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RESEARCH ARTICLE

IN VITRO BIO EFFICACY OF MICROBIAL ANTAGONISTS AGAINST FUSARIUM *OXYSPORUM* F.SP. *VASINFECTUM*

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ABSTRACT

The bio efficacy of *T. viride* and *P. fluorescens* isolates were tested in *in vitro* against *Fusarium oxysporum* f. sp. *vasinfectum* causing wilt of cotton. Among the *T. viride* isolates, Tv_7 and *P.fluorescens* isolates, Pf_5 significantly inhibited the growth of *F. oxysporum* f.sp. *vasinfectum* tested by the dual culture technique than the control. Similarly, the culture filtrate of Tv_7 and Pf5 isolates were significantly inhibits the growth of *F. oxysporum* f.sp. *vasinfectum*. The reduction of mycelial growth of fungus was directly correlated with the concentration of Tv_7 and Pf_5 culture filtrates in poisoned food technique. The combination of culture filtrate of *T. viride* (Tv_3) and *P. fluorescens* (Pf_7) recorded the maximum germination percentage of 95.75% and increased the shoot and root length to the maximum of 14.10 and 20.29 cm, respectively and recorded a higher vigour index of 3292.84 in roll towel method.

INTRODUCTION

Cotton (*Gossypium spp*) is one of the important fibre crops playing a key role in the economic and social affairs of the world, providing basic input to the textile industry. It is the oldest among the commercial crops of the world and is regarded as "white gold" (Shah *et al.*, 2011; Akhtar *et al.*, 2013). In India, the productivity of cotton is very low due to many constraints including diseases. Diseases are inherent compounds of agro ecosystem that must be dealt continuously and on knowledge basis. The crop is affected by various diseases caused by fungi, bacteria and viruses. Of these pathogens, ***Fusarium oxysporum*** f.sp. ***vasinfectum*** is an important pathogen, distributed worldwide. The pathogen, a soil-borne fungus, can survive for several years in a dormant state (chlamydospores) in plant debris or in the soil. It invades the plants through the roots, and subsequently infects the vascular system, resulting in wilt symptoms (Hillocks, 1992). Management of *F. oxysporum* f. sp. *vasinfectum* using chemical fungicides has been the prevailing control method for over fifty years. Though effective fungicides are available to manage the soil borne diseases, they will not be reliable as a long term solution because of the concerns about exposure risks, health and environment hazards. As a result, in recent years, the biological control especially using fungal and bacterial antagonists against fungal plant pathogen has gained considerable attention and appears to be promising as a viable supplement to chemical control (Papavizas, 1985: Howell *et al*., 1987).

Hence, the possibility of exploiting the native antagonistic potential was thought off for the management of *Fusarium* wilt disease which may represent a potential eco-friendly strategy.

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MATERIALS AND METHODS

Isolation of *Fusarium oxysporum* f. sp. *vasinfectum*

The pathogen *F. oxysporum* f.sp. *vasinfectum* was isolated from the diseased roots of cotton plants showing the typical wilt symptoms by tissue segment method (Rangaswami, 1972). Infected roots and stems were washed in tap water and cut into small pieces. The pieces were surface sterilized in 1 per cent sodium hypochlorite ($NaOCl_2$) solution for 30 sec. and washed serially in sterile distilled water to remove the traces of sodium hypochlorite and then transferred to sterilized Petri plate containing potato dextrose agar (PDA). The Petri plates were incubated at room temperature (28±2°C) for 5-7 days. Hyphal tips growing from infected bits were transferred to PDA slants and the fungus was purified by using hyphal tip technique (Rangaswami, 1972) and were preserved in a refrigerator at 4°C and used for further studies. The pathogen *F. oxysporum* f.sp. *vasinfectum* was identified with the help of the descriptions by Booth (1971) and Singh (1987). The pathogenicity of the isolates was proved by Koch's postulates.

Isolation of native antagonists from rhizosphere soil *Trichoderma* spp.

