EFFECT OF SEED BACTERIZATION AND METHODS OF APPLICATION OF *PSEUDOMONAS FLUORESCENS* ON THE CONTROL OF *ROTYLENCHULUS RENIFORMIS* INFECTING TOMATO

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Summary. A glasshouse study was conducted in which the efficacy of *Pseudomonas fluorescens* strain Pf_1 was evaluated as seed bacterization, soil drench, and bare root dip alone or seed bacterization followed by soil drench or bare root dip on tomato. Seed bacterization resulted in 90% germination compared to 81% in non-bacterized seeds. Seedling weight and shoot length were improved by 54 and 18%, respectively, 21 days after sowing. Root colonization by the rhizobacterium in terms of cfu/g and cm fresh root of the seedlings raised from bacterized seed increased with increasing observation time. Further, it was found that the strain Pf_1 when applied as seed bacterization, soil drench and bare root dip either singly or in combination caused a significant reduction in nematode penetration of *Rotylenchulus reniformis* compared to the untreated control. The maximum reduction of 55% of nematode penetration occurred when the bacterium was applied as seed bacterization followed by a soil drench. Application of the bacterium alone or in combination with the seed bacterization and soil drenching/root dip improved growth characters of the tomato plants, except root length. The multiplication rate (Pf/Pi) of the nematode was also significantly reduced in all of the treatments receiving bacteria with the maximum (45%) in the treatment that received seed bacterization followed by a soil drench.

The reniform (*Rotylenchulus reniformis*) and root-knot (*Meloidogyne* spp.) nematodes are considered to be important limiting factors in the production of tomato in India. To combat losses caused by *R. reniformis* several management strategies have been developed.

As well as the use of nematicides, the focus of researchers has shifted to the exploitation of rhizobacteria for nematode control. There have been several reports of reduced plant damage by nematodes using plant growth promoting rhizobacteria (PGPR) due to growth promotion and/or biological control (Becker *et al.*, 1988; Oostendorp and Sikora, 1989; Kloepper *et al.*, 1992; Racke and Sikora, 1992; Kluepfel *et al.*, 1993; Neip and Becker, 1999). Most of the work on PGPR as biocontrol agents has been done on root-knot and cyst nematodes (Tian and Riggs, 2000). The results of an *in vitro* study on the efficacy of *Pseudomonas fluorescens* in reducing hatching and mobility of *R. reniformis* have shown promise for biocontrol potential (Niknam and Dhawan, 2002).

In this study, information is presented on the efficacy of three application methods of the Pf_1 isolate against *R. reniformis* on tomato.

MATERIALS AND METHODS

Egg masses of *R. reniformis* Linford *et* Oliveira were hand-picked from the naturally infected roots of castor (*Ricinus communis* L.) plants and pure cultures of the nematode were cultured by inoculating a single egg mass in the rhizosphere of cowpea [*Vigna unguiculata* (L.) Walp.] plants (cv. Pusa Komal).

Pseudomonas fluorescens Migula, isolate Pf_1 originally isolated from the rhizosphere of tomato plants, was obtained from Tamil Nadu Agricultural University, Coimbatore. Re-culturing was done by streaking on King's B medium in Petri plates and incubated at 28 °C for 48 h. The process was repeated at least three times to obtain a pure culture. Thereafter, the pure cultures were maintained in test tube slants, stored at 4-5 °C in a refrigerator and subcultured at monthly intervals. The cell density in the suspension (cfu/ml) was estimated using a dilution plating technique (Cappucinno and Sherman, 1983).

Seeds of tomato, *Lycopersicon esculentum* Mill., cv. Pusa Ruby were surface-sterilized with 2.4% sodium hypochlorite (NaOCl) solution for 2-3 min, followed by rinsing in sterile distilled water and dried for 2 h in a sterile air stream (laminar flow). The bacterial inoculum to be used for seed bacterization was prepared by inoculating plates of King's B medium with a bacterial suspension of *P. fluorescens*. These were incubated at 28 °C for 2-3 h to stimulate bacterial growth. Plates were then injected at the centre with 0.2 ml of sterile streptomycin sulphate solution containing 100 µg of active ingredient per ml to obtain an antibiotic resistant mutant. These were again incubated at 28 °C for 48 h. The stability of the antibiotic resistance trait was confirmed for the bacterium by streaking three times on the medium (without antibiotic). Subsequently, a single colony of the bacterium was streaked on the medium containing 100 ppm streptomycin sulphate and incubated at 28 °C for 48 h to compare the growth of mutants with that of the wild types (original cultures).

