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Isolation and screening of effective *Trichoderma* spp. against the root rot pathogen *Macrophomina phaseolina*

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The main emphasis of the present study was screened and evaluation of effective *Trichoderma* spp. for biocontrol of *Macrophomina phaseolina* the causative agent of root rot of groundnut. Five *Trichoderma* spp. were isolated from the rhizosphere soil of healthy groundnut plants, identified using morphological and microscopic characteristics and were evaluated for *in vitro* antifungal activity against *M. phaseolina* by dual culture plate technique and bioassay methods (*in vitro* antibiosis). Scanning electron microscopy was used to study the conidial surface of *T. harzianum*. Among the five isolates *T. harzianum* (T3), *T.viride* (T1) had maximum antifungal activity against *M.phaseolina* compared to the other *Trichoderma* spp. In dual culture technique *T. viride* and *T. harzianum* reduced mycelial growth by 61.1% and 64.4% respectively. Based on the dual culture technique, *T. harzianum* (T3), *T. viride* (T1) were selected for further research. Metabolites released from *T. harzianum*, *T.viride* were tested in culture medium against *M.phaseolina*. Cell free metabolites of *T. viride, T. harzianum* inhibited the growth of *M. phaseolina in vitro* and appeared to be fungicidal. The inhibition varied depending on the *Trichoderma* species producing metabolites, *T. viride* inhibited fungal growth upto 69% and *T. harzianum* upto 72.7% in nonvolatile and 47%, 64.7% in volatile metabolites respectively. The medium was optimized for mass multiplication of *Trichoderma* spp. *T. harzianum*, *T. viride* were tested for their ability to protect groundnut plants from disease caused by *M. phaseolina* in pot culture experiment. The growth of groundnut plants with the antagonist alone or in combination with pathogen was greater than in plants inoculated with pathogen alone.

Key words: *T. harzianum, T. viride, M. phaseolina,* biological control, groundnut

Introduction

Groundnut (*Arachis hypogaea* .L) is one of the chief commercial crops of India. It is the primary source of vegetable oil and contributes to about 43% of the total oil production in India. Species of *Trichoderma, Gliocladium and Aspergillus* etc. have been found effective in reducing the sheath blight and extensively explored for the control of soil borne plant pathogens (Khan and

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Sinha, 2005). *Trichoderma* sp. is one of the most important biocontrol agent used for management of different diseases (Harman, 2004). *Trichoderma* spp. are free living fungi that are common in soil and root ecosystems and promote plant growth (Yedidia, 2001). *Trichoderma* spp. are effective in control of soil/seed-borne fungal diseases in several crop plants (Kubicek *et al*., 2001). Different isolates of *P. fluorescens* and *Trichoderma* spp. were identified as biocontrol agents of groundnut stem rot and other soil-borne diseases (Podile and Kishore, 2002). These free-living fungi are ubiquitous in the soil environment and are being successfully used and commercialized to combat a broad range of phytopathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum*, and *Botrytis cinerea* (Hjeljord *et al*., 2000; Desai *et al*., 2002; Fravel, 2005). *Trichoderma* spp. can directly impact other fungi, after sensing a suitable fungal host, *Trichoderma* sp. responds with the production of antibiotic compounds, formation of specialized structures, and degradation of the host's cell wall, followed by the assimilation of its cellular content, a process known as mycoparasitism (Chet and Chernin, 2002; Steyaert *et al*., 2003; Benitez *et al*., 2004). The mechanisms of mycoparasitism, antibiosis and competition afforded by *Trichoderma spp.* have been widely studied (Howell, 2003; Harman *et al*., 2004b). *T. harzianum* has been evaluated for the control of charcoal stem root rot of melon in Egypt (Khalifa and Linddel, 1995). *Trichoderma harzianum* (Rifai) is a most potent biocontrol agent which inhibits the growth of *F. solani*, causal organism of wilt of brinjal, efficiently both *in vitro* and in field condition (Chakraborty, 2005). Seed treatment with *T. harzianum* and *T.viride* was more effective incontrolling *R. solani* in *Phaseolus vulgaris* both under greenhouse and field condition (Robert *et al*. 1993). Recently, several attempts have been undertaken to survey *Trichoderma* spp. promotion of seedling establishment, enhancement of plant growth and elicit plant defense reaction in some crops such as cotton (Shanmugaiah *et al*., 2009), bean (Hoyos-Carvajal *et al*., 2009). Using *T. harzianum* control of soil borne plant pathogens has been reported by many investigators (Ulkhede 1992, Ziedan *et al*., 2005) but there is little information was available on the use of *T. viride* and *T. harzianum* as biocontrol agents against *M.phaseolina* in groundnut plants. The objective of the present investigation was isolation and screening of effective *Trichoderma* spp. against the root rot pathogen *M. phaseolina* and optimization of cultural conditions for the growth of *Trichoderma* spp.