Cotton rhizosphere soil samples collected from different locations were used for the isolation of *Trichoderma* isolates by soil dilution plating technique using *Trichoderma* selective medium (TSM) (Elad and Chet, 1983). These strains of *Trichoderma* spp. were, purified following single hyphal tip method and maintained in TSM slants at 4^0C in refrigerator with periodical sub-culturing. *Trichoderma* spp., thus isolated was subjected for identification based on the key to species suggested by Domsch *et al*. (1980).

***Pseudomonas* spp.**

Pseudomonas spp. were isolated from the rhizosphere soil collected during the survey. The soil along with root bits was mixed thoroughly and one g of rhizosphere soil was processed following serial dilution. One ml of 10^{-5} dilution was plated on King's B (KB) agar medium and incubated at room temperature (28 ± 2^0C) for 48 hours (Aneja, 2003) to isolate *Pseudomonas*. The colonies fluorescencing under UV light were picked up, purified and maintained in KB slants. The efficient *Pseudomonas* strains identified from the *in vitro* dual culture assay were examined for the colony morphology, growth, pigmentation, cell shape and gram reaction as per the standard procedures given by Barthalomew and Mittewer (1950). The isolate obtained from the culture collection center of the Department of Plant Pathology, Faculty of Agriculture, Annamalai University was used for the comparison.

Efficacy of *Trichoderma* spp. against *F. oxysporum* f.sp. *vasinfectum* (*in vitro*)

The antagonistic activity of bio control agents against *F. oxysporum* f.sp. *vasinfectum* was tested by dual culture technique (Dennis and Webster, 1971). At one end of the sterile Petri dish containing 15 ml of sterilized and solidified PDA medium a 9 mm mycelial disc obtained from five day old culture of *Trichoderma* spp. was placed under aseptic conditions. Similarly, at the opposite end approximately 75 mm away from the *Trichoderma* culture disc, a 9 mm culture disc of *F. oxysporum* f.sp. *vasinfectum* was placed and incubated. A control was maintained by inoculating *F. oxysporum* f.sp. *vasinfectum* alone at one end of the Petri dish. The plates were incubated at room temperature (28 ± 2°C) for seven days. In case of *P. fluorescens* one cm long streak was gently made onto the medium using two days old culture. The radial growth (in mm) of the pathogen and the test antagonists and the extent of the inhibition zones (in mm) developed between the two colonies were measured. The effective antagonists were identified based on the inhibition of the growth of the pathogen. The radial mycelial growth of the pathogen and per cent reduction over control was calculated by using the formula (Vincent, 1927)

Per cent inhibition (I) = C-T/C × 100

Where, C- mycelial growth of pathogen in control

T- mycelial growth of pathogen in dual plate

Bioassay of culture filtrate of the antagonists on the mycelial growth of *F. oxysporum* f.sp. *vasinfectum*

Preparation of the culture filtrates of *T. viride*

The effective *T. viride* isolates were grown for 10 days at room temperature (28 ± 2°C) in Erlenmeyer flasks containing 50 ml of sterilized potato dextrose broth. The cultures were filtered under vacuum through bacteriological filter to remove the mycelium and spores. The filtrate thus obtained was used for the studies.

Preparation of the culture filtrate of *P. fluorescens*

The effective *P. fluorescens* isolates were inoculated into Erlenmeyer flasks containing 50 ml of sterile King's B broth and kept on a rotary shaker at 100 rpm for 48 h. Then the cultures were filtered through bacteriological filter under vacuum and the filtrates thus obtained were used for the studies.

Effect of culture filtrates on the mycelial growth of *F. oxysporum* f.sp. *vasinfectum* (Poisoned food technique)

The culture filtrates of the antagonists were separately incorporated into sterile PDA medium at 10, 20 and 30 per cent by adding the calculated quantity of the culture filtrates to the medium by means of a sterile pipette. The PDA medium without the culture filtrate served as control. The amended media were transferred to sterile Petri dishes separately @ 15 ml and allowed to solidify. Each plate was inoculated at the centre with a five day old (9 mm) PDA culture disc of *F. oxysporum* f.sp. *vasinfectum*. Three replications were maintained for each treatment. The diameter of the mycelial growth (in mm) of *F. oxysporum* f.sp. *vasinfectum* was measured when the mycelial growth fully covered the control plates.