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The bacterial growth of the mutants was scraped off from the agar surface with a sterile needle and mixed with 10 ml of a 0.1% (w/v) sterilized aqueous methyl cellulose solution in sterile distilled water as an adhesive and preservative. The surface sterilized seeds were exposed to the bacterium suspension (10^{10} cells/ml) for 30 min and then dried in a laminar flow for at least 3 h. Immediately after seed bacterization, cfu/seed were determined by washing ten seeds after dipping them in sterile distilled water using the dilution plating method. Final bacterial load obtained was approximately 10^8 cells/seed. Surface sterilized seeds treated with sterile methyl cellulose served as non-bacterized controls.

Bacterized and non-bacterized seeds were sown separately in a sterilized soil-sand mixture of 3:1 contained in 30 cm diam. earthen pans. Data on seedling germination were recorded daily up to 11 days. Total fresh weight, length of shoots, and roots of 20 randomly selected seedlings raised from both bacterized and non-bacterized seeds were recorded 21 days after sowing at the time of transplanting.

In addition, ten random seedlings, raised from bacterized seeds with streptomycin-resistant mutants, were carefully removed 7, 14 and 21 days after sowing. The bacterial populations on the roots were ascertained by cutting them into small segments of 1 cm length. One g of root segments was shaken vigorously in 10 ml sterile solution of 0.1 M $MgSO_4$ for 10 min to disperse the bacteria in the solution. The required dilution of isolate Pf_1 suspension was then streaked on KB medium containing 100 µg ml^{-1} streptomycin sulphate in Petri plates. These were incubated at 28 °C for 48 h and then bacterial populations of Pf_1 were determined by counting cfu using the dilution plating technique.

Also, the population of the mutant per cm length of the roots of ten seedlings raised from bacterized seeds with streptomycin-resistant mutants selected randomly was also estimated 21 days from sowing. Root segments of 1.0 cm length were cut from four regions (immediately near the seed, the main root, the main root tip and a secondary root) of each plant separately and weighed. Root lengths from each region were mixed separately and examined by shaking them vigorously in 10 ml of sterile solution of 0.1 M $MgSO_4$ for 10 min to disperse the bacteria into the $MgSO_4$ solution for estimation of cfu per cm using the dilution plating method.

The experiment comprised twelve treatments (Table III).

Seven seedlings of uniform size raised from bacterized and non-bacterized seeds were individually transplanted, 21 days after sowing, in 15 cm earthen pots containing a mixture of autoclaved soil and sand in the ratio of 3:1. The pots were kept randomly in a glasshouse. Again at transplanting time the following bacterial inoculation was done:

- at transplanting time roots of tomato seedlings, raised from bacterized seeds with streptomycin-resistant mutants, were immersed separately in a suspension of the isolate Pf_1 (10^{10} cells/ml) for 2 h each. Treated seedlings were immediately transplanted into 15 cm earthen pots containing 1 kg steam-sterilized soil-sand mixture. Roots of seedlings raised from non-bacterized seeds and dipped only in sterile distilled water for 2 h served as controls.
- twenty-five ml of bacterial suspension (10^{10} cells/ml) were poured into the soil around each plant at transplanting time as a soil drench and covered with soil. The bacterial suspension of Pf_1 was prepared by scraping the bacterial colonies from the media and thoroughly mixing in sterile distilled water. The cfu were determined using the dilution plating technique. Plants receiving 25 ml of sterile distilled water served as controls.

A week after transplanting, the soil top layer around the plant base was carefully removed and the exposed roots were inoculated with two immature females and males nematode/g soil using a pipette. After inoculation, the soil was replaced and regular watering was done.

