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Mycoparasitic capabilities of diverse native strain of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract: The *Fusarium* wilt of tomato (*Lycopersicon esculentum* Mill.) caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen is recognised as one of the most devastating disease and major yield limiting factor in tomato growing regions worldwide. For eco-friendly and sustainable management of the disease, 19 *Trichoderma* native isolates belonging to 3 species of the genus, *T. harzianum, T. asperellum* and *T. virens* were evaluated *in vitro* against the pathogen using dual culture method. Out of 19 isolates, 8 isolates showed mycoparasitism, 8 isolates showed antibiosis and remaining showed lysis. Microscopic observations of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) growth in dual cultures revealed that growth inhibition occurred just before near to contact with the antagonist. All *T. harzianum* isolates tested exhibited coiling around the hyphae of FOL. Isolates of *T. harzianum*, showed good coiling and growth inhibition of the pathogen. The *T. harzianum* strains did not differ in coiling pattern and gave somewhat equal coiling performances. Strains of *T. asperellum*, showed coiling but the coiling pattern of all these strains was different. Only one strain of *T. virens* showed coiling out of 2 strains. Among them *T. harzianum* (SVPUTh91) showed the best performance *in vitro* as biological control agent against FOL followed by *T. asperellum* and *T. virens*, resulting in 83, 73 and 65% reduction in colony growth, respectively.

Keywords: Antibiosis, Coiling, *Fusarium*, Lysis, *Trichoderma*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the world's largest vegetable crop after potato and sweet potato, grown in all parts of India. Its popularity is due to its high nutritive value, diversified use, and nutritional significance as a source of vitamins A, C potassium, minerals and fibers (Sundaramoorthy and Balabaskar, 2013). It occupies number one position in its nutrient contribution to human diet. India ranks second in the area as well as in production of tomato, and share 7.6% of the world total production, at present the estimated area under cultivation and production is 8.82 lakh ha and 187.4 lakh tonnes, respectively (NHB Database, 2014). Tomato is affected by various biotic diseases, causing negatively effect on plant growth and the produced yield.

Fungal phytopathogens pose serious problems world-wide in the cultivation of economically important plants, especially in the subtropical and tropical regions (Brimner and Boland, 2003). Out of these, pathogenic fungi especially, the wilt caused by species of *Fusarium* remain to be a challenging task in terms of management (Agrios, 2005; Srinon *et al.,* 2006). At present, around 30% of all plant species are affected by pathogens that cause disease and death of individual plants. Wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen is one of the most important diseases world-wide (Cal *et al.,* 2004). Pesticides and organic compounds that are widely used to control plant pathogens do not degrade completely and leave toxic residues in food chain (Chet, 1987; Lynch, 1990). Their applications have an abuse as favoured the development of fungicidal resistant in pathogens. Another risk potential have been observed as the greater probability of decreasing the effect through genetic shifts in the population, whereas fungicides of broad spectrum produce adverse consequences on non-target organisms (Tjamos *et al.,* 1992).

Biological control of plant disease is the suppression of disease symptoms and disease incidence by the application of a biological agent, usually a microorganism (Avis *et al.,*2001). Several fungal and bacterial biocontrol agents have been used for achieving plant disease control, amongst them *Trichoderma* group has been found effective against aerial, root and soil pathogens (Weller, 1988; Whipps *et al.,* 1993; Elad *et al.,* 1998 a, b; van Loon *et al.,* 1998; Elad, 2000; Harman *et al.,* 2004; Chaube *et al.,* 2002). *Trichoderma* species have

