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Combined application of endophytic *Fusarium solani* and *Pseudomonas aeruginosa* for the suppression of *Meloidogyne javanica* in tomato

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Summary. The impact of two application rates of *Fusarium solani* strain Fs5 (3.5×10^6 and 2.2×10^7 cfu ml^{-1}) and *Pseudomonas aeruginosa* strain IE-6S$^+$ (2.8×10^7 and 1.3×10^8 cfu ml^{-1}) on various population densities of *Meloidogyne javanica* in tomato was tested in pots under greenhouse conditions. Whilst combined applications of *F. solani* and *P. aeruginosa* caused a marked suppression of root knot 6 and 12 weeks after nematode addition, *F. solani* alone failed to inhibit root-knot infection after 6 weeks, and *P. aeruginosa* alone was ineffective after 12 weeks. Both biocontrol agents were frequently isolated from the inner root tissues of tomato without any detrimental effect on plant growth. High inoculum levels of *M. javanica* not only increased root knot but also promoted inner root colonization by *F. solani* and *P. aeruginosa*. Inner root colonization by *F. solani* was greatly reduced when high inoculum levels of the bacterium and low application rates of the fungus were used together. Likewise, *P. aeruginosa* at low inoculum levels in the presence of high dosages of *F. solani* led to the complete absence of the bacterium from the inner root tissues. *F. solani* parasitized eggs and females of *M. javanica* but *P. aeruginosa* did not. Egg parasitism increased with increasing fungal inoculum levels. Six weeks after nematode inoculation, *F. solani* at the low inoculum level and *P. aeruginosa* at both dosages greatly reduced fungal egg parasitism rates, whereas 12 weeks after nematode inoculation, both organisms used together promoted fungal parasitism. Under field conditions at Karachi and Gharo, combined application of *P. aeruginosa* and *F. solani* caused greater suppression of root-knot development due to *M. javanica* than either antagonist alone. Inner root colonization by *F. solani* and *P. aeruginosa* was higher in Karachi than in Gharo. When the microorganisms were applied alone, egg parasitism rates by *F. solani* were similar in both localities, but when they were applied together, the egg parasitism rate was slightly higher in Karachi than in Gharo.

Key words: biological control, tomato, root-knot nematode, endophytes.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are economically important pests that cause severe damage to a wide variety of crops, in particular tomato. Various techniques, including crop rotation, the planting of resistant cultivars, and nematicide application have been used to combat these nematodes but yield losses persist. Use of nematicides is limited for safety and economic reasons, resistance can be affected by shifts in nematode races, and crop rotation is often effective but may be restricted by the need to select for certain crops, by the increase of other nematode populations, and by the ability of the eggs to remain dormant for years (Rodríguez-Kábana, 1992; Young, 1992; Meyer *et al*., 1998). There is, therefore, growing interest in alternative control approaches for use in in-

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tegrated pest management programs. Existing management procedures could be enhanced by the development of biocontrol strategies.

Biocontrol strategies generally involve the application of an agent that is antagonistic to a single pathogen. This often results in failure because the agent's activity varies with the soil environment and may not perform as well against other pathogens attacking the host. One way to control a wide range of pathogens and to achieve better control of a single pathogen is to employ mixtures of antagonists with superior biocontrol potential. Among several possible strategies, a mixture of antagonists with different mechanisms of disease control is appealing and often successful (Raupach and Kloepper, 1998). Under natural conditions, too, the most common form of biocontrol is with a mixture of agents rather than a single antagonist. Mixtures of fungi and rhizobacteria have generally given promising results (Pierson and Weller, 1994; Siddiqui *et al.,* 2000).

Despite being successful against a variety of pathogens, only a limited number of compatible and effective mixtures of biocontrol agents have been discovered, and this presumably for several reasons. Most mixtures have no benefit, or are even detrimental to biocontrol activity (Dandurand and Knudsen, 1993). A mixture that is effective under one set of conditions or on one particular host may have a negative effect under another set of conditions or on a different host. From an economic viewpoint, a mixed biocontrol product may be disadvantageous, because its mass production is likely to be more expensive than that of a single agent (Raupach and Kloepper, 1998). Nevertheless, greater emphasis should be placed on developing mixtures of biocontrol agents because they may achieve better plant colonization, are better adapted to changing environmental conditions over a growing season, present a larger number of pathogen-suppressive mechanisms, and/or protect against a broader range of pathogens (Backman *et al*., 1997).

