ROLE OF IRON IN RHIZOBACTERIA-MEDIATED SUPPRESSION OF ROOT-INFECTING FUNGI AND ROOT-KNOT NEMATODE IN TOMATO

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Summary. The comparative efficacy of *Pseudomonas aeruginosa* strain IE-6 and its streptomycin resistant strain IE-6 S^+ was assessed to cause suppression against root-infecting fungi including *Macrophomina phaseolina, Fusarium solani* and *Rhizoctonia solani* and the root-knot nematode *Meloidogyne javanica* in tomato. IE-6 and IE-6S^+ markedly suppressed nematode population densities in root and subsequent root-knot development. Strain IE-6S^+ significantly suppressed *R. solani* infection. Both the bacteria failed to suppress *M. phaseolina* and *F. solani*. Whereas bacterial populations declined in the rhizosphere and inner root tissues of tomato, the colonization pattern of the two bacteria was identical. When iron concentration in the soil was lowered by the addition of an iron chelator, suppression of *F. solani* and *R. solani* by strains IE-6 and IE-6S^+ was substantially increased. However, against *M. phaseolina* and *M. javanica*, efficacy of the two strains was uninfluenced by the addition of the iron chelator.

The vast majority of microorganisms produce siderophores (Winkelmann, 1991; Barton and Hemming, 1993), a diverse class of high-affinity iron chelating compounds produced in response to iron limitation, and exported from the cell where they chelate ferric iron. Ferric-siderophore complexes are recognized and bound by specific outer-membrane receptor proteins, and iron is transported into the cell where it becomes available for metabolic functions. Certain strains of rhizosphere bacteria can suppress plant diseases by inducing resistance responses in the plant through a process termed induced systemic resistance (ISR) (Alström, 1991; van Peer *et al.*, 1991; Wei *et al.*, 1991).

In a previous report, siderophores including pyrrolnitrin were present in the cell extracts from the two *Pseudomonas cepacia* strains whereas phenazine-1-carboxylic acid and pyocyanine, respectively, were produced by two strains of *P. aeruginosa* responsible for the antifungal activity against *Rhizoctonia solani*, the rice sheath blight pathogens (Rosales *et al.*, 1995). Likewise, *P. aeruginosa* strain 7NSK2 produced three siderophores under iron limitation, pyoverdin, pyochelin and salicylic acid, which induced systemic resistance against leaf infection by *Botrytis cinerea* on bean (De Meyer and Höfte, 1997).

The aims of the present study were to compare the efficacy of *P. aeruginosa* strain IE-6 and its spontaneous streptomycin resistant strain IE-6S^+ in the suppression of root-infecting fungi including *Macrophomina phaseolina, Fusarium solani* and *Rhizoctonia solani* and root-knot nematode, *Meloidogyne javanica* and to ascertain the possible relationship between iron availability and suppression of soil-borne root-infecting fungi and root-knot nematode by strains IE-6 and IE-6 S^+ in tomato.

MATERIALS AND METHODS

The experiment was designed to compare the efficacy of *P. aeruginosa* (Schroeter) Migula strain IE-6 and its spontaneous streptomycin resistant strain IE-6 S^+. Strains IE-6 grown in KMB and IE-6 S^+ multiplied in KMB amended with 50 µg/ml of streptomycine for 48 h at 25 °C with shaking at 200 rpm. Strains were centrifuged and resuspended in physiological saline (0.14 M NaCl). Soil used for the experiment was a sandy loam (pH 8.1, moisture holding capacity of 40%) obtained from the experimental field of the Department of Botany, University of Karachi and put into 8 cm diam plastic pots at 350 g/pot. The soil had a natural population of 3-9 sclerotia/g of soil of *M. phaseolina* (Tassi) Goid., as estimated by a wet sieving and dilution technique (Sheikh and Ghaffar, 1975); 5% colonization of *R. solani* Kuehn on sorghum seeds used as bait (Wilhelm, 1955); and 3000 cfu g^{-1} of soil of mixed population of *Fusarium* spp., as estimated by soil dilution technique (Nash and Snyder, 1962). Soil was excavated to a depth of 2 cm and 25 ml aqueous cell suspension of IE-6 and IE-6 S^+ containing 1.9×10^8 and 2.2×10^8 cfu/ml respectively, or saline (control) were drenched before three tomato (*Lycopersicon esculentum* Mill.) seedlings cv. SUN (6002) PVP were planted in each pot. One week after the seedlings were transplanted, roots were infested with 2000 freshly hatched juveniles of *M. javanica* (Treub.) Chitw. Treatments and control were replicated four times and arranged on a glasshouse bench. The experiment was terminated 44 days after the addition of nematodes and plant growth parameters, including plant height and fresh weight of shoot, were recorded. The numbers of galls induced by *M. javanica* on the entire root system were counted under low magnification (x6). To determine nematode penetration, fresh

