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Effects of cell suspension and cell-free culture filtrate of *Pseudomonas aeruginosa* in the control of root rot-root kont disease complex of tomato (*Lycopersicon esculentum* Mill.)

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Summary

The plant growth-promoting rhizobacterium *Pseudomonas aeruginosa* strain IE-6 was tested for antagonistic activity towards *Meloidogyne javanica*, the root-knot nematode and soilborne root-infecting fungi viz., *Macrophomina phaseolina*, *Fusarium solani* and *Rhizoctonia solani* under laboratory and greenhouse conditions. Cell-free culture filtrate of the bacterium caused significant reduction in egg hatching of *M. javanica* and inhibited radial growth of fungi *in vitro*. Cell-free culture filtrate also caused lyses in mycelium of *F. solani*. Under greenhouse conditions, soil drenches with the aqueous cell suspension or cell-free culture resulted in a considerable reduction in nematode population densities in soil and subsequent root-knot development due to *M. javanica*. In addition to nematode control, rhizobacterium application also inhibited root-infection caused by soilborne root-infecting fungi with significant enhancement of growth of tomato seedlings.

Key words: *Pseudomonas aeruginosa*, cell-free culture filtrate, root-infecting fungi, root-knot nematode.

INTRODUCTION

The rhizosphere is an intense zone of microbial activity and the reduction in the population of plant-parasitic nematodes and soilborne root-infecting fungi in soil is sometimes attributed to soil microorganisms. However, the use of these

microorganisms in practical biological control has been limited because of their poor establishment in the rhizosphere, requirement of the energy source and lack of specificity. Repellent or lethal effects to nematodes have been demonstrated for the non-pathogenic actinomycetes, bacteria and fungi (M o r i, 1961; K a t z n e l s o n et al., 1964; A l i et al., 1989) and the metabolic products of these microorganisms (S a k h u j a et al., 1978; A l i et al., 1990).

During the 1970s, specific rhizosphere bacteria applied to seeds were reported to colonize roots and promote plant growth (K l o e p p e r, S c h r o t h, 1978) and were termed "plant growth-promoting rhizobacteria" (PGPR). Studies of mechanisms indicated that PGPR promote plant growth directly by production of plant growth regulators or stimulating nutrient uptake (L o p e r, S c h r o t h, 1986) or indirectly by production of siderophores or antibiotics to protect plants from soilborne pathogens or deleterious rhizobacteria (W e l l e r, 1988; S c h i p p e r s et al., 1987). Experiment were therefore carried out to examine the effects of aqueous cell suspension of *Pseudomonas aeruginosa*, the plant growth-promting rhizobacterium and its culture filtrate on soilborne root-infecting fungi such as *Macrophomina phaseolina, Fusarium solani* and *Rhizoctonia solani* and the root-knot nematode (*Meloidogyne javanica*) in tomato.

MATERIALS AND METHODS

Pseudomonas aeruginosa strain IE-6, isolated from the rhizosphere of sunflower was maintained on King's B medium at room temperature (28±1°C). From a five-day-old culture, the bacterium was scrapped from the medium surface using a sterilized bent glass rod after adding 10 ml sterilized distilled water. Aqueous cell suspension so obtained was stored in a refrigerator prior to use.

For the preparation of the cell-free culture filtrate, the bacterium was grown in King's B liquid medium at 30°C for 48 h in dark and centrifuged twice at (2,800 x g for 20 min). Pellets were discarded and culture supernatant was collected in a beaker. The extract was designated as (N) concentration. Further dilutions (N/10, N/100 and N/1000) were prepared after adding requisite amount of sterilized distilled water.

In vitro experiments

To determine the effects of culture filtrate on egg hatching, two medium size egg masses of *Meloidogyne javanica* were placed in glass cavity slides containing 2-ml of different dilutions of the culture filtrate. Cavity glass slides containing 2-ml KB liquid medium served as controls. Each treatment was replicated four times and kept at 28°C. After 72 h, the number of hatched juveniles counted. The egg-masses were then transferred from the culture filtrate to sterile distilled water to ascertain whether the egg-masses kept in filtrate had been temporarily or permanently inactivated. The emergence of juveniles was again recorded after 72 h.

To determine the effects of culture filtrate on root-infecting fungi, 5-mm-diam. discs of sterilized Whatman No. 1 filter paper soaked separately in 15-ml of different dilutions of the culture filtrate were placed onto Czapek's Dox Agar medium on a Petri

dish in a clockwise manner according to the concentration. Disc inoculated with KB liquid medium served as control. A 5-mm-diam. Disc of the actively growing test fungus maintained on Potato Dextrose Agar medium was inoculated at the center of the dish. The treatments were replicated three times. The dishes were incubated at 28°C and zone of inhibition (if any) was recorded after 7 days.

