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IN VITRO ANTAGONISM OF INDIGENOUS TRICHODERMA ISOLATES AGAINST PHYTOPATHOGEN CAUSING WILT OF LENTIL

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ABSTRACT

The basic aim of the work was to assess the indigenous potential of bio-agents and their antagonistic potential against phytopathogen viz: *Fusarium oxysporum f.sp. lentis; causing* wilt of lentil*,* a disease prevalent in Bihar. In this research work nineteen isolates of *Trichoderma* were isolated and these were ascribed to three species namely: *Trichoderma harzianum* (Th), *Trichoderma viride* (Tv) and *Trichoderma koningii (*Tk***).** Efficacy of these bio-antagonists were investigated in *in vitro* conditions by employing dual culture technique and liquid culture filtrate assay. The outcome of *in vitro* dual culture testing revealed that among the different isolates of *Trichoderma* isolate-5 and 7 of Th, 2 and 18 of Tv and isolate 9 of Tk were found to be more efficient amongst all, as they showed better antagonism against the tested phytopathogen.. The isolate Th-5 caused maximum inhibition (82.8%) followed by Th-7 (82.3%), Tv-2(79.2%) Tv-18 (74.4%) and Tk-9 (71.0%). Rest isolates were moderate in activity. Metabolites extracted from liquid culture filtrates also depicted almost the same trend of superiority as mentioned in dual culture i.e. the same isolates further proved its better potentiality when compared with rest, Th-5 with superior bio-antagonistic potential.

Keywords: *Trichoderma* spp. *Fusarium* sp. antagonistic activity, phytopathogen, wilt of lentil.

INTRODUCTION

Lentil (*Lens culinaris* Medikus) commonly known as "Poor man's meat ,as it is one of affordable protein rich legume. It is a rich source of protein, minerals (k,P,Fe,Zn) and vitamins for human nutrition (Bhatty, 1998). Additionally, because of its high lysine and tryptophane contents, its consumption with wheat or rice provides a balance in essential amino acids in human nutrition. Lentil straw is also a valued animal feed (Erskine et al, 1990).Among the several biotic stresses limiting lentil yield, wilt of lentil is one of serious diseases caused by *Fusarium oxysporum* f.sp. *lentis* and plays a major role in reducing lentil yield (Hamali and Hassanein 1996).The disease may cause complete crop failure under favorable conditions for disease development and can be the major limititing factor for lentil cultivation in certain areas (Chaudhary and Amerjit,2002).The disease appears in either the early stage of crop growth (seedling) or during the reproductive stage (adult stage)(Khare,1981; Stoilova and Chavdarov, 2006). Although chemical sprays offers reasonable management disease but now their diverse harmful effects are well cited. In recent times, a change has gradually taken place with respect to the perception of priorities. Under this concept, bio-antagonists or biological control agents offers a great promise and thus given priority over chemical control. Despite, substantial progress that has been made in this field, its application in fields is still doubtful.

Trichoderma species are considered as promising biological control agents against numerous phytopathogenic fungi including *F. oxysporum (*Sarhan et al*, 1999). Trichoderma* species have shown efficiency on biocontrol of plant pathogens (Chet and Baker, 1980; Elad et al, 1980; Lifshitz et al, 1986; Mehta et al, 1995; Viterbo et al, 2002; Benitez et al, 2004; Harman et al, 2004 ;Etebarian 2006).Their efficacy is closely related with local conditions. Thus in present study we concentrated on isolation, identification of *Trichoderma* isolates existing in few regions of Bihar Lentil fields. The antagonist potential of the best active strains have been identified with the *in vitro* assays.

MATERIALS AND METHODS

1. Collection of Plant Pathogen: *(F. oxysporum f. sp. lentis).*

Root samples of ten lentil plants which were infected with wilting were collected and brought to the laboratory. Root samples were cut into approximately 1.5 cm length, surface sterilized for 30-40 seconds. These were placed onto Potato Dextrose Medium (PDA) in Petri-plates and incubated for 7 days at 28±2°C. Fungal isolates appearing on the root pieces were identified and transferred to fresh PDA medium.

