Survival and colonization of rhizobacteria in a tomato transplant system

Zhinong Yan, M.S. Reddy, and Joseph W. Kloepper

Abstract: Plant-growth-promoting rhizobacteria (PGPR) are used on crops most often as seed treatments; however, an alternative application method for transplanted vegetables is mixing PGPR into the soilless medium in which the transplants are grown. Studies were undertaken to compare root colonization and persistence of rifampicin-resistant mutants of PGPR strains *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61, SE34r and 89B61r, on tomato as a function of application method. When the bacteria were incorporated into Promix™ soilless medium at log 6, 7, and 8 colony-forming units/g, populations of strain SE34r per gram of medium maintained the initial inoculum densities, while populations of 89B61r decreased approximately one to two orders of magnitude by 4 weeks after planting. The populations of each PGPR strain colonizing roots after application into the soilless medium showed a similar pattern at 6 weeks as that at 4 weeks after planting, with higher populations on the whole roots and lateral roots than on the taproots. Strain SE34r but not 89B61r moved upwards and colonized the phyllosphere when incorporated into the soilless medium. Following application as seed treatment, populations of SE34r were significantly higher on upper roots and on the taproot than were populations following application through the soilless medium. Conversely, populations were higher on lower roots and lateral roots following application through the soilless medium than were populations following application as seed treatment. While strain SE34 enhanced plant growth with application both to the medium and as seed treatment, the level of growth promotion was significantly greater with application in the soilless medium. The results indicate that PGPR can be successfully incorporated into soilless media in vegetable transplant production systems.

Key words: rhizobacteria, plant colonization, *Bacillus pumilus*, *Pseudomonas fluorescens*.

Résumé : Les rhizobactéries favorisant la croissance des plantes (PGPR) sont la plupart du temps utilisés pour traiter les semences des cultures; toutefois, une autre méthode d'application pour des légumes transplantés est de mélanger les PGPR au milieu sans terre dans lequel les transplants sont cultivés. Nous avons entrepris des études comparatives de la colonisation des racines et de la persistance de souches mutantes des PGPR *Bacillus pumilus* SE34 et *Pseudomonas fluorescens* 89B61, SE34r and 89B61r, résistantes à la rifampicine, chez la tomate, en fonction de la méthode d'application. Lorsque les bactéries ont été incorporées au milieu sans terre Promix™ à des log de 6, 7 et 8 unités formant des colonies/g, les populations de SE34r par gramme de milieu ont conservé leurs densités initiales d'inoculum, alors que les populations de 89B61r ont diminué d'environ un à deux ordres de grandeur, 4 semaines après la plantation. Les populations de chacune des souches de PGPR colonisant les racines suite à l'application dans le milieu sans terre ont démontré un profil semblable, 6 semaine après la plantation, avec des populations plus importantes sur les racines entières et les racines latérales que sur les racines pivotantes. Lorsqu'elle fut incorporée dans le milieu sans terre, la souche SE34r, mais non la 89B61r, s'est déplacée vers le haut et a colonisé la phyllosphère. À la suite de l'application comme traitement des semences, les populations de SE34r étaient significativement plus élevées sur les racines supérieures et sur la racine pivotante par rapport aux populations dénombrées à la suite de l'application via le milieu sans terre. Inversement, les populations étaient plus élevées sur les racines inférieures et latérales à la suite d'une application via le milieu sans terre qu'à la suite d'une application sur les semences. Bien que la souche SE34 ait stimulé la croissance végétale aussi bien après une application dans le milieu que comme traitement des semences, le taux de stimulation de la croissance était significativement supérieur avec l'application dans le milieu sans terre. Les résultats indiquent que des PGPR peuvent être incorporés avec succès dans des milieux sans terre pour usage dans des systèmes de productions de transplants de légumes.

Mots clés : rhizobactéries, colonisation des plantes, *Bacillus pumilus*, *Pseudomonas fluorescens*.

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Introduction

Plant-growth-promoting rhizobacteria (PGPR) can be applied to a wide range of plants, resulting in growth promotion and disease control (Kloepper 1994; Kapulnik 1996; Bowen and Rovira 1999; Tilak et al. 1999; Whipps 2001). Although many rhizobacteria exhibit significant growth promotion and biological control activities, some fail under various conditions. The variability in yield increase and disease control observed in previous bacterization studies was probably due in part to the failure of the introduced bacteria to colonize the rhizosphere (Kloepper et al. 1980; Paulitz 1997; Benizri et al. 2001; Walker et al. 2002).

