EFFECT OF PGPR DOSAGE ON PLANT GROWTH PROMOTION AND INDUCED SYSTEMIC RESISTANCE

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Abstract

The effects of PGPR dosage on plant growth promotion, induced systemic resistance (ISR), and colonization of tomato plants by PGPR was investigated. Two PGPR strains, *Bacillus pumilus* SE-34 and *Pseudomonas fluorescens* 89B-61, with known plant growth promotion and ISR activities were used in these studies. PGPR strains were incorporated into soil-less media at different population densities, and the effects on plant growth promotion and systemic protection against late blight disease were measured with tomato. Regression analyses demonstrated a significant linear relationship between the initial population density of the two PGPR and tomato seedling growth or late blight disease reduction. With strain SE-34, $Y = 1.53 + 0.16\ X$ ($R^2 = 0.89$, 38 days after planting) defined the relation between initial population density and tomato growth, while $Y = 6.96X - 3.2$ ($R^2 = 0.96$) described the relationship between population density and reduction of late blight disease. With strain 89B-61, $Y = 3.0 + 0.10X$ ($R^2 = 0.92$, 38 days after planting) and $Y = 6.7X - 5.3$ ($R^2 = 0.94$) defined the relation between population density and growth and reduction of late blight disease, respectively. Regression analyses also demonstrated a significant correlation between tomato seedling growth and late blight disease suppression. $Y = 0.89X + 7.7$ ($R^2 = 0.91$) and $Y = 1.2X + 3.5$ ($R^2 = 0.95$) represented these relations for strains SE-34 and 89B-61, respectively. Persistence of the two strains in soil-less media and colonization of tomato plants were monitored. When mixed into soil-less medium, populations of SE-34 maintained the initial population densities 6 weeks after planting, while populations of 89B-61 decreased. Populations of both strains significantly increased in the spermosphere and then decreased after roots started to grow, suggesting the early stage of colonization is important for tomato growth and disease reduction. Colonization studies showed that mixing PGPR into soil-less media was a good delivery system for tomato transplants, as population densities in the soil-less media and in the rhizosphere remained relatively constant during the production time of transplants. Unlike past studies with seed treatment using these strains, strain SE-34, but not 89B-61, colonized the phyllosphere when incorporated into the soil-less media. The effect of phyllosphere colonization on disease is unknown at this time.

Introduction

Plant growth promotion and biological control are often both induced by the same strain of PGPR (Kloepper, 1993). However, whether plant growth promotion alone could result in biological control of plant disease remains unclear.

Rhizobacteria-mediated biological control has been reported to be dependent on dosage of bacteria applied (Bull et al.1991). Similar investigations on the effect of PGPR inoculum density and ISR have not been extensively conducted. Raaijmakers et al (1995) demonstrated a significant non-linear asymptotic relation between the rhizosphere

population density of *P. putida* WCS358 or *P. fluorescens* WCS374 and the level of disease suppression of Fusarium wilt of radish. Also, the relation between inoculum density and growth promotion mediated by PGPR has rarely been demonstrated.

Some dose-response studies on PGPR and biological control or growth promotion have been conducted. Raijmakers et al. (1995) reported that a threshold of approximately 10^5cfu/g root was required for strains WCS358 and WCS374 to provide significant suppression of Fusarium wilt of radish. Suslow (1982) reported that 10^5 cfu/seed or 10^7 cfu/ g dry inoculum were required for plant growth promotion of sugar beet. Determining the threshold of PGPR inoculum density for each system is a necessary step to help ensure the consistency of plant growth promotion and disease protection by PGPR.

It is generally accepted that colonization of plant roots plays an essential role in biological control (Weller, 1988). With ISR, however, prolonged colonization may be less critical than for other mechanisms of biological control (van Loon 1998).

With different application methods of PGPR, the effects of growth promotion and disease reduction vary (Schroth and Becker, 1990). For production of vegetable transplants, mixing rhizobacteria into the soil-less media used to grow the transplants is a commercially feasible delivery system. Population studies comparing colonization dynamics from PGPR applied as mixes into transplant media vs. applied as seed treatments have not yet been conducted.