been increasingly used as biological control agents and a few of the isolates are commercially available (Henis, 1984; Chet and Baker, 1980; Chet and Inbar, 1994). Results from different studies showed that several strains of *Trichoderma* exerted significant reducing effects on plant diseases caused by pathogens such as *R. solani, S. rolfsii, Pythium aphanidermatium, Fusarium oxysporum, F. culmorum, Collectotrichum falcatum* and *Gaeumannomyces graminis* under greenhouse and field conditions (Sivan and Chet, 1993; Inbar *et al.,* 1994; Chet and Baker, 1981; Prasad *et al.,* 2010). Isolates of *Trichoderma* spp. can produce lytic enzymes (Haran *et al.,* 1996), and antifungal antibiotics (Dennis and Webster, 1971a; Brewer *et al.,* 1987; Almassi *et al.,* 1991), which inhibit the growth of pathogenic fungi. However, it is reported that all the isolates of *Trichoderma* spp. are not equally effective in control of pathogen *in vitro* and *in vivo* conditions to control diseases (Biswas and Das, 1999; Ramezani, 2008). Therefore, specific isolates of *Trichoderma* spp. are needed for successful control of a particular pathogen. The present study on the efficacy of the mycelial growth inhibition of *F. oxysporum* f. sp. *lycopersici* by *Trichoderma* spp. and investigating the mechanisms involved in inhibition under microscopic observation that could be used in the future for *F. oxysporum* control.

MATERIALS AND METHODS

Collection of samples: Soil samples were collected from different geographical areas of Western region of India. Soil samples (50 g each) were collected at four corners and the centre of the different crop fields from various places. The samples were collected from top 2-5 cm depth of rhizospheric soils. The soil samples were then transferred into sterile polyethylene bags and transported to the laboratory and kept at room temperature for further use.

Isolation and identification of *Trichoderma* spp.: One gm of the soil sample was taken and added to 1ml of sterilized distilled water to make a dilution of 10^{-1}. The soil suspension was left during 5 min. at ambient temperature to release conidia and hyphae adhering to soil particles. This suspension was then subjected to serial dilutions and a dilution of 10^{-5} was attained. One ml of each dilution viz., 10^{-3} to 10^{-4} was poured on to potato dextrose agar (PDA) medium supplemented with 500 mg/l of antibiotic Chloramephenicol. The plates were then incubated at 27±2°C for one week. The distinct colonies were picked and transferred in to PDA containing petriplates at least two times to obtain pure cultures using single spore culture. These isolates were identified on the basis of their morphological (Rifai, 1969) and molecular characterization (Prasad *et al.,* 2012) using ITS1 and ITS4 markers (White *et al.,* 1990). Cultures were identified according to conidiophore, shape of the phialides and emergence of phialophores and phialospores. The purified and identified cultures of *Trichoderma* spp. were maintained on Potato Dextrose Agar (PDA) medium and stored at 4°C for further use and sub cultured every two months intervals. For long terms storage strains were conserved in 50% glycerol at -80°C.

Plate confrontation assays: Variation in the antagonistic activity of 19 isolates belonging to *T. harzianum*, *T. asperellum* and *T. virens* were evaluated *in vitro*, soil-borne plant pathogen *Fusarium oxysporum* f. sp. *lycopersici* and the ability of *Trichoderma* spp. to coil around *F. oxysporum* in dual culture technique (Dennis and Webster, 1971). Dual culture was carried out by using one week-old culture of *F. oxysporum* and *Trichoderma* on PDA. The agar medium was inoculated with a 5 mm diameter disc of antagonist positioned diametrically opposite a 5 mm diameter disc of pathogen. The distance between discs was approx. 5 cm. The plates were incubated at 27±2°C in BOD and measurements were taken after four days. In the control treatment, a sterile agar disc (0.5 mm diam.) was placed instead of *Trichoderma* isolates. There were three replicates for each treatment. At the end of incubation period, redial growth was measured. Types of interactions in dual culture were studied seventh day after inoculation. After both the fungi came in contact with each other, the contact/inhibition zone mounted under lectophenol-cotton-blue over a clean glass slide and observations were made under optical microscope. The reduction in mycelial growth was recorded and the percentage of inhibition over control for each treatment was calculated in this dual plate culture test as given in Table 1. The efficacy of *Trichoderma* spp. in suppressing redial growth was calculated as follows:

% Reduction in growth = (X-Y) / (X) × 100

X= Growth of pathogen alone without antagonist (control)

