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Evaluation of Combined Efficacy of *Pseudomonas fluorescens* and *Bacillus subtilis* in Managing Tomato Wilt Caused by *Fusarium oxysporum* f. sp. *lycopersici* (Fol)

S. Sundaramoorthy and P. Balabaskar
Department of Plant Pathology, Faculty of Agriculture, Annamalai University,
Chidambaram-608 002,Tamil Nadu, India

Abstract: The objective of this study was to evaluate the individual and combined efficacy of *Pseudomonas fluorescens* (EPI-5, KPI-7, ANR-2 and RTM-3) and *Bacillus subtilis* (KGI-4, PYR-3 and OCM-6) strains to promote the growth and yield parameters of tomato and to manage *Fusarium* wilt disease under *in vitro* and greenhouse conditions. The dominant pathogen which causes *Fusarium* wilt of tomato, was isolated and identified as *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Twenty five native bacterial antagonists were isolated from healthy tomato rhizosphere soil in different geographical regions. Under *in vitro* conditions, the results revealed that the combined application of EPI (Pf-5)+KGI(Bs-4)+KPI (Pf-7) was found to effectively inhibit the mycelial growth of the pathogen (by 40%) when compared to application of individual strains of the bacterial antagonists. The above strains of *P. fluorescens* and *B. subtilis* were found compatible. Under greenhouse conditions, the combined application of EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) exhibited the highest disease reduction. Also, tomato plants treated with EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) strains showed a significant stimulatory effect on plant height and increased the dry weight of tomato plants up to 27% in comparison to the non-bacterized control. The combined strains also increased tomato fruit weight. It could be concluded that synergistic consortia of beneficial bacteria isolated from rhizosphere soil are perfectly able to promote plant growth and could be exploited for sustainable management of soil borne diseases especially, *Fusarium* wilt of tomato.

Key words: Biological control, *Fusarium oxysporum* f. sp. *lycopersici*, tomato *Fusarium* wilt, *Pseudomonas fluorescens*, *Bacillus subtilis*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetables grown and consumed worldwide. Its popularity is due to its high nutritive value, diversified use and nutritional significance as a source of vitamins A and C. It occupies number one position in its nutrient contribution to human diet. Tomato is cultivating in an area of 22,433 ha, with a production of 2, 82, 912 tonnes and a productivity of 12.61 tonnes ha^{-1} in Tamil Nadu (Anonymous, 2007). The crop is known to be affected by a number of diseases among which wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen causes yield losses up to 50% (Lukyanenko, 1991). Wilt caused by species of *Fusarium* is one of the most serious disease problems of several agricultural, vegetable and fruit crops (Kloepper, 1991). The disease manifests as yellowing of the older leaves on one side of the stems close to fruit maturity. In the wilted plants, vascular tissue was dark brown and discoloration extended to the apex (Sheu and Wang, 2006). Presently, the management of tomato *Fusarium* wilt disease has been done with the application of chemical fungicides. However, it may not be sustainable in the longer run as chemical fungicides are known to cause residual toxicity, toxicity to non-target organisms and other environmental hazards and this has stimulated the search for biological options. Hence, recent attempts have been spotlighted on developing environmentally safe, long lasting and efficient management strategy for the management of plant diseases (Latha *et al*, 2009; Sundaramoorthy *et al*., 2012, 2013; Sundaramoorthy and Balabaskar, 2012).

Some priming strains of fluorescent Pseudomonads are also known as Plant Growth-promoting Rhizobacteria (PGPR) as they promote plant growth by secreting auxins, gibberellins and cytokinins (Dubeikovsky *et al*., 1993). Rhizobacterial strains of *Pseudomonas* and *Bacillus* spp. are also been used to reduce disease caused by a variety of soil borne pathogens (Larkin and Fravel, 1998). Several bacterial biocontrol agents including

Corresponding Author: S. Sundaramoorthy, Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Chidambaram-608 002, Tamil Nadu, India

Pseudomonas fluorescens (Migula), *Pseudomonas putida* (Trevisan) and *Bacillus subtilis* (Ehrenberg) have been demonstrated to have promising biocontrol against *F. oxysporum* f. sp. *lycopersici* (Monda, 2002). Recently, Sundaramoorthy *et al*. (2012, 2013) and Sundaramoorthy and Balabaskar (2012) reported that combination of *P. fluorescens* strain (Pf1) and *B. subtilis* strain (EPCO16) effectively inhibited the growth of *F. solani* and *Sarocladium oryzae* in chilli and rice plants, respectively. Therefore, the present study was designed to evaluate the combined efficacy of *Pseudomonas fluorescens* and *Bacillus subtilis* strains against *Fusarium* wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*.