The objectives of the research reported here were: 1) to determine the relation between inoculum density of select PGPR strains and growth promotion and induced systemic resistance mediated by PGPR; 2) to determine if plant growth promotion by PGPR is related to ISR; 3) to determine population dynamics of the select PGPR strains on tomato roots and the distribution of select PGPR strains on tomato plants when incorporated into soil-less media; and 4) to compare seed application and mixing into soil-less media for tomato growth promotion and colonization.

Materials and Methods

The relation of inoculum density and growth promotion by PGPR. Two PGPR strains, *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61, were used in the experiments. SE34 and 89B61 were grown on TSA for 48 h. Different amounts of bacterial cell suspensions were mixed into 480 ml distilled water and then incorporated into 600 g "Speedling" soil-less growing media to reach rates of 10^3, 10^5, 10^7 and 10^9cfu/g. Distilled water was used as a non-treated control. Plant growth was measured each week from the tenth day after planting. The total seedling fresh weight and seedling height were collected.

The relation of inoculum density and induced systemic resistance by PGPR. Four weeks after planting, tomato seedlings were transplanted into 10-cm diameter pots and grown in a growth room for additional 2 weeks. *Phytophthora infestans* tomato race 1, was used to inoculate plants. Plants were challenged with 5 x 10^3 zoospores/ml of *P. infestans* until run-off. Disease was rated by leaf area covered with late blight lesions 7 days after challenge.

Population dynamics of PGPR strains in soil-less mix and tomato roots. To monitor the population of strains SE34 and 89B61 in "Speedling" mix and on tomato roots, rifampicin (Rif) resistant mutants of the both strains, SE34r and 89B61r, were used in the

experiments. Bacterial cells were incorporated into 'Speedling mix' soil-less media at the rates of 10^6, 10^7, and 10^8cfu/g. SDW was used as a non-treated control. For population studies in soil-less media, two grams of "Speedling" mix were taken from each cavity and shaken in 100 ml SDW for 1 h. The samples were taken at 0, 1, 2, 3, and 4 weeks after planting. For spermosphere colonization, three tomato seeds were randomly taken for each replicate from "Speedling mix" at 0, 1, 3 and/or 5 days after planting. The pooled seeds were rinsed with SDW and ground in a Kleco tissue pulverizer. For root colonization, samples were taken each week until 4 weeks after planting. The roots were excised, washed with tap water and dried with tissue paper. A total 150 to 200 mg root from each replicate was ground in Kleco tissue pulverizer.

Distribution of select PGPR strains on tomato plants. Experiments were designed to determine the distribution of the two select PGPR strains on the whole tomato plant. Both strains SE34r and 89B61r were mixed into "Speedling" mix to reach 10^8cfu/g mix. Six weeks after planting, tomato seedlings were sampled. The roots, stems, cotyledons and leaves were separated and weighed. The populations of SE34r and 89B61r on different parts of tomato seedlings were estimated using the same procedure as above for population dynamics studies. The populations on different parts of the roots when strain SE34r was incorporated into soil-less media were also detected. SE34r was mixed into "Speedling" mix at 10^8 cfu/g. Six weeks after planting, twenty roots were pooled and then separated into three groups: total roots, tap roots, and lateral roots.

Comparison of application methods on tomato growth promotion and colonization on tomato roots. To determine which application method provides better performance on tomato growth promotion, seed application and mixing into soil-less media with strain SE34r were compared. When planting, 1 ml of SE34r containing 10^9cfu/ml was pipetted onto each seed and "Speedling" media was mixed with SE34r at 10^8 cfu/g. Growth promotion was determined by measuring the fresh weight six weeks after planting. Meanwhile, roots were separated into taproots, lateral roots, and total roots. The distribution of SE34r along the roots was detected by estimating the populations in the upper half and lower portions of root systems.