Y= Growth of pathogen along with the antagonist

RESULTS AND DISCUSSION

Isolation and identification of *Trichoderma* isolates: A total of 24 isolates, in which 8 strains of *T. harzianum*, 9 strains of *T. asperellum*, 2 strains of *T. virens,* 2 strains of *Hypocrea jecorina,* 1 strain of *T. hamatum,* 1 strain of *T. aureoviride* and 1 strain of *H. rufa* isolated from various soil samples which were collected from different places and crops like- potato, tomato and cabbage. The rDNA-ITS-PCR amplified nucleotide sequences of these *Trichoderma* isolates have been deposited in the GenBank database under accession numbers KC 312632.1 to KC 312652.1 (Table 1). Colony morphology of all the isolates was more or less similar showing sparse to thin colony mycelial mass with whitish border in some cases. Sporulation started after 48h of incubation at 27±2°C for all the isolates. Out of 24 isolates, 11 produced dark green colour colonies, eight yellowish green and the remaining five light green. Eight isolates produced defuse mycelial growth, while the rest were of cottony

white type. Reverse side colour was yellow green in 10 isolates, light drab in 4 and no pigmentation was found in 10 isolates (Table 2). The same findings like pale or yellowish colour of reverse of colonies, rapid growth of *Trichoderma* isolates were recorded by several workers (Samuels *et al.,* 2002; Sharma and Singh, 2014). Morphological characterization was conventionally used in the identification of *Trichoderma* sp., and it remains as a potential method to identify *Trichoderma* sp. (Gams and Bissett, 2002; Anees *et al.,* 2010).

***In vitro* antagonism test:** In the present study 19 out of 24 *Trichoderma* spp., isolated from different geographical region were selected for screening against fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici.*

Mycelial growth inhibition of *F. oxysporum* f. sp. *Lycopersici:* Differential action of the biocontrol agents was noticed on mycelial growth of the *F. oxysporum*. A reduction in the growth of *F. oxysporum* was evidenced when it was paired with antagonists. Among all the treatments, fast mycelial growth (7.5 cm) was recorded from the isolate- SVPUTh91 of *T. harzianum* followed by isolate SVPUTh10 (7.3 cm), SVPUTh38 (7 cm), SVPUTh47, SVPUTh36 (6.9 cm) and SVPUTh82, SVPUTh94, SVPUTh98 (6.7 cm) (Fig. 1). The results indicate that among the nineteen species of *Trichoderma* tested for their *in vitro* antagonism, the *T. harzianum* (SVPUTh91 and SVPUTh10) was consistently found to be the most effective with 83% and 82 %, respectively, reduction in radial growth of pathogen over control and was significantly superior to all other isolates. *T. harzianum* (SVPUTh38) is the next best and it reduced the growth of pathogen to 80% followed by *T. harzianum* (SVPUTh54 and SVPUTh74). *T. asperellum* isolates (SVPUAsp47, SVPUAsp58) showed 70% reduction in mycelial growth of pathogen. *T. virens* (SVPUTv56 and SVPUTv82) was the least effective with 65% reduction to the test pathogen (Table 3). The present finding was supported by several workers (Choudary *et al.,* 2007; Ramezani, 2010). Joshi *et al.,* (2010) found the antagonistic variability in different isolates of *Trichoderma* spp. collected from different places of India. Siameto *et al.,* (2011) also revealed that sixteen isolates of *T. harzianum* collected from different lands were found highly antagonist against five test fungus (*Rhizoctonia solani, Pythium spp., Fusarium graminearum, F. oxysporum* f. sp. *phaseoli* and *F. oxysporum* f. sp. *lycopersici*).

Mycoparasitism assays: A second mechanism of pathogen control was mycoparasitism. In dual cultures of *F. oxysporum* and the antagonists several morphological changes were seen when inhibition zone were analyzed under optical microscope. Microscopic observation of the interaction region between *F. oxysporum* with *T. harzianum* showed that *T. harzianum* hyphae coiled around those of *F. oxysporum*. Lysis of hyphae of *F. oxysporum* with close contact of *T. harzianum* hyphae was observed. Penetration of *Trichoderma* hyphae of strain SVPUTh91 was observed in this study (Fig. 2). All the observations were compared

Table 1. Morphological and Molecular Identified isolates of *Trichoderma* spp. used in this study.