Efficacy of biocontrol agents on plant growth promotion (*in vitro*)

Preparation of inoculum of the antagonists

The effective isolate of *P. fluorescens* was grown in conical flasks (250 ml) containing 100 ml of King's B for 48 h. on a rotary shaker (100 rpm) at 28 ± 2ºC. Cells were removed by centrifugation at 8000 rpm for ten min. at 4°C and washed in sterile water. The pellet was resuspended in small quantity of sterile dist. water until to obtain bacterial colonies of 10^7 cfu ml^{-1} measured by dilution plate technique.

The effective fungal antagonist *T. viride* isolate was multiplied by inoculating a disc of actively growing mycelial disc in TSM broth and incubated for 15 days. Then the mycelial mats along with spores were harvested by filtration through What man No.1 filter paper. Then the contents were mixed with sterile distilled water and adequate number of colony forming units (10^7) was checked through dilution plate technique. For testing the combination effect of the most effective isolates of antagonists the culture suspensions of *T. viride* (Tv_3) and *P. fluorescens* (Pf_7) were mixed @ 1:1 ratio and used for the study.

Seed treatment with antagonist

Seeds of cotton (MCU 7) were surface sterilized with two per cent sodium hypochlorite for 30 sec., rinsed in sterile dist. water and dried overnight. Ten ml of antagonist inoculum was taken in a Petri dish. To this, 100 mg of carboxy methyl cellulose (CMC) was added as an adhesive material. Seeds were soaked in antagonistic suspension for 2 h. and air dried overnight in a sterile Petri dish.

Plant growth promotion (*In vitro*)

Plant growth-promoting activity of the antagonists was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Twenty five seeds treated with antagonists were kept over the presoaked germination paper. The seeds were held in position by placing another presoaked germination paper strip over it and gently pressed. The sheets along with seeds were then rolled and incubated in growth chamber for 10 days. Three replications were maintained for each treatment. The root length and shoot length of individual seedlings were measured and the per cent germination of seeds was also calculated. The seedling vigour index was calculated by using the formula as described by Abdul Baki and Anderson (1973).

Vigour Index = (Mean root length + Mean shoot length) × Germination (%)

RESULTS AND DISCUSSION

Efficacy of native fungal biocontrol agents against *F. oxysporum* f.sp. *vasinfectum* (Dual culture)

In general all the native *Trichoderma* spp. tested significantly inhibited the mycelial growth of *F. oxysporum* f.sp. *vasinfectum* (Table 1). However, among the isolates, the isolate Tv_3 showed the maximum inhibition and significantly inhibited the growth of *F. oxysporum* f.sp. *vasinfectum* (30.47 mm), which was 66.14 per cent reduction on the growth of the pathogen when compared to control. This was followed by the isolates $Tv_1$and Tv_5 in the decreasing order of merit, which inhibited the growth of *F. oxysporum* f.sp. *vasinfectum* by 64.72 and 63.88 per cent over control. The standard isolate used for comparison recorded 63.77per cent reduction on the growth of the pathogen over control.

The least growth inhibition of the pathogen (46.88%) was exhibited by the isolate Th_{10}. The results of the present study correspond with Muthukumar (2002) who reported that the *T. viride* reduced the growth of pathogen *F. oxysporum* to an extent of 30.60mm over control in tuberose. Kalaivani 2006 reported that *T. viride* isolate TvV1 was most effective recording the higher inhibition of mycelial growth of *F. oxysporum* (72 per cent) in tuberose.

