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International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975–9174, Vol. 3, Issue 2, 2011, pp-79-84
Available online at http://www.bioinfo.in/contents.php?id=27

BIOLOGICAL CONTROL OF *F. OXYSPORUM* f. sp. *LYCOPERSICI* CAUSING WILT OF TOMATO BY *PSEUDOMONAS FLUORESCENS*

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Received: Received: June 07, 2011; Accepted: August 03, 2011

Abstract- *Pseudomonas fluorescens* is one of the major fungal biocontrol agents found in the soil and the rhizosphere of various crop systems. Ten isolates of *P.fluorescens* were isolated from rhizosphere soil samples collected from various tomato-growing fields and evaluated for their efficacy in increasing seed quality variables of tomato and in inhibiting the mycelial growth of *Fusarium oxysporum*. *Pseudomonas* isolate 2 produced effective results and was selected and mass multiplied. Talc and sodium alginate formulations of mass multiplied using different agents were prepared and evaluated for their effects against fusarium wilt under greenhouse conditions. Fresh cultures of Pf2 isolate was found to increase seedling emergence and reduce fusarium wilt disease incidence when compared to the control and the formulations.
Keywords: *Pseudomonas fluorescens;* formulation; mass production; seed quality; tomato wilt control

INTRODUCTION

The production of tomato is of worldwide agricultural importance. Tomato (*Lycopersicon esculentum* Mill.) is one of the most popular important commercial vegetable crops rich in vitamins A, B, and C grown throughout the world. In India, it occupies an area of 0.54 million ha with a production of 7.60 million tons [1]. Many diseases and disorders can affect tomatoes during the growing season. *Fusarium oxysporum* f.sp. *lycopersici* (FOL) is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves and minimal or absent crop yield. There may be a 30 to 40% yield loss [2].

Management of seed-borne and soil-borne diseases such as wilt caused by *Fusarium* species has always been problematic [3, 4]. Soil solarization/disinfection, crop rotation and mixed cropping are the best ways of eliminating soil borne pathogens [5]. Seed treatment with synthetic fungicides such considerably reduce wilt incidence in tomato. However, their use is costly as well as environmentally undesirable [6]. The use of resistant varieties is one of the most effective alternative approaches to controlling wilt disease [7]. But, due to breakdown of resistance in the face of high pathogenic variability in the pathogen population, the usefulness of many resistant cultivars is restricted to only a few years. Thus there is a need to develop alternative strategies to provide durable resistance over a broad geographic area. In this context, biocontrol is an eco-friendly way of managing fusarium wilt in tomato which offers an alternative to fungicides [8]. *Pseudomonas* sp. are the most extensively studied plant growth-promoting rhizobacteria (PGPR), and are known to protect the plant from many deleterious soil and foliar plant pathogenic microorganisms [9, 10]. There is evidence that *Pseudomonas* have a role in the suppressiveness of certain soils to Fusarium wilt of flax radish and cucumber [11]. *Pseudomonas fluorescens isolate Pf1* biovar I showed the lowest disease incidence in tomato and hot pepper respectively, and increased the plant growth under green house conditions. Powder formulations of Pf1 biovar1 was effective in controlling the disease and promoted the plant growth under field conditions [12].

The major constraint for extensive use of biological control under field conditions is a lack of knowledge concerning how to mass produce and properly deliver biocontrol agents [13]. In the present study, *Pseudomonas fluorescens* isolates were isolated from rhizosphere, checked for their *invitro* antagonistic activity, formulated and evaluated for their ability to control fusarium wilt and promote growth of tomato plants under greenhouse conditions. The media chosen for study were talcum powder and sodium alginate formulations.

MATERIALS AND METHODS

Seed material

Tomato seeds of PKM variety were obtained from local seed agencies, Mysore district, Karnataka state, India. The seeds were surface- sterilized with 0.5% sodium

hypochlorite for 3 min followed by repeated washes in distilled water.

Isolation of *Fusarium oxysporum and pathogenicity testing*

Surface sterilized Tomato seeds were plated onto wet blotter disc following the standard blotter method [14]. The plates were incubated for 7 days at 22°C and under 12h/ L: 12h D. After incubation, fungi developed on each seed were examined under different magnifications of a stereomicroscope and identified. The identification was based on the characteristic growth of the fungus on seeds and the morphological characters of conidia (observed under a compound microscope). Pure cultures were maintained on Potato Dextrose Agar (PDA) slants at 4°C. The pathogenecity test was conducted using Tomato PKM under greenhouse conditions to confirm the pathogenecity of the isolated strain.