Materials and methods

Isolaton of Biological control agents from soil by serial dilution method: Different strains of *Trichoderma* were isolated from rhizosphere soil of healthy groundnut plants by serial dilution technique on *Trichoderma* specific medium.

($MgSO_4.7H_2O$-0.2g, K_2HPO_4-0.9g, KCl-0.15g, NH_4NO_3-3.0g, Glucose-3.0g, Agar-15g, Rosebengal-0.15g, Chloramphenicol-0.25g, Distilled water-1000ml, pH-6.5) (Elad, 1980).

Isolation of Macrophomina phaseolina

Grounndnut (*Arachis hypogaea* .L) plants showing root rot symptoms were identified, collected for the isolation of the pathogen . The isolation of the pathogen from diseased plant roots was performed on potato dextrose agar (Potatoes-200g, Dextrose- 20g, Agar- 20g, Distilled water-1000ml) medium and identified according to its morphology and colony characteristics.

Scanning electron microscopy: To study the surface of conidia of *T. harzianum*: Material for SEM examination of conidial surfaces was obtained from cultures that were grown on PDA for 10 days at 30°C. Agar blocks were cut into small pieces with conidiating hyphae and fixed in 2% glutaraldehyde in 0.1 M $NaPO_4$ buffer and post fixed in buffered 1% O_5O_4 for 2 hours. The material was then dehydrated in an ethanol series (10, 25, 60, 75, 95 and 100%) with 15 min per change. The specimens were dried in a critical point drying apparatus and viewed using a scanning electron microscope (Itamar Soares de Melo, 2004).

Dual culture technique

Dual culture plate technique (Webster, 1971b) was used to study the antagonistic effects of the *Trichoderma* isolates on *M.phaseolina*. All antagonistic pathogen combinations were examined on 20ml of PDA in 9-cm petriplates, with four replicate plates per treatment. For dual culture technique, a mycelial plug (0.5cm in diameter), taken from actively growing 3 day old culture of *M. phaseolina* and *Trichoderma* isolates placed 8cm apart from each other on the PDA. For control treatments, a plug of *M. phaseolina* was placed on the PDA medium. The plates were incubated at 28°C. Observations on the antagonistic activities of *Trichoderma* isolates on *M. phaseolina* were recorded after every 24hr for 5days and inhibition percentage was calculated using the following formula (Edington, 1971).

Inhibition percentage (%) = A_1-A_2/A_1 x 100

Where, A1 is the colony area of uninhibited *M. phaseolina* in the control, and A2 is the colony area of *M. phaseolina* in dual culture.

Effect of non-volatile compounds produced by antagonist(s) on the radial growth of Macrophomina phaseolina

The effect of culture filtrate of the fungal antagonist(s) on the growth of *M. phaseolina* was studied by method described by (Bruce, 1984; Dennis, Webster, 1971b). *Trichoderma* isolates were grown in potato dextrose broth at 27^{o}C with intermittent shaking at 150rpm. Metabolites were collected after 10days and filtered through the Whatmann No.1 filter paper and centrifuged at 2000 rpm for 10 min. The supernatant was filtered through Sartorius Millipore (0.4µ) filter. Filtrates were amended in PDA to make 5%, 10%, 20%, 40% concentration in petriplates. Solidified agar plates were inoculated at the centre with 6mm diameter mycelial disc of pathogen and incubated at 28^{o}C for 5 days. Plates without filtrate served as control.