Colonization of plant roots by rhizobacteria plays an essential role in biological control and growth promotion (Kloepper and Beauchamp 1992). In a study of biological control by PGPR, Bull et al. (1991) demonstrated an inverse linear relationship between the doses of *Pseudomonas fluorescens* 2-79, which produces the antibiotic phenazine-1-carboxylic acid, and disease incidence of take-all in wheat caused by *Gaeumannomyces graminis* var. *tritici*. They also reported a direct linear relation between the population of *P. fluorescens* 2-79 on the root of wheat and the initial doses on seeds. Parke (1990) found that colonization of the spermosphere by the biocontrol agent was important for controlling damping-off in pea. Understanding the dynamics of root colonization by specific microbial components of the rhizosphere is basic to the effective use of beneficial microorganisms for plant growth enhancement and disease control.

The application method used to treat crops with PGPR affects the distribution and population size of PGPR on plant roots (Weller 1988; Schroth and Becker 1990; Young and Burns 1993). Seed treatment is the most common application method for bacterial agents. However, population sizes of PGPR on plant roots following seed treatment are often highly variable and exhibit lognormal distribution (Loper et al. 1984). An alternative application system is to apply PGPR into the soilless medium used to grow vegetable transplants (Nemec et al. 1996; Paulitz 2000; Kloepper et al. 2003). Studies aimed at comparing colonization and efficacy of PGPR applied as seed treatments with that of PGPR applied in soilless media have not been reported.

This study focuses on how the application method affects (*i*) the persistence of PGPR in soilless mix, (*ii*) colonization dynamics on roots, and (*iii*) distribution of rhizobacteria along roots. The objectives of this research were to (*i*) determine the population dynamics of select PGPR strains on tomato roots and the distribution of selected PGPR strains on tomato plants when incorporated into a soilless medium and (*ii*) compare the application of PGPR as a mixture in soilless medium with PGPR as a seed treatment for plant growth promotion and root colonization.

Materials and methods

Soilless medium

The soilless medium used throughout this study was Speedling Mix™, which is a commercial product (Speedling, Inc., Sun City, Fla.). The mix contains peat, perlite, and ver-miculite in a proportion that is not specified on the commercial label.

Bacterial strains and inoculation preparation

Wild-type strains *Bacillus pumilus* SE34 and *P. fluorescens* 89B61 have exhibited plant growth promotion and induced systemic protection on several crops (Yan et al. 1998). Rifampicin (Rif)-resistant mutants of both strains, SE34r and 89B61r, were used in all experiments to differentiate inoculated PGPR from indigenous rhizobacteria. SE34r and 89B61r were obtained by growing strains SE34 and 89B61 on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) amended with 100 µg/mL Rif (Rif TSA) and by selecting the colonies with growth rates similar to the wild-type strains. The stability of the Rif resistance was confirmed by serial culturing on Rif TSA. For long-term storage, SE34r and 89B61r were maintained at –80°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) that contained 20% glycerol. Inoculum for the treatment of soil and tomato seeds was prepared by streaking strains from storage onto Rif TSA plates and incubating at 28°C for 24 h. Bacterial cells were harvested from the plates in sterile distilled water (SDW) to yield 10^{11} colony forming units (CFU)/mL.

Enumeration of introduced bacterial strains

Unless otherwise stated, bacterial populations in the tomato spermosphere and rhizosphere and populations on the roots, stems, cotyledons, and leaves were determined as follows. Plant tissues were washed, carefully blotted with tissue paper to not disrupt adhering medium, weighed, and homogenized in a Kleco tissue pulverizer (Kleco 4200, Visalia, Calif.) for 30 s in 2 mL of SDW. The homogenates were then serially diluted with SDW, and selected dilutions were spiral plated (model D, Spiral Plater, Spiral Systems, Inc., Cincinnati, Ohio) on Rif TSA amended with 100 ppm nystatin. Plates were incubated at 28°C for 24 h, and the numbers of CFU were counted.