Collection and screening of soil samples for biocontrol agents

Ten rhizosphere soil samples of tomato fields were collected from different agro climatic regions of Karnataka to isolate *P.fluorescens*. To screen them for the presence of potentially antagonistic strains of bacteria, one gram of soil sample was placed in a 250 ml conical flask containing 100ml of sterile distilled water (SDW) and mixed thoroughly. Different dilutions of working samples were prepared by serially diluting the stock solutions. Fifty microlitres of each of the dilutions was spread on King's medium B (KB) agar [15] for isolation for *P.fluorescens*, for each dilution three plates were maintained. *P.fluorescens* was isolated by incubating plates at 28±2°C for 48 h. After incubation the plates were examined under UV light and screened for fluorescing bacterial colonies. Pure cultures were setup on KB agar slants and confirmed by biochemical tests following the standard methods as described by [16]. Cultures maintained on KB agar slants at 4°C.

Antagonism studies

P.fluorescens isolates were screened for their antagonistic activity against the wilt pathogen by employing the dual culture technique. The interaction was studied in 90-mm diameter petriplate containing PDA. Four isolates were point inoculated on each plate. Plates were incubated for 2 days at 15°C and one 5-mm diameter agar disc of *F.oxysporum* from a 1-week-old PDA culture was placed in the centre of the plate. After 7 days of incubation at 28±2°C, the width of the inhibition zone was measured for each *P.fluorescens* isolate and the inhibition was calculated according to the formula given by [17].

$$I=100-(100\ R_2/R_1)$$

Where 'I' is the degree inhibition of vegetative growth of the fungi, R_1 is the radius of the control colony in mm, and R_2 is the distance traveled by the *F.oxysporum* colony. The experiment was conducted in triplicate and was repeated twice.

Seed treatment with biocontrol agents and its effect on seed quality variables

The isolates of *P.fluorescens* were inoculated into KB broth and incubated at 28±2°C for 36h on a rotary shaker at 150 rpm. Bacteria were subsequently pelleted by centrifugation at 8000 rpm for 10 min in a bench top refrigerated centrifuge .The pellets were washed with sterile distilled water (SDW) three times, and the concentration of cells adjusted to 1×10^8 cfu/ml by dilution to give suspensions with an optical density of 0.45 (A 610nm) using UV-visible spectrophotometer [18].

Seeds of tomato (PKM) were soaked in a culture suspension amended with 0.2% caboxymethyl cellulose (CMC) to facilitate adherence of the biocontrol agent to seeds and then incubated at 28±2°C in an incubator rotary shaker at 150 rpm for 6 h. After incubation, the seeds were air dried aseptically. Seeds soaked in distilled water amended with 0.2% CMC served as control.

Germination testing was carried out using the paper towel method [14] employing eight replicates of 50 seeds each. Fifty seeds of tomato were placed equidistantly on the paper towel pre soaked in distilled water, and then covered with another presoaked paper towels. The paper towel with seeds were rolled up along with polythene wrapping to prevent drying of towels, and were incubated in an incubation chamber for 10 days at 28±2°C . Paper towels were unrolled after incubation and the number of germinated seeds counted. Seedling vigor was analyzed by using the method as described by [19]. To assess vigor, the length of the root and that of the shoot were measured for individual seedlings. The vigor index (VI) was calculated using the formula: VI= (mean root length+ mean shoot length) (% germination).

Preparation of formulations and seed treatment

Formulations of biocontrol agents were prepared on two carrier materials-talcum powder and sodium alginate. One hundred grams of each carrier material was placed in a metal tray under aseptic conditions and the pH adjusted to 7 by adding $CaCO_3$ at the rate of 15 g/kg. CMC was then added at the rate of 10g/kg and mixed well. Finally, the mixtures were packed into a polythene bag and autoclaved for 1 h at 121°C (15lb/inch^2). After autoclaving, 400 ml of bacterial suspension (1×10^8 cfu/ml) was added to the sterilized carrier material (1kg) followed by thorough mixing under sterile conditions. The formulations thus prepared were allowed to dry aseptically and were then ground to powder. They were then packed in sterile polythene bags and stored at 4°C. Just before sowing, seeds were coated with 0.2% CMC as an adhesive and treated with formulations at the rate of 5g/kg and 10g/kg seeds. Seeds treated with sterile distilled water amended with CMC served as a negative control and seeds treated with fresh cultures amended with CMC served as positive control.