Effect of volatile compounds produced by antagonist(s) on the radial growth of Macrophomina phaseolina

The effects of volatile metabolites from Trichoderma isolates were tested. Fungal biocontrol agent(s) were grown on petriplates containing PDA medium for 48hr. The lid of each petriplate was replaced with the bottom position of a petriplate dispensed with PDA and inoculated with *M. phaseolina* at the centre. The bottom of the petriplate containing centrally inoculated mycelial disc of test fungus (*M. phaseolina*) was kept inverted to the petriplate containing PDA media only which served as control. The pairs of each plate were attached together with cellophane adhesive tape. Observations on the radial growth of the test pathogen were recorded after 24, 48 and 96 hr of incubation at 28$\pm$ 1^{o}C. The colony diameter of the test fungus in the treatment in comparison with that of control gave growth inhibition percent.

Influence of various nutrients on the growth of Trichoderma species

In all nutritional studies aliquots of 50ml of the medium were taken into 250ml conical flasks, plugged with cotton and sterilized. The basal medium used was Czapek-Dox medium of the above composition. The initial pH of all the media was adjusted to pH 5.0 and the cultures were incubated for 10 days at room temperature as stationary cultures. A 0.5cm disc of actively growing mycelium cut from the periphery of a 7 day old culture with the help of a sterile cork borer and inoculated on to czapek dox medium. The flasks were incubated for 10 days at 28$\pm$2^{o}C as stationary culture. A number of carbon, nitrogen, sulphur and phosphorus compounds were tested for their effect on growth of the *Trichoderma* species (Grondona, 1997).

Pot culture technique

Oatmeal sand was prepared in 250ml conical flasks. Each flask inoculated with *T. viride, T. harzianum, M. phaseolina* separately and incubated at 28± 2°Cfor 10 days. 1kg of soil and 1kg of sand was taken into polythene bags and sterilized at 121°C for 30min at 15lbs 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. viride, T. harzianum* separately and in combinations. Replicates were maintained for each treatment. Control pots also maintained without any fungal cultures. On germination of seeds symptoms, root/shoot length, dry weights were recorded.

Results and discussion

Isolation and Identification of Trichoderma isolates

Trichoderma species were isolated according to Elad *et al.* (1981) method using *Trichoderma* selective medium (TSM) and then subcultured on potato dextrose agar. Based on the conidiophore morphology, the isolates were designated as T_1, T_2, T_3, T_4, and T5. Based on the mycelial characteristics, the fungus was identified as *Macrophomina phaseolina* (Dhingra and Sinclair, 1973). Under scanning electron microscope the fine structure of *T. harzianum* conidial surfaces and conidial masses on a phialide was observed (Fig. 1). The ability of *Trichoderma* isolates to inhibit the mycelial growth of *M. phaseolina* in dual culture was determined on PDA medium.

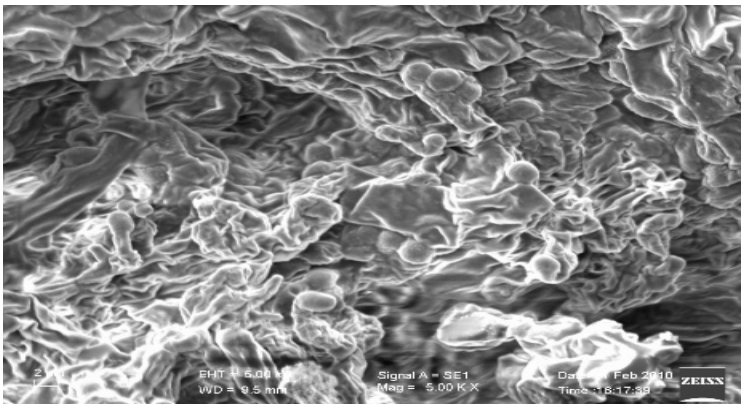

Fig. 1. Scanning microscopy of fixed conidia and conidial masses of *T.harzianum*.

Effect of Trichoderma species on Mycelial growth of Macrophomina phaseolina in vitro