MATERIALS AND METHODS

Isolation and purification of pathogens: Infected vascular tissues from stem and root regions of tomato cultivar (PKM 1) showing wilt symptoms were collected separately from farmer's field. Tissue bits were surface sterilized with 10% sodium hypochlorite for 5-10 min. and subsequently three washings with sterile distilled water. Then, they were placed on Potato Dextrose Agar (PDA) medium separately and incubated at the laboratory conditions at 25±3°C for five days (Fig. 1a). The fungi were purified separately by transferring the tip of the mycelia into PDA slants and maintained as stock cultures for further studies (Fig. 1b).

Isolation and maintenance of bacterial native antagonists from tomato rhizosphere soil: Rhizosphere soil from healthy tomato plants were collected from different locations. The identified bacterial antagonists, *viz*., *Pseudomonas* spp. and *Bacillus* spp. were isolated by serial dilution technique using King's B medium (King *et al*., 1954) and nutrient agar media (Allen, 1953), respectively. The bacterial antagonists were further purified on their respective media and compared with the isolates maintained in laboratory.

Compatibility among bacterial strains: The isolates of *Pseudomonas* and *Bacillus* were tested for their compatibility among each other following the method of Fukui *et al*. (1994). The compatibility was determined for *P. fluorescens* and B. *subtilis* strains using NA medium. The bacterial strains were streaked horizontally and vertically to each other. The plates were incubated at room temperature (28±2°C) for 72 h and observed for the inhibition zone. Absence of inhibition zone indicates the compatibility with respective bacterial strains and the presence of inhibition zone indicated the incompatibility.

***In vitro* effect of individual and combined bacterial native antagonists against FOL:** Dual culture technique described by Nandakumar *et al*. (2001, 2002) was adopted to study the effect of antagonists *Pseudomonas* spp. and *Bacillus* spp. against *F. oxysporum* f. sp. *lycopersici*. Nine mmillimeter disc of fifteen days old fungal

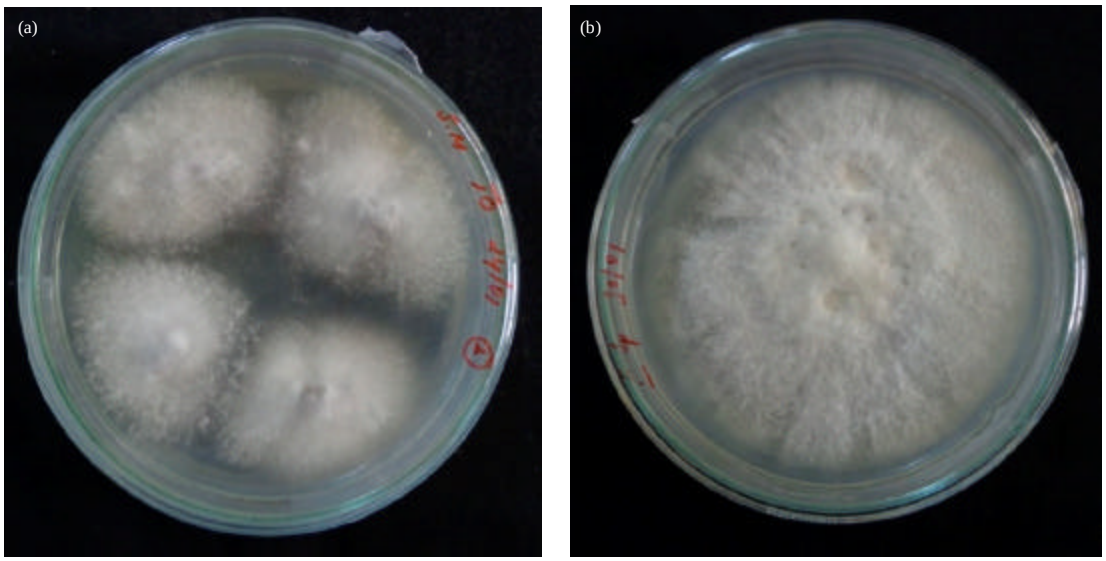

Fig. 1(a-b): Isolation and purification of *Fusarium oxysporum* f.sp. *lycopersici* (FOL) from wilt infected tomato tissue bits, (a) Isolation of FOL and (b) Axenic culture of FOL