All these earlier reports are in line with the present findings. Bell *et al.* (1982) classified *Trichoderma* isolates based on their ability to overgrow the hyphae of *R. solani*. They considered an isolate of *Trichoderma* to be antagonistic to the pathogen only if it overgrew on the pathogen in the dual culture. In the present study also, *Trichoderma* isolate Tv_3 put forth copious overgrowth and sporulated on *F. oxysporum* f.sp. *vasinfectum* in dual culture. These earlier reports lend support to the present findings. A multiplicity of mechanisms involving mycoparasitism, antibiosis, lysis and hyphal interference could be attributed to the reduction in the mycelial growth of *F. oxysporum* f.sp. *vasinfectum*.

Effect of culture filtrate of native *Trichoderma* isolates on the mycelial growth of *F. oxysporum* f.sp. *vasinfectum* (*in vitro*) (poison food technique)

The results presented in Table 1 showed that all the *Trichoderma* isolates significantly inhibited the growth of *F. oxysporum* f.sp. *vasinfectum* when compared to control and generally an increase in the concentration of the culture filtrate showed enhanced inhibition on the mycelial growth of the pathogen. Among the isolates tested, the isolate Tv_3 was found to be most inhibitory to the growth of *F. oxysporum* f.sp. *vasinfectum* by recording the least mycelial growth with 28.10, 16.87 and 0.00 mm at 10, 20 and 30 per cent concentration of the culture filtrate, respectively in poison food technique.

This was followed by Tv_5, Tv_1, and Tv_9 in the decreasing order of merit. The isolate Th_{10} exhibited the least inhibitory effect. *Trichoderma* spp. are known to produce large quantities of fungistatic metabolites such as trichodermin, dermin, trichovirdin, trichobrachin, chitinase, β-1,3 glucanase, protease etc. (Elad *et al*., 1982; Bruchkner *et al.,* 1990), which were active against many soil borne pathogens. Such fungistatic metabolites and other enzymes produced by the native isolate of *T. viride* could be attributed as the reduction in the mycelial growth.

Efficacy of native bacterial isolates against *F. oxysporum* f.sp. *vasinfectum* (Dual culture)

The results presented in Table 2 revealed varying degree of antagonism by the isolates of *Pseudomonas* against *F. oxysporum* f.sp. *vasinfectum*. Among the *Pseudomonas* isolates, Pf_7 produced significantly the maximum inhibition zone (12.67 mm) and minimum mycelial growth (21.35 mm) accounting for 76.05 per cent reduction on the mycelial growth of *F. oxysporum* f.sp. *vasinfectum* over control. This was followed by isolate Pf_5 which recorded an inhibition zone of 11.20 mm accounting for 72.96 per cent reduction on the mycelial growth over control. The isolate Pf_{10} was the least effective among *Pseudomonas* isolates as it recorded the minimum inhibition zone.

Ben *et al.* (1993) reported that *P. fluorescens* (strain Wc S417) significantly reduced the carnation wilt caused by *F. oxysporum* f. sp. *dianthi* which involved multiple mechanisms like induced resistance, siderophore – mediated competition for iron and possibly antibiosis. Yeole and Dube (2000) reported that *F. oxysporum* and *F. solani* were effectively controlled by *P. fluorescens* strain COR B2.

The *P. fluorescens* inhibited the growth of *F. oxysporum* f. sp. *lycopersici, Phytophthora nicotianae, Pythium debaryanum* and *P. ultimum in vitro* (Sarath Chandra *et al.,* 1993). *B. subtilis* inhibited the growth of *F. udum, F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *vasinfectum in vitro* and field conditions (Podile and Dube, 1985).