Greenhouse experiments

Sandy-loam soil of pH 8.1 with moisture holding capacity of 40% obtained from the experimental field of the Department of Botany, University of Karachi was used. The soil was passed through 2-mm sieve to discard non-soil particles and 2-kg sample was transferred in 21-cm-diam. earthen pots. The soil was naturally infested with 2-7 sclerotia g^{-1} of soil of *M. phaseolina* as estimated by wet sieving and dilution technique (S h e i k h, G h a f f a r, 1975); 6.8% colonization by *R. solani* was stated on sorghum seeds used baits (W i l h e l m, 1955); and 3000 cfu g^{-1} of soil of *F. solani* as determined by soil dilution technique (N a s h, S n y d e r, 1962). Seeds of tomato cv 'SUN 6002 (PVP)' after surface sterilization with 1% $Ca(OCl)_2$ were washed thoroughly with running tap water and allowed to dry under a laminar flow hood. The seeds were sown in a 5-L. capacity tank containing steam-sterilized soil. Three-week-old seedlings were used for the experiments.

To determine the effects of cell suspension and the cell-free culture filtrate of the bacterium, upper soil surface from each pot was removed to a depth of 5-cm and the following treatments were applied separately in soil: i) 200-ml aqueous cell suspension at 3.3 x 10^8 cfu ml^{-1}; ii) 200-ml of different dilutions of cell-free culture filtrate separately (N, N/10, N/100 and N/1000); iii) 200-ml KB broth; and iv) 200-ml sterilized distilled water was used as controls. Treated soil was accordingly replaced and three tomato seedlings of almost equal size were transplanted in each pot. Treatments were replicated three times and the pots were arranged in randomized complete block design. One week after seedlings transplantation, roots in each pot was infested with 2000 freshly hatched juveniles (5-day-old) of *M. javanica* into three holes made around the seedlings.

The experiment was terminated 45 days after the addition of nematode and plant growth parameters were recorded. To determine the incidence of fungi, 5-mm-long root pieces from each plant after surface sterilization with 1% $Ca(OCl)_2$ for three min were plated onto PDA plates supplemented with penicillin (100, 000 units/l.) and sterptomycine sulfate (0.2 g/l.) at 5 pieces per plate. After 5-days incubation at 28±1°C, incidence of root-infecting fungi was recorded as follows:

$$\text{Infection \%} = \frac{\text{Number of plants infected by a fungus}}{\text{Total number of plants}} \times 100$$

The remaining root samples were chopped into small segments, mixed thoroughly and stored in a refrigerator for other tests. To determine the nematode invasion, one-g sub sample wrapped in muslin cloth was dipped for 3-4 min in boiling 0.25% acid fuchsine with few drops of lactic acid. The roots after washing in running tap water were macerated in an electric grinder for 45 seconds. The macerate was

suspended in 100-ml water and number of juveniles that had penetrated the roots were counted in 5 samples of 5-ml. To determine the nematode population, soil from each replicate of a treatment was collected in a plastic container, mixed thoroughly and 5 samples of 50-g were used for the extraction of nematode using modified Baerman funnel technique.

The data were analysed and subjected to one way analysis of variance (ANOVA) followed by the standard error of the difference between means (SED). Treatment means were also compared following Duncan's multiple range test according to Gomez, Gomez (1984). Data were transformed to $\log_{10}$ x+1 where necessary.

RESULTS

In vitro experiments

Cell-free culture filtrate of *P. aeruginosa* caused significant reduction ($p<0.001$) in egg hatching of *M. javanica*, as less number of juveniles emerged from the egg masses kept in culture filtrate as compared to untreated KB broth. Reduction in egg hatching varied from 15 to 97% depending upon filtrate concentration. After 72 h when egg masses were transferred from culture filtrate to distilled water, more juvenile emergence was observed as compared to untreated control or KB broth. After 6 days interval, pure culture filtrate (N) and its (N/10) dilution caused 87 and 63% inhibition of egg hatching respectively (Table 1).

Table 1.

