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Biocontrol of *Fusarium* Wilt in Tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*

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ABSTRACT

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Efficacy of biocontrol agents and organic amendments was evaluated for their potential to manage the *Fusarium* wilt of tomato (*Lycopersicon esculentum* L.) caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Yeast, *Trichoderma viride*, *T. harzianum* and *Pseudomonas* spp. were collected from tomato growing areas of Tamil Nadu, India, and tested for antagonistic activity against the pathogen using a dual culture technique in Petri dishes. Yeast 1 was best in inhibiting mycelial growth of FOL (69.59%), followed by *Trichoderma viride* 1 which inhibited mycelial growth by 68.50%. Among oil cakes and plant oil extracts tested, neem cake extract (5%) and neem oil (3%) reduced growth of FOL. The effective antagonists and organic amendments screened *in vitro* were confirmed in pot culture. In pot culture soil application of Yeast 1 @ 2.5 kg ha⁻¹ was the most effective. Combinations screened in laboratory and pot culture conditions were tested against FOL under field conditions. The field experiment confirmed that Yeast 1 SA @ 2.5 kg ha⁻¹ provided the best disease reduction over control and increased fruit yield.

Introduction

Tomato (*Lycopersicon esculentum* L.) suffers significant losses in greenhouse and field production due to Tomato Wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) (Borrero *et al.*, 2004; Nusret Ozbay and Steven, 2004; Kirankumar *et al.*, 2008). Di Pietro *et al.*, (2003) reported that FOL is identified based mainly on morphology of sexual and asexual spores and spore bearing structures. Rozlianah and Sariah (2010) differentiated twenty-two isolates of *Fusarium* from tomato based on cultural and morphological characteristics.

Agricultural producers have become dependent on use of agrochemicals as a reliable method of crop protection. However, increased use of chemical inputs can cause development of pathogen resistance to the applied agents and can detrimentally affect the environment. Alternative treatments for control of plant diseases are needed. The use of microorganisms to control plant pathogens is a method of biological control. It is accepted as an alternative, or a supplemental way, to reduce use of chemicals against plant diseases (Compant *et al.*, 2005). Biocontrol preparations of fungi, bacteria, and yeast have been applied to seed, seedlings and planting

media to reduce tomato wilt disease under greenhouse and field condition with various degrees of success (Sabuquillo *et al.*, 2006). Yeast specie of *Saccharomyces cerevisiae* have been used as a biocontrol agent against soil-borne fungal plant pathogens *F. solani* and *Rhizoctonia solani* causing root-rot disease (Shalaby and El-Nady, 2008). The plant growth promoting yeasts, *S. cerevisiae*, *Candida sake* and *Pichia membranifaciens*, used as biocontrol agents, were effective against *Fusarium* wilt of tomato under greenhouse conditions (Kamal *et al.*, 2009). Dual inoculation of *Trichoderma viride* and FOL to tomato plants increased DHA activity and microbial flora in the rhizosphere than use of individual organisms (Morsy and Ebtsam, 2005; Zaghloul *et al.*, 2007). A *Pseudomonas fluorescens* strain, possessing multiple mechanisms of broad spectrum antagonism and PGP activities, can be used as a biocontrol agent against Solanaceous phytopathogens. Zaidi and Dar (2002) reported that neem oil cake and neem leaves, as soil amendments, were effective against *Fusarium* spp. in okra.

Materials and Methods

Isolation of pathogen

The FOL was isolated from wilted tomato plants and maintained in pure culture on Potato Dextrose Agar (PDA) (Chakraborty and Chatterjee, 2007). Infected portions of diseased plants were cut into small pieces using a sterilized scalpel and then surface sterilized with 0.1% mercuric chloride for one min, washed three times in sterile distilled water, and placed on solidified PDA in Petri dishes. The plates were incubated at room temperature (28+2°C) for five days. Fungal hyphal tips were transferred aseptically to PDA slants for maintenance of the culture. The fungi were identified based on cultural and morphological characters.