2. Isolation and Identification of Trichoderma Species

Soil samples were collected from rhizosphere of local Lentil crop fields from five different areas of Bihar. For rhizospheric soil, plants were gently uprooted, soil tightly adhering the roots were collected, mixed and composite mixture of soil of the region was obtained. The pH of soil was determined in 1:2 (soil: water) ratio, keeping 30 minutes of equilibration. Collected soil samples were air dried for 4 hour and isolation was done by serial dilution technique. Trichoderma Selective Medium (TSH) was used for identification of the isolates of *Trichoderma* (Elad Y et al, 1983).1 ml of soil suspension was taken with the help of 5ml sterilized pipette and poured on the Petri- plate seeded with TSM. The plates were incubated at 28 ± 2°C for 5 days. Observation on the appearance of colonies was recorded from 3rd to 5th day. Individual colonies were picked up and maintained in pure culture for further study. *Trichoderma* species were identified and examined under compound microscope on the basis of their cultural and morphological character (Park M.S. et al, 2005) and were maintained on PDA slants at 4°C for subsequent studies.

3. Antagonistic Assessment

3.1.First assessment

The antagonistic potential of indigenous isolates of *Trichoderma* were evaluated against *F. oxysporum f. sp. Lentis* using dual culture technique.(Dhingra and Sinclair 1995) with slight modifications. Five millimeter diameter mycelial disc of each test antagonist (*Trichoderma* isolates) taken from 7 day old culture was paired against same sized mycelial disc of *Fusarium oxysporum f.sp. lentis* at opposite end on PDA (20 ml) contained in 90 mm diameter Petri- plates. The pathogen and antagonist disc were place at equal distances from the periphery of the petriplate. The PDA plates inoculated only with either antagonists and phytopathogen served as control. The plates were incubated at 28±2°C. The experiment was conducted under Completely Randomized Design (CRD). The growth of the pathogen in both test and control experiments were recorded. Data were obtained for percent inhibition of radial growth (PIRG) = $(R_1–R_2)/R_1$ x 100. Where R_1 = radial growth of pathogen in control. R_2 = radial growth of pathogen in dual culture experiments with antagonists.

3.2. Second Assessment

The numbers of days taken by Trichoderma isolates to completely overlap the pathogen colony were recorded. The isolate taken the shortest number of days was counted for signifying good antagonistic properties.

4. Culture filtrate assay

The inhibition of the mycelial growth of plant pathogen was tested by metabolites secreted by *Trichoderma* in liquid medium. It was determined as follows: one hundred milliliters (ml) of potato dextrose broth (PDB) were dispensed into 250 ml

Erlenmeyer flasks and inoculated with 5 mm diameter disc from edge of 7 days old culture of the *Trichoderma* isolates. Each flask was inoculated with three discs of each in triplicate and set up was shaken at 100 rpm for 15 days at 28 $\pm$ 2°C on Thermostat Culture Shaker. After the optimum period, the cultures were filtered through Whatman No.1 and sterilized by millipore membrane filtration of 0.25 µm and stored at 4°C for further use. The sterilized filtrate were amended in PDA to make three concentrations (25%, 50% and 100%) in Petri plates. 5mm wide mycelial discs of the pathogen were placed at the centre of solidified agar plates and incubated at optimum temperature for 7 days. Plates devoid of culture filtrates served as control. Radial growth of *F oxysporum f sp. Lentis* was measured and its inhibition percentage of mycelial growth was calculated using the formula I= [(C2−C1)/C2)] ×100 [Edington et al, 1971] Each experiment was performed in triplicate. I = percentage inhibition of radial mycelial growth, C2 is radial growth measuring pathogen in control, C1 is radial growth of pathogen in treated plates.

5. Slide culture method

For each *Trichoderma* isolate – pathogen interaction, a clean slide was placed in 9 cm diameter plates and sterilized. Then a small amount of autoclaved melted Potato Dextrose Agar medium was spread over the slide to make a thin PDA film on the slide. 3 mm disc of both pathogen and antagonists isolates were paired on slide 3 cm apart on PDA surface. Distilled water was poured in Petri plates to, avoid drying (incubated at 28 $\pm$ 2°C for more than a week, according to interactions). Meeting area was observed microscopically by staining with Lactophenol and Cotton blue for presence of mycelia penetration and cell wall disintegration in the area of interaction.

RESULTS

Species identification: Ninteen *Trichoderma* isolates were identified according to the identification key (Rifai 1969) based on branching of conidiophores, shape of the phialides, emergence of phialospores, and shape of phialospores. These isolates were identified into three species,viz; *T harzianum* (08 isolates), *T. viride* (04 isolates), *T. koningii* (05 isolates) and unknown species (02) (Table -1)

Table-1 : ***Identification of indigenous (Bihar) Trichoderma isolates .***

S.N.	*Trichoderma* species	*Trichoderma* isolates
1	*T. harzianum*	Th_3, Th_4, Th_5, Th_6, Th_8, Th_{10}, Th_{14}, Th_{16}
2	*T. viride*	Tv_1, Tv_2, Tv_{18}, Tv_{13}
3	*T. koningii*	Tk_7, Tk_9, Tk_{11}, Tk_{12}, Tk_{15}
4	Unknown spp.	T_{19}, T_{17}

Percent Inhibition of Radial Growth (PIRG) and colony overgrowth assessment :
First assessment : Table-2, Fig-1, Fig. 3.