Strain	Crop	Morphological identification[a]	Genbank accession no. (ITS Sequence)	Molecular identification	Definitive identification
SVPUTh10	POTATO	*H. lixii*	KC 312632	*H. lixii*	*H. lixii*
SVPUTh38	POTATO	*H. lixii*	KC 312633	*H. lixii*	*H. lixii*
SVPUTh50	POTATO	*T. harzianum*	KC 312634	*T. harzianum*	*T. harzianum*
SVPUTh94	POTATO	*H. lixii*	KC 312639	*H. lixii*	*H. lixii*
SVPUTh77	POTATO	*H. lixii*	KC 312640	*H. lixii*	*H. lixii*
SVPUTh91	POTATO	*H. lixii*	KC 312644	*H. lixii*	*H. lixii*
SVPUTh96	POTATO	*H. lixii*	KC 312645	*H. lixii*	*H. lixii*
SVPUTh98	POTATO	*H. lixii*	KC 312646	*H. lixii*	*H. lixii*
SVPUAsp47	POTATO	*T. asperellum*	KC 312631	*T. asperellum*	*T. asperellum*
SVPUAsp32	POTATO	*T. asperellum*	KC 312635	*T. asperellum*	*T. asperellum*
SVPUAsp51	POTATO	*T. asperellum*	KC 312638	*T. asperellum*	*T. asperellum*
SVPUAsp55	POTATO	*T. asperellum*	KC 312641	*T. asperellum*	*T. asperellum*
SVPUAsp58	POTATO	*T. asperellum*	KC 312643	*T. asperellum*	*T. asperellum*
SVPUAsp82	POTATO	*T. asperellum*	KC 312647	*T. asperellum*	*T. asperellum*
SVPUAsp93	POTATO	*T. asperellum*	KC 312649	*T. asperellum*	*T. asperellum*
SVPUAsp94	TOMATO	*T. asperellum*	KC 312650	*T. asperellum*	*T. asperellum*
SVPUAsp99	POTATO	*T. hamatum*	KC 312636	*T. hamatum*	*T. hamatum*
SVPUAur57	POTATO	*T. aureoviride*	KC 312637	*T. aureoviride*	*T. aureoviride*
SVPUJec45	POTATO	NI	KC 312642	*H. jecorina*	*H. jecorina*
SVPUJec27	POTATO	NI	KC 312651	*H. jecorina*	*H. jecorina*
SVPUHam54	POTATO	*T. hamatum*	KC 312636	*T. hamatum*	*T. hamatum*
SVPUTv56	POTATO	*H. virens*	KC 312648	*H. virens*	*H. virens*
SVPUTv82	CABBAGE	*H. virens*	KC 312653	*H. virens*	*H. virens*
SVPUTr22	CABBAGE	NI	KC 312652	*H. rufa*	*H. rufa*

a NI: not identified

Table 2. Colony diameter and morphological characteristics of *Trichoderma* isolates.