Population dynamics of PGPR strains in a soilless medium and on tomato roots

Bacterial suspensions were incorporated into the soilless medium at the rates of 10^6, 10^7, and 10^8 CFU/g. The moisture level of the medium was 90% (water/mix, w/w). SDW was used as a nontreated control. The PGPR-inoculated medium was placed into the cells (cavities) of the Styrofoam™ flats. Each flat contained 121 cells, and each cell was 2.5 cm². One set of flats was used to monitor bacterial population in the soilless medium, and the second set to study colonization of tomato roots. One tomato seed ('Solar Set') was planted in each cell of the planting flats. Flats were kept on a bench in a controlled growth room maintained at 21–26°C, 70% relative humidity, and 12 h : 12 h light:dark photoperiod. Both sets of flats were arranged in a randomized complete block design with four treatments and four replications of each treatment. Flats were watered gently with a hand sprayer. Tomato seedlings were fertilized with soluble 10:15:15 once a week, beginning at 2 weeks after planting (WAP).

Bacterial populations in the soilless medium were determined using randomly selected 2-g samples taken from each well. The samples were shaken at 150 rpm in a sterilized flask (500 mL) with 100 mL SDW for 1 h. A suspension of

1 mL was taken from each flask for serial dilution. The population was determined by spiral plating. Samples were taken at 0, 1, 2, 3, and 4 WAP. Four replicates were used at each sampling time for each concentration.

For assessing spermosphere colonization, we randomly selected three tomato seeds from each replicate containing soilless medium at 0, 1, 3, and 5 days after planting (DAP). The pooled seeds were rinsed slightly with SDW and ground in a Kleco tissue pulverizer (Kleco 4200).

For root colonization, samples were taken weekly until 4 WAP. The roots were excised, washed slightly with tap water, and dried with tissue paper. Between 150 and 200 mg of fresh roots for each replicate was ground in a Kleco tissue pulverizer. For each replication, three plants were sampled for the first 2 weeks to increase precision in estimating PGPR populations on small plants. For the remaining sampling times, one plant was sampled from each replication.

### Distribution of selected PGPR strains on tomato plants

Experiments were designed to monitor the distribution of SE34r and 89B61r on different parts of the roots and the whole tomato plant. Both strains were mixed into soilless medium at 10^8 CFU/g. Tomato seedlings were sampled at 6 WAP. To detect bacterial colonization along tomato roots, the roots from 20 tomato seedlings were excised, rinsed in SDW, pooled, and then separated into three groups: total roots, taproots, and lateral roots. Each group was divided into five replicates. The roots, stems, and leaves were separated at 6 WAP to determine the distribution of SE34r and 89B61r. There were ten replicates, each with one plant.

Comparison of application methods on tomato growth promotion and colonization on tomato roots

Spore-forming bacilli retain viability in dried formulations, while pseudomonads do not. Hence, *Bacillus pumilus* SE34 was used in an experiment designed to compare the effects of using a seed treatment with the effects of a treatment mixed into soilless medium. Soilless medium was placed into Speedling™ trays at the time of planting. One tomato seed was seeded in each cell of the flat. For seed treatment, 1 mL of suspension of SE34r containing 10^8 CFU/mL was drop inoculated onto each seed. For application into the soilless medium, strain SE34r was incorporated thoroughly into the medium at 10^8 CFU/g (90% moisture), and the mixture was placed into Speedling™ trays. One seed was sown into each cell. Soilless medium treated with SDW served as control. Trays were arranged in a randomized complete block design with three treatments and five replicates of each treatment. The flats were kept in a controlled growth room maintained at 26°C, 70% relative humidity, and 12 h : 12 h light:dark cycle.

Effects of SE34r on tomato seedling growth were determined at 6 WAP. Five seedlings were randomly sampled from each replicate. The roots were washed and blotted dry with paper towels. The total fresh weight and height of each seedling were measured. To determine the distribution of SE34r along the roots, we randomly sampled three seedlings from each replicate. The roots were washed, dried with paper tissue, and divided into upper and lower portions. Three additional seedlings were taken from each replicate to sample total root population. Populations on taproots and lateral