roots were cut into small segments, reweighed, wrapped in a muslin cloth and dipped for 3-5 min in boiling 0.25% acid fuchsin with lactic acid. Roots were washed in running tap water to remove the excess stain and comminuted in an electric grinder for 45 seconds. The homogenate was suspended in 100 ml water and *M. javanica* females and juveniles in five samples of 5 ml were counted with the aid of a low power microscope (x6). To determine the incidence of fungi, 3 mm-long root pieces from the tap root, surface disinfested with 1% $Ca(OCl)_2$ for three min, were plated on potato dextrose agar plates (5 pieces/plate) supplemented with penicillin (100,000 units/l) and streptomycin sulfate (0.2 g/l) to avoid bacterial contamination. After five days incubation at 28 °C, incidence of root-infecting fungi was recorded. Infection % was calculated as follows: Infection % = (Number of plants infected by a fungus/Total number of plants) x 100.

An experiment was conducted to determine the colonization pattern of the two bacteria in the rhizosphere and inner root tissues of tomato. The experimental design was a 3 x 4 factorial arranged in a randomized complete block design. The factors were three levels of bacterial treatments (untreated control, IE-6 and IE-6S$^+$) and five sampling time (0, 7, 14, 21 and 28 days after planting). The bacteria and nematodes were introduced into the soil in the same manner as mentioned earlier. Treatments and control were replicated four times. Bacteria were re-isolated from the rhizosphere and inner tissues of the roots by the modified method of Pillay and Nowak (1997). Rhizosphere populations of *P. aeruginosa* were isolated by putting root samples with adhering soil into a 250 ml Erlenmeyer flask containing 10 ml of 0.1M $MgSO_4$ solution (pH 6.5) plus 0.02% Tween-20. Ten-fold serial dilutions of the suspension were prepared and 50 µl aliquots from the appropriate dilutions were plated on to KMB for IE-6 and KMB supplemented with streptomycin for IE-6S$^+$. For the assessment of endophytic populations of bacteria, fresh roots were surface disinfected with 1% $Ca(OCl)_2$ for 1 min, rinsed twice with sterile distilled water, submerged for 30 s in 15% H_2O_2 and rinsed twice again in sterile distilled water. The tissue was then comminuted in 10 ml of 0.1M $MgSO_4$ solution (buffered to pH 6.5) with 0.02% Tween-20. Ten-fold serial dilutions of the suspension were prepared and 50 µl aliquots from the appropriate dilution were plated onto KMB for IE-6 and KMB supplemented with streptomycin for IE-6S$^+$. The plates were incubated at room temperature (25±3 °C) for 48 h and the number of cfu recorded. Plants grown in soil not inoculated with *P. aeruginosa* was also examined for the presence of fluorescent pseudomonads. Since *P. aeruginosa* was not isolated from the untreated plants, it was not included in the statistical analysis.

Another experiment was designed to determine if lowered iron concentrations in the soil influenced biological control activity of the two bacteria. Tomato plants were treated biweekly with 10 µM ethylenediamine di(o-hydroxyphenylacetic acid) (EDDHA) solution as a soil drench. Fe-EDDHA has a formation constant of K 33.9 and decreases iron available to bacteria in soil (Press *et al.*, 2001). The experiment was a 3 x 2 x 2 factorial arranged as a randomized complete block design. The factors include three levels of treatments (untreated control, IE-6 or IE-6S$^+$), two levels of EDDHA treatment (0 and 10 µM) and two levels of sampling time (22 and 44 days after nematode addition). The bacteria and nematode were inoculated in the same way as described above. Each treatment and control was replicated four times. At each sampling time, incidence of the root-infecting fungi and nematode penetration rates were determined as described previously.

The data were subjected to analysis of variance (ANOVA) or factorial analysis of variance (FANOVA) in accordance with Sokal and Rohlf (1995). Populations of *P. aeruginosa* were transformed to $\log_{10}$ (x+1) before analysis.