The particular stage in the nematode life cycle that is attacked by the control agent has a profound effect on the nematode damage caused to the crop and on the amount of population control achieved. Trapping fungi and rhizosphere bacteria that attack only second-stage juveniles of cyst and root-knot nematodes may improve crop growth but is unlikely to prevent nematode populations from increasing, especially those belonging to species that produce more than one generation in a growing season. In contrast, parasites that attack developing females and eggs act like a partially resistant cultivar in that initial nematode invasion and plant damage is not prevented but later multiplication of the nematode is significantly reduced. Root colonizing fungi such as the mycorrhizae, and endophytic species such as *Fusarium* spp., reduce both the invasion and the development of nematodes (Kerry, 1997).

The present paper investigates the potential of *Fusarium solani* and *Pseudomonas aeruginosa* at two inoculum levels, singly and in combination, against two different population densities of *Meloidogyne javanica*, and examines infection levels of subsequent generations of nematodes on tomato under greenhouse conditions. The effectiveness of the two biocontrol agents singly and in mixture was also evaluated under different field conditions.

Materials and methods

Organisms

The biocontrol agents used were *F. solani* strain Fs5, originally isolated from a female of *M. javanica* and the spontaneous streptomycin-resistant strain IE-6 S^+ of *P. aeruginosa*. The wild-type strain IE-6 was originally isolated from the rhizosphere of sunflower where it produced significant suppression of root-infecting fungi and root-knot nematodes (Siddiqui *et al*., 2001). Strain Fs5 of *F. solani,* which is also antagonistic to *M. javanica* in tomato (Amer-Zareen *et al*., 2001), was maintained on potato dextrose agar (PDA) (Difco, Detroit, MI, USA), and *P. aeruginosa* on King's B medium (KB) (King *et al*., 1954) at 28°C.

Greenhouse experiments

Unsterilized sandy-loam soil of pH 8.1 (organic matter 0.3%, water holding capacity 37.8%) was obtained from the experimental field of the Crop Disease Research Institute (CDRI, Karachi, Pakistan) and screened through a 2-mm mesh sieve to discard pebbles and gravel. The soil was autoclaved and placed in 8-cm-diam. plastic pots at 350 g pot^{-1}.

The effectiveness of high and low inoculum levels of *F. solani* and of *P. aeruginosa* individually or in combination, against high and low population densities of *M. javanica* was evaluated at two time

intervals. This experiment was planned as a 3×3×3×2 factorial design with four replicates for each harvest (a total of eight pots per treatment). The factors comprised 3 inoculum concentrations of *F. solani* (0, 10^6 or 10^7 cfu ml^{-1}); 3 inoculum concentrations of *P. aeruginosa* (0, 10^7 or 10^8 cfu ml^{-1}); 3 population densities of *M. javanica* (0, 500 or 2000 juveniles/plant); and two sampling dates (6 weeks and 12 weeks after inoculation). The upper soil was removed to a depth of 3 cm and a 25-ml conidial suspension of *F. solani* in sterile distilled water containing 3.5×10^6 or 2.2×10^7 (cfu ml^{-1}) was deposited in each pot. Other pots received a 25-ml cell suspension of *P. aeruginosa* containing either 2.8×10^7 or 1.3×10^8 (cfu ml^{-1}). In a third set of pots *F. solani* and *P. aeruginosa* were applied together (at both concentrations); in this case the amount of each was reduced by half, *i.e.* to 12.5 ml of each organism. Pots not receiving any organisms served as controls. After treatment the previously removed soil was replaced. Each pot was planted with two three-week-old tomato seedlings, which were allowed to become established for one week, when they were inoculated with 0, 500 or 2000 freshly hatched *M. javanica* juveniles. The pots were fertilized twice a month with urea at 0.10 g kg^{-1} of soil. The plants from four pots per treatment were harvested 6 weeks after nematode inoculation, those from the remaining four pots after a further 6 weeks (12 weeks after nematode inoculation).