Seeds treated with biocontrol agents were sown in pots filled with autoclaved sand, soil and farmyard manure in the ratio 2:1:1. Prior to planting each pot received 200ml of tap water and was incubated for 2 days at room

temperature. Five seeds were sown 2cm deep in each pot. Five pots computed one replicate and there were five replicates for each treatment. Various methods of treatment included, T_1=the plants raised from seeds treated with fresh culture, T_2=the plants raised from seeds treated with talc formulation (5g/kg of seeds), T_3= the plants raised from seeds treated with talc formulation (10g/ kg of seeds), T_4= the plants raised from seeds treated with sodium alginate formulation (5g/kg of seeds), T_5= the plants raised from seeds treated with sodium alginate formulation (10g/kg of seeds), T_6 = healthy control. Treatments were labeled and the pots randomly arranged. Each pot received 25 ml of 1/3-(v/v) strength Hoagland's solution once a week. The percentage of seeds emerging up to 45 days after sowing, were recorded. One more set of treated and control seeds was sown in pots without the pathogen for comparison.

Effect of seed treatment with PGPR strains on *Fusarium* wilt incidence in tomato under greenhouse conditions

Seedlings were raised as explained earlier. Fifteen day-old-seedlings were challenge inoculated with conidial suspension (1,000 micro conidia/ml) of *F.oxysporum.* Wilt incidence was recorded up to 30 days after challenge inoculation. Ten pots per replication were maintained. The pots were arranged in a randomized manner with three replications. The experiment was performed thrice.

Data analysis

Data from laboratory and greenhouse studies were analyzed separately for each experiment and were subjected to arcsine transformation and ANOVA (SPSS Software version II). Treatment means were separated by Tukey's HSD test.

RESULTS AND DISCUSSION

Tomato cultivation is affected by a number of diseases, among them; wilt disease caused by *F.oxysporum* is a serious disease in major tomato growing areas. A new approach in crop protection to reduce the disease damage level using several PGPR strains and microorganisms are reported in many crops for the control of fungal pathogens [20].

P.fluorescens isolated from rhizosphere soil samples of tomato were employed against an important seed and soil borne pathogen of tomato, *F.oxysporum. P.fluorescens* bacteria are important rhizosphere microbes and are efficient in suppressing root and foliar diseases of several plant species [8, 9, 21, 22, 23-24].

Fusarium oxysporum was isolated from infected seeds of PKM I which showed 7% incidence. A pure culture of *F.oxysporum* was maintained on PDA slants at 4^{o}C. Among the twenty different soil samples screened for biocontrol agents, 10 isolates of *P.fluorescens* showed characteristic fluorescence under UV. These were cultured and maintained on King's B medium at 4^{o}C.

Pseudomonas spp. has been reported as being antagonistic to several plant pathogenic organisms such as, *Macrophomina phaeseolila* [25], *Pythium* sp. [26], *F.oxysporum* f.sp. *lycopersici* [27], *Rhizoctonia solani* [28], *Scerotium rolfsii* [29], *Phytopthora capsici* [17], *Gaeumannomyces graminis* var. *tritici* [30]. In our studies all isolates of *P.fluorescens* inhibited growth of *F.oxysporum* to various extents. A maximum inhibition zone of 2.2cm was measured for *P.fluorescens* isolate 2 and minimum zone of 0.2cm was found for isolate 8 (table 1).

Treatment with PGPR increased germination percentage, seedling vigor, emergence, plant stand, and root growth, shoot growth, total biomass of the plants, seed weight, early flowering, increased grain, fodder, fruit yields etc. [31, 32-33]. The isolates of the biocontrol agent evaluated were effective in increasing the seed germination and seedling vigor of tomato compared to the control. Germination was significantly increased up to 89 and 88% in treated seeds. Germination in the control was 78%. Similarly, the highest VI values were found for seedlings treated with Pf2 isolate (VI=879) compared to control (VI=729) Based on the results of the antagonism study, and the effect of biocontrol agent on seed quality variables Pf2 isolate was selected for formulation experiments (table 2).