In dual culture test, each of all tested *Trichoderma isolates* differentially limited the colony growth of the pathogen. Among these, with individual variability the best antagonistic potential was displayed by Th5,Th7,Tv2,Tv18 and Tk9(Table-2,Fig:1,Fig:3)Th5,Th7andTv2 were the most effective isolates of T.harzianum and T.viride species,where the maximum percent growth inhibition of 82.8%,82.3% and 79.2% respectively was recorded. Next to it were Tv18 and Tk9. Based on the PIRG values five best antagonistic isolates in series were Th_5> Th_7> Tv2> Tv_{18}> Tk_9 isolates. Isolate Tk_{15} showed the least amount . of inhibitory effect on the test pathogen.

Second assessment:Table-2, Fig-1.

Table -2: ***Antagonistic activity of Trichoderma strains against Fusarium oxysporum f. sp. lentis. dual culture technique.***

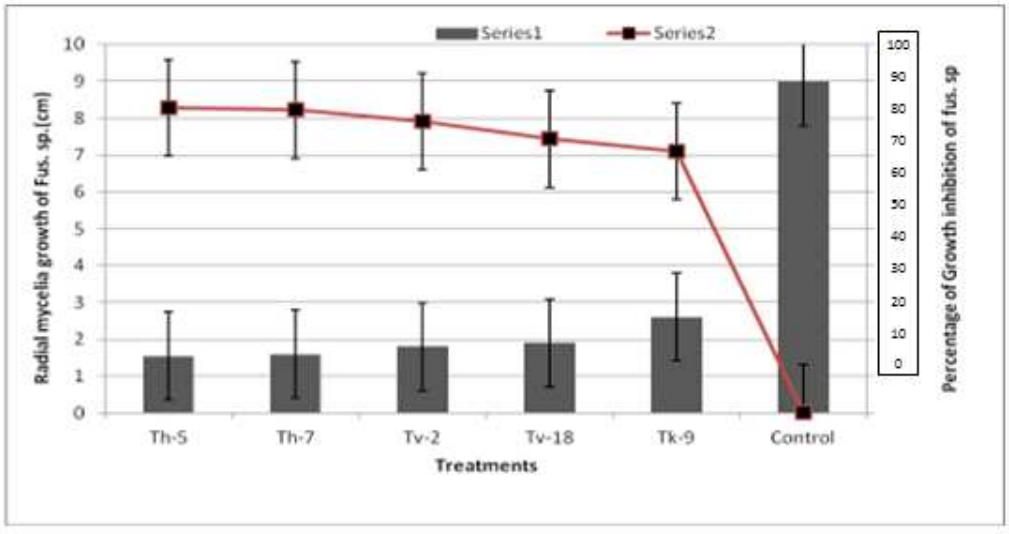
Sl. No.	Antagonistic isolates	Radial growth (cm)	% inhibition of growth (PIRG)	Time of overgrowth (days)of *Trichoderma* spp	Scale of Antagonistic activity
1.	Th-5	1.54	82.8%	8	++++
2.	Th-7	1.59	82.3%	8	++++
3.	Tv-2	1.80	79.2%	10	++++
4.	Tv-18	1.90	74.4%	11	+++
5.	Tk-9	2.60	71.0%	14	+++
6.	Control	9.00	0.00%	-	-

Descriptive assessment of the antagonistic activity was scaled as follows (Soy tong 1988).

++++	=	***very high antagonistic activity***	***(> 75 PIRG)***
+++	=	***high antagonistic activity***	***(61 – 75 PIRG)***
++	=	***moderate antagonistic activity***	***(51 – 60 PIRG)***
+	=	***low antagonistic activity***	***(<50 PIRG)***
_	=	***no antagonistic activity***	

This assessment was taken up with the best selected isolates in dual culture method. In this cultural study, each of *Trichoderma i*solates (Th_5 , Th_7 , Tv_2 ,Tv_{18}, Tk_9) completely inhibited or overgrew the test pathogen colony with a colony degradation ranging from 8 to 14 days. Th_5 & Th_7 took 08 days to overlap the pathogen ,further Tv_2 and Tv18 overgrew in 10 and 11days respectively. Tk_9 took 14 days until the whole plate was covered.