Trichoderma isolates	Diameter (cm) at 96 h	Morphological characteristics of *Trichoderma* isolates at various duration		
		After 36 h	After 60 h	After 90 h
SVPUTh38 SVPUTh50	4.5	Normal off white growth appears inoculum, very thin mycelium surround the inoculum	Compact fluffy light green sporulation on older region	Light green away from inoculums inner circle sparse and outer circle with dense growth, encircled dense with fluffy mycelium
SVPUAsp47 SVPUAsp58 SVPUTr22	3.0	Normal trasulant white defused mycelial growth on inoculums	Around inoculum light yellow green mycelium growth after dark green sporulation	On inoculum yellow growth surrounded by dirty green growth 2 cm encircled by dark green mycelium
SVPUAur57 SVPUHam54	3.7	Cottony off white mycelial growth on inoculums	Around the inoculums very light green fluffy mycelium encircled by 2 cm dense light green sporulation	Around inoculum 5 cm dia white mycelial growth surrounded by whitish green mycelium 2 cm dia
SVPUAsp51 SVPUAsp55	4.5	Cottony white mycelial growth on the inoculums	Around inoculums dark green mycelium encircled by 2 cm light green sporulation	Around inoculums 3 cm dia dark green after 4 cm dia dense fluffy off white mycelium then dark green
SVPUAsp82 SVPUAsp93 SVPUAsp32	3.4	Cottony white mycelial growth on the inoculum sparse very thin mycelium	Sparse 4 cm mycelium growth, media become yellow around inoculum encircled with compact dark green sporulation	Around inoculums 5 cm dia white mycelial growth surrounded by sparse whitish green mycelium 1.5 cm encircled by dark green 0.5 cm dia.
SVPUTv56 SVPUTv82	3.9	Cottony white mycelial growth on the inoculums	On the inoculum white mycelial growth light fluffy green sporulation 4 cm	Around inoculum 4 cm dia white mycelium growth surrounded by light green bands encircled by off white mycelium
SVPUJec27 SVPUJec45	4.3	Off white mycelial growth on the inoculum very thin mycelium surround the inoculums	White mycelium on inoculums 2.7 cm after raised cottony off white growth 8.7 cm	Inoculum covered with snow white mycelium surrounded sparse growth later thick dirty green slightly fluffy raised 1.5 cm then light green
SVPUTh10 SVPUTh91 SVPUTh94	5.2	Normal watery trasulant off white defused mycelial growth around the inoculums	Off White mycelium on inoculums 2 cm after raised cottony light green growth 6.7cm	Inoculum covered with snow white mycelium surrounded sparse growth light green slightly fluffy raised 2.5 cm then light green
SVPUAsp94 SVPUAsp99	3.2	Sparse whitish thick growth	On inoculums white growth dense light sparse fluffy green sporulation 4.5 cm media becomes yellow around inoculums	Inoculum covered with yellow growth surrounded dirty green growth 2 cm, encircled with slightly raised growth
SVPUTh77 SVPUTh96 SVPUTh98	4.0	Yellow growth on inoculums surrounds sparse cottony white growth	Yellow growth on the inoculums sparse light green sporulation appears white dense growth at periphery media becomes yellow	Inoculums covered with pale yellow or dark brown growth surrounded dirty green growth 2.5 cm encircled with slightly raised growth

with the control plate study of *F. oxysporum*. All isolates of *T. harzinum* showed antibiosis and lysis mechanism of parasitism. The least growth inhibition efficacy showed by *T. virens* isolates. The *in vitro* culture of *F. oxysporum* and *T. harzianum* together led to a variety of interactions. *F. oxysporum* growth was generally inhibited, the hyphae lysed on dual culture media and hyphae were intensely parasitized by *T. harzianum*. Highest rapid growth (7.5 and 7.3 cm) of the antagonist isolate of *T. harzianum* (SVPUTh91 and SVPUTh10, respectively) compared with slow growth of the *F. oxysporum* isolate (1.5 cm) and showed the high growth inhibition efficiency 83 and 82% respectively, showed the lysis of the *Fusarium* mycelium, could have been caused by nutrient depletion, and therefore mycelial growth inhibition. The inhibition could also be caused by production of toxic substances like enzymes, metabolites, antibiotics, volatile and non -volatile substances, released by the antagonist, as has been shown in other studies with phytopathogenic fungi (Askew and Laing, 1994; Kay and Steward, 1994). The pathogen mycelia then disintegrated suggesting enzyme action. Metcalf and Wilson (2001) demonstrated possible role of chitinolytic and/or glucanases