Statistics analysis of data. All the data were analyzed by JMP program (SAS Institute Inc. Cary, NC) using one-way ANOVA test. LSD values at P=0.05 level were used to separate treatment means when ANOVA indicated a significant F value. The correlations between dose and growth and disease reduction were done by JMP using correlation analysis. All the experiments described above were conducted at least twice.

Results

The relation of inoculum density and growth promotion by PGPR. The relation between concentrations of two PGPR strains and seedling fresh weight increase are presented in Figs. 1 and 2. The graphs showed that there were significant correlations between doses of these two PGPR strains and tomato growth promotion, indicated by seedling fresh weight, at all sampling times.

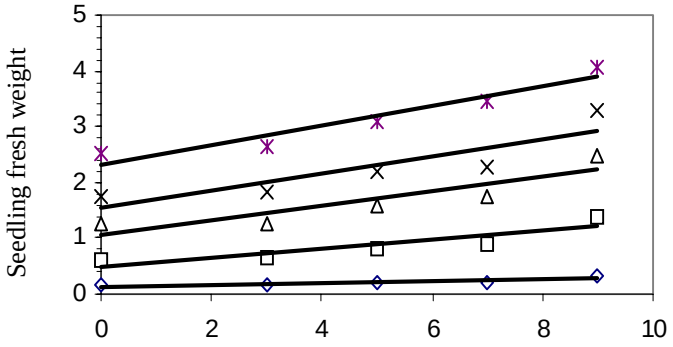

Figure 1. Correlation between doses of SE34 and tomato seedling fresh weight.
Regression analysis demonstrated significant relations between the doses of SE34 and tomato growth. The correlation were calculated as $Y = 0.13 + 0.016X$ ($R^2 = 0.80$, $p = 0.042$), $Y = 0.51 + 0.08X$ ($R^2 = 0.77$, $p = 0.048$), $Y = 1.05 + 0.13X$ ($R^2 = 0.80$, $p = 0.039$), $Y = 1.53 + 0.16X$ ($R^2 = 0.78$, $p = 0.047$), for 10, 17, 24, 31, and 38 days after planting, respectively. ◊, , Δ, X,∗ represent sampling times of 10,17, 24, 31, and 38 days after planting, respectively.

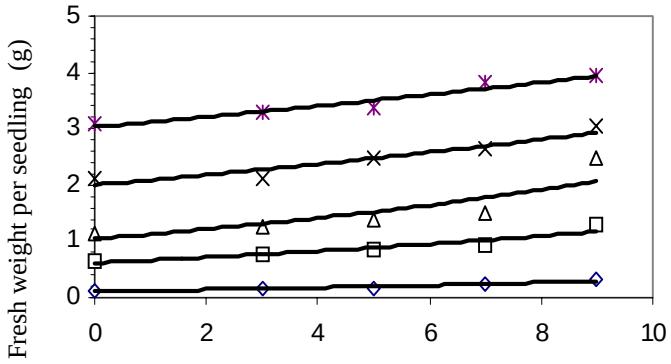

Figure 2. Correlation between doses of 89B61 and tomato seedling fresh weight.
The regression analysis demonstrated a significant relation between doses of 89B61 and tomato seedling fresh weight. The correlation were: $Y = 0.10 + 0.022X$ ($R^2 = 0.84$, $p = 0.029$), $Y = 0.59 + 0.065X$ ($R^2 = 0.85$, $p = 0.025$), $Y = 0.95 + 0.13X$ ($R^2 = 0.80$, $p = 0.049$), $Y = 1.97 + 0.11X$ ($R^2 = 0.91$, $p = 0.013$), and $Y = 3.0 + 0.10X$ ($R^2 = 0.92$, $p = 0.01$) at 10, 17, 24, 31, and 38 DAP. ◊, , Δ, x,∗: indicate sampling times of 10, 17, 24, 31, and 38 days after planting, respectively.