Isolation of antagonists from the rhizosphere region

Antagonistic fungi and bacteria were isolated from the rhizosphere soil collected from tomato growing areas of Tamil Nadu, India. Plants were gently removed from the soil with intact roots and soil adhering to roots was removed gently. Ten-g of rhizosphere soil was transferred to 250 ml Erlenmeyer flasks containing 100 ml of sterile distilled water. After a thorough shaking, the organisms in the suspension were isolated by serial dilution. From the 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} dilutions, one-ml aliquots were removed by pipette and placed separately in sterilized Petri dishes containing *Trichoderma* special medium (TSM), King's B medium (King *et al.*, 1954) or nutrient agar medium (Allen, 1953) and gently rotated clockwise and counterclockwise for uniform distribution and incubated at room temperature (28+2°C) for 24 hrs. Colonies with characteristics of *Bacillus* spp. or *Pseudomonas* spp. were isolated individually and purified with the streak plate method (Rangaswami, 1993) on nutrient agar medium and King's B medium. *Trichoderma* spp. was isolated from TSM medium and purified on PDA. Pure cultures were maintained on respective agar slants at 4°C.

Isolation of yeast antagonists from the rhizosphere

Antagonistic yeast fungi were isolated from the rhizosphere soil (Azeredo *et al.*, 1998) using serial dilution in saline solution (NaCl 0.85%) and plating in the (YEPD) culture media (1% yeast extract, 2% peptone, 2% glucose, 2% agar, 0.01% ampicilin, 0.01% nalidixic acid). Inoculated plates were incubated at 25°C for 3-7 days and colonies of yeast were identified by cell characteristics and isolated and purified in YEPD medium. Colonies were maintained in agar slants at 4°C.

In vitro* screening of fungal and bacterial antagonists against *Fusarium oxysporum* f. sp. *lycopersici

Two isolates of *T. viride* and *T. harzianum* were screened against FOL. *Trichoderma* spp. were placed opposite of FOL near the periphery of the Petri plate and incubated at room temperature (28±2°C). After four days mycelial growth of the pathogen and the size of the inhibition zone measured in treated and control plates. Percent inhibition (PI) of mycelia growth was calculated using the formula of Pandey *et al.*, (2000). Overgrowth and zones of inhibition of antagonists over the pathogen was measured seven days after incubation.

The bacterial isolates were tested for their inhibitory effect on growth of FOL using a dual culture technique (Dennis and Webster, 1971). Bacterial isolates were streaked on one side of the Petri dish (1 cm from the edge of the plate) on PDA medium and a mycelial disc (8 mm dia) of five-day-old FOL was placed on the opposite side of the Petri dish perpendicular to the bacterial streak. The plates were incubated at room temperature (28±2°C) for 4 days and pathogen growth and inhibition zones measured (Table 1).

Efficacy of oil cake extracts against *Fusarium oxysporum* f. sp. *lycopersici* in *in vitro*

Preparation of aqueous extracts from oil cakes

One-g quantities of each oil cake was made into powder, soaked in 1.25 ml of sterile distilled water and kept overnight. The material was ground using a pestle and mortar and filtered through muslin cloth and the filtrate centrifuged at 10,000 rpm for 15 min. The supernatant served as the standard extract solution (100%) (Dubey and Patel, 2000).

Antifungal activity of oilcake extracts against *Fusarium oxysporum* f. sp. *lycopersici*

The efficacy of oil cake extract was tested against FOL using the technique of Schmitz (1930). Fifty-ml of freshly prepared PDA was placed in conical flasks. Aqueous extracts of oil cake (5 ml) was mixed with the PDA medium to obtain a 5% concentration and sterilized.

The sterilized PDA medium (15 ml/Petri dish) was poured in sterile Petri dishes and allowed to solidify. A nine mm mycelial disc of FOL was taken from an actively growing culture, placed at the centre of each Petri dish and incubated at room temperature.

The PDA medium without oil cake extract served as control. Radial growth of FOL was recorded after seven days of incubation (Table 2).

Antifungal activity of plant oils against *Fusarium oxysporum* f. sp. *lycopersici* in *in vitro*

The efficacy of plant oils was tested against FOL using the technique (Schmitz, 1930). Thirty-ml of freshly prepared PDA was placed in conical flasks.