Talc based formulation has been reported for the management of several crop diseases in India [34]. Talc based formulation of *P.fluorescens* was found to be effective for seed treatment and foliar application on the control of rust and leaf spots of groundnut [35]. In our study all isolates of *P.fluorescens* were found to be effective in increasing seed quality variables to various extents upon seed treatment. *Pseudomonas fluorescens* was mass multiplied in nutrient broth and subsequently used to prepare suitable formulations [36]. The major factor determining the success of a biocontrol agent is delivered into the environment [8]. Bio formulations of *P.fluorescens* using bacterial suspension, was found to be effective in reducing the tomato wilt under greenhouse conditions when applied as a seed treatment. This may have been due to their ability to inhibit the growth of *F.oxysporum* in the rhizosphere, which we demonstrated in our in vitro antagonism assay.

The fresh culture and formulations (Talc and Sodium alginate) of *P.fluorescens isolate* 2, significantly increased seed germination and seedling vigor over the control. Such plant growth promotion activities of *P.fluorescens* was reported in crops such as, rice, wheat, sorghum pearl millet, tomato, brinjal, chilli, redgram, cucumber and sunflower [25, 37, 38, 39, 21- 40].

The fresh cultures of the biocontrol agent were found to be best in reducing fusarium wilt under green house conditions. The fresh cultures contain bacteria are in the active state and, after application they immediately colonize the emerging root. However, in the case of formulations, the growth of biocontrol agents is negligible; it should be stimulated by root exudates [28]. Among the different formulations tested, fresh cultures of

isolate Pf2 increased seedling emergence and reduce wilt incidence, followed by talc and sodium alginate formulations. Seedling emergence increased significantly up to 85% in seeds treated with fresh culture of Pf2 when compared to the control, in which seedling emergence remained at 63% in pots with the pathogen (P^+) (Table 3). The same fresh cultures of *P.fluorescens* isolate 2 increased seedling emergences up to 90 and 85%, respectively, when compared to the control (76%) in pots without *F.oxysporum* (P^-). Talc formulation also increased seedling emergence, but the effect was not significantly greater than the fresh culture (Table 3). The fresh culture of Pf2 isolate significantly (P=0.05) reduced disease incidence up to 12% compared to talc and sodium alginate formulations where disease incidence was 20 and 31%, respectively. In the corresponding controls, initial seedling emergence was 63% in pots with *F.oxysporum* (P+); however disease incidence increased up to 71% after 45 d of sowing.

Seeds treated with the isolate Pf2 as fresh suspensions and powdered formulations showed positive growth responses among all parameters recorded under greenhouse conditions compared with the non bacterized control. The highest plant height (16.3cm) was resulted from seeds treated with fresh suspension of pf2 and followed by talc at 10g/kg seed, 5g/kg seed (15.5, 14.6 cm) and Sodium alginate formulations at 10g/kg seed, 5g/kg seed (14.9, 14.3 cm) respectively compared with untreated control (8.1cm) (table4). Similarly seed treatment with fresh suspensions as well as powdered formulations of Pf2 significantly increased plant height, fresh weight and dry weight over control (table 4). Similar results were reported by [36], who used *P.fluorescencens* strains that inhibited mycelial growth of *F.udam*, in the form of formulations against fusarium wilt in pegionpea successfully through seed treatment.

Various formulations of *fluorescent pseudomonads* are available in the market in India that have proven to be efficient in the control of bacterial and fungal pathogens of various crops [36, 41-42].

The results of the present investigation suggest that there was no negative effect when the *P.fluorescencens* is applied as biocontrol agent; on the contrary, they exhibited synergism in promoting crop growth and yield of tomato besides controlling the fusarium wilt disease. Hence the Talc and Sodium alginate formulations of *P.fluorescencens* can be recommended to the farmers as one of the crop protection strategies for the management of Fusarium wilt of tomato and this practice may also be extended to other crops.