Fig. 1. Persistence of plant-growth-promoting rhizobacteria strains *Bacillus pumilus* SE34r (A) and *Pseudomonas fluorescens* 89B61r (B) in soilless medium following incorporation at various dosages. Different letters indicate statistically significant differences ($P = 0.05$) among doses at each sampling time. Vertical bars indicate standard error of means pooled from three experiments.
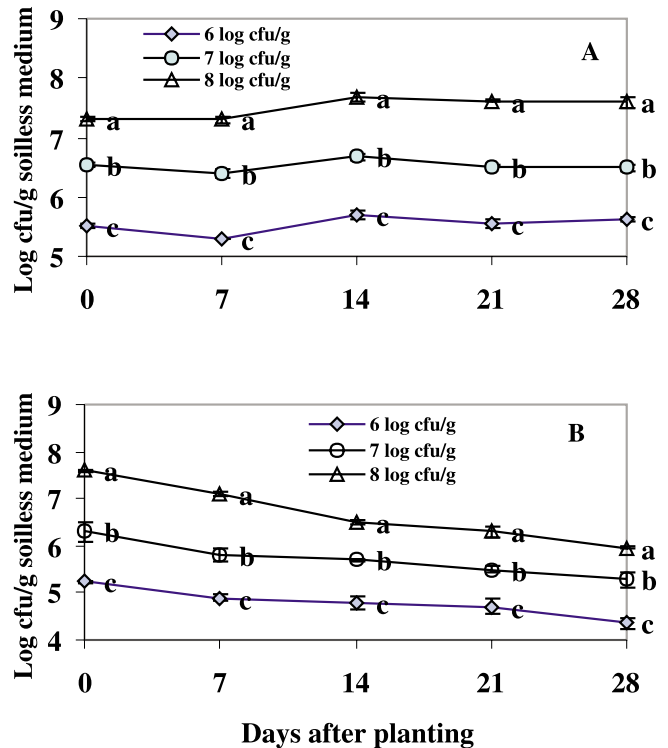
roots were determined by taking 200–300 mg of root per sample. Populations were determined as described above.

Statistical analysis

All data were analyzed by the JMP program (SAS Institute Inc., Cary, N.C.), using the one-way ANOVA test. Least significant difference values at $P = 0.05$ level were used to separate treatment means when ANOVA indicated a significant F value. All experiments were conducted three times. Pooled data were used for statistical analysis.
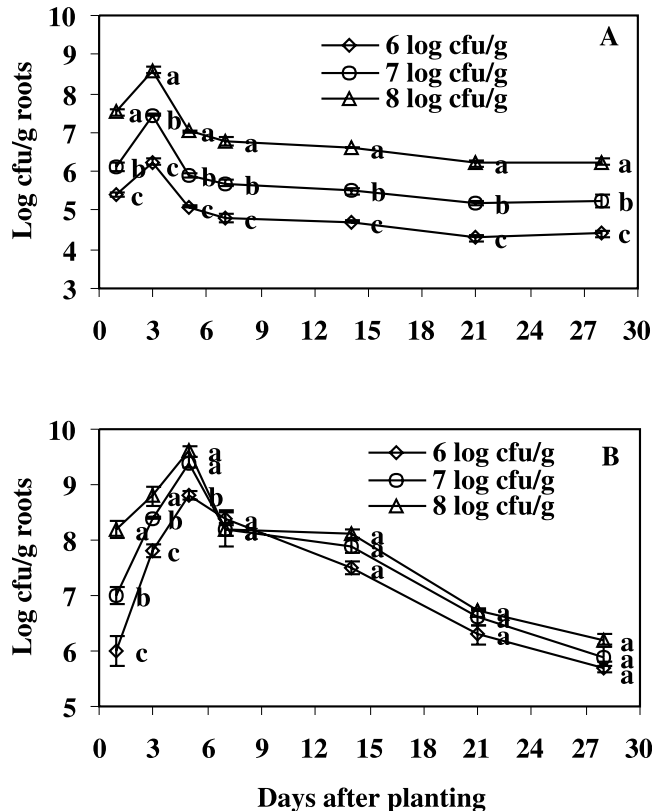
Results

Population dynamics of PGPR strains in soilless mix and on tomato roots

Populations of PGPR strains SE34r and 89B61r in soilless medium are presented in Fig. 1. At 4 WAP, populations of SE34r at all three inoculum densities maintained the same levels as the initial inoculation densities. At all three inoculum levels, strain 89B61r decreased by about 1 log unit at 4 WAP.