cultures were placed on PDA medium one cm away from the edge of the plate, separately. Bacterial antagonists were streaked separately at opposite side of the Petri plate (Vidhyasekaran *et al*., 1997). Plates were incubated at 25±3°C for seven days. For each fungus and each antagonist three replicated plates were maintained. Per cent inhibition over control was calculated (Vincent, 1927) as per the equation:

$$PI = \frac{C - T}{C} \times 100$$

Where,

PI = Per cent inhibition over control
C = Growth of test pathogen with absence of antagonist (mm)
T = Growth of test pathogen with antagonist (mm)

Preparation of individual and mixture of PGPE and PGPR bioformulations: A loopful of *P. fluorescens* and *B. subtilis* were inoculated into the sterilized KB and nutrient broth, respectively and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28±2°C). After 48 h of incubation, the broth containing 9×108 cfu mL^{-1} was used for the preparation of talc-based formulation. To 400 mL of bacterial suspension, 1 kg of talc powder (sterilized at 105°C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and Carboxy Methyl Cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions, following the method described by Nandakumar *et al*. (2001). After shade drying overnight, it was packed in polypropylene bag and sealed. At the time of application the population of bacteria in talc formulation was not less than $2.5\text{-}3\times10^{8}$ cfu g^{-1}. For bacterial strain mixture, the bacterial strains were grown separately and the strains that are going to make up the mixture were added equally (v/v) and finally mixed with talc powder, $CaCO_3$ and CMC (Nandakumar *et al*., 2001).

Efficacy of bacterial native antagonistic mixture on the incidence of *Fusarium* wilts disease under greenhouse conditions: A pot culture study was conducted to test the antagonistic potential of native antagonists against *F. oxysporum f. sp. lycopersici*. Potting mixture (red soil: sand: decomposed FYM at 1:1:1 w/w/w) was prepared and autoclaved 1 hr for two consecutive days and filled in earthern pots of 5 kg capacity. Tomato seeds (cv. PKM1) were sown in autoclaved pot mixture in earthern pots. After 25 days, the seedlings were pulled out from the pots and dipped in their respective formulation for 2 h ensuring that the roots alone were immersed in the inoculum and then transplanted in pots at the rate of four seedlings per pot (5 kg capacity). Bacterial native strains EPI (Pf-5), KPI (Pf-7), KGI (Bs-4), EPI (Pf-5)+KGI (Bs-4), KGI (Bs-4) +KPI (Pf-7) and EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) were effective against *F. oxysporum f. sp. lycopersici in vitro* were selected. Soil application with the formulation was done 15 days and 30 days after transplantation. The *F. oxysporum f.sp. lycopersici* mass multiplied on sand maize medium was incorporated in to the pots at 5% cent (w/w). The observation on the per cent disease incidence was recorded at the time of harvest. Each treatment was replicated thrice in Randomized Block Design (RBD). The scheduled treatment details were as given below:

Trt. No.	Designation of PGPR strains	Treatment details
T1	EPI (Pf-5)	(Seedling dip @ 0.2 %+Soil application at 15 and 30 DAT @ 0.2%)
T2	KPI (Pf-7)	(Seedling dip @ 0.2%+Soil application at 15 and 30 DAT @ 0.2%)
T3	KGI (Bs-4)	(Seedling dip @ 0.2%+Soil drenching at 15 and 30 DAT @ 0.2%)
T4	EPI (Pf-5)+KGI (Bs-4)	(Seedling dip @ 0.2%+Soil application at 15 and 30 DAT @ 0.2%)
T5	KGI (Bs-4)+KPI (Pf-7)	(Seedling dip @ 0.2%+Soil application at 15 and 30 DAT @ 0.2 %)
T6	EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7)	(Seedling dip @ 0.2%+Soil application at 15 and 30 DAT @ 0.2%)
T7	Carbendazim (0.1%)	(Seedling dip+Soil application at 15 and 30 DAT)
T8	Healthy control	
T9	Inoculated control	

Statistical analysis: The data were statistically analyzed (Gomez and Gomez, 1984) and the treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for a nalysis was IRRI-Stat version 92-a developed by International Rice Research Institute Biometrics Units, The Philippines.