Table 1. Screening of *Trichoderma* isolates against *F. oxysporum* f. sp. *Vasinfectum*

T. No.	Isolates	Dual culture technique		Poisoned food technique					
				Mycelial growth (mm) in different culture filtrate conc.					
		Mycelial growth (mm)	Per cent inhibition over control	10%	Per cent inhibition over control	20%	Percent inhibition over control	30%	Percent inhibition over control
1	Tv_1	31.75	64.72	34.40	65.11	23.16	74.26	10.17	88.07
2	Tv_2	38.40	57.33	40.41	60.44	28.94	81.25	18.14	79.84
3	Tv_3	30.47	66.14	28.10	66.18	16.87	67.84	0.00	100.00
4	Tv_4	45.60	49.33	45.60	53.88	33.42	62.86	21.36	76.26
5	Tv_5	32.50	63.88	31.40	60.66	19.31	78.54	7.12	92.08
6	Tv_6	41.40	54.00	41.50	55.10	30.29	66.34	18.21	79.76
7	Tv_7	41.70	53.66	43.40	59.33	31.51	64.98	19.73	78.07
8	Th_8	38.10	57.66	36.60	60.55	24.37	72.92	13.81	84.65
9	Th_9	35.40	60.66	35.50	51.77	23.84	73.51	12.26	86.37
10	Th_{10}	47.80	46.88	46.70	48.11	34.96	60.15	23.91	73.43
11	Comparison isolate	32.60	63.77	32.41	63.98	21.92	75.64	8.14	90.95
12	Control	90.00	-	90.00	-	90.00	-	90.00	-

Table 2. Screening of *P. fluorescens* isolates against *F. oxysporum* f. sp. *vasinfectum*

T. No.	Isolates	Dual culture technique			Poisoned food technique					
					Mycelial growth (mm) in different culture filtrate conc.					
		Mycelial growth (mm)	Per cent inhibition over control	Inhibition zone (mm)	10%	Per cent inhibition over control	20%	Percent inhibition over control	30%	Percent inhibition over control
1	Pf1	38.74	56.95	06.15	37.70	58.11	24.30	73.00	28.56	82.26
2	Pf2	40.66	54.82	05.78	40.95	54.50	26.75	70.27	32.40	64.00
3	Pf3	35.36	60.71	07.11	36.55	59.38	23.50	73.88	24.70	72.88
4	Pf4	33.70	62.55	07.73	35.72	60.31	20.70	77.00	20.80	76.88
5	Pf5	24.33	72.96	11.20	23.46	73.93	14.60	83.77	4.50	95.00
6	Pf6	32.46	63.93	08.75	30.66	65.93	18.62	79.31	16.46	81.71
7	Pf7	21.35	76.05	12.67	20.56	77.15	11.55	87.16	0.00	100.00
8	Pf8	25.60	71.55	10.15	25.60	71.55	15.66	82.60	8.60	90.44
9	Pf9	29.55	67.16	08.00	28.70	68.11	16.70	81.44	12.70	85.88
10	Pf10	42.65	52.61	04.10	42.10	53.22	28.40	68.49	35.44	60.62
11	Comparison Pf	23.88	73.46	10.11	26.80	70.22	13.14	85.40	3.20	96.44
12	Control	90.00	-	-	90.00	-	90.00	-	90.00	-

Table 3. Effect of biocontrol agents on plant growth promotion (Roll towel method)

Treatments	Seed germination (%)	Shoot length (cm)	Root length (cm)	Vigour index
T. viride	92.25	12.10	16.47	2635.58
P. fluorescens	91.65	13.75	18.37	2943.79
T. viride + *P. fluorescens*	95.75	14.10	20.29	3292.84
Carbendazim 50% WP	95.55	9.97	14.41	2329.50
Control	89.90	8.13	13.00	1899.58

The inhibitory action might be due to production of antifungal or antibacterial agents (Lambert *et al*., 1987; Leyns *et al*., 1990; Maurhofer *et al*., 1998), antibiotics *viz*, HCN, Pyrrolinitrin, Phenazine and 2,4- diacetyl phloroglucinol and lytic enzymes as observed in endophytic nature of Pf1 against pathogens (Ramamoorthy and Samiyappan, 2001; Viswanathan and Samiyappan 2001), siderophore production (Schroth and Hancock, 1981; Duijff *et al*., 1993).