The plant oils (3 ml) was mixed with the 30 ml of PDA medium to obtain a 3% concentration and sterilized. The sterilized PDA medium (15 ml/Petri dish) was poured in sterile Petri dishes and allowed to solidify.

A nine mm mycelial disc of FOL was taken from an actively growing culture and placed at the centre of each Petri dish and incubated at room temperature. The PDA medium without plant oils served as the control. Radial growth of FOL was recorded after seven days of incubation (Table 3).

Efficacy of biocontrol agents, organic amendments and chemicals against wilt incidence of tomato in pot culture

The biocontrol potential of *Trichoderma* spp., *Pseudomonas* spp. and yeast was studied in pot culture conducted in a greenhouse. Talc based formulation of the antagonistic bacteria and fungi were delivered as soil applications at 30 and 60 days after sowing. The FOL multiplied on sand maize medium and incorporated in the pots at 5% (w/w).

The treatments were: T1 = Yeast 1 talc based SA @ 2.5 kg·ha⁻¹; T2 = Yeast 2 talc based SA @ 2.5 kg·ha⁻¹; T3 = *T. viride*1 talc based SA @ 2.5 kg·ha⁻¹; T4 = *T. harzianum*1 talc based SA @ 2.5 kg·ha⁻¹; T5 = Yeast 3 talc based SA @ 2.5 kg·ha⁻¹; T6 = Yeast 4 talc based SA @ 2.5 kg·ha⁻¹; T7 = *P. fluorescens*1 talc based SA @ 2.5 kg·ha⁻¹; T8 = *P. fluorescens*2 talc based SA @ 2.5 kg·ha⁻¹; T9 = Neem cake @ 150 kg·ha⁻¹ SA; T10 = Mahuva cake @ 150 kg·ha⁻¹ SA; T11 = Gingelly cake @ 150 kg·ha⁻¹ SA; T12 = 0.1% carbendazim as a soil drench, and T12 = Untreated control. Percent wilt disease incidence were determined. Each treatment was replicated three times (Table 4).

Effect of biocontrol agents, organic amendments and chemicals on wilt incidence and yield of tomato in field condition

A field experiment was conducted during 2011-2012 to examine management practices against tomato wilt disease. Effective treatments tested under pot culture were evaluated in the field. Seedling of tomato dvs. PKM 1 and PKM 2 were used. The experiment was conducted in a Completely Randomized Block Design replicated three times. After leveling the soil, composted materials and fertilizers were applied at recommended rates (Horticulture Crop Production Guide, 2008) and seedlings planted

in rows with 45 × 15 cm spacing and later thinned. Plants were irrigated after planting. Irrigation occurred again three days after planting and thereafter plots were irrigated at weekly intervals. Observations on disease incidence and yield were made from 10 to 85 DAS (Table 5).

Results and Discussion

Among the isolates of Yeast screened for antifungal activity against FOL, Yeast 1 had the most reduction of mycelial growth and largest inhibition zone inhibition zone followed by *T. viride*. El-Mehalawy (2004) found that the two species of rhizosphere yeast fungi *S. unispora* and *Candida steatolytica* have antagonistic and inhibitory effects on growth of *F. oxysporum* of kidney bean Soyong *et al.*, (2005) reported that *Trichoderma* spp. control FOL. Among the *Trichoderma* spp., *T. viride* showed the best performance *in vitro* for control of FOL followed by *T. harzianum* (Sahi and Khalid, 2007).

Neem cake had the most reduction of mycelial growth over the control followed by Mahuva cake. The least reduction was in the vermicomposting extracts. The neem oil had the most reduction of mycelial growth over control followed by mahuva oil. The least reduction was for peanut oil. The highest inhibition of FOL growth was recorded in neem cake followed by Mahuva cake (Padmodaya and Reddy, 1999).

Paul and Sharma (2002) reported the aqueous extracts of neem inhibited growth of the soil-borne fungi. *F. moniliforme*, *Macrophomina phaseolina* and *Rhizoctonia solani*. Dry neem seed extract completely inhibited mycelial growth of *F. oxysporum* (Agbenin and Marley, 2006). Thiruvudainambi *et al.*, (2010) used neem cake and talc formulations of the bioagent to controlled *F. oxysporum*.