RESULTS

Compatibility among bacterial strains: Twenty five native bacterial antagonists were isolated from healthy tomato rhizosphere soil in different geographical regions (Table 1). *P. fluorescens* (EPI-5, KPI-7, ANR-2 and RTM-3) and *B. subtilis* (KGI-4, PYR-3 and OCM-6) strains were tested for their compatibility *in vitro*. None of the antagonistic bacteria inhibited each other, suggesting that these bacterial native antagonists were compatible with each other.

***In vitro* screening of bacterial native antagonists against the radial mycelial growth *F. oxysporum f. sp. lycopersici*:** Among the twenty five bacterial native antagonists, seven promising bacterial antagonistic *P. fluorescens* and *B. subtilis* strains (Table 2) were tested individually and in combination to assess the

Table 1: Bacterial native antagonists isolated from rhizosphere soil of tomato

Place of collection	Colony color	*PGPR
Edappadi	Light yellow	*Pseudomonas* sp.
Edappadi	Light yellow	*Pseudomonas* sp.
Edappadi	White	*Pseudomonas* sp.
Edappadi	White	*Bacillus* sp.
Edappadi	Brownish green	*Pseudomonas* sp.
Ramanathapuram	Green	*Pseudomonas* sp.
Ramanathapuram	Light brown	*Pseudomonas* sp.
Oddanchatram	White	*Bacillus* sp.
Oddanchatram	Brownish green	*Pseudomonas* sp.
Kovilpatti	Brownish green	*Pseudomonas* sp.
Kovilpatti	Light brown	*Bacillus* sp.
Palur	Whitish grey	*Bacillus* sp.
Palur	White	*Pseudomonas* sp.
Annamalai Nagar	Cream	*Pseudomonas* sp.
Annamalai Nagar	Brown	*Bacillus* sp.
Paiyur	Orange	*Pseudomonas* sp.
Paiyur	Brown	*Pseudomonas* sp.
Paiyur	White	*Bacillus* sp.
Thondamuthur	Brownish green	*Pseudomonas* sp.
Thondamuthur	Brownish green	*Pseudomonas* sp.
Krishnagiri	White	*Bacillus* sp.
Krishnagiri	Brownish green	*Pseudomonas* sp.
Pondicherry	White	*Pseudomonas* sp.
Pondicherry	White	*Pseudomonas* sp.
Pondicherry	Cream	*Pseudomonas* sp.

*PGPR-Plant growth promoting rhizobacteria

Table 2: Selected priming bacterial native antagonists isolates from tomato rhizosphere soil

Name of agro climatic zone	*PGPR Bacterial native antagonists	Designation of PGPR strains	Colony color
Edappadi	*Pseudomonas fluorescens*-5	EPI (Pf-5)	Brownish green
Ramanathapuram	*Pseudomonas fluorescens*-3	RTM (Pf-3)	Green
Oddanchatram	*Bacillus subtilis*-6	OCM (Bs-6)	White
Kovilpatti	*Pseudomonas fluorescens*-7	KPI (Pf-7)	Brownish green
Annamalai Nagar	*Pseudomonas fluorescensi*-2	ANR (Pf-2)	Cream
Paiyur	*Bacillus subtilis*-3	PYR (Bs-3)	White
Krishnagiri	*Bacillus subtilis*-4	KGI (Bs-4)	White

*PGPR- Plant growth promoting rhizobacteria

Table 3: Effect of bacterial native antagonists on the mycelia growth of *F. oxysporum* f. sp. *lycopersici*

Trt. No.	Treatments Bacterial native antagonists	*Mycelial growth	Inhibition zone (mm)	Percent Inhibition over control
T1	EPI (Pf-5)	58.00b^{cd}	13.10^{e}	35.55^{cd}
T2	KPI (Pf-7)	60.30^{cde}	11.50^{f}	33.00^{cde}
T3	KGI (Bs-4)	61.00^{de}	10.00^{g}	32.22^{de}
T4	ANR (Pf-2)	63.50^{ef}	8.70^{h}	29.44^{ef}
T5	PYR (Bs-3)	65.60^{f}	8.60^{h}	27.11^{f}
T6	RTM (Pf-3)	70.40^{g}	7.00^{i}	21.77^{g}
T7	OCM (Bs-6)	72.30^{g}	5.55^{j}	19.67^{g}
T8	EPI (Pf-5)+ KGI (Bs-4)	56.20^{bc}	15.20^{d}	37.55^{bc}
T9	KGI (Bs-4) +KPI (Pf-7)	54.10^{b}	19.50^{c}	39.89^{b}
T10	EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7)	54.00^{b}	23.50^{b}	40.00^{b}
T11	Carbendazim (0.2%)	49.00^{a}	26.00^{a}	45.55^{a}
T12	Control	90.00^{j}	0.00	0.00