Colonization dynamics of the two strains in tomato spermosphere and rhizosphere are shown in Fig. 2. Spermosphere populations of strain SE34r at all three inoculum levels increased rapidly from 1 to 3 DAP. Rhizosphere populations significantly declined by about 2 log units at 28 DAP. At each sampling time, populations of the three different inoculation densities were significantly ($P = 0.05$) different from each

Fig. 2. Colonization of tomato roots by plant-growth-promoting rhizobacteria strains *Bacillus pumilus* SE34r (A) and *Pseudomonas fluorescens* 89B61r (B) following incorporation into soilless medium at various dosages. Different letters indicate statistically significant differences ($P = 0.05$) among doses at each sampling time. Vertical bars indicate standard error of means pooled from three experiments.

Fig. 3. Colonization of total root system, taproots, and lateral roots of tomato seedling transplant by plant-growth-promoting rhizobacteria strains *Bacillus pumilus* SE34r and *Pseudomonas fluorescens* 89B61r at 6 weeks after seeding when incorporated into soilless medium at 10^8 CFU/g mix. Different letters indicate statistically significant difference ($P = 0.05$) among populations on whole roots, taproots, and lateral roots. Vertical bars indicate standard error of means pooled from three experiments.
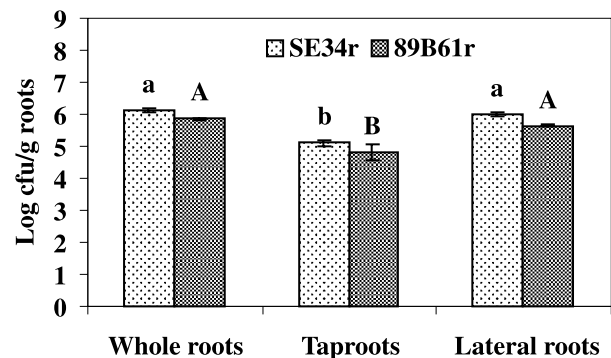
Fig. 4. Distribution of plant-growth-promoting rhizobacteria strains *Bacillus pumilus* SE34r and *Pseudomonas fluorescens* 89B61r on different parts of tomato seedlings at 6 weeks after seeding when incorporated into soilless medium. Different letters indicate statistically significant difference ($P = 0.05$) among populations on roots, stems, and leaves. Vertical bars indicate standard error of means pooled from three experiments.


other, showing a parallel colonization pattern (Fig. 2A). Spermosphere populations of strain 89B61r at the three inoculation densities increased significantly from 1 to 5 DAP. The rhizosphere populations began to decrease at 5 DAP and significantly declined by about 3 log units and were comparable with spermosphere populations at 28 DAP (Fig. 2B). At the three inoculation densities, the pattern of population dynamics of strain 89B61r was different from that of strain SE34r. At 1 and 3 DAP, populations in the spermosphere were significantly different from each other; while from 7 to 28 DAP, no difference in populations in the rhizosphere was found among the densities (Fig. 2B).

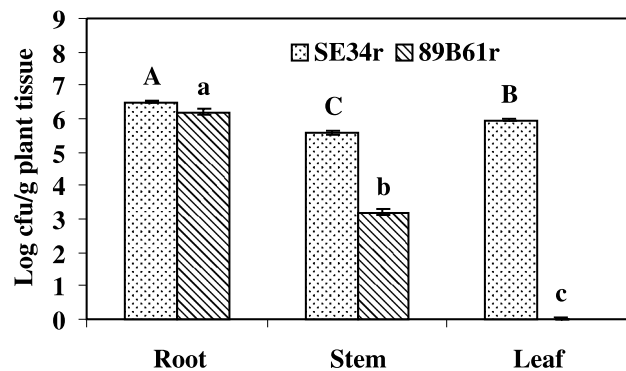### Distribution of select PGPR strains on tomato plants

When incorporated into soilless medium, both SE34r and 89B61r were detected on total roots, taproots, and lateral roots (Fig. 3). Populations of both strains in lateral roots did not differ from those on whole roots, but populations on taproots were significantly less than populations on lateral roots and whole root systems.