Three plants from each treatment were removed carefully seven days after nematode inoculation, and their roots separated from the seedlings. The roots were washed free of soil in tap water, weighed, cut into 1 cm segments, and stained in an acid fuchsin solution (Bridge *et al.,* 1982). The number of nematodes attached to the roots was counted using a stereomicroscope (x 120) by pressing stained root segments in between two glass slides containing a thin film of glycerine.

Data on fresh lengths of shoot and root and their fresh and dry weights, number of egg masses/plant, eggs/egg mass, final nematode population in the soil and its multiplication rate (Pf/Pi) in the remaining four plants were recorded 83 days after sowing. The data collected were subjected to analysis of variance (ANOVA) using MSTAT-C software (Michigan State University Version 2.10) and differences among treatment means were determined with Duncan's multiple range test at a probability level of 5%.

RESULTS

The germination of bacterized seeds was 91% compared to 81% with non-bacterized seeds. Significant improvements in seedling weight (54%) and shoot length (18%) were observed in the treatments that received bacterized seeds compared to non-bacterized seeds. The increase in the seedling weight and shoot length recorded was approximately 54 and 18%, respectively with bacterized seeds over non-bacterized seeds. However, differences in root length were not significant among treatments but numerically greater values for root length were recorded with bacterized seeds than non-bacterized seeds (Table I).

The bacterial cfu/g fresh root, after 7, 14 and 21 days of germination, was 2.2×10^4, 2.8×10^4 and 1.5×10^5,

respectively; these were substantially less than the initial inoculum level of 10^8 cells/seed. The bacterial cfu on different parts of the root varied and ranged between 1.9 x 10^5 and 7.2 x 10^6/cm near the seed; 7.2 x 10^3 to 2.3 x 10^5/cm on the main root; 1.3 x 10^3 to 4.8 x 10^3 /cm on secondary roots and 5 x 10^3 to 7.1 x 10^4/cm on the main root tip

Root penetration by the nematode was significantly reduced in all the treatments receiving the bacterium compared to the control. However, there were no significant differences between the different treatments receiving the bacterium. The greatest reduction in penetration was 55% in seedlings raised from bacterized seed followed by the soil drench and the least (37%) in seedlings raised from non-bacterized seeds followed by the bare root dip (Table II).

Increases in shoot length were not significant in any of the treatments receiving the bacterium compared to treatments without the bacterium. This increase was, however, not significant. The highest increase in shoot length (27.1%) was recorded in treatments that received bacterized seeds followed by bare root dip as well as bacterized seeds alone, and the lowest (12.8%) was caused by non-bacterized seeds control (Table III). However, fresh shoot weight was significantly increased in treated plants. The greatest increase (25%) in fresh shoot weight occurred with bacterized seeds alone and bacterized seeds followed by the bare root dip, while the least effect (15%) was in the treatment receiving bacterized seeds + nematode compared to non-bacterized seeds (Table III).

Dry shoot weights increased markedly in all the treatments that received the bacterium over non-bacterized seeds. Also, only one treatment that received the bacterized seeds alone significantly increased dry shoot weight over the treatments receiving non-bacterized seeds alone and non-bacterized seeds and the nematode (Table III).

No uniform trend emerged on root length. All treatments, irrespective of the presence or absence of the

Table I. Effect of seed bacterization with *Pseudomonas fluorescens* on growth characters of tomato seedlings 21 days after sowing.

Treatment	Seedling weight (g)	Root length (cm)	Shoot length (cm)
Bacterized seed	1.1 a*	2.2	9.2 a
Non-bacterized seed (Control)	0.7 b	1.9	7.8 b
LSD (0.05)	0.2	N.S.	0.6

Within a column, data followed by the same letter are not significantly different ($P \geq 0.05$).

Table II. Penetration of immature females of *Rotylenchulus reniformis* into roots of tomato seedlings bacterized with *P. fluorescens.*

Treatment	Penetration	% Reduction over control
Bacterized seed	32 a* (5.65)	42.5
Bacterized seed + soil drenching	25 a (4.97)	55.0
Bacterized seed + bare root dip	28 a (5.26)	50.0
Non-bacterized seed + soil drenching	34 a (5.76)	39.0
Non-bacterized seed +bare root dip	35 a (5.93)	36.5
Non-bacterized seed (Control)	56 b (7.39)	-
LSD (0.05)	(1.39)	-

Figures in parentheses represent square-root transformed values; * within a column, data followed by the same letter are not significantly different ($P \geq 0.05$).