All the five isolates of *Trichoderma* spp. exhibited antibiotic potential against *M. phaseolina* by inhibiting its mycelial growth (Fig. 2). Five days after inoculation, growth of *M.phaseolina* was found to be inhibited by T1, T2, T3, T4, T5 isolates and attained a growth of 5.5cm, 5.0cm, 5.8cm, 5.1cm, and 4.8 cm respectively. This indicates that T3 (*T. harzianum*) and T1 (*T. viride*) isolates had maximum antifungal activity against *M. phaseolina* compared to the other *Trichoderma* spp (Table 1). Based on microscopic characters, conidiophore branching patterns and conidium morphology T3 and T1 isolates indentified as *T. harzianum, T. viride* respectively (Rifai, 1969). Majumdar *et al*. (1996) observed that *T. viride* and *T. harzianum* were inhibitory to *M. phaseolina* causing blight of mothbean. Based on dual culture technique, *T. harzianum, T. viride* species were used for further research. Culture metabolites of *T.viride* at 40% inhibited the growth of *M. phaseolina* upto 69% where as the culture metabolites of *T. harzianum* inhibited *M.phaseolina* by 72.7% at 40% (Fig. 3). Antibiotic substances were produced in sufficient concentration to affect the growth of soil fungi *M. phaseolina* by *Trichoderma* spp (Table 2). The effect of non volatile culture metabolites on *M. phaseolina* revealed that *T. harzianum*, *T. viride* isolates were effective for inhibition of mycelial growth of *M. phaseolina* (Dubey and Dwivedi, 1988; Mathur and Bhatnagar, 1994). 24hrs old culture of *T. harzianum* produced maximum volatile metabolites which accounted for 64.7% inhibition of growth of *M. phaseolina* followed by *T. viride* (47%) (Fig.4, Table 3). Volatile and non volatile antibiotics produced by *T. harzianum* are responsible for the inhibitor action against root pathogen, *Fusarium culmorum* (Iqbal *et al.,* 1994) and *F. oxysporum* (Michrina *et al.,* 1995).

Optimization of cultural conditions for the growth of Trichoderma species

All *Trichoderma* isolates were able to grow in the presence of different carbon sources. Of different carbon sources tested, sucrose was proved to be the best source of carbon, followed by glucose, cellulose, starch, and lactose. Poor growth was observed on fructose, glycerol and pectin. Among different nitrogen sources tested, sodium nitrate and potassium nitrate supported good growth of the *Trichoderma* isolates; where as moderate growth was observed in ammonium nitrate, magnesium nitrate and urea. Among the different sulphur sources, sodium sulphate and potassium sulphate yielded maximum growth of the *Trichoderma* isolates, while the others supported only moderate growth (Table 4).

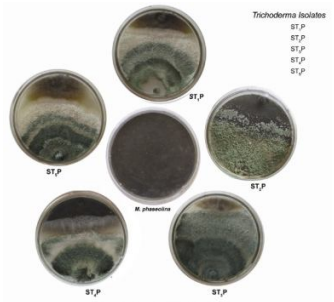

Fig. 2. Dual culture technique.

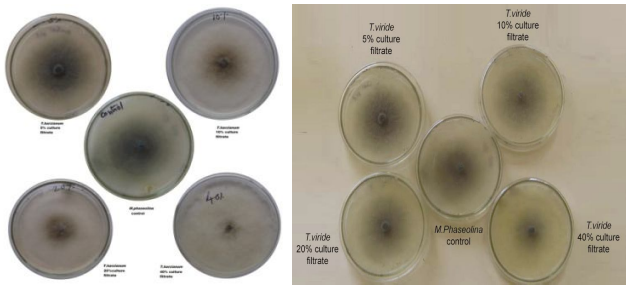

Fig. 3. Effect of non-volatile compounds produced by antagonist on the radial growth of *Macrophomina phaseolina*.

Table 1. Dual culture technique.

Treatments	Radial growth of bioagents (cm)	Growth of pathogen(cm)	% of Inhibition
T1+*M. phaseolina*	5.5±0.04	3.5±0.05	61.1%
T2+*M. phaseolina*	5.0±0.05	4.0±0.04	55.5%
T3+*M. phaseolina*	5.8±0.05	3.2±0.05	64.4%
T4+*M. phaseolina*	5.1±0.06	3.9±0.05	56.6%
T5+*M. phaseolina*	4.8±0.04	4.2±0.1	53.3%
Control(*M. phaseolina)*	-	9.0	-

Each value is an average of 3 replicate samples, ± Standard error,
T1, T2, T3, T4, T5=*Trichoderma* isolates.

Table 2. Effect of non-volatile compounds produced by antagonist(s) on the radial growth of *Macrophomina phaseolina*.

