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Article

Plant Growth Promotion and Suppression of Root Disease Complex due to *Meloidogyne incognita* and *Fusarium oxysporum* by Fluorescent Pseudomonads in Tomato

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While screening for nematicidal activity of bacterial origins, various pseudomonads strains were inhabited in tomato rhizosphere. One isolate designated as PE_{10} was selected for studies on nematicidal properties and plant growth-promoting (PGP) activity and was identified as *Pseudomonas aeruginosa* based on morphological features, biochemical and physiological tests, and carbohydrate utilization. To investigate nematicidal activity, *Meloidogyne incognita* juvenile mortality was determined using PE_{10} culture filtrate. Inhibition of strain PE_{10} against *Fusarium oxysporum* was observed using dual culture technique. Strain PE_{10} showed good siderophore activity, HCN and IAA production abilities, and growth and development enhancement of tomato.

Key words: Meloidogyne incognita, Fusarium oxysporum, Pseudomonas aeruginosa, *Disease complex, Antagonism, Biological control*

The association of root knot nematode *Meloidogyne* spp. with root-infecting fungi such as *Fusarium* spp. results in greater damages to crops including vegetables than those produced by either pathogen alone.[1)] Among the nematode-fungus associations infecting the tomato, disease-complex caused by root knot nematode and wilt fungi occurs most frequently in nature.

Fluorescent pseudomonads have been widely recognized as potential biocontrol agents against cyst and root knot nematodes.[2)] In addition, the culture filtrate of *P. fluorescens* showed root knot and cyst nematode juvenile mortalities.[3)] Many species of pseudomonads were also found to promote plant growth and reduce the root knot nematode population.[4,5)]

Earlier, seed bacterization by fluorescent *Pseudomonas* was proved to be a potential method for the enhancement of plant growth and suppression of plant pathogenic fungi, *Rhizoctonia solani* and *Sclerotium rolfsii*.[6)]

Among the root knot nematodes, *Meloidogyne* spp. is the most predominant one, causing severe damages to most vegetable crops including tomato. Although nematicides are used for the control of root knot nematode, due to their residual effects and health hazardous nature, an alternative in the form of biological control, which could provide substantial protection against root disease in tomato, is required.

Present work was aimed at investigating the ability of fluorescent *Pseudomonas* PE_{10} to colonize tomato roots with special reference to its effects on growth promotion and inhibition against *M. incognita* and the fungal pathogen *F. oxysporum* causing root disease in tomato.

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Material and Methods

Bacterial inoculum. The fluorescent Pseudomonads strains were isolated from the rhizosphere of mature tomatoes (*Lycopersicon esculentum*). Appropriate serial dilutions of soil suspension in sterile water were spread on tryptic soy agar medium (TSM), and the plates were incubated at 28 ± 1°C for 24-48 h. The fluorescent pigment-producing bacterial colonies were carefully collected and streaked on to a TSM medium supplemented with 100 μg · ml^{-1} streptomycin to evaluate the antibiotic-resistant strain. Pseudomonads strains were maintained on TSM slants at 4°C. Morphological and biochemical characterizations of the bacterial strains were carried out as outlined earlier.[7)]

F. oxysporum was isolated from diseased roots of tomato plant using the dilution plate technique and identified according to the method of Barnett and Hunter.[8)] A culture was maintained on potato dextrose agar (PDA).

Plant growth-promoting activities of fluorescent pseudomonads were determined by growing log phase (24 h-old) culture of different strains of pseudomonads. The production of siderophore was estimated qualitatively on Chrom-Azurol agar medium (CAS), a universal medium for siderophore detection, according to the method of Schwyn and Neilands as follows.[9)] The strains were separately spotted on CAS agar medium, and the plates were incubated at 28 ± 1°C for 48 h. Hydrocyanic acid production (HCN) was determined by the modified method of Miller and Higgins[10)] while Indole acetic

acid (IAA) production was observed according to the method of Gupta.[11]

***In vitro* interaction between juveniles and bacterial culture filtrate.** The egg masses of *M. incognita* were handpicked from galled roots of tomato grown in a culture pot surface-sterilized with 0.1% $HgCl_2$ for 60 s. They were then rinsed, placed in distilled water, and incubated at room temperature (25-30°C) for 24 h. After hatching, the juveniles were collected, and a suspension of juveniles in distilled water was prepared. *Pseudomonas* strains were grown in Erlenmeyer flasks containing Nutrient Broth (NB) at 28°C. After 24 h, the bacterial culture was centrifuged at 7000 g for 15 min at 4°C to obtain cell-free culture filtrate. The bacterial pellet was used for seed bacterization. To determine the effect of culture filtrate on juveniles of *M. incognita*, 2 m*l* of 24 h-old bacterial culture filtrate (viz. 25, 50, 75 and 100%) was transferred on to a cavity glass slide, to which 1 m*l* juveniles (40-45 surface sterile juvenile/m*l*) was added in quintuplicates. The numbers of dead juveniles were counted, and mean percentage of the dead larvae was observed after 12 and 24 h.[12] The culture filtrates were heat-treated by boiling in water bath for 5 min and subsequently tested for the nematicidal activity using the method described by Ali.[4]