Table.1 Effect of different isolates of biocontrol agents against *Fusarium oxysporum* f. sp. *lycopersici* *in vitro*

S. No	Treatments	Mycelial growth(cm)**	Per cent reduction over Control	Inhibition zone (mm)
1.	<i>Trichoderma viride</i> (Tv1)	2.80	68.50	1.32
2.	<i>Trichoderma harzianum</i> (Th1)	3.18	64.22	1.96
3.	Yeast 1	2.73	69.59	1.28
4.	Yeast 2	2.98	66.66	1.23
5.	Yeast 3	3.45	61.19	1.10
6.	Yeast 4	3.52	60.40	0.96
7.	<i>Pseudomonas fluorescens</i> (Pf1)	4.95	44.38	0.85
8.	<i>Pseudomonas fluorescens</i> (Pf2)	4.74	46.74	0.92
9.	<i>Bacillus subtilis</i> (Bs1)	5.43	39.32	0.69
10.	<i>Bacillus subtilis</i> (Bs2)	5.56	37.87	0.77
11.	Control	8.89	-	-
CD(P=0.05)		0.21		

* Mean of five replications

Table.2 *In vitro* efficacy of different oil cakes on the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici*

S. No.	Treatments	Mycelial growth (cm)* 7DAI**	Per cent reduction over control
1	Neem cake (5%)	3.16	64.04
2	Mahua cake (5%)	4.21	55.05
3	Gingelly cake (5%)	4.25	52.80
4	Castor cake (5%)	4.38	50.56
5	FYM (5%)	4.43	49.43
6	Cotton seed cake (5%)	5.06	42.69
7	Coconut (5%)	5.13	41.57
8	Vermicompost (5%)	5.43	38.20
9	Control	8.92	-
CD (P=0.05)		0.19	-

* Mean of three replications

** DAI – Days after inoculation

Table.3 *In vitro* efficacy of different plant oils on the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici*

S. No.	Treatments	Mycelial growth (cm)* 7 DAI**	Per cent reduction over control
1	Neem oil (3%)	4.21	52.80
2	Mahua oil (3%)	5.26	41.57
3	Gingelly oil (3%)	5.84	34.83
4	Coconut oil (3%)	6.13	30.33
5.	Castor oil (3%)	6.42	28.08
6.	Groundnut oil (3%)	6.53	26.96
7.	Control	8.92	-
CD(P=0.05)		0.29	

*Mean of three replications; ** DAI – Days after inoculation

Table.4 Effect of biocontrol agents, organic amendments and chemical on wilt incidence of tomato plants in pot culture