*Mean of three replications; In a column, mean followed by a common letter are not significantly different at the 5% level by DMRT

radial growth of FOL. All the treatments were effective in reducing the mycelia growth of the pathogen. However, the combined application of EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) resulted in the least mycelia growth with 54.00 mm (Fig. 2). The control plates recorded the highest mycelia growth of 90.00 mm (Table 3).

Effectiveness of bacterial native antagonists on wilt incidence and yield parameters under glasshouse conditions: The application of bacterial native antagonists through seedling dip and soil application was found effective in suppressing wilt incidence (by 12.52-25.50%). Conspicuously, a combined application of EPI (Pf-5)+ KGI (Bs-4)+KPI (Pf-7) antagonistic bacterial formulation was recorded least wilt incidence (by 12.52%) followed by KGI (Bs-4)+KPI (Pf-7) by (15.33%) and EPI (Pf-5)+ KGI (Bs-4) by (17.45%) than any of the strains treated individually (Fig. 3b, Table 4). Among the treatments, Carbendazim (0.1%) was found to be the most effective and recorded the least wilt incidence of 10.26% compared to control (57.75%). Also, the results of this experiment revealed that the combined application of EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) antagonistic bacterial formulation significantly increased the plant height

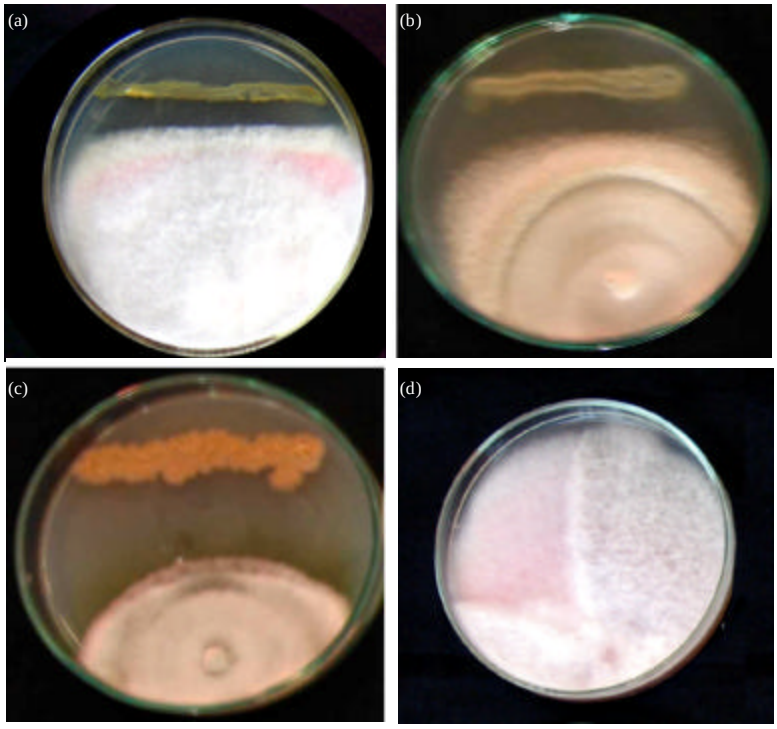

Fig. 2 (a-d): Combined efficacy of bacterial native antagonistic activity against wilt pathogen (FOL) under *in vitro* condition, (a) EPI (Pf-5)+KGI (Bs-4), (b) KGI (Bs-4)+KPI (Pf-7), (c) EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) and (d) Control

Table 4: Efficacy of bacterial native antagonistics against *fusarial* wilts of tomato (cv. PKM1) under greenhouse conditions