Antagonism *in vitro*. Antagonistic properties of bacterial strains were tested against *F. oxysporum* on tryptic soy agar medium (TSM) plates using a dual culture technique.[13] Agar blocks containing 5 days old mycelia were placed on TSM plate, and the log phase (24 h-old) bacterial strains were spotted 2 cm apart from each other. After incubation at 28 ± 1°C for 5 days, the growth inhibition was calculated by measuring the distance between the bacterial and fungal colonies as compared to the control.

Preparation of *F. oxysporum* inoculum. The fungus inoculum was prepared by culturing the isolate in potato dextrose broth for 15 days at 25 ± 2°C. The mycelium was collected on blotting paper to absorb excess water, and the inoculum was prepared by comminuting 100 g mycelium in 1000 m*l* distilled water, using 10 m*l* suspension to provide 1 g mycelium per pot for *Fusarium*-infested soil.

Seed bacterization. The pellet obtained by PE_{10} was washed with sterile distilled water (SDW) and re-suspended in SDW to obtain a population density of 1×10^8 CFU ml^{-1}. The suspension was mixed with 1% carboxymethylcellulose (CMC) solution. The slurry was coated on to the surface of sterilized seeds. Seeds were allowed to air-dry overnight under aseptic condition. Surface-sterilized seeds coated with CMC slurry (without *Pseudomonas* PE_{10}) served as the control.

Bacterized and non-bacterized tomato seeds were sown separately in 15-cm diameter pots containing 1 Kg steam-sterilized soil (77.3% sand, 13.6% silt, 11.7% clay, 0.097% total organic C, pH 6.4 and 36% water-holding capacity) subjected to the following treatments: 1. Soil inoculated with *F. oxysporum* plus non-bacterized seeds (treatment I) 2. Bacterized seeds with *Pseudomonas* PE_{10} (treatment II) 3. Soil inoculated with *F. oxysporum* plus bacterized seeds (treatment III) 4. Soil inoculated with *F. oxysporum* plus bacterized seeds with MTCC strain (treatment IV) 5. Control (Non-bacterized seeds in soil devoid of *F.oxysporum*) (treatment V).

One week after seedling emergence, each pot was inoculated with 2000 freshly hatched second stage juveniles of *M. incognita*. Effect of treatments on the plant growth (shoot length, shoot fresh weight, shoot dry weight), root knot index, and fungal infection were recorded after 60 days. Pots were arranged in a randomized block design, and each treatment was replicated three times.

Root colonization. Tomato seedlings grown from seeds bacterized with *Pseudomonas* PE_{10}^{strep+} were sampled after 30 and 60 days, and the bacterial population on roots was determined. The roots were cut into 1-cm long segment, and 1 g root segment was dipped in 5 ml sterile distilled water and vortexed 4-5 times to release the rhizosphere bacteria into water. A dilution of the suspension was poured in-to Petri plates containing TSM medium supplemented with 100 μg · ml^{-1} streptomycin to evaluate the population of *Pseudomonas* PE_{10}^{strep+}. After 24 h incubation at 28 ± 1°C, CFU's per gram root segment were counted.

Root knot index. At the harvesting stage of the crop, the roots were washed to remove the adhering soil and the number of root knots were counted. The root knot index was determined by recording the severity of disease on a scale of 0 to 7, where 0 and 7 indicate no root knot formation and severe infestation of root knot, respectively.

Assessment of fungal infection. To assess the incidence of root-infecting fungi, five 1-cm long root pieces from each plant were cut, surface-sterilized with 1% $Ca(OCl)_2$ for 2 min, and transferred on to PDA plates containing penicillin (100,000 units/litre) and streptomycin (0.2 g/litre). After incubation for 5 days at 28°C, the incidence of root-infecting fungi viz. *F. oxysporum* was recorded.[14]

Results and Discussion

Isolation of microorganism. Among ten strains of fluorescent pseudomonads isolated, *Pseudomonas* PE_{10} was selected as the most promising strain, because it produced more siderophore, HCN, and IAA in comparison to those of other isolates and showed antagonism against *F. oxysporum* (Table 1).