Table III. Effect of three application methods of *P. fluorescens* on growth characters of tomato infested with *R. reniformis*.

Treatment	Shoot			Root		
	Fresh length (cm)	Fresh weight (g)	Dry weight (g)	Fresh length (cm)	Fresh weight (g)	Dry weight (g)
Bacterized seed (Bacterized seedlings)	92.4	44.5 a*	4.7 a	19.8	14.8	2.10 a
Bacterized seed + nematode	82.2	41.0 ab	4.3 ab	18.2	14.8	1.77 ab
Bacterized seed + soil drenching (at transplanting time)	91.7	43.7 a	4.1 ab	17.8	17.5	2.32 a
Bacterized seed + soil drenching (at transplanting time) + nematode	90.3	44.0 a	4.2 ab	17.8	17.3	2.27 a
Bacterized seed + bare root dip (at transplanting time)	92.4	44.5 a	4.3 ab	16.3	15.3	2.20 a
Bacterized seed + bare root dip (at transplanting time) + nematode	86.0	42.0 ab	4.2 ab	19.8	15.1	1.92 ab
Non-bacterized seed + nematode	67.5	34.2 c	3.2 c	18.5	9.6	1.10 b
Non-bacterized seed + soil drenching (at transplanting time)	91.1	44.2 a	4.1 ab	17.2	16.6	2.10 a
Non-bacterized seed + soil drenching (at transplanting time) + nematode	86.2	41.7 ab	4.1 ab	19.0	16.7	2.00 a
Non-bacterized seed + bare root dip (at transplanting time)	89.5	43.7 a	4.2 ab	17.6	16.1	2.07 a
Non-bacterized seed + bare root dip (at transplanting time) + nematode	84.0	41.2 ab	4.1 ab	18.0	15.6	1.90 ab
Non-bacterized seed (Control)	72.7	35.5 bc	3.7 bc	17.2	10.3	1.12 b
LSD (0.05)	N.S.	6.03	0.65	N.S.	N.S.	0.75

* Within a column, data followed by the same letter are not significantly different ($P \geq 0.05$).

Table IV. Influence of three application methods of *P. fluorescens* cell suspension (10^{10} cells/ml) on the multiplication of *R. reniformis* on tomato after 83 days of sowing.

Treatment	Nematode multiplication				
	No. of egg masses/root	No. of eggs/egg mass	Soil population/ kg soil	Total	Multiplication rate (Pf/Pi)
Bacterized seed + nematode*	96	96	6442	17262 b**	8.6 b
Bacterized seed + soil drenching (at transplanting time) + nematode	83	74	5822	12076 a	6.0 a
Bacterized seed + bare root dip (at transplanting time) + nematode	95	88	6737	15065 ab	7.5 ab
Non-bacterized seed + soil drenching (at transplanting time) + nematode	99	93	6070	15246 ab	7.6 ab
Non-bacterized seed + bare root dip (at transplanting time) + nematode	100	97	6942	16787 ab	8.3 ab
Non-bacterized seed + nematode (Control)	121	115	7376	21690 c	10.8 c
LSD (0.05)	N.S.	N.S.	N.S.	4387	2.19

* Original nematode inoculum: 2 nematode/g soil; ** within a column, data followed by the same letter are not significantly different ($P \geq 0.05$).

bacterium, had no significant effect in increasing root length and these treatments were at par with each other.

An increase in fresh root weight was observed in all the treatments that received the bacterium. The highest increase was 70% in the treatment that received bacterized seed and a soil drench, and the smallest (44%) in bacterized seed alone with or without nematode treatments (Table III).

Dry root weights were significantly increased in treatments receiving either single or double applications of the bacterium compared to treatments without the bacterium and with or without nematode. The nematode decreased root dry weights compared to their corresponding treatments without nematode. The greatest increase (107%) in dry root weight was caused by the treatment that received bacterized seed and the soil drench, and the smallest (58%) in the treatment that received bacterized seed + nematode in comparison to the control (Table III).