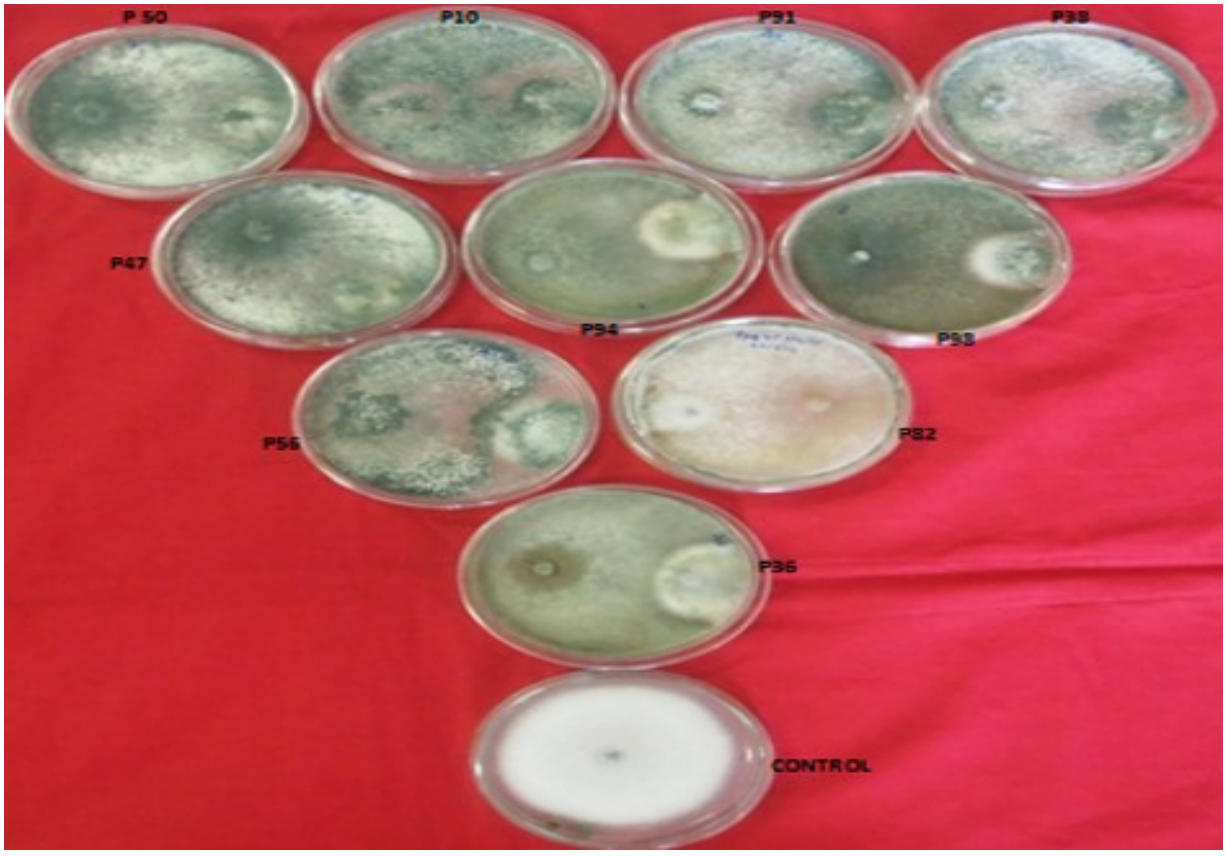

Fig. 1. *Antagonism between Trichoderma isolates and Fusarium oxysporum f. sp. lycopersici at 7 Days after inoculation. Each Petridishes containing Fusarium oxysporum (pathogen) at the right and Trichoderma spp. (antagonists) at the left side. Last Petriplate containing Fusarium colony only (control).*

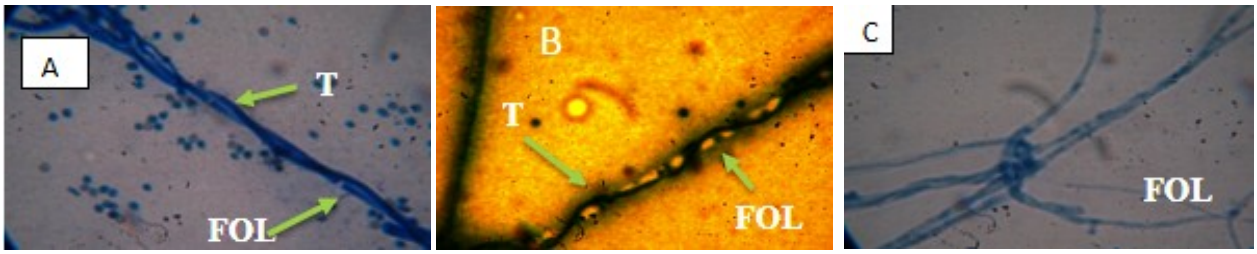

Fig. 2. *Trichoderma harzianum undergoes with some mechanisms inhibiting the mycelial growth of Fusarium oxysporum. (A) T. harzianum (T) mycelium coiling around Fusarium oxysporum f. sp. lycopersici (FOL) mycelium, (B) Lysis of hyphae of Fusarium oxysporum, (C) Fusarium oxysporum mycelium (control culture).*

enzymes in biocontrol by *Trichoderma*. Ojha and Chatterjee (2011) recorded *T. harzianum* to be the best in rank with *T. viride* and *T. harzianum* towards their antagonist activity against *F. oxysporum* and production of lytic enzymes, glucanase and chitinase. These enzymes function by breaking down the polysaccharides, chitin, and fungal cell wall, thereby destroying cell wall integrity limiting the growth of the pathogen (Siameto *et al.,* 2011).