Strain PE_{10} was motile, Gram-negative, aerobic, non-spore forming rod, and formed small, round colonies with smooth margins on TSM plates after 24 h incubation at 28 ± 1°C. The strain was positive for oxidase, catalase, indole production and starch hydrolysis. Other physiological properties and carbon utilization of the isolate PE_{10} are shown in Table 2. Comparison of these characteristics with those of the known strain *P. aeruginosa* (MTCC strain 1934) revealed that PE_{10} belongs to *Pseudomonas aeruginosa*.

Siderophore production was evaluated by observing the orange zone around the inoculated strain PE_{10} on chrome-

Table 1. Characteristics of isolated strains of fluorescent pseudomonads from tomato rhizosphere

Strains	CAS blue agar		TSM agar fluorescent pigment production[c]	HCN production	IAA production[d]	Antagonism against *F.oxysporum*[e]	Inhibition (%)
	Growth[a]	Halo formation[b]					
PE_1	++	+	+	+c	-	-	-
PE_2	++	+	+	-	+	-	-
PE_3	++	++	+	-	+	-	-
PE_4	++	++	+	-	-	-	-
PE_5	++	+++	+	+a	+	+	32.6
PE_6	+	++	+	-	-	-	-
PE_7	++	+++	+	+a	+	+	54.4
PE_8	++	++	+	+c	-	-	-
PE_9	++	+	+	-	-	-	-
PE_{10}	++	+++	+	+a	+	+	60.0
MTCC 1934	++	+	-	-	-	-	-
Control	-	-	-	-	-	-	-

Table 2. Comparison of bacterial characteristics of PE_{10} and *Pseudomonas aeruginosa* MTCC 1934 strains

Test/ characteristics	PE_{10}	MTCC 1934
Cells short rod	+	+
Fluorescent pigment	+	+
Gram reaction	-	-
Flagellar attachment	+	+
Motility	+	+
Starch hydrolysis	-	-
Gelatin hydrolysis	+	+
Oxidase	+	+
Catalase	+	+
Indole production	+	+
Polyβ-hydroxybutyrate hydrolysis	-	-
Growth at 4°C	-	-
Growth at 41°C	+	+
Utilization of:		
Glucose	+	+
Mannose	-	-
Ribose	+	+
L-Arginine	+	+

(-) Negative response, (+) Positive response

Azurol agar medium. Changing of the color of the filter paper from yellow to reddish brown after 2-3 days of incubation by strain PE_{10} confirmed strong HCN production. IAA production by strain PE_{10} was observed in tryptophan-free medium as evidenced by the production of a pink-colored product.

***In vitro* interaction between juveniles and bacterial culture filtrate.** The preliminary screening of 10 *Pseudomonas* strain isolates was performed *in vitro*. Interaction study indicated that strain PE_{10} showed substantial nematicidal ($p < 0.05$) effect, killing the second stage juveniles of *M. incognita*. PE_{10} strain caused highest juvenile mortalities of 57 and 72% at the maximum concentration (100%), whereas the lowest juvenile mortalities of 14 and 23% at the minimum treated concentration (25%) of culture filtrate after 12 and 24 h, respectively. Strain PE_{10} showed significantly higher ($p < 0.05$) mortality at much lower concentration than MTCC strain (100%) after 24 h. These data revealed a significant difference in mortality after 24-h exposure at all concentrations of the PE_{10} culture filtrate as compared to the control. Nematode mortality increased with increasing exposure period and concentration of the culture filtrate. On the other hand, the nematicidal activity decreased considerably in the heat-treated culture filtrate (Table 3), indicating the extra-cellular and heat-sensitive nature of the nematicidal substance of PE_{10}.

Antagonism *in vitro*. *Pseudomonas* PE_{10} strongly inhibited the growth of *F. oxysporum* on TSM plates at 28 ± 1°C. Maximum growth inhibition of *F. oxysporum* was observed after 5 days of incubation. Increase in the incubation time increased the zone of inhibition up to 5 days, thereafter the growth of mycelia towards the interaction zone stopped, and the mycelia gradually lost vigour. *Pseudomonas* PE_{10} caused hyphal shriveling, and mycelial and conidial deformities, leading to *F. oxysporum* lysis.

Pot experiments. The bacterized seeds showed significant ($p < 0.01$) increases in shoot length, shoot fresh weight and shoot dry weight in comparison to those of non-bacterized seeds, with maximum increases observed in seedlings raised with PE_{10} bacterized seeds as compared to the control (non-bacterized) (Table 4).

A drastic decline (96%) in the incidence of fusarial wilt was found when bacterized seeds were sown in *F. oxysporum*-treated soil. Strain MTCC decreased the incidence of disease by 84%.