The relation of inoculum density and induced systemic resistance by PGPR.
Different concentrations of bacteria in soil-less media significantly affected the disease reduction, as measured by % of leaf area covered with late blight lesions (Table 3). With both strains, 10^5cfu/g mix was the minimum bacterial population which significantly exhibited ISR activity against late blight compared to the non-treated control. Regression

analysis defined the relations between inoculation densities and late blight disease reduction as Y=6.96X-3.24 (R^2=0.96, *P*=0.045) and Y=6.69X – 5.3 (R^2=0.94, *P*=0.02) for SE34 and 89B61, respectively (Fig.3).

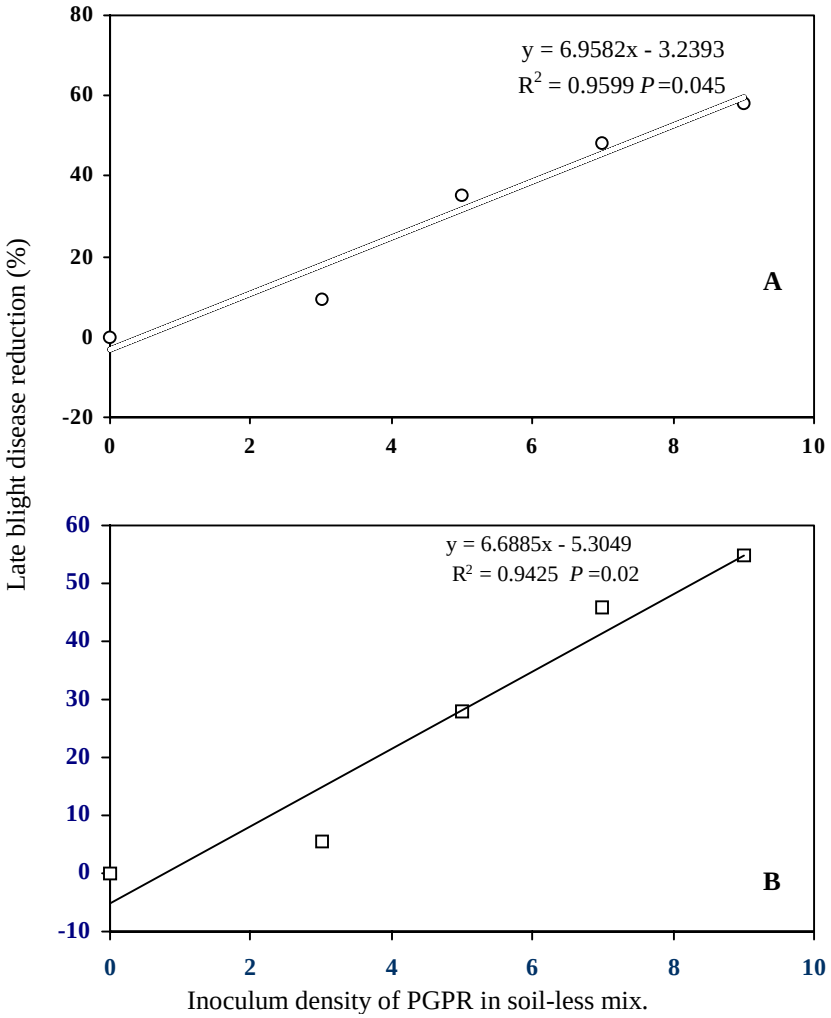

Fig.3. Correlation between inoculum densities of PGPR in soil-less mix and tomato late blight disease control. A: SE34; B: 89B61. X-axis: different inoculum densities presented as log cfu/g mix. Y-axis: % of enhanced tomato seedling growth measured by fresh weight.

The correlation of growth promotion and induced systemic resistance by PGPR. With both PGPR strains SE34 and 89B61, induced systemic resistance against tomato late blight was significantly correlated with enhanced tomato seedling growth measured by fresh weight (Fig.4). The correlations were Y= 0.8906 X +7.7066 (R^2=0.9068, *P*=0.03) and Y=1.1881 X +3.4 (R^2=0.9495, P=0.04) for SE34 and 89B61, respectively.