Plant growth parameters such as plant height, root length and fresh weight of the shoots and roots were recorded. The galls produced by *M. javanica* on the root system were counted under low magnification (×6). The root system in each set of pots was chopped into small segments, divided into three equal portions and kept in a refrigerator prior to use. To determine nematode invasion of the root, one of the root portions was blotted dry, reweighed, wrapped in muslin cloth and dipped for 3–4 min. in boiled 0.25% acid fuchsin with lactic acid. It was then washed in running tap water to remove excess stain and cooled in vials containing 1:1 glycerol:water plus a few drops of lactic acid. Roots were macerated in an electric grinder for 45 s. The macerate was suspended in 100 ml water and invaded females of *M. javanica* counted in 5 samples of 5 ml each with the aid of a low-power microscope (×6). To determine fungal parasitism, ten egg-masses per treatment were randomly selected. Each egg-mass was crushed in a drop of 0.01% sodium hypochlorite solution to dissolve the gelatinous matrix. Eggs were washed three times in distilled water, dispersed in 3 ml water and 0.5 ml of each suspension was plated onto 0.8% water agar. The dishes were incubated at room temperature (27±3°C). After 3 days, 100–200 eggs on each dish were examined for growth of fungal hyphae from the eggs. Ten females were hand-picked, surface sterilized with 0.5% $Ca(OCl)_2$, washed thoroughly and plated onto 0.8% water agar to observe parasite invasion.

To assess colonization of *F. solani* in tomato roots, 5-mm-long root-pieces (five pieces per plate) from one of the stored samples were surface-sterilized with 1% $Ca(OCl)_2$ for three minutes and placed on PDA supplemented with penicillin (100,000 units l^{-1}) and streptomycin sulphate (0.2 g l^{-1}). After incubation for 5 days at 28°C, the colonization percentage was calculated as follows:

$$\text{Colonization (\%)} = \frac{\text{No. of root pieces colonized by } \textit{F. solani}}{\text{Total No. of root pieces}} \times 100$$

To determine the endophytic populations of *P. aeruginosa*, the remaining root samples with adhering soil were reweighed, washed to eliminate the soil, surface sterilized with 1% Ca $(OCl)_2$ for 1 min and rinsed twice with sterile distilled water. The sterilized roots were submerged for 30 s in 15% H_2O_2 and again rinsed twice in sterile distilled water. The tissue was then macerated in 10 ml of 0.1 M $MgSO_4$ solution (buffered to pH 6.5) with 0.02% Tween-20. Ten-fold serial dilutions of the suspension were prepared and 100-µl aliquots from the appropriate dilution were plated onto KB medium amended with 100 µl streptomycin sulphate. The plates (9 cm diam.) were incubated at room temperature (25±3°C) for 48 h and the cfus were counted (Pillay and Nowak, 1997). Plants grown in soil not inoculated with *P. aeruginosa* were also examined for fluorescent pseudomonads. The colonies of streptomycin-resistant *P. aeruginosa* that appeared on the plates were compared with the culture of IE-6S$^+$ with regard to morphological and biochemical characteristics.

Field trials

Field trials were conducted in 2×1 m microplots at two different sites, one at the experimental field

of the CDRI in Karachi and the other at Gharo, about 40 miles east of Karachi city. The soil characteristics of the two sites are given in Table 1.