Fig. 1 ***Effect of Trichoderma isolates on the growth of Fusarium sp. (fus)***



Series 1 – Radial mycelia growth of Fusarium sp. in cm.
Series 2 – Percentage of growth inhibition of Fusarium sp. by Trichoderma spp.

Table-3 : ***Radial Mycelial Growth (RMG) and Percent Inhibition Mycelial growth (PIMG) of test pathogen in different concentrations of Trichoderma Liquid Culture Filtrate (LCF)***

Selected Trichoderma isolates	LCF (%)	RMG (mm)	PIMG (%)
T_{h-5}	10	31	65.5
	25	25	72.2
	50	15	83.3
T_{h-7}	10	32	64.4
	25	27	70.0
	50	16	82.00
T_{v-2}	10	34	62.2
	25	29	67.7
	50	21	76.6
T_{v-18}	10	39	56.06
	25	30	66.06
	50	25	72.2
T_{k-9}	10	45	51.1
	25	38	57.8
	50	30	66.7
Control	---	90	---

Effect of Liquid Culture Filtrate (LCF) of different Trichoderma isolates on the radial growth of Fusarium oxysporum f. sp. lentis (after 7 days of incubation).

In vitro antagonistic activity of undertaken culture filtrates of *Trichoderma* species significantly and variably reduced the radial colony growth of test pathogen. It was observed that all culture filtrates of *Trichoderma* isolates at 50% concentration showed strongest inhibition (Table-3.Fig-2.) *T.harzianum* isolate Th_5 showed the highest radial inhibition of 83.3% followed by Th_7 *as* 82.0% in performance and decreased colony growth from 90mm (control) to 15 mm (LCF 50% of Th_5). Rest of species of *Trichoderma* viz Tv_2,Tv_{18} & Tk_9 in 50% LCF reduced the colony growth of *F. oxysporum* with percent mycelia inhibition of 76.6%,72.2%,66.7% respectively. The inhibition decreased with decrease in LCF concentration in each case.

Slide culture assay

In slide culture, meeting area of *Trichoderma* isolates and *Fusarium oxysporum* sp was observed under light microscope, the presence of coiling structures for wall disintegration was well observed.

DISCUSSION

Lentil is an economically important crop which undergoes huge loss with wilt diseases caused by various *Fusarium* species. Biological control in recent times have been accepted as more natural and environmentally acceptable alternative to the existing chemical treatments. (Elad 2000; Howell 2003; Ezlashi et al; 2007 Shalini and Kotasthane 2007;). In this purview *Trichoderma* spp has been investigated as an important antagonistic soil fungus having the ability to reduce disease incidence caused by the phytopathogenic fungi, particularly the soil borne fungi (Freeman et al; 2004; Ashrafizadeh et al., 2005; Dubey et al 2007) Rhizosphere competence of antagonists is a pre-requisite for the biological control of soil borne plant pathogens. Several research papers reveal the fact that success of bio agent introduced in soil does not guarantee the control of target pathogen(s) because plants, physicochemical and biological factors affect establishment and antagonism of introduced bio agents .In this context it is presumed

that to ensure success of introduced bioagents, they should be isolated from the local area where they exist. Similar efforts have been tried here forth (Kucuk and Kivanen, 2003;Chang et al, 2006.). The present study is focused on this theme, that after isolation and identification of local rhizospheric fungi and testing their antagonistic potential under *in vitro* conditions they can be prescribed for application in fields which is already a known and conducive environment. Among the nineteen isolates of *Trichoderma* spp from rhizosphere of Lentil crop, five isolates of three species showed excellent capability to inhibit the growth of pathogen in *in vitro* conditions. The results reported, suggest that the isolates of *T.harzianum* and *T. viride* were more capable of influencing the growth of tested pathogen in dual culture. Similarly, isolates of different *Trichoderma* species to control soil borne phytopathogens have been reported to differ in their effectiveness (Ram Bhandra Raju M *et al* 2000;Anand S and Jayarama Reddy 2009;). This result is a pioneer information that particular isolate from a particular location can be employed in bulk for treatment of disease incidence. Furthermore, it can be tested against other pathogens. Efforts are onto to evaluate the performance of promising isolates in fields conditions. The radial mycelial growth inhibition of *Fusarium oxysporum f. sp. lentis* by liquid culture filtrate varied from 51.1% to 83.3% among *Trichoderma* isolates and species. This is in accordance with Sivasithamparam and Ghisalberti, 2002, which indicated that different species of the same family and different strains of the same species, often can produce significantly different compounds which suggests that secondary metabolites express the individuality of species in chemical terms.