Treatments	Concentration of Metabolite(filter sterilized)							
	5%		10%		20%		40%	
	Pathogen growth (cm)	% Inhibition	Pathogen growth (cm)	% Inhibition	Pathogen growth (cm)	% Inhibition	Pathogen growth (cm)	% Inhibition
T. viride+ *M. phaseolina*	3.6±0.02	34.5	3.2±0.03	41.8	2.3±0.02	58.1	1.7±0.05	69
T. harzianum+ *M. phaseolia*	3.5±0.05	36.3	3.0±0.05	45.4	2.0±0.05	63.6	1.5±0.02	72.7
Control (*M. phaseolina*)	5.5±0.05	-	5.5±0.1	-	5.5±0.04	-	5.5±0.05	_

Each value is an average of 3 replicate samples
± Standard error

Table 3. Volatile compounds produced by *Trichoderma* spp. against *Macrophomina phaseolina.*

Treatments	Radial growth of *Macrophomina phaseolina* (cm)			% inhibition		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
T.harzianum+M.phaseolina	0.6±0.1	1.9±0.1	2.6±0.1	64.7	42.4	39.5
T.viride+ M.phaseolina	0.9±0.05	2.5±0.1	3.7±0.05	47	21.2	13.9
Control(*M.phaseolina*)	1.7±0.05	3.2±0.05	4.3±0.05	-	-	-

Each value is an average of 3 replicate samples, ± Standard error.

Table 4. Effect of Carbon compounds on the growth of *Trichoderma* isolates.

Carbon sources	Average dry weight in mg/50ml				
	T1	T2	T3	T4	T5
Glucose	650	570	980	800	120
Lactose	490	120	100	50	280
Fructose	160	90	60	110	60
Cellulose	890	650	590	580	880
Starch	610	280	650	160	350
Pectin	20	40	80	30	70
Glycerol	220	60	120	160	50
Sucrose	1000	10	1230	480	260
Nitrogen sources					
Ammonium nitrate	170	100	300	200	60
Potassium nitrate	240	200	450	140	250
Sodium nitrate	1000	560	1200	480	260
Urea	140	120	110	150	120
Magnesium nitrate	180	145	185	137	140
Sulphur sources					
Magnesium sulphate	880	530	780	830	970
Copper sulphate	300	340	450	690	620
Sodium sulphate	860	900	890	980	810
Potassium sulphate	910	840	850	910	710
Phosphorus sources					
Potassium phosphate	540	900	870	850	620
Sodium phosphate	870	880	820	600	880
Calcium phosphate	400	700	880	530	670
Ammonium phosphate	840	730	670	790	640

T1, T2, T3, T4, T5=*Trichoderma* isolates.

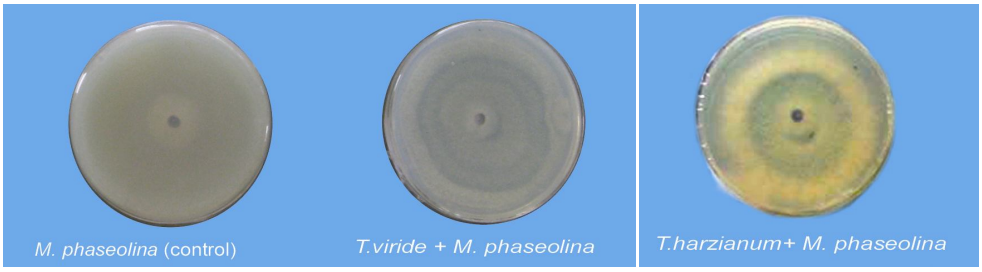

Fig. 4. Volatile compounds produced by *Trichoderma* spp. against *Macrophomina phaseolina*.

Biological control of Macrophomina phaseolina on groundnut in plot culture technique

Maximum root length was recorded in *T. harzianum, T. harzianum+M. phaseolina, T. viride,* and *T. viride+M. phaseolina* treated plants compared to *M. phaseolina* inoculated plants (Table 5). Maximum shoot length was recorded in *T. harzianum, T. harzianum+M. phaseolina, T. viride,* and *T. viride+M. phaseolina* treated plants compared to *M. phaseolina* inoculated plants (Table 6). Similarly, *T. harzianum* and *T. viride* and their fusants enhanced rice and tomato shoot and root lengths (Balasubramanian, 2003).

Table 5. Effect of *Trichoderma* spp. and *M.phaseolina* on root length (cm) of groundnut at different stages of growth.