After removing the soil to a depth of 5 cm, a conidial suspension of either Fs5 yielding 1.3×10^7 cfu ml^{-1} or of IE-6S$^+$ containing 2.1×10^8 cfu ml^{-1} was deposited in a furrow at a rate of 300-ml m^{-1}. In other plots, the soil was drenched with a mixture of both the *P. aeruginosa* and the *F. solani* strain (150+150 ml l^{-1} m^{-1} furrow). Soil drenched with 300-ml l^{-1} m^{-1} sterile distilled water served as control. After drenching, the removed soil was replaced and six three-week-old tomato seedlings were planted. One week after transplantation, these seedlings were inoculated with 1000 freshly hatched *M. javanica* second-stage juveniles. The juveniles were suspended in 25-ml water and poured into three holes around the tomato roots. Each treatment and control was replicated three times. The microplots were arranged in a randomized complete block design.

The plants were watered at two-day intervals and harvested 60 days after nematode inoculation. The data recorded were: plant height, f wt of shoots and roots, number of galls and egg masses produced on the root system. Reisolation of Fs5 from females and eggs was performed as above.

Statistical analysis

Data were analyzed using factorial analysis of variance (FANOVA) employing the Statistica 5.0 package, StatSoft, Inc. (1995, Tulsa, OK, USA). Treatment means were compared using the least significant differences test (Sokal and Rohlf, 1995). The data of bacterial colonization were transformed to $\log_{10}$ x+1 prior to analysis.

Table 1. Soil characteristics of the two locations (Karachi and Gharo) where the field experiments were conducted.

Soil characteristic	Location	
	Karachi	Gharo
Maximum water holding capacity %	35.7	26.2
Moisture content of soil % (November)	8.6	8.3
pH	8.1	7.8
Organic matter %	0.5	0.2
K^+ ppm	24	17
$CaCO_3$ %	10.1	12.4

Results

Green-house experiment

Six weeks after nematode inoculation, in soils infested with 500 *M. javanica* juveniles/pot, *F. solani* applied alone at either dosage failed to control root-knot development from *M. javanica*, while *P. aeruginosa* used alone, or in conjunction with *F. solani* (at either dosage), significantly reduced galling intensity ($P<0.05$) (Table 2). By contrast, in pots with 2000 juveniles, *F. solani* alone at high level, or *P. aeruginosa* at either dosage alone or in combination with *F. solani*, caused significant suppression of root-knot development ($P<0.05$). At 12 weeks, *F. solani* applied alone at either concentration, or *P. aeruginosa* at the high concentration, or both agents combined at all concentrations significantly reduced galling intensity in soils with 500 juveniles/pot. At 2000 juveniles/pot, both *F. solani* and *P. aeruginosa* alone, and also the two in combination, greatly reduced galling rates. *P. aeruginosa* was more effective in suppressing galls than *F. solani* at 6 weeks, but the reverse was true at 12 weeks. The combined application of *F. solani* and *P. aeruginosa*, particularly at the higher concentrations, was considerably more effective than the application of each singly, at both sampling dates. Not unexpectedly, nematode invasion levels closely followed the pattern of galling intensity.

At both sampling dates (6 and 12 weeks after nematode inoculation), the higher concentration of *F. solani* plus the higher nematode population density resulted in increased fungal colonization of the inner root tissues of tomato (Table 3). At 6 weeks, *P. aeruginosa* at the higher concentration in the presence of the lower concentration of *F. solani* reduced fungal inner root colonization, while both these organisms at their higher dosage enhanced the endophytic colonization capability of the fungus. By contrast, at 12 weeks after nematode inoculation, the bacterium at both concentrations greatly curtailed the internal root colonization of *F. solani*, regardless of nematode density.

At both sampling dates, *P. aeruginosa* and *F. solani* used together at both dosages strongly reduced bacterial inner-root colonization (Table 3). *P. aeruginosa* at low dosage but with a high dosage of *F. solani* resulted in complete absence of the bacterium from the inner root tissues. *P. aeruginosa* levels in the inner root tissues declined ten-fold at 12 weeks compared with its levels at 6 weeks.

Table 2. Number of galls per root system and number of juveniles g^{-1} of root extracted from tomato roots harvested 6 and 12 weeks after inoculation with 0, 500 or 2000 *Meloidogyne javanica* juveniles per pot and treated with *Fusarium solani* at rates of 10^6 (FL) or 10^7 (FH) cfu ml^{-1} suspension, or with *Pseudomonas aeruginosa* at rates of 10^7 (PL) or 10^8 (PH) cfu ml^{-1} suspension, or with combinations of the two (FL+PL; FL+PH; FH+PL; FH+PH), or left untreated (n=4).