Trt. No.	Treatments	FOL[1]	*Percent disease incidence (PDI)	*Plant height (cm)	Dry weight (mg)	*Fruit yield g plant^{-1}
T1	EPI (Pf-5)	+	22.50^{d}	65.91^{e}	110	187.86^{e}
T2	KPI (Pf-7)	+	25.50^{e}	63.27^{f}	106	186.78^{e}
T3	KGI (Bs-4)	+	25.10^{e}	61.76^{g}	103	185.97^{e}
T4	EPI (Pf-5)+ KGI (Bs-4)	+	17.45^{c}	70.63^{c}	120*	228.43^{d}
T5	KGI (Bs-4)+ KPI (Pf-7)	+	15.33^{b}	71.25^{b}	124*	271.21^{b}
T6	EPI (Pf-5)+ KGI(Bs-4)+ KPI (Pf-7)	+	12.52^{b}	73.62^{a}	127*	288.38^{a}
T7	Carbendazim (0.1%)	+	10.26^{a}	67.00^{d}	106	235.48$^{c d}$
T8	Healthy control	-	25.80^{e}	60.98^{h}	100	164.86^{f}
T9	Inoculated control	+	57.75^{f}	57.73^{i}	101	120.68^{g}

[1]*Fusarium oxysporum* f.sp. *lycopersici* ('+' Presence; '-' Absence), *Mean of three replications, In a column, mean followed by a common letter are not significantly different at the 5% level by DMRT

(by 73.62 cm), Dry weight (by 127 mg) and fruit yield (by 288.389 g) when compared to individual strains and untreated control (Fig. 3a, Table 4).

DISCUSSION

Accumulating evidence from literature has shown that compatible multiple strains appear to be an important pre-requisite for the desired effectiveness of strains and more consistent disease suppression (Ganeshamoorthi *et al*., 2008; Latha *et al*., 2009; Sundaramoorthy *et al*., 2012, 2013, Sundaramoorthy and Balabaskar, 2012). In the present study, about twenty five isolates of PGPR were isolated from the rhizosphere of tomato. Similarly, most of the research workers have isolated PGPR from varying ecosystems. *P. fluorescens* was isolated predominantly from suppressive soils for the management of soil borne diseases (Ongena *et al*., 1999; Zehnder *et al*., 2000; Weller *et al*., 2002). The present *in vitro* study clearly showed that the bacterial antagonists *P. fluorescens* (EPI-5, KPI-7, ANR-2 and RTM-3) and *B. Subtilis* strains (KGI-4, PYR-3 and OCM-6) were compatible and effectively inhibited the growth of FOL. The inhibitory effect of *Pseudomonas fluorescens* and *Bacillus subtilis* against *F. oxysporum* f. sp. *lycopersici* under *in vitro* condition has been reported by several workers (Podile and Dube, 1985;

Fig. 3(a-b): Effect of combined application bacterial antagonistic in the management of tomato wilt disease under greenhouse conditions, (A) Control, (B) EPI (Pf-5)+KGI (Bs-4), (C) KGI (Bs-4)+KPI (Pf-7) and (D) EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7)

Sarathchandra *et al*., 1993). Several strains of *Pseudomonas* and *Bacillus* spp. have been reported to produce wide array of antibiotics viz., 2,4, diacetylphloroglucinol, oligomycin, phenazine, pyoluteorin, pyrolnitrin, pyocyanin, iturin, bacillomycin, zwittermycin A and surfactin which are responsible for their antifungal action (Yu *et al*., 2002). Results from the present study clearly indicated maximum reduction in mycelia growth due to the combination of biocontrol strains than individual strains suggesting the synergism among bacterial native antagonistics in reducing the mycelia growth of the pathogen.

The treatment with combination of EPI (Pf-5)+ KGI (Bs-4)+KPI (Pf-7) increased the plant growth tomato more than did individual biocontrol strains. Similar results on increased plant growth due to combined application of Pf1+Py15+Bs16+Zimmu in tomato (Latha *et al*, 2009) and EPCO16+EPC5+Pf1 in chilli and tomato (Sundaramoorthy *et al*., 2012; Sundaramoorthy and

Balabaskar, 2012) were also reported. In the greenhouse studies also the treatment with combination of EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) resulted in a significantly least wilt disease incidence than any of the strains treated individually. Although, the chemical treatment with Carbendazim (0.1%) recorded the least disease incidence it is noteworthy to observe that the treatment with combination with EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) also produced almost comparable results in reducing the disease incidence.

CONCLUSION

Obviously, the results of the present study demonstrated that combined application of bacterial native antagonistic EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) is a promising approach for the eco friendly management of *Fusarium* wilt disease caused by *F. oxysporum f. sp. lycopersici* and enhancing the growth of the tomato plants.

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