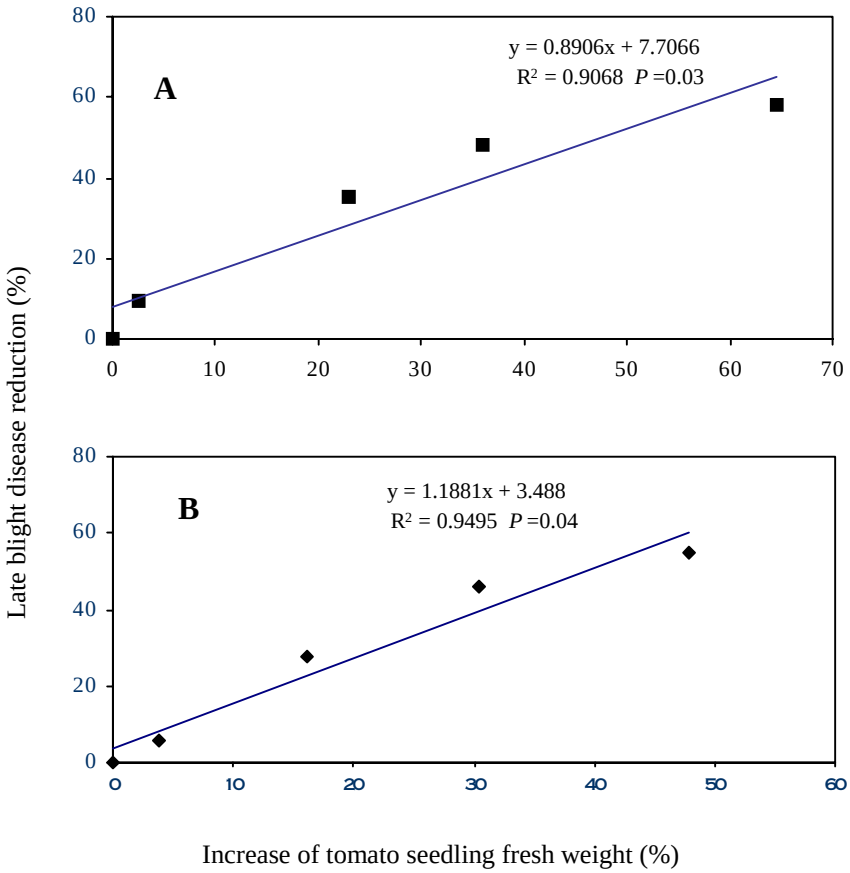

Fig. 4. Correlation of growth promotion and induced systemic resistance mediated by PGPR strains. A. Strain SE34; B. Strain 89B61. Y-axis represented percent of disease reduction compared to control. X-axis represented percent of increased tomato seedling growth measured by fresh weight.

Table 3. Dose response of SE34 and 89B61 on tomato late blight control

Concentration of SE34 (cfu/g mix) [1]	Leaf area covered with late blight lesions (%)[2]	Concentration of 89B61 (cfu/g mix)[1]	Leaf area covered with late blight lesions (%)[2]
Control	41.5 ± 3.1a[2]	Control	39.0 ± 4.2a
10^3	37.8 ± 3.3a	10^3	40.4 ± 1.9a
10^5	25.1 ± 3.9b	10^5	28.7 ± 4.0b
10^7	19.2 ± 2.5b	10^7	22.3 ± 2.9b
10^9	16.5 ±2.4b	10^9	20.8 ± 1.7b
$LSD_{0.05}$	8.97	$LSD_{0.05}$	8.96

[1] Populations of bacteria mixed "Speedling" soil-less media. Control consisted of sterile distilled water mixed into "Speedling" soil-less media.

[2] Means ± SD were the means from 8 replicates. Data within columns followed by different letters differ at P=0.05 level according to one-way ANOVA test.