Effect of culture filtrate of native bacterial isolates on the mycelial growth of *F. oxysporum* f.sp. *vasinfectum* (poison food technique)

The mycelial growth of *F. oxysporum* f.sp. *vasinfectum* was found reduced with an increase in the concentration of culture filtrates of all the isolates of the antagonists tested and the reduction was significantly the maximum in the case of *Pseudomonas* isolate Pf_7 with 20.56, 11.55 and 0.00 mm at 10, 20 and 30 per cent concentration of the culture filtrate respectively as against the maximum growth of 90 mm in the control in poison food technique (Table 2). This was followed by the isolate Pf_5.

The fungistatic activity of the fluorescent *Pseudomonas* based on the inhibition of mycelial growth of the pathogen was well established by several workers (Samiyappan, 1988; Revathy and Muthusamy, 2003; Vivekanandhan *et al*., 2004; Surjit Sen *et al*., 2006). *P. fluorescens* were known to produce an array of low molecular weight metabolites some of which were potential antifungal agents (O' Dowling and O'Gara, 1994). The *in vitro* inhibition of *Rhizoctonia* sp. by DAPG (Michereff *et al*., 1994) and *M. phaseolina* by Pyrolnitrin (Karunanithi, 1996), the antibiotics produced by *P. fluorescens* have been reported. Such antifungal metabolites produced by *P. fluorescens* might be attributed as the reason for the reduction in the growth of the pathogen.

Efficacy of antagonists on the cotton seed germination and plant growth promotion under *in vitro* conditions

The data on the effect of the culture filtrates of the antagonists on cotton seed germination and growth promotion are presented in table 3. The culture filtrates of none of the antagonists showed any inhibitory effect on the germination of cotton seeds and in general all the treatments induced the plant growth promotion *viz.,* shoot and root length significantly over untreated check as revealed by the data. Among the biocontrol agents tested individually and in combination, the combination of culture filtrate of *T. viride* (Tv_3) and *P. fluorescens* (Pf_7) recorded the maximum germination per cent of 95.75 per cent, which was on par with that of Carbendazim treatment. The same treatment increased the shoot and root length to the maximum with 14.10 and 20.29 cm, respectively and recorded a vigour index of 3292.84. This was followed by individual antagonistic treatment, among which the isolate *P. fluorescens* (Pf_7) recorded numerically superior values with 91.65 per cent germination and 13.75 and 18.37 cm shoot and root length, respectively and a vigour index of 2943.79. The untreated control recorded the lowest shoot and root length of 8.13 and 13.00cm, respectively. The carbendazim treatment recorded 95.55 per cent germination, 9.97 and 14.41 cm shoot and root length, respectively and a vigour index of 2329.50.

The growth promotion might be probably due to the production of growth promoting substances by the antagonists in the culture medium as observed by Chang *et al.* (1986) and also the growth promoting substances produced by *T. viride* and *P. fluorescens* might have exerted a synergistic action and enhanced the growth of groundnut. Similarly, the combinations *T. harzianum* and *P. fluorescens* increased greengram plant growth in pot culture and in the field more than did individual biocontrol strains (Thilagavathi *et al.,* 2007). Also, the combinatorial efficacy of *P. fluorescens* and *T. harzianum* in enhancing the biometrics of biometrics of sweet pepper (Sunilkumar *et al.,* 2012) was reported. *P. fluorescens* might have stimulated plant growth by improving uptake of minerals into the host plants particularly phosphate (Kloepper *et al.,* 1980), siderophore mediated iron uptake (Jurkevitch *et al.,* 1988), association with N_2 fixation (Hong *et al*., 1991), production of IAA (Dubeikovsky *et al.,* 1993), promotion of mycorrhizal function (Garbaye, 1994) and solubilizing nutrients such as phosphorus (Whitelaw, 2000). Thus, the results of the present study and the earlier reports have confirmed that the growth promoting substances produced by *P. fluorescens* and *T. viride* (Tv_3+Pf_5) would have exerted a synergism in promoting the growth parameters of cotton.

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