.) showed increased penetration of fungal hyphae to the plant roots as well as colonization of the root epidermis and cortex cells but not the vessels at early stages of safflower root growth (Fig. 6). Fungal growth was observed mainly in intercellular spaces, and fungal colonization was not associated with disruption of the host cell wall, in contrast to reports for various plant-pathogen interactions (Stergiopouls *et al.*, 2003). A similar behaviour was reported for cucumber roots after inoculation with *T. harzianum* strain T-203, i.e., extensive colonization of the root surface, appressoria-

like structures, and hyphal filaments penetrating the root epidermis between adjacent cells, generating a lytic zone around the penetrated area (Yedidia *et al.*, 2001). Plants inoculated with *T. harzianum* Th4d, *T. asperellum* Tv5 and *T. asperellum* TaDOR7316 developed better plant growth promotion than control safflower plants under greenhouse conditions (Table 4) and these strains were found to be effective against *M. phaseolina* and *F. oxysporum carthami* of safflower and better yield was observed under field conditions (Table 5). Among all treatments, Captan @ 0.2% and *T. harzianum* Th4dWP showed least disease incidence of wilt and root. Highest seed yield was observed with Th4d WP (3150 kg/ha) followed by *T. asperellum* TaDOR 7316 WP (3005 kg/ha) and captan @ 0.2% (2783 kg/ha). Similar work carried out by Yedidia *et al.*, (2001) on colonization of cucumber roots by *T. asperellum* has shown significant increase in dry weight, shoot length and leaf area.

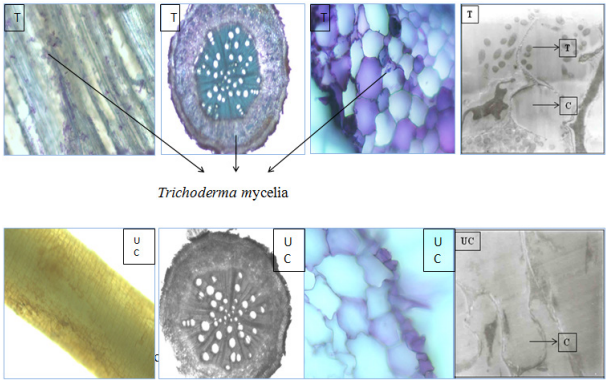

Fig. 6. TaDOR 7316 of safflower at 40X and 20X; TEM of safflower root 24 hr after inoculation with *Trichoderma asperellum*, TaDOR 7316.

Table 5. Management of *Fusarium* wilt and *Macrophomina phaseolina* of safflower by potential *Trichoderma* isolates

Treatments	Wilt (%)*	Root rot (%)	Seed Yield (Kg/ha)
Carbendazim + Mancozeb (saff) @0.2%	16.3 (23.80)	07.4 (15.7)	2783
Carbendazim @ 0.2%	20.2 (26.70)	06.2 (14.4)	1816
Captan @ 0.2%	10.3 (18.70)	08.2 (16.6)	2822
Cymoxanil 8% + Mancozeb 64% @ 0.2%	18.8 (25.70)	10.3(18.7)	2111
T. harzianum Th4d WP @ 10 g/kg	12.9 (21.00)	08.9 (17.3)	3150
T. asperellum TaDOR 7316 WP @ 10 g/kg	18.3 (25.30)	11.0 (19.3)	2661
T. asperellum Tv5 WP @ 10 g/kg	24.3 (29.50)	13.4 (21.4)	3005
Pathogen check	53.3 (46.89)	25.6 (30.4)	1911
CD ($P = 0.05$)	2.7	1.2	
CV%	7.1	9.1	

*Values in parenthesis are arcsine transformed values

The results showed that *T. harzianum* Th4d and *T. asperellum* TaDOR7316 were able to colonize roots and promote plant growth. Furthermore, these isolates produced mycolytic and defense enzymes at appreciable rate playing key role to stimulate the defense mechanism and aid in disease management. Nevertheless, there is need for more studies aimed at gaining insight into the signalling transduction pathways, related to defence and develop-

Table 4. Effect of biocontrol agent's seed treatment on seedling emergence, plant height and different growth parameters of safflower against *Macrophomina phaseolina* root - rot of safflower under greenhouse conditions

Treatments	Germination (%)	Shoot length (cm)	Root length (cm)	Vigour Index	Dry weight of root/plant (gm)	Dry weight of shoot/ plant (gm)	Mean branches/ plant	Mean no. of leaves/ Plant	Total no. of heads/ plant	Mean no. of seeds/ head
T. harzianum Th4d	100	57.5	14.6	7210	3.5	09.0	14.0	65.0	14.0	7.0
*T. asperellum*TaDOR7316	100	58.7	15.4	7410	3.2	09.0	19.0	69.0	18.0	6.0
T.asperellum Tv5	100	60.2	14.2	7440	3.5	08.1	16.0	63.0	16.0	5.0
Carbendazim	100	57.0	14.6	7160	3.2	09.7	14.0	65.0	14.0	4.0
Pathogen check	080	39.0	10.8	5176	1.4	08.0	03.0	50.0	05.0	5.0
Untreated control	100	52.1	12.2	6430	1.8	10.1	04.0	54.0	06.0	6.0
CD ($P = 0.05$)	-	6.30	5.2	-	1.5	01.7	03.2	01.7	05.2	1.1
CV%	-	6.00	4.2	-	8.6	06.7	12.5	06.7	04.2	9.0

Mean - denotes mean value of 3 replications.

ment, resulting from *Trichoderma*-plant interactions in the presence of pathogens and actual distinguish between competition for root colonization and locally induced systemic resistance is to be done.

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