Antibiosis/ lysis was another mechanism used by *Trichoderma* to control pathogen. Inhibition by antibiotic have been documented by Claydon *et al.* (1987), Dubey and Suresh (2006), Kucuk and Kivanc (2003) and Lynch (1990). Claydon *et al.* (1987) reported inhibition due to antibiotics trichodermin, harzianum A and harzianolide. Dubey and Suresh (2006) reported that non-volatile substances produced by *T. harzianum* are inhibitory to *F. oxysporum* f. sp. *ciceris* causing chickpea wilt.

Conclusion

Biological control is a promising tool to maintain current level of agriculture production while reducing the release of polluting chemical pesticides to the environment.. In this study, the mycoparasitism inhibitory effects of 19 *Trichoderma* isolates belongs to three species (*T. harzianum, T. virens* and *T. asperellum*) on the growth of *Fusarium oxysporum* f. sp. *lycopersici* were investigated. The maximum and minimum inhibitory effect was caused by *T.*

Table 3. Growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* in dual culture by different isolates of *Trichoderma* spp.

***Trichoderma* isolates**	**Growth inhibition efficiency of *Trichoderma* spp. in dual culture**						**Type of Inhibition**
	5 days after inoculation		**6 days after inoculation**		**7 days after inoculation**		
	Linear Growth (cm)	**Growth Inhibition (%)**	**Linear Growth (cm)**	**Growth Inhibition (%)**	**Linear Growth (cm)**	**Growth Inhibition (%)**	
SVPUTh38	2.33	43.5	2.00	70.0	1.76	80.4	Antibiosis
SVPUTh50	2.76	33.0	2.36	64.5	1.96	82.2	Lysis
SVPUTh54	2.26	45.1	2.03	69.5	1.80	80.0	Antibiosis
SVPUTh74	2.43	41.1	2.13	68.0	1.80	80.0	Antibiosis
SVPUTh91	2.66	35.5	2.43	63.5	1.60	83.5	Lysis
SVPUTh94	2.66	35.5	2.36	64.5	1.50	82.2	Antibiosis
SVPUTh10	2.63	36.2	2.33	65.0	1.56	82.6	Lysis
SVPUTh98	2.66	35.5	2.40	64.0	1.83	79.6	Antibiosis
SVPUAsp47	3.03	26.6	2.86	57.0	2.63	70.7	Mycoparasitism
SVPUAsp32	2.83	31.4	2.66	60.0	2.36	73.7	Mycoparasitism
SVPUAsp51	2.63	36.3	2.53	62.0	2.16	76.6	Mycoparasitism
SVPUAsp55	3.00	27.4	2.70	59.9	2.36	76.3	Antibiosis
SVPUAsp58	3.00	27.4	2.46	62.9	2.10	71.5	Mycoparasitism
SVPUAsp82	2.66	35.5	2.46	62.9	2.13	76.3	Antibiosis
SVPUAsp93	3.10	25.0	2.83	57.5	2.56	73.7	Mycoparasitism
SVPUAsp94	2.70	34.7	2.40	64.0	2.13	76.3	Antibiosis
SVPUAsp99	3.26	20.9	2.63	60.5	2.36	73.7	Mycoparasitism
SVPUTv56	3.66	11.2	3.50	47.5	3.13	65.0	Mycoparasitism
SVPUTv82	2.93	29.1	3.03	54.5	3.03	66.3	Mycoparasitism
CONTROL	4.13		6.66		9.00		
C.D.	0.20		0.19		0.176		
C.V.	4.31		4.49		4.554		

harzianum and *T. virens,* respectively. It was concluded that a novel strain of *T. harzianum* (SVPUTh91) was a good candidate for biological control compare to other *Trichoderma* spp. due to the different modes of action the fungus employs in inhibiting the growth of other fungi, and indicates this isolate of *T. harzianum* can be used for production and development of *T. harzianum* based biocontrol formulation to control *Fusarium* wilt diseases of plants and serve as a model for environment friendly biocontrol agent.

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