RESULTS AND DISCUSSION

A comparative evaluation of the efficacy of strain IE-6 and its spontaneous streptomycin resistant strain IE-6S$^+$ revealed that both strains significantly increased plant height ($p<0.01$) and fresh weight of shoot ($p<0.05$) compared with the controls (Table I). However, bacterial strains did not show significant difference in this respect. Galling intensity ($p<0.001$) and the nematode population in the root ($p<0.05$) were significantly reduced by both the strains of *P. aeruginosa*.

Table I. Comparative efficacy of *Pseudomonas aeruginosa* strain IE-6 and its streptomycin resistant strain IE-6 S$^+$ on root-knot nematode and root-infecting fungi and growth of tomato.

Treatments	Plant height (cm)	Shoot weight (g)	Galls/ root system	Nematode population per g root	*M. phaseolina*	*F. solani*	*R. solani*
					Infection %		
Control	13.6	1.2	73	111	46	92	37
IE-6	16.8	1.8	45	65	39	54	16
IE-6 S$^+$	16.9	1.4	52	69	37	62	8
$LSD_{0.05}$	1.5	0.4	9	27	43	42	27

Among the three root-infecting fungi, only *R. solani* infection was significantly ($p<0.05$) suppressed following application of strain IE-6S$^+$.

The population sizes of the strains IE-6 and IE-6S$^+$ were compared in order to determine if lack of suppression of the root-infecting fungi and root-knot nematodes could be attributed to a reduction in root population size of the bacterial strains. Rhizosphere and inner root population sizes of IE-6 and IE-6S$^+$ did not differ significantly at any of the sampling periods (Table II). However, it is noteworthy that bacterial populations (rhizosphere and inner root) declined significantly ($p<0.001$) with time. Rhizosphere population of strains IE-6 declined from 7.59 (day 0) to 5.19 log cfu (day 28) and that of IE-6S$^+$ from 7.35 to 5.06 log cfu. Likewise, the inner root population of IE-6 declined from 4.98 (day 7) to 3.40 log cfu (day 28) while that of IE-6S$^+$ from 4.95 to 3.36 log cfu.

To assess the role of iron concentration on strains IE-6 and IE-6S$^+$ mediated suppression of two groups of plant pathogens (fungi and root-knot nematodes), iron concentration of the soil was allowed to decrease with the addition of an iron chelator (EDDHA). Whereas the application of EDDHA alone did not significantly suppress either of the pathogens over the controls at any sampling time, when applied together with either of the bacterial strains the nematode penetration and fungal colonization were both markedly suppressed (P at the most 0.05) over the controls, although the suppressive effect of the combination against root-knot nematode and *M. phaseolina* was not different from that of the bacteria alone (Table III).

Table II. Comparative efficacy of *P. aeruginosa* strain IE-6 and its streptomycin resistant strain IE-6S$^+$ on rhizosphere and inner root colonization in tomato.

Treatments	Bacterial colonization $\log_{10}(x+1)$									
	Rhizosphere					Inner root tissue				
	Sampling time (days)									
	0	7	14	21	28	0	7	14	21	28
IE-6	7.59	6.69	6.50	5.52	5.19	0	4.98	4.95	4.10	3.40
IE-6 S$^+$	7.35	6.83	6.54	5.44	5.06	0	4.95	4.91	4.06	3.36
$LSD_{0.05}$:										
Strains			0.14					0.10		
Time			0.23					0.16		

Table III. Effect of EDDHA on the efficacy of *P. aeruginosa* strain IE-6 and strain IE-6S$^+$.

Treatments	Nematode population per g root		*M. phaseolina*		*F. solani*		*R. solani*	
	Infection %							
	Sampling time (days)							
	22	44	22	44	22	44	22	44
Control	45	103	66	75	83	92	41	58
EDDHA	43	88	66	66	75	83	58	66
IE-6	23	56	50	50	50	58	25	50
EDDHA + IE-6	27	60	33	41	33	33	17	25
IE-6 S$^+$	28	53	33	58	41	50	25	41
EDDHA + IE-6 S$^+$	30	48	33	50	17	25	8	17
$LSD_{0.05}$:								
Bacterial application	6		12		13		11	
EDDHA	7		15		15		13	
Sampling time	6		12		13		11	

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