Effects of the cell-free culture filtrate of *Pseudomonas aeruginosa* strain IE-6 on egg-hatching activity of *Meloidogyne javanica* and radial growth of *Macrophomina phaseolina*, *Fusarium solani* and *Rhizoctonia solani*

Treatments	Number of eggs hatched in		Type of reaction/zone of inhibition (mm)		
	Culture filtrate	Distilled† Water	*M. phaseolina*	*F. solani*	*R. Solani*
Control	260a	41d	*	*	*
KB broth	222b	43d	*	*	*
N	8d	32d	3	***	**
N/10	16cd	95c	2	***	**
N/100	29cd	139b	2	***	**
N/1000	44c	178a	***	***	**

† After a 48 h hatching period in the culture filtrate, egg masses were transferred to sterile distilled water.

Data in columns followed by the same letters are not significantly ($p<0.05$) different according to Duncan's multiple range test; n=4.

* No inhibition

** Test fungus inhibited but later overgrew

*** Colony of the test fungus met with the disc containing culture filtrate. No further growth of the test organism was observed.

**** After inhibition, fungal mycelium lysed.

Culture filtrate of *P. aeruginosa* also inhibited radial growth of root- infecting fungi such as *M. phaseolina*, *F. solani* and *R. solani*. Zones of inhibition of 3, 2 and 2-mm were produced against *M. phaseolina* where pure culture filtrate (N) and its (N/10 or N/1000) dilutions were used respectively. Culture filtrate at all the dilutions resulted in complete distortion in mycelium of *F. solani*. Similarly, growth of *R. solani* was initially inhibited by the culture filtrate but later the test fungus overgrew discs containing culture filtrates (Table 1).

Greenhouse experiments

Aqueous cell suspension and the cell-free culture filtrate considerably reduced nematode population on root and in soil and subsequently reduced root-knot disease severity in tomato. Aqueous cell suspension was found more effective as compared to the culture filtrate of the bacterium. Nematode population density in root and soil was inversely proportional to the filtrate concentration of the bacterium; higher the filtrate concentration lower was the population density. The greatest reduction in gall formation (38%), nematode population in soil (28%) and root (54%) was recorded following treatment with aqueous cell suspension (Table 2).

Table 2.

Effects of aqueous cell suspension and the ecll-free culture filtrate (C.F.) of *Pseudomonas aeruginosa* strain IE-6 on the development of root rot-root knot disease complex and growth of tomato

Treatments	Galls/ root system	Nematode population		Infection %			Plant height (cm)	Shoot weight (g)
		soil 250 g	root one-g	*M. phaseolina*	*F. solani*	*R. solani*		
Control	74	4920 (3.69)	154 (2.18)	33	77	55	14.4	1.9
KB broth	68	4840 (3.67)	117 (2.05)	22	22	55	19.0	2.8
Cell suspension	46	3540 (3.53)	71 (1.85)	11	22	11	23.2	7.0
C.F. (N)	59	3680 (3.55)	104 (2.00)	0	11	22	22.6	2.5
C.F. (N/10)	68	4280 (3.61)	117 (2.05)	11	22	11	20.1	2.1
C.F. (N/100)	69	4660 (3.66)	113 (2.01)	33	22	11	23.3	4.2
C.F. (N/1000)	67	4500 (3.63)	128 (2.10)	22	28	11	19.5	1.8
SED	6	(0.11)	(0.05)	23	16	18	2.1	0.9
Significance level (p)	<0.05	N.S.	N.S.	N.S.	<0.05	N.S.	<0.05	<0.001

Values in the brackets are transformed to log10 x+1.

N.S. non-significant.

A complete suppression of *M. phaseolina* infection was observed following application of a pure culture filtrate. Similarly *M. phaseolina* infection was inhibited by more than 66% when aqueous cell suspension or N/10 dilution of the culture filtrate was used. All the treatments reduced *F. solani* infection by more than 63% as compared to untreated controls or KB broth. *R. solani* infection was suppressed by more than 80% following application of aqueous cell suspension or cell-free culture filtrate (Table 2).

Aqueous cell suspension and cell-free culture filtrate of the bacterium enhanced plant height ($p<0.05$) and fresh weight of shoot ($p<0.001$) with maximum plant height was stated in the treatment where N/100 dilution of culture filtrate was used. Maximum fresh weight of shoot was observed in the treatment where aqueous cell suspension was used (Table 2).

DISCUSSION

In the present study, aqueous cell suspension and the cell-free culture filtrate of *P. aeruginosa* showed complete inactivation of eggs of *M. javanica* and inhibited radial growth of soilborne root-infecting fungi viz., *M. phaseolina, F. solani* and *R. solani* producing zones of inhibition *in vitro*. It is interesting to note that cell-free culture filtrate of *P. aeruginosa* at all the concentration levels resulted in the lyses of *F. solani* hyphae. There are reports where the pseudomonads produce chelators that make iron unavailable to other microorganisms. Hubbard et al., (1983), observed lyses in germlings from spores of *Trichoderma hamatum* by this lack of iron.