Bacterial application did not cause a significant reduction in the number of egg masses/root, eggs/egg mass and nematode population in the soil (Table IV). However, the total population was significantly reduced in all treatments that received the bacterium compared to the control, with the greatest effect in the treatment using bacterized seeds and a soil drench. This treatment differed significantly from the bacterized seed treatment, but it was similar to other treatments that received single or dual application of the bacterium.

DISCUSSION

Three application methods are usually used for bacterization of plant materials i.e., seed bacterization, soil drenching and bare root dip, because these methods of application place rhizobacteria directly at, or in close proximity to, the infection site of the nematode targeted for control (Sikora and Hoffmann-Hergarten, 1993). The concentration of initial inoculum used for seed bacterization normally does not stick to the seed surface completely, but declines after bacterization (Suslow, 1982; Suslow and Schroth, 1982). A similar trend was also observed in the present study in the reduction of 10^{10} cells/ml to 10^8 cells/seed. Application of the bacterium as seed bacterization increased seed germination, seedling fresh weight and shoot length indicating the plant growth promoting nature of the rhizobacterium. This is in agreement with other workers who reported increases in plant growth (Suslow and Schroth, 1982; Kumar and Dubey, 1992).

One of the important characteristics of rhizobacteria as a biological control agent is their root colonization capability. The isolate showed that its cfu/g fresh root of the seedlings raised from bacterized seed increased from 10^4 to 10^5; with an increase in observation time from 7 to 21 days, however, it was much less than the initial inoculum of 10^8 cells/seed. This supports the findings of Neipp and Becker (1999) who reported similar levels of bacterial population per g root. The least

cfu/cm root was counted on secondary roots followed by the main root tip. The maximum cfu/cm fresh root, 21 days after seeding, was found in the hypocotyl region. Poor colonization of the distal parts of the root has been frequently observed in seed inoculation treatments (Bahme and Schroth, 1987). The results demonstrate that the bacterium is a successful root colonizer.

Investigations on the bacterium using three methods of bacterial application either as seed bacterization alone or in combination with soil drench/bare root dip revealed a significant reduction in nematode penetration compared to the control. The maximum reduction (55%) was recorded when the bacterium was applied twice as seed bacterization followed by a soil drench. The reduced penetration by the nematode into tomato roots may be due to prior colonization by Pf_1, thus blocking the sites for nematode penetration or alter root exudates that are either toxic or repellent to the nematode.

Furthermore, application of the bacterium by any method improved the growth of tomato seedling compared to the control. The maximum growth enhancement by the bacterium occurred when seed bacterization was followed by a soil drench. This may have resulted in a greater and more uniform distribution of bacterial cells in the root zone during the entire growth period. However, the exact mechanism(s) of growth improvement is not known, but it may be due to solubilization of some essential nutrients for the plant and/or indirectly through reducing nematode infections. The results confirm the reports of Oostendorp and Sikora (1989), Siddiqui and Mahmood (1995), and Khan and Tarannum (1999).

The influence of the bacterium on *R. reniformis* multiplication was observed after 83 days from seeding at an initial inoculum level of two immature females and males/g soil. The results indicated a reduction in the nematode multiplication rate by all three methods of application. However, seed bacterization followed by a soil drench was found significantly more effective in reducing the rate of nematode multiplication than seed bacterization alone. The results are in agreement with Becker *et al.* (1988), Siddiqui and Mahmood (1993) and Bansal *et al.* (1999). However, the reduction in the number of egg masses/root, eggs/egg mass and nematode population in the soil was not significant in the present study, while the reduction in the total population and nematode multiplication rate was significant compared to the control. The explanation for no significant reduction may be that during the 83 days of the experiment, the nematode completes almost three generations on tomato; its penetration was greatly reduced when the bacterium was added at the time of transplanting but the effect was not sustained.

It can be concluded from this study that seed bacterization followed by soil drenching of *P. fluorescens* was the most effective method in reducing penetration and multiplication of *R. reniformis* and for the improvement of plant growth. Therefore, the bacterium can be considered as a satisfactory bioagent against the reniform nematode.

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