F. solani and/or *P. aeruginosa* treatment	*M. javanica* galls/root system						*M. javanica* juveniles/g root					
	6 weeks			12 weeks			6 weeks			12 weeks		
	0	500	2000	0	500	2000	0	500	2000	0	500	2000
Control	0	33	127	0	68	182	0	38	181	0	77	279
FL	0	29	119	0	42	131	0	44	208	0	56	191
FH	0	32	104	0	30	117	0	33	167	0	39	152
PL	0	20	94	0	61	162	0	29	146	0	71	259
PH	0	18	81	0	50	140	0	26	138	0	64	213
FL+PL	0	20	109	0	36	128	0	30	173	0	60	186
FL+PH	0	16	78	0	37	118	0	25	157	0	53	174
FH+PL	0	22	89	0	48	104	0	30	141	0	55	161
FH+PH	0	16	78	0	35	95	0	17	119	0	34	142
$LSD_{0.05}$												
F. solani			7.5						6.6			
P. aeruginosa			7.5						6.6			
M. javanica			7.5						6.6			
Time			6.3						5.8			

Table 3. Inner root colonization by *Pseudomonas aeruginosa* and *Fusarium solani* and fungal infection in *Meloidogyne javanica* females and eggs extracted from tomato roots harvested 6 and 12 weeks after inoculation with 0, 500 or 2000 *M. javanica* juveniles per pot and treated with *F. solani* at rates of 10^6 (FL) or 10^7 (FH) cfu ml^{-1} suspension, or with *P. aeruginosa* at rates of 10^7 (PL) or 10^8 (PH) cfu ml^{-1} suspension, or with combinations of the two (FL+PL; FL+PH; FH+PL; FH +PH), or left untreated (n=4).

Treatment	*F. solani* colonization (%)						*P. aeruginosa* colonization [(log cfu g^{-1} f wt)+1]					
	6 weeks			12 weeks			6 weeks			12 weeks		
	0	500	2000	0	500	2000	0	500	2000	0	500	2000
Control	0	0	0	0	0	0	0	0	0	0	0	0
FL	18.7	37.5	46.6	21.8	46.8	46.8	0	0	0	0	0	0
FH	25	25	40.6	40.6	50	59.3	0	0	0	0	0	0
PL	0	0	0	0	0	0	3.2	3.2	3.4	2	2.3	2.4
PH	0	0	0	0	0	0	3.9	3.9	4	2.3	2.5	2.7
FL+PL	12.5	12.5	21.8	18.7	22.1	37.6	3.1	3.2	3.2	n.d.	2.1	n.d.
FL+PH	12.5	31.1	37.5	18.7	21.8	40.6	3.7	3.4	3.6	2.2	2.3	2.4
FH+PL	24.8	28.1	43.7	37.5	37.5	46.8	2.2	2.4	2.5	n.d.	n.d.	n.d.
FH+PH	25.2	34.3	50	22.1	46.8	46.8	2.5	3.1	3.0	n.d.	2	2
$LSD_{0.05}$												
F. solani			4.7						0.2			
P. aeruginosa			4.7						0.2			
M. javanica			4.7						0.2			
Time			4						0.2			

n.d., not detected.

In general, at both sampling dates, an increase in the fungal inoculum level and in the population density of *M. javanica* was accompanied by an increase in the female parasitism rate by *F. solani* (Table 4). At 6 weeks, *F. solani* at the lower concentration combined with either high or low levels of *P. aeruginosa* enhanced female parasitism, while the fungus combined with the higher concentration of *P. aeruginosa* did not affect the rate of fungal egg parasitism. At 12 weeks, when nematode levels were low, *F. solani* at low concentration in the presence of the low level of *P. aeruginosa* enhanced the female parasitism rate, while the higher level of the bacterium inhibited egg parasitism, whereas when nematode infestation was high, the lower level of *F. solani* with either level of *P. aeruginosa* enhanced fungal parasitism. At low nematode infestation, *F. solani* at the higher concentration with either dosage of *P. aeruginosa* reduced the female parasitism rate, while at high nematode infestation, *F. solani* at the higher concentration with the lower concentration of *P. aeruginosa* reduced parasitism, but with the higher concentration of *P. aeruginosa* increased it.