Treatments	Stage1	Stage2	Stage3
T.viride	6.5±0.05	14.5±0.04	18.3±0.1
T.viride+M.phaseolina	7.2±0.05	12.7±0.1	17.4±0.05
T.harzianum	9.3±0.01	15.8±0.05	23.2±0.06
T.harzianum+M.phaseolina	8.6±0.1	14.3±0.1	20.6±0.05
M.phaseolina	3.5±0.05	6.8±0.08	14.7±0.04
Control	6.5±0.05	14.2±0.05	16.3±0.05

Each value is an average of 3 replicate samples, ± Standard error.

Table 6. Effect of *Trichoderma* spp.and *M.phaseolina* on shoot length(cm) of groundnut at different stages of growth.

Treatments	Stage1	Stage2	Stage3
T.viride	16.4±0.2	27.3±0.7	37.4±0.5
T.viride+M.phaseolina	14.3±0.05	24.2±0.5	38.3±0.9
T.harzianum	19.2±0.1	28.5±0.3	39.2±0.4
T.harzianum+M.phaseolina	17.5±0.4	26.3±0.5	38.3±0.7
M.phaseolina	7.5±0.7	16.2±0.08	17.3±0.5
Control	16.2±0.5	22.4±0.4	27.5±0.09

Each value is an average of 3 replicate samples, ± Standard error.

Biological control of plant pathogens by microorganisms has been considered as a natural and environmentally acceptable alternative to the existing chemical treatment methods (Baker, Paulitz, 1996). Intensified use of fungicides resulted in the accumulation of toxic compounds potentially hazardous to humans and environment (Cook, Baker, 1983). The *T. harzianum* (T3), *T. viride* (T1) isolates were better potential biocontrol agents against *M. phaseolina in vitro* compared to other isolates of *Trichoderma* spp. According to Hermosa *et al* (2000) *T. harzianum* had a potential biocontrol activity in a dual culture studies against the phytopathogenic fungi of *Phoma betae*, *Rosellinia necatrix*, *Botrytis cinerea* and *Fusarium oxyporum* f. sp. *dianthia* in the three different media. The present work of *in vitro* plate assays showed that *T. harzianum* is more effective in suppressing the growth of *M. phaseolina* followed by *T.viride*. With the increase in concentration of culture filtrates of the bioagent, the radial growth of test pathogen was proportionally decreased. Maximum inhibition of the mycelial growth of *M. phaseolina* was observed with the culture filtrate of *T. harzianum* used as 40% concentration. Seshagiri and Eswaran (2002) reported that mycelial growth of pathogen decreases with the increase in the concentration of the culture filtrate produced by fungal antagonists from 10 to 40 percent and no growth at 50%. *T. harzianum* overgrew *M. phaseolina* and suppressed its growth *in vitro* (Patel and Anahosur 2001).Volatile toxic substances produced by antagonists could diffuse easily through their filled pores of the soil and inhibited the soil borne pathogen *M. phaseolina* specially suppressing the sclerotia formation. Kukuk and Kvanc (2004) reported that *T. harzianum* strains produced a metabolite that inhibit growth of plant pathogenic fungi. Similar results reported that seed treatment with *T. harzianum* and *T.viride* was more effective in controlling *Rhizoctonia solani* in *Phaseolus vulgaris* under both green house and field conditions (Prashanthi *et al* 1997). Biological activity of antagonistic fungi and bacteria may partially be associated with production of antibiotics (Etebarian *et al.*, 2000; Faull *et al.,* 1994; Pusey and Wilson, 1984). The present investigation suggests that metabolites released by this *Trichoderma* spp. are toxic and fungistatic to *M. phaseolina.* The growth of the groundnut plants in the *T. viride* + *Macrophomina, T. harzianum*, *T. harzianum* + *M. phaseolina* was greater than for *M. phaseolina* alone and demonstrated the best result in the control of root rot in groundnut. In pot culture assay soil treatment with *T.harzianum, T. viride* was found to enhance the root/shoot length. Increased growth by *Trichoderma* sp. was also induced by a diffusible growth-regulating factor (Windham *et al*., 1986). *R. solani* infection was completely inhibited by *T. harzianum* (Malik *et al.*, 2005). Spraying of Talc based formulations of bioagents *T. harzianum* and *T.virens* were found quite effective against sheath

blight (Khan and Sinha, 2006). In conclusion, the *Trichoderma* isolates reduced disease severity in groundnut plants.

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