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Table 1-Invitro antagonism assay against F.oxysporum

P. fluorescens Isolates	*P. fluorescens* Inhibition zone (cm)
Pf1	0.9±0.02^{e}
Pf 2	2.2±0.11^{a}
Pf 3	1.8±0.04^{b}
Pf 4	1.8±0.02^{b}
Pf 5	1.5±0.03^{bc}
Pf 6	1.0±0.05^{de}
Pf 7	0.2±0.01^{f}
Pf 8	1±0.11^{de}
Pf 9	0.8±0.04^{e}
Pf 10	1.3±0.02^{cd}

Values are the means ± SE of three replicates. The values in the column followed by the same letter(s) are not significantly different according to Tukey's HSD test at P=0.05.

Table 2- Effect of seed treatment with P.fluorescens on seed quality variables.

Isolates	Germination(%)	Vigor Index
Control	78±0.57^{b}	729±16.7^{b}
Pf 1	86±1.15^{ab}	810±23^{ab}
Pf 2	89±0.86^{a}	879±17.3^{a}
Pf 3	86±3.46^{ab}	821±23^{ab}
Pf 4	83±4.61^{ab}	785±31.7^{ab}
Pf 5	86±0.57^{ab}	806±25.9^{ab}
Pf 6	81±0.28^{ab}	778±28.86^{ab}
Pf 7	83±0.11^{ab}	798±29^{ab}
Pf 8	80±0.33^{ab}	842±24^{ab}
Pf 9	86±3.46^{ab}	865±20^{a}
Pf 10	81±1.15^{ab}	843±20^{ab}

Values are the means ± SE of four replicates f 100 seeds each. VI, Vigor index. The values in the column followed by the same letter(s) are not significantly different according to Tukey's HSD test at *P*=0.05.

Table 3- Effect of seed treatment with bioformulations on seedling emergence and disease incidence in greenhouse conditions

Treatments	Seedling emergence (P^{-}) (%)	Seedling emergence (P^{+}) (%)	DI (P^{+}) (%)
Control	76±3.46^{a}	63±2.3^{B}	71±1.15^{a}
Fresh culture of Pf2	90±2.8^{a}	85±2.8^{a}	12±0.57^{e}
talc formulation of Pf2 (5g/ kg of seeds)	83±2.3^{a}	80±4.4^{a}	20±0.34^{d}
talc formulation of Pf2 (5g/ kg of seeds)	85±2.8^{a}	81±4.4^{a}	19±0.11^{d}
Sodium alginate formulation of Pf2 (10g/ kg of seeds)	78±4.0^{a}	78±3.4^{ab}	36±0.57^{b}
Sodium alginate formulation of Pf2 (5g/ kg of seeds)	81±3.4^{a}	79±2.3^{ab}	31±0.28^{c}

DI, Disease incidence; P^{+}, pots with pathogen; P^{-}, pots without pathogen. Values are the means ± SE of five replicates five pots each. The values in the column followed by the same letter(s) are not significantly different according to Tukey's HSD test at *P*=0.05.

Table 4- Effect of seed treatment with bioformulations on plant growth under greenhouse conditions

Treatments	Plant height(cm)	Fresh weight(g)	Dry weight(g)
Control	8.1±0.05^{f}	0.444±0.057^{d}	0.052±0.012^{d}
Fresh culture of Pf2	16.3±0.17^{a}	0.781±0.05^{a}	0.115±0.02^{a}
talc formulation of Pf2 (5g/ kg of seeds)	14.6±0.23^{cd}	0.710±0.31^{ab}	0.079±0.009^{b}
talc formulation of Pf2 (5g/ kg of seeds)	15.5±0.17^{ab}	0.773±0.04^{a}	0.110±0.010^{a}
Sodium alginate formulation of Pf2 (10g/ kg of seeds)	14.3±0.17^{bc}	0.611±0.05^{c}	0.068±0.015^{bc}
Sodium alginate formulation of Pf2 (5g/ kg of seeds)	14.9±0.17^{bc}	0.643±0.04^{bc}	0.075±0.002^{b}

Values are the mean with in the column sharing the same letters are not significantly different according to Tukey's HSD at P≤0.05. Plant growth promotion studied under green house conditions were done using 30-day-old–seedlings grown from bacterized and formulation treated seeds. Distilled water treated seeds served as control..