In general, percent egg infection increased with increasing fungal concentration. At 6 weeks, in the presence of low nematode populations, fewer eggs were infected when the lower level of *F. solani* was applied alone (Table 4), while at higher nematode infestation, *F. solani* at low concentration combined with *P. aeruginosa* at either application level reduced the egg parasitism rate by *F. solani*. At 12 weeks, when population densities of *M. javanica* were low, egg parasitism by the fungus increased when *F. solani* at either dosage was combined with the lower concentration of *P. aeruginosa*, while the higher concentration of the bacterium decreased egg parasitism. In the presence of high nematode densities, egg parasitism by the fungus was higher when *P. aeruginosa* and *F. solani* were applied together.

Field experiment

At both the Karachi and the Gharo fields, the application of *F. solani* and *P. aeruginosa* individually or in combination significantly ($P<0.05$) suppressed root-knot development (Table 5). At both sides the combined application of the two organ-

Table 4. *Fusarium solani* infection in *Meloidogyne javanica* females and eggs extracted from tomato roots 6 or 12 weeks after inoculation with 500 or 2000 *M. javanica* juveniles per pot and treated with *F. solani* at rates of 10^6 (FL) or 10^7 (FH) cfu ml^{-1} suspension, or with *Pseudomonas aeruginosa* at rates of 10^7 (PL) or 10^8 (PH) cfu ml^{-1} suspension, with combinations of the two (FL+PL; FL+PH; FH+PL; FH +PH), or left untreated (n=4).

Treatment	Female infection (%)						Egg infection (%)					
	6 weeks			12 weeks			6 weeks			12 weeks		
	0	500	2000	0	500	2000	0	500	2000	0	500	2000
Control	0	0	0	0	0	0	0	0	0	0	0	0
Carbofuran	0	0	0	0	0	0	0	0	0	0	0	0
FL	0	3	10	0	15	18	0	1.4	10.6	0	11.4	23.4
FH	0	10	18	0	28	30	0	0	20.4	0	11.4	29.9
PL	0	0	0	0	0	0	0	0	0	0	0	0
PH	0	0	0	0	0	0	0	0	0	0	0	0
FL+PL	0	13	18	0	23	23	0	0	8.3	0	20.9	26.6
FL+PH	0	5	10	0	10	23	0	0	3.9	0	8.7	28.4
FH+PL	0	10	10	0	18	28	0	0	20.5	0	18.5	30.1
FH+PH	0	10	18	0	20	35	0	0	17.6	0	9.1	28.6
$LSD_{0.05}$												
F. solani			1.6						0.8			
P. aeruginosa			1.6						0.8			
M. javanica			1.6						0.8			
Time			1.3						0.6			

Table 5. Effect of *Fusarium solani* (strain Fs5) alone or in combination with *Pseudomonas aeruginosa* (strain IE-6S$^+$) on the development of root-knot infection, nematode invasion, and root colonization by *F. solani*, and on female and egg parasitism by the fungus and growth of tomato plants under field conditions at Karachi (K) and Gharo (G).