Under greenhouse conditions, *P. aeruginosa* significantly reduced population densities of *M. javanica* in soil and root and consequent root-knot disease in tomato. Rhizosphere bacteria have been reported to produce nematicidal compounds that affected the vitality of second stage juveniles of *M. incognita* (Becker et al., 1988), inhibited egg hatching (Westcott et al., 1993) or by the release of repellent reduced nematode penetration to its hosts (Oostendorp, Sikora, 1989). It is interesting to note that *P. aeruginosa* also effectively controlled root infection caused by soilborne root-infecting fungi. *Pseudomonas fluorescens* and *P. putida* which colonize roots of a wide range of crop plants are reported to be antagonistic to soilborne plant pathogens like *Rhizoctonia solani, Pythium ultimum, P. aphanidermatum* and *P. debaryanum* (Suslow, Schroth, 1982). Likewise root treated with *Pseudomonas* sp., strain PsJN enhanced *Verticillium* wilt resistance in tomato (Sharma, Nowak, 1998).

In the present study, aqueous cell suspension of *P. aeruginosa* showed better biocontrol and growth promoting effects as compared to the cell-free culture filtrate of the bacterium, indicating that the respective activity was of extracellular origin. It was also observed that the bacterium produced compounds in culture media that reduced egg-hatching activity of *M. javanica in vitro*. The O-antigenic chain of outer membrane lipopolysaccharides (LPS) from *Pseudomonas fluorescens* WCS417r and WCS374 appears to be responsible for induced systemic resistance (ISR) to *Fusarium* wilt in radish (Leeman et al., 1995). The ability of bacteria to envelop or bind to root

surface lectins possibly interfere with nematode-host-recognition and therefore, penetration (Oostendorp, Sikora, 1990). It is also speculated that compounds required for the growth promotion and suppression of deleterious microorganisms were not produced in sufficient quantity in culture media as produced by the bacterial cell suspension in the rhizosphere, or there might be a difference in the quality of compounds released in culture media and in the rhizosphere. In addition, the soil used in the present study was a sandy-loam. It is also possible that inhibitory compounds might have been adsorbed by soil colloidal particles and therefore could provide better control in sandy soils containing little organic matter.

In the present study, aqueous cell suspension and the cell-free culture filtrate significantly promoted growth of tomato seedlings, which could be due to direct or indirect mechanisms like synthesis of siderophore sequestering iron from the soil for plant use, solubilization of minerals including phosphorus, or the production of plant growth regulators (PGRs) such as auxins, cytokinins or ethylene synthesis inhibitors, which act directly on the plant itself and affect growth (Glick, 1994; Kloepper et al., 1986; Davison, 1988; Hussain, Vancura, 1970).

CONCLUSION

The results of the present study suggest that *Pseudomonas aeruginosa* has a great potential in the suppression of soilborne plant pathogens that may result in an increase in plant growth and yield.

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Wpływ zawiesiny komórek i filtratu kultury bez komórek *Pseudomonas aeruginosa* w ochronie przed zgnilizną korzeni i guzowatością – kompleksem chorób pomidora (*Lycopersicon esculentum* Mill.)

Streszczenie

W warunkach laboratoryjnych i szklarniowych była testowana bakteria ryzosferowa *Pseudomonas aeruginosa* szczep IE-6 oddziaływująca korzystnie na wzrost roślin, dla określenia antagonistycznej aktywności względem *Meloidogyne javanica* nicienia powodującego guzowatość korzeni oraz takich grzybów glebowych porażających korzenie jak *Macrophomina phaseolina*, *Fusarium solani* i *Rhizoctonia solani*. Filtraty kultur bez komórek bakterii powodowały znaczną redukcję wylęgania się z jaj larw *Meloidogyne javanica* i hamowały promienisty wzrost grzybów w warunkach *in vitro*. Filtraty kultur bez komórek powodowały także lizę strzępek

F. solani. W warunkach szklarniowych zanotowano znaczną redukcję gęstości populacji nicieni w glebie, a następnie rozwoju guzów na korzeniach powodowanych przez *M. javanica*, gdy do gleby wprowadzono wodną zawiesiną komórek lub filtrat bez komórek. Zwalczanie nicieni oraz zastosowanie bakterii ryzosferowej ograniczało porażenie korzeni także przez grzyby przeżywające w glebie oraz wspomagało wzrost siewek pomidora.