Strains SE34r and 89B61r exhibited different patterns of distribution on the whole tomato plant. When inoculated into soilless medium at 10^8 CFU/g mix, SE34r colonized tomato roots, stems, and leaves at 6 WAP (Fig. 4). Populations were as high as $10^{6.5}$ CFU/g root, 10^6 CFU/g leaf, and $10^{5.5}$ CFU/g stem. Populations on whole roots were significantly higher
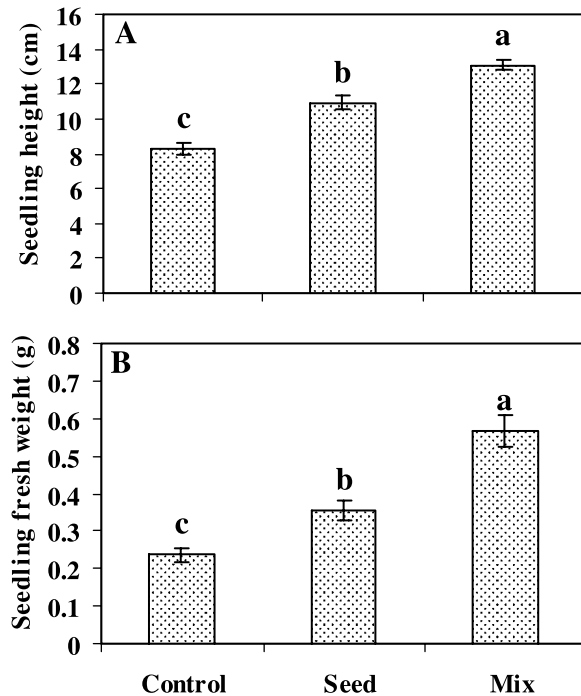
than on stems and leaves, and populations on leaves were significantly higher than on stems. In contrast, 89B61r did not colonize tomato leaves when inoculated into soilless mix, although bacteria were found on roots and in stems with populations of 10^6 CFU/g and 10^3 CFU/g, respectively (Fig. 4).

Comparison of application methods on tomato growth promotion and colonization of tomato roots

Application of SE34r in soilless medium significantly ($P = 0.05$) improved tomato seedling growth, as measured by seedling fresh weight and seedling height, compared with application as a seed treatment. Compared with the nontreated control, both application methods significantly ($P = 0.05$) enhanced tomato plant growth (Fig. 5).

Colonization and distribution of SE34r along tomato roots following the two application methods are shown in Fig. 6. No significant differences ($P = 0.05$) were found between

Fig. 5. Effect of two application methods on tomato seedling growth by plant-growth-promoting rhizobacteria strain *Bacillus pumilus* SE34r. Seed indicates drop inoculation of 1 mL of bacterial suspension (10^8 CFU/mL) onto each seed when seeding. Mix indicates mixing of bacterial suspension thoroughly into soilless medium at 10^8 CFU/g prior to seeding. (A) Seedling height. (B) Seedling fresh weight. Different letters indicate statistically significant difference ($P = 0.05$) among treatments. Vertical bars indicate standard error of means pooled from three experiments.

Fig. 6. Effect of two application methods on colonization of tomato roots by plant-growth-promoting rhizobacteria strain *Bacillus pumilus* SE34r 6 weeks after planting. Seed indicates seed treatment, which was conducted by drop inoculation of 1 mL of bacterial suspension (10^8 CFU/mL) onto each seed when seeding. Mix indicates application into soilless medium by mixing bacterial suspension thoroughly into soilless medium at 10^8 CFU/g prior to seeding. Whole roots indicates population on the whole root system. Upper roots and lower roots indicate populations on the upper and lower halves of the whole roots. Taproots indicates populations on roots after removing all lateral roots. Lateral roots indicates populations on all lateral roots without the taproot. Different letters indicated significant differences among different locations of plant at $P = 0.05$. Vertical bars indicate standard error of means pooled from three experiments.
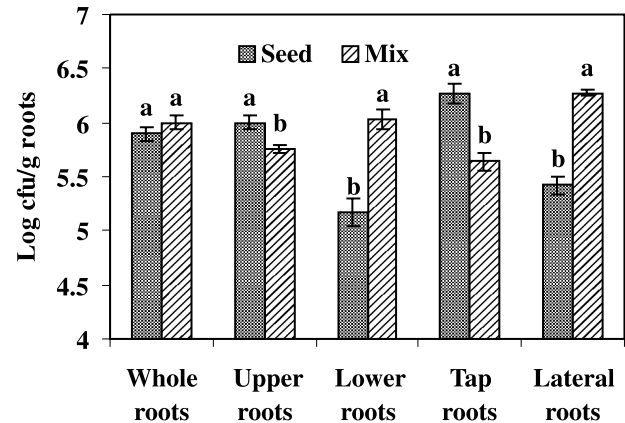

the two application methods when populations in the whole root systems were compared. However, in the upper part of the roots, populations of SE34r applied as a seed treatment were significantly ($P = 0.05$) higher than those resulting from incorporation into soilless medium. In the lower part of the roots, populations from incorporation into the medium were significantly ($P = 0.05$) higher than those from seed treatment. Interestingly, seed treatment provided significantly higher populations in taproots than incorporation into the medium, although the reverse was true with populations on lateral roots.