Treatment	*M. javanica* galls/root system[a]		Nematode colonization by *F. solani* (%)[a]		*F. solani* parasitism (%)[a]				Plant height (cm)[a]		Shoot weight (g)[a]		Root weight (g)[a]	
					Female		Egg							
	K	G	K	G	K	G	K	G	K	G	K	G	K	G
Control	73	93	25	18	0	0	3	0	14.2	13.5	16.3	14.5	1.8	2.3
F. solani (Fs5)	59	68	43	32	18	10	21	18	16.8	14.9	18.4	14.9	1.4	1.8
P. aeruginosa (IE-6 S$^+$)	46	63	0	0	3	0	2	0	19.2	17.8	23.1	20.1	1.4	2
Fs5 + IE-6	43	56	57	29	23	17	25	20	18.6	18.1	17.9	19.8	1.5	1.8
$LSD_{0.05}$														
Treatments	11		9		12		14		4.3		3.4		0.8	
Localitions	9		6		7		8		3.1		2.7		0.5	

[a] All surveys were carried out in two locations: K, Karachi, and G, Gharo.

isms caused greater suppression of the root-knot nematode than when only one was applied. However, the suppressive effect was not significantly different between application of the bacterium alone and that of the two antagonists together. Root knot was more severe in Gharo than in Karachi (P<0.05) whether or not the biocontrol agent(s) were present. Compared to the application of *F. solani* alone or to the untreated controls, combined treatment of *F. solani* and *P. aeruginosa* at Karachi resulted in significantly (P<0.01) greater colonization of the inner root tissues by the fungus. Similarly, at both locations, *F. solani* and *P. aeruginosa* applied together resulted in a greater inner root colonization than when *P. aeruginosa* was applied alone. However, inner root colonization by the bacterium was significantly (P<0.01) greater in Karachi than in Gharo (Fig. 1). Though the difference was not significant, the antagonists applied together caused greater female parasitism by *F. solani* than when each was applied alone at both locations, with a slightly higher parasitism rate in Karachi than in Gharo. A few females were also parasitized by *F. solani* when *P. aeruginosa* was applied alone in Karachi. Egg parasitism by *F. solani* was also greater in both locations when both organisms were applied together. With respect to egg parasitism, there was no marked difference between the two locations when *F. solani* was used alone, but when the two organisms were applied together egg parasitism was slightly higher in Karachi than in Gharo. *P. aeruginosa* either alone or in combination with *F. solani* caused a significant (P<0.05) increase in plant height over the untreated controls at both locations, whereas *P. aeruginosa* applied alone caused a significant (P<0.01) increase in the f wt of the shoot. Untreated plants had greater root weights than treated plants (Table 5).

Discussion

Whereas at 12 weeks after nematodes inoculation *F. solani* was more effective when large amounts of egg masses were developed, with increased chances of fungal infection, at the first sampling date the fungus failed to prevent the nematodes from invading the roots. *F. solani* should therefore be regarded as a facultative parasite of the root-knot nematode rather than a true parasite. Since opportunistic and ectoparasitic fungi do not produce structures for the capture of nematodes, the constant movement of nematodes hampers sustained contact and penetration of the fungi into live juveniles (Freire and Bridge, 1985). Females and eggs, which are sedentary stages of the nematode, are more easily penetrated by the fungus. The fungus has a parasitic phase (on root-

knot nematodes) as well as a saprophytic phase. In the saprophytic phase its growth and survival are dependent on nutrients other than those provided by the nematode host. Furthermore, the fungus is also an endophyte utilizing the nutrients being absorbed by the plant. In its parasitic phase the fungus infects the eggs laid by root-knot nematodes in egg masses produced on the root surface (Amer-Zareen *et al.,* 2001).