T. No.	Treatments	*Disease incidence (%)						Mean Disease incidence (%)*	Per cent reduction over control (%)
		10 DAS	25 DAS	40 DAS	55 DAS	70 DAS	85 DAS		
T1	Yeast 1(Y1)SA @ 2.5 kg/ha	0.86 (5.26)	2.68 (9.60)	6.99 (15.74)	8.51 (15.67)	7.67 (16.26)	9.13 (16.74)	6.78	85.58
T2	Yeast 2 (Y2)SA @ 2.5 kg/ha	2.24 (8.58)	4.73 (12.47)	6.91 (15.27)	8.85 (17.74)	10.61 (19.03)	11.12 (19.46)	7.10	83.25
T3	<i>Trichoderma viride</i> (Tv1) SA @ 2.5 kg/ha	0.92 (5.73)	2.66 (9.06)	6.15 (15.91)	8.47 (16.16)	7.68 (15.87)	9.64 (17.63)	7.24	84.39
T4	<i>Trichoderma harzianum</i> (Th1) SA @ 2.5 kg/ha	2.50 (8.85)	10.10 (18.02)	12.37 (20.35)	14.28 (22.80)	11.37 (23.95)	17.15 (24.37)	9.46	79.89
T5	Yeast 3 (Y3) SA @ 2.5 kg/ha	2.36 (8.87)	6.59 (14.67)	10.19 (18.65)	13.15 (21.34)	15.33 (22.06)	20.39 (25.39)	11.02	76.57
T6	Yeast 4 (Y4) SA @ 2.5 kg/ha	2.30 (8.64)	6.26 (14.23)	14.62 (22.56)	18.78 (25.45)	19.92 (26.32)	28.25 (32.43)	14.04	69.03
T7	<i>Pseudomonas fluorescens</i> (Pf1) SA @ 2.5 kg/ha	2.33 (8.76)	6.47 (14.79)	9.55 (17.97)	12.34 (20.56)	14.7 (22.57)	16.49 (23.89)	10.31	78.08
T8	<i>Pseudomonas fluorescens</i> (Pf2) SA @ 2.5 kg/ha	2.20 (7.46)	6.46 (14.73)	9.4 (17.98)	12.29 (20.67)	14.57 (22.58)	16.29 (22.89)	10.20	78.32
T9	Neem cake @ 150 kg/ha	3.06 (10.06)	8.39 (16.84)	13.27 (21.26)	16.88 (24.28)	21.28 (27.54)	20.26 (16.59)	13.86	70.54
T10.	Mahuva cake @ 150 kg/ha	2.89 (9.79)	6.17 (15.07)	13.69 (21.72)	16.56 (24.12)	21.26 (27.48)	22.15 (28.25)	13.79	70.69
T11	Gingelly cake @ 150 kg/ha	3.02 (10.02)	8.46 (16.78)	14.59 (22.54_)	17.89 (24.87)	20.59 (27.32)	22.64 (28.67)	16.53	67.11
T12	Carbendazim soil drenching 0.1%	2.76 (19.78)	2.79 (9.21)	6.84 (15.03)	8.58 (17.05)	7.74 (16.18)	10.63 (18.86)	8.16	82.65
T13	Untreated Control	9.45 (11.92)	37.97 (42.56)	40.85 (43.32)	54.77 (46.68)	67.41 (54.78)	71.87 (57.34)	47.05	-

*Mean of three replications *Figures in the parentheses are arc sine transformed values

DAS= Days After Sowing

CD (P=0.05)

Treatments = 0.38

Days = 0.26

Treatments × Days = 1.24

Table.5 Effect of biocontrol agents, organic amendments, chemical and their combinations on wilt incidence of tomato plants in field condition

T. No.	Treatments	**Disease incidence (%)						Mean Disease incidence (%)*	Per cent reduction over control (%)	Plot yield (20 m ²) kg	Yield t/ha
		10 DAS	25 DAS	40 DAS	55 DAS	70 DAS	85 DAS				
T1	Yeast1(Y1)SA @ 2.5 kg/ha	3.45 (10.56)	8.30 (16.78)	9.47 (17.36)	11.65 (19.71)	13.45 (21.67)	14.18 (22.12)	8.88	80.55	73.00	36.5
T2	Yeast2 (Y2)SA @ 2.5 kg/ha	3.13 (10.56)	5.45 (13.92)	7.09 (14.23)	9.21 (18.64)	12.21 (21.26)	15.22 (23.12)	10.57	77.84	70.00	35.0
T3	<i>Trichoderma viride</i> (Tv1) SA @ 2.5kg/ha	2.39 (8.92)	2.55 (9.13)	6.97 (15.76)	9.42 (17.87)	8.29 (16.98)	9.82 (18.04)	9.42	79.06	71.00	35.5
T4	<i>Pseudomonas fluorescens</i> (Pfl) SA @ 2.5 kg/ha	3.37 (10.87)	8.25 (16.45)	9.08 (17.09)	10.86 (18.89)	11.35 (19.67)	12.12 (20.32)	10.05	77.42	65.00	32.5
T5	Neem cake @ 150 kg/ha	2.67 (9.52)	5.78 (14.45)	12.89 (20.75)	11.18 (19.06)	13.42 (21.43)	17.12 (24.87)	10.51	76.40	62.00	31.0
T6	Carbendazim soil drenching 0.1%	2.24 (8.47)	4.89 (12.38)	6.88 (15.56)	8.36 (16.87)	10.67 (18.79)	12.38 (20.47)	9.84	77.91	64.00	32.0
T7	T1 + T2 (1:1)	3.06 (10.09)	5.54 (13.78)	8.59 (16.94)	10.42 (18.72)	12.23 (20.05)	12.53 (20.56)	9.65	78.53	61.00	30.5
T8	T1 + T3 (1:1)	3.45 (10.67)	5.22 (13.37)	8.07 (16.09)	10.57 (19.06)	13.71 (21.94)	13.36 (21.52)	9.72	78.18	58.00	29.0
T9	T1 + T4 (1:1)	3.66 (10.76)	7.70 (15.89)	8.49 (16.96)	12.13 (20.05)	10.58 (18.93)	24.75 (30.03)	11.22	74.81	55.00	27.5
T10	T2 + T3 (1:1)	3.76 (11.28)	8.64 (17.28)	14.52 (22.21)	16.73 (23.43)	18.18 (25.37)	22.62 (28.46)	12.08	72.88	53.00	26.5
T11	T2 + T4 (1:1)	3.42 (10.46)	8.70 (17.06)	18.23 (25.48)	21.12 (27.23)	23.62 (29.25)	25.76 (30.29)	14.81	66.75	50.00	25.0
T12	T1 + T2 + T3 + T4 (1:1:1:1)	2.24 (8.86)	4.21 (12.24)	6.83 (14.75)	8.56 (17.04)	14.21 (16.78)	15.45 (19.97)	9.16	79.47	64.00	34.0
T13	Untreated Control	8.73 (17.28)	22.12 (28.36)	46.75 (40.67)	54.86 (47.89)	67.73 (54.78)	78.13 (63.39)	44.55	-	-	-