The marker strain *Pseudomonas* PE_{10}^{strep+} showed positive root colonization of tomato (Table 5). Higher root colonization was observed by *Pseudomonas* PE_{10}^{strep+} in *F. oxysporum* - infested soil as compared to *Pseudomonas* PE_{10}^{strep+} alone (5.0×10^5 and 7.5×10^4 respectively) after 60 days of germination. The colony-forming unit (CFU) increased with observation

Table 3. Heat-treated and -untreated culture filtrates of *Pseudomonas aeruginosa* PE_{10} and MTCC 1934 strains on mortality of *Meloidogyne incognita* infecting tomato

Culture filtrate conc. (%)	Culture filtrate of strain PE_{10}		Culture filtrate of MTCC 1934		Heat- treated culture filtrate of strain PE_{10}		Heat treated culture filtrate of MTCC 1934	
	% mortality after an exposure of		% mortality after an exposure of		% mortality after an exposure of		% mortality after an exposure of	
	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
25	14	23	12	14	12	17	12	15
50	29	40	23	37	14	15	13	16
75	43	49	37	45	12	13	10	14
100	57	72	43	58	15	18	12	12
Control (NB)	11	20	11	20	11	20	11	20
$LSD_{0.05}$	12	16	10	14	4	6	3	5

NB = Nutrient Broth

$LSD_{0.05}$ means least significant difference at 0.05 probability.

Table 4. Effect of *Pseudomonas* EP_{10} on root disease complex and growth of tomato after 60 days

Treatment	Plant length (cm)	Plant fresh weight (g)	Shoot dry weight (g)	Root knot index	Infection (%) of F. oxysporum
F. oxysporum	26.5	18.0	4.5	6.0	100
Pseudomonas PE_{10}	55.0 **	51.0 **	15.0 **	3.0	-
Pseudomonas PE_{10} + *F. oxysporum*	58.5 **	55.0 **	17.5 **	2.5	4
MTCC 1934 + *F. oxysporum*	51.0 **	48.0 **	13.0 **	4.0	16
Control	32.8	27.5	6.5	7.0	-
CD @ 1 %	17.5	15.0	9.25		
± SEM	4.12	3.75	1.98		

**$p < 0.01$

CD @ 1 % means critical difference at 1% probability.

± SEM means standard error of mean.

period from 30 and 60 days. Strain PE_{10} with or without *F. oxysporum* decreased the root knot index.

P. aeruginosa PE_{10} produced siderophore, HCN, and IAA *in vitro*. In an earlier study, Gupta isolated *P. aeruginosa* GRC_1 from potato rhizosphere that also displayed the above characteristics and possessed strong antagonistic activity against important fungal pathogens, viz. *F. oxysporum* and *Macrophomina phaseolina*.[11] *P. aeruginosa* PE_{10} inhibited the mycelial growth of *F. oxysporum* as well as *M. incognita in vitro*. Fluorescent *Pseudomonas* are known to have a significant role in the suppression of fungal pathogen,

Table 5. Population dynamics of *Pseudomonas* EP_{10}^{strep+} in rhizosphere of tomato

Treatment	Bacterial population (CFU/g of root)	
	After 30 Days	After 60 Days
Pseudomonas PE_{10}	1.5×10^{3}*	7.5×10^{4}**
Pseudomonas PE_{10}+*F. oxysporum*	3.0×10^{4}**	5.0×10^{5}**
MTCC 1934	2.5×10^{3}*	6.5×10^{4}**

CFU means colony forming unit.

*$p < 0.05$, **$p < 0.01$ (ANOVA). Data are the means of three replicates.

apparently via the production of antifungal metabolites such as antibiotics, siderophore, and HCN, whereas these rhizobacteria reduced the hatching and invasion due the production of toxic metabolites, nematicidal components, and alteration of specific root exudates that altered the nematode behavior.[15-20] Our results showed that the culture filtrate of strain PE_{10} caused significant juveniles mortality, as also similarly observed by Ali,[4] whereas heat-treated culture filtrate lost the nematicidal activity. *P. aeruginosa* PE_{10} inhibited the growth of *F. oxysporum in vitro* in pot experiment, subsequently enhancing the plant growth as compared to the control. Ali demonstrated that seed treatment with *P. aeruginosa* significantly reduced the population of *Meloidogyne javanica* and the severity of the subsequent root knot disease, and enhanced the mungbean yield.[4] Our strain PE_{10} has merit over other strains, being a successful colonizer of the spermosphere, and increased seedling emergence and its establishment in the rhizosphere of tomato against *F. oxysporum* and root knot nematode.

Plant growth-promoting rhizobacteria suppress plant parasitic nematode population through blockage of the receptors on roots and modification of root exudates of the host plant, thus hindering the attraction, hatching, and penetration behaviors of nematodes.[21] *Pseudomonas* PE_{10}

promotes the growth of plants and reduces the nematode multiplication, and have high potential as biological control agents against wilt as well as root knot disease.

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