Population dynamics of PGPR strains in soil-less mix and tomato roots. Populations of PGPR strains SE34r and 89B61r in soil-less media are presented in Fig.5. Populations of SE34r did not decrease 4 weeks after planting, while 89B61r decreased about one log unit 4 weeks after planting. Populations of both strains in tomato roots are shown in Fig.6. With both strains, populations in the spermosphere increased rapidly from day 1 to day 3 after planting. The populations started to decrease from day 3 after planting with SE34r and from day 5 after planting with 89B61r.

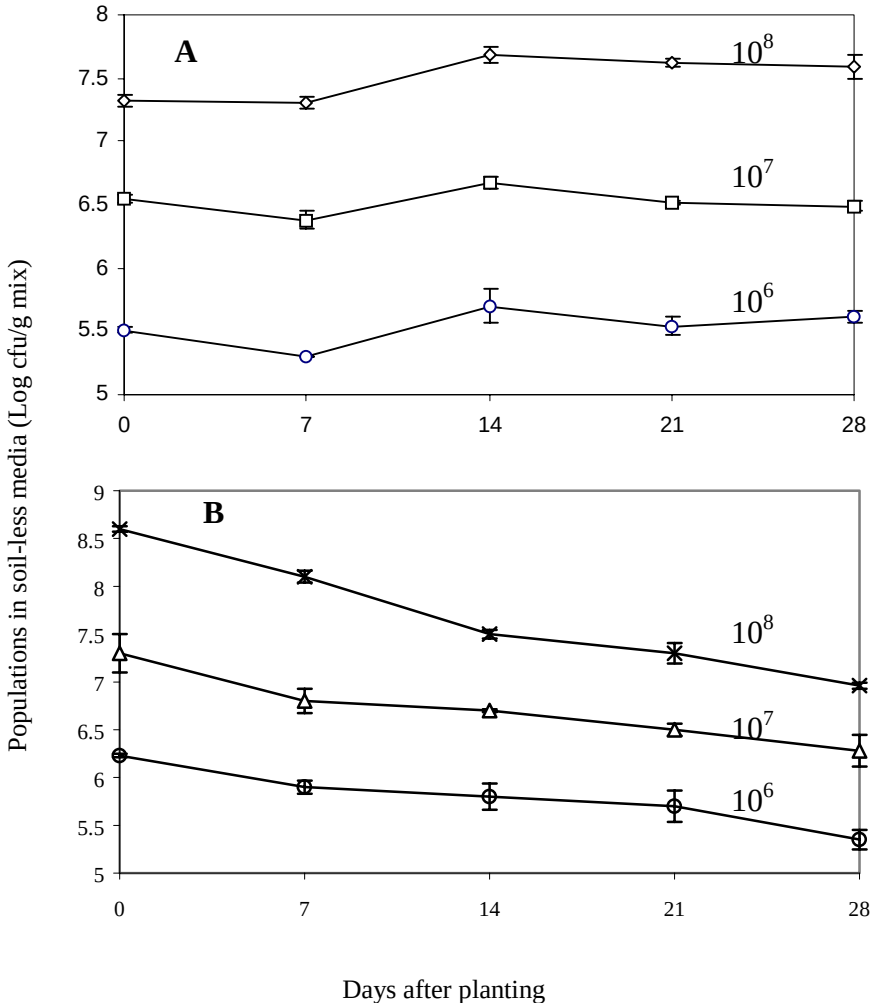

Fig. 5. Population dynamics of PGPR strain SE34r and 89B61r when mixed into "Speedling" soil-less media. A. SE34r; B. 89B61r.