*Mean of three replications

* Figures in the parentheses are arc sine transformed values

**DAS = Days After Sowing

CD (P=0.05)

Treatments = 0.21

Days = 0.14

Treatments × Days = 0.51

Among the treatments tested, Yeast 1 SA @ 2.5 kg·ha⁻¹ caused less percent disease incidence an 85.58% disease reduction followed by *T. viride* SA @ 2.5 kg·ha⁻¹, a reduction of 84.39%; soil drenching with carbendazim 0.1% produced an 82.65%

reduction of the disease. The plant growth promoting yeasts, *S. cerevisiae*, *C. sake* and *P. membranifaciens*, as biocontrol agents, were effective against *Fusarium* wilt of tomato disease under greenhouse conditions (Kamal *et al.*, 2009). Hashem (2009)

confirmed that biological methods can be used to control FOL under greenhouse conditions. The *Trichoderma* spp. used alone, protected tomato seedlings against *Fusarium* wilt. Plants treated one week before inoculation with the pathogen appeared healthy and with no wilting symptoms in pots (Ali *et al.*, 2009).

Tomato root disease incidence was most reduced by application of Yeast 1 SA @ 2.5 kg·ha⁻¹ at 85 DAS followed by combinations of Yeast1 SA @ 2.5 kg·ha⁻¹ + Yeast 2 SA @ 2.5 kg·ha⁻¹ + *T. viride* SA @ 2.5 kg·ha⁻¹ + *P. fluorescens* SA @ 2.5 kg·ha⁻¹ at 85 DAS. Untreated controls had the least at 85 DAS. Percent mean disease incidence and percent reduction over control was greatest with Yeast 1 followed by combination Yeast 1 + Yeast 2+ *T. viride* 1 + *P. fluorescens* 1. Bastasa and Baliad (2005) reported that *Trichoderma* and yeast isolates were the most antagonistic against *F. oxysporum* f. sp. *cubense*. Control of the disease provided by *T. viride* and Yeast 1 was equivalent to 81.76 and 82.82%, respectively. Shalaby and El-Nady (2008) reported *S. cerevisiae* used as biocontrol agent against soil-borne fungal plant pathogens causing root-rot disease by *F. solani* and *R. solani*. Dry yeast combined with *T. viride* inoculated potato plants increased yield compared to controls and reduced disease incidence and severity under field conditions (Andera *et al.*, 2008). Kamal *et al.*, (2009) reported that strains of *S. cerevisiae*, *P. branifaciens* and *C. sake* reduced disease severity and increased tomato yield relative to control infected with FOL under field conditions and increased yield.

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