Discussion

A soilless growth medium provides an ideal carrier for biological control agents (Schroth and Becker 1990; Young and Burns 1993). In our study, persistence of two PGPR strains in a peat-based soilless growth medium was dependent on the initial inoculum densities. Our results, showing that the population density of SE34r remained stable while populations of 89B61r decreased at 4 WAP, indicated that the spore-forming *Bacillus* strain survived better in the growing medium than the pseudomonad strain.

When strains were introduced into the soilless medium, populations of both strains in the spermosphere increased rapidly and then declined as tomato roots grew (Fig. 2). This finding is similar to typical colonization curves by seed in-

oculation, in which the population grows exponentially for the first several weeks and then begins a steady decline until detection limits are reached (Parke 1990; Hebbar et al. 1992; Kluepfel 1993). The difference in population increases in the spermosphere suggests that pseudomonad bacteria might multiply more rapidly than bacilli bacteria (Fig. 2). This result confirms previous findings that pseudomonad strains were the most predominant spermosphere colonizer (Parke 1990; Kluepfel 1993; Deacon 1994).

Regardless of the initial inoculum applied in soilless mix, no differences were found for populations of strain 89B61r on tomato roots at sampling times starting at 5 DAP. This finding suggests a carrying capacity of tomato roots in which final colonization densities are independent of initial inoculum densities.

In contrast, colonization dynamics on tomato roots by strain SE34r were different from strain 89B61r. At each sampling time, significant differences were detected among the three initial inoculum densities in colonization of tomato roots, indicating an initial inoculum-density-dependent relationship with strain SE34r. A similar density-dependent relationship was reported by Hebbar et al. (1992) with *Burkholderia cepacia* applied on maize seeds.

When incorporated into soilless mix, the distribution of both strains along tomato roots showed that lateral roots were colonized with higher populations than taproots. This observation was different from the colonization patterns observed when bacteria were introduced as seed treatment. When inoculated into soilless mix at 10^8 CFU/g, strain SE34r moved upward to tomato leaves. A population of

10^6 CFU/g was detected at 6 WAP in tomato leaves. Populations of 89B61r were not detected on tomato leaves, although about 10^3 CFU/g were found in stems. Populations of both strains on tomato cotyledons were monitored at 3 WAP (data not shown). Strain SE34r colonized tomato cotyledons at about 10^6 CFU/g and strain 89B61r colonized them at 10^3 CFU/g.

We conducted a separate study of both strain populations on tomato roots and stems by surface sterilizing the roots and stems and comparing them to nonsterilized ones. No significant difference was found between populations of sterilized and nonsterilized treatments (data not shown), indicating that strains SE34r and 89B61r moved upwards internally. These results are similar to those reported in endophyte studies on sweet corn, cotton, red bean, and tomato plants with the same strains used in this study (McInroy and Kloepper 1995; Hallman et al. 1997) and to reports by Kluepfel (1993) that *Pseudomonas aureofaciens* L11 readily moved into leaves and stems of corn, wheat, and soybean through vascular systems. As suggested by Raaijmakers et al. (1995) and Kluepfel (1993), populations in aboveground portions might also be due to contact with bacterial cells when seed germinated in soilless medium.

The application method is an important factor to ensure the consistent performance of PGPR in growth promotion and biological control activities. Results from this study demonstrate that mixing PGPR strain SE34r into soilless medium provides significant growth promotion compared with seed treatment. The effect of the two application methods on plant growth promotion may be partially accounted for by the difference of colonization patterns on tomato roots. Seed treatment resulted in significantly higher populations of SE34r in upper roots and taproots, while mixing into soilless medium provided uniform colonization of whole root systems, especially on lateral roots and lower portions of roots (Fig. 6). When SE34r was incorporated into the soilless medium, populations of SE34r in lateral roots were significantly higher than in taproots (Fig. 3).

Applying PGPR for growth promotion and biological control would seem ideally suited for transplant systems. The environmental conditions during transplant production are more uniform than field conditions, thereby allowing consistent colonization of plants by introduced PGPR.

Acknowledgements

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