It is interesting to note that, when applied alone, *P. aeruginosa* was more effective in the suppression of root-knot nematode at the first sampling date, whereas *F. solani* afforded better protection at the second sampling. Therefore the fungus alone cannot be used to prevent initial root damage. However, the combined application of both biological control agents caused significant suppression of the root-knot nematode at both sampling dates. Since the establishment of the bacterium in the rhizosphere is a prerequisite for the control of plant pathogens, the failure of *P. aeruginosa* to suppress *M. javanica* at the second sampling date could be seen as the result of a depleted *P. aeruginosa* population in the rhizosphere at that time. Despite the reduction, the final population remained at a fairly high level, presumably because of the short duration of the colonization of the bacterium, resulting in delayed nematode hatching and eventual penetration. In addition, only a portion of the root and root tips was colonized with the bacterium and a certain amount of root penetration therefore always occurred. These results accord well with our previous finding that *P. aeruginosa* caused greater suppression of the root-infecting fungi and root-knot nematode in tomato and mungbean at the first sampling date than at the second (Siddiqui *et al.,* 2001). This interactive dynamic of *P. aeruginosa* and *M. javanica* explains why *F. solani* was more effective at the second sampling date. Strain Fs5 of *F. solani* used in this study is a facultative parasite on eggs and females of *M. javanica* (Amer-Zareen *et al.,* 2001), but the juveniles, because they are mobile, escape fungal attack. It is likely that due to the prevalence of *P. aeruginosa* in the rhizosphere, fewer juveniles (females) migrated to the roots, producing fewer egg masses. This created an opportunity for the fungus to grow and multiply on and in the roots while not seriously attacking the nematode. However, after the first sampling date, more juveniles invaded the host producing more egg masses. It is likely that the fungus infected these egg masses, the aggregation of the eggs facilitating the spread of infection, and this reduced the reproductive potential of the females in subsequent generations, thus preventing severe root damage at the second sampling date.

Plant-parasitic nematodes, fungi and bacteria are common coinhabitants of the rhizosphere environment, interacting with each other in negative, synergistic, or positive ways, often by the release of toxic substances. Therefore, apart from parasitism by *F. solani* on the eggs and females of *M. javanica*, a possible role of toxic compounds in the suppression of the root-knot nematode must also be considered. *Fusarium* spp. are pathogens and endophytes that produce a wide variety of mycotoxins (Bacon and Hilton, 1996; Leslie *et al.,* 1996). Among the more significant toxins that are produced by *Fusarium* spp., and that are nematicidal in action, are T-2 toxin, zearalenone, moniliformin, and fusaric acid (Ciancio, 1995). Recently it has been found that fusaric acid is produced by all strains of *Fusarium* (Bacon *et al*., 1996). In addition to its ubiquity and potential toxicity, fusaric acid may also act synergistically with other toxins, including bacterial toxins, to elicit toxic responses in a variety of organisms (Smith *et al*., 1997; Smith and Seddon 1998). Rhizobacteria are also known to produce some toxic compounds inhibitory to plant-parasitic nematodes (Oostendorp and Sikora, 1990; Devidas and Rehberger, 1992).

It is interesting to note that at both sampling dates, *P. aeruginosa* and *F. solani* used together at both dosages markedly reduced bacterial inner root colonization. This inhibitory effect was even more pronounced at the higher concentration of *F. solani* and the lower concentration of *P. aeruginosa*. In a previous study, a negative correlation between *F. oxysporum* infection and bacterial inner root and shoot colonization was recorded in tomato (Siddiqui and Ehteshamul-Haque, 2000). In other studies, fusaric acid at concentrations as low as 0.12 $\mu g\, mg^{-1}$ repressed production by *Pseudomonas fluorescens* CHA0 of the antibiotic 2,4-diacetylphloroglucinol, a key factor in the biocontrol activity of the bacterium (Duffy and Defago, 1997).

In this study, *P. aeruginosa* (at high inoculum level) and *F. solani* (at low inoculum level) adversely affected fungal inner root colonization. The vast majority of microorganisms including certain plant-

associated bacteria, produce siderophores (Winkelmann, 1991; Barton and Hemming, 1993), a diverse class of high-affinity iron chelating compounds. This makes iron unavailable to other organisms so that they are unable to grow in these environments; germlings of many fungi lyse under these conditions. In some studies, siderophores produced by strains of *P. aeruginosa* were reported to display antifungal activity (Rosales *et al*., 1995; De Meyer and Höfte, 1997).

The ability of *P. aeruginosa* (by preventing initial root damage) and *F. solani* (for long-term nematode control by parasitizing eggs and females) to suppress root-knot nematode may contribute to the effectiveness of these organisms as biocontrol agents used in a mixture. But before application of either organism, their pathogenicity to human beings should be investigated. The results presented here indicate that mixtures of endophytic *F. solani* and *P. aeruginosa* can provide enhanced disease protection and improve the consistency of biological control.

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