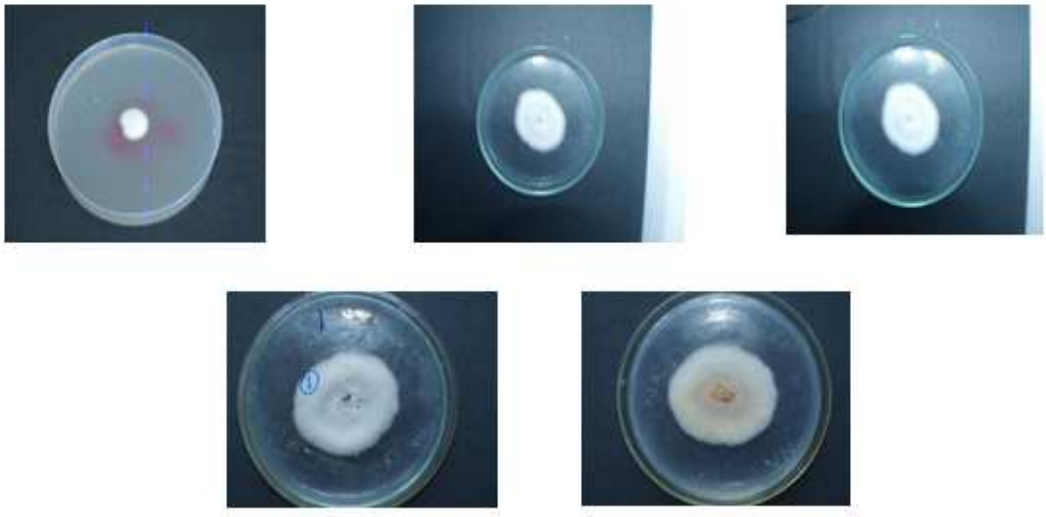


Fig. 2
Inhibitory effect of Liquid Culture Filterate Metabolites on radial mycelial growth of Fusarium sp.
Top : left to right Th-5, Th-7, TV-2.
Bottom : left to right TV-18, TK-9.

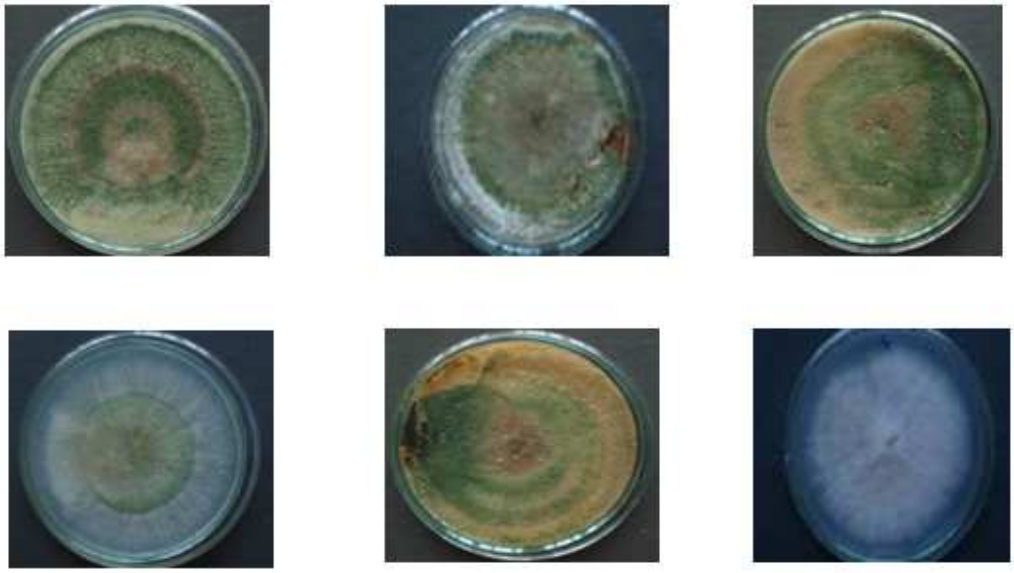


Fig. 3
Inhibitory effect of different isolates of *Trichoderma spp* on mycelial growth of *Fusarium sp.* in dual culture test.
Top : left to right Th-5, Th-7, TV-2.
Bottom : left to right TV-18, TK-9., Control.

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