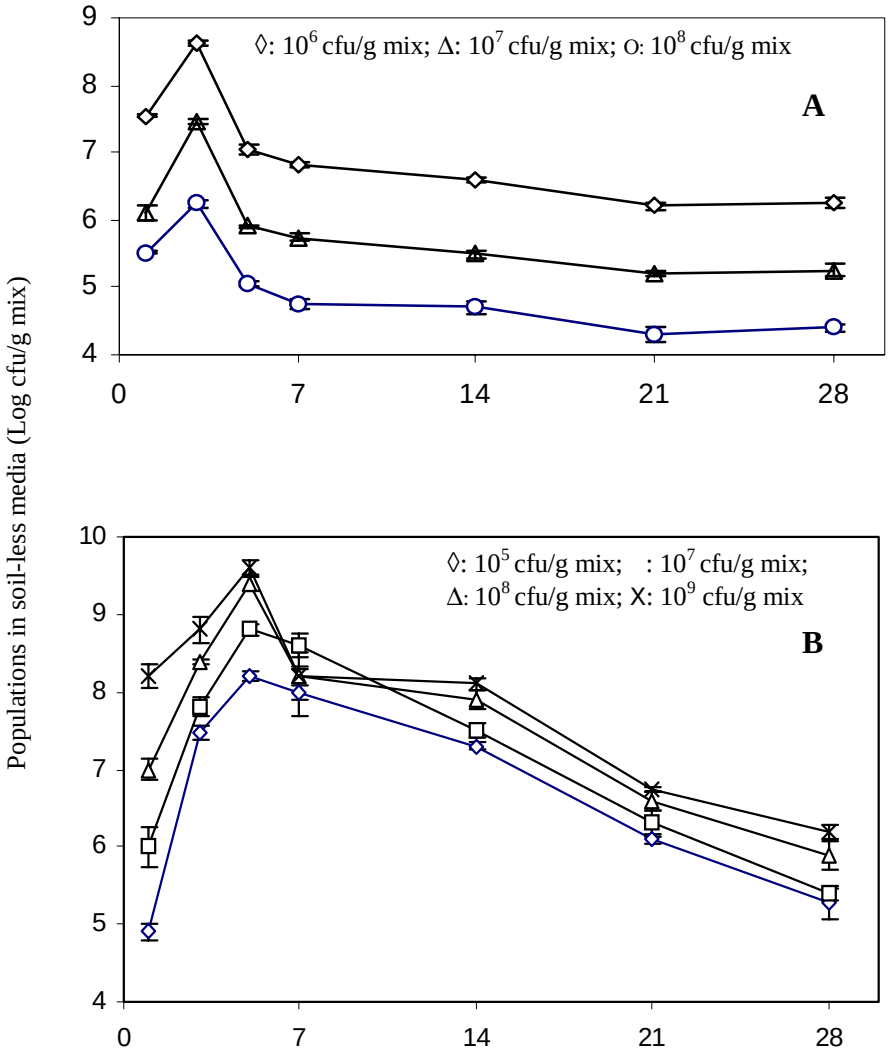

Fig. 6. Population dynamics of PGPR strains SE34r and 89B61r in tomato roots when mixed into "Speedling" media. A. SE34r; B. 89B61r

Distribution of select PGPR strains on tomato plants. PGPR strains, SE34r and 89B61r, exhibited different colonization patterns on the whole tomato plants. SE34r inoculated into soil-less media at 10^8 cfu/g mix colonized tomato roots, stem, cotyledons and leaves detected 6 weeks after planting (Fig.7). However, with 89B61r inoculated into "Speedling" mix, no bacteria were detected on leaves although they were found in cotyledons (Fig.8). When strain SE34r was mixed into "Speedling" soil-less media, bacteria were detected in total roots, tap roots and lateral roots (Fig.9). Populations in lateral roots did not significantly differed from total root, but populations in taproots were significantly less than populations in lateral roots and total roots.

Comparison of application methods on tomato growth promotion and colonization on tomato roots. Effects of two application methods on tomato seedling growth and

colonization were studied. Results on tomato seedling growth showed that mixing PGPR strain SE34r into soil-less media enhance tomato seedling growth at a significantly greater level that did seed application (Fig.10). The colonization and distribution of SE34r along tomato roots are showed in Fig. 11. In mix, applying SE34r into soil-less media gave significant higher populations than seed application. In the upper parts of roots, populations with seed application were significantly higher than with the mixing application, while in lower parts of roots, populations with the mixing application were significantly higher than with seed application. Interestingly, seed application provided significant higher populations in tap roots than mixing application, while mixing application gave significant higher populations in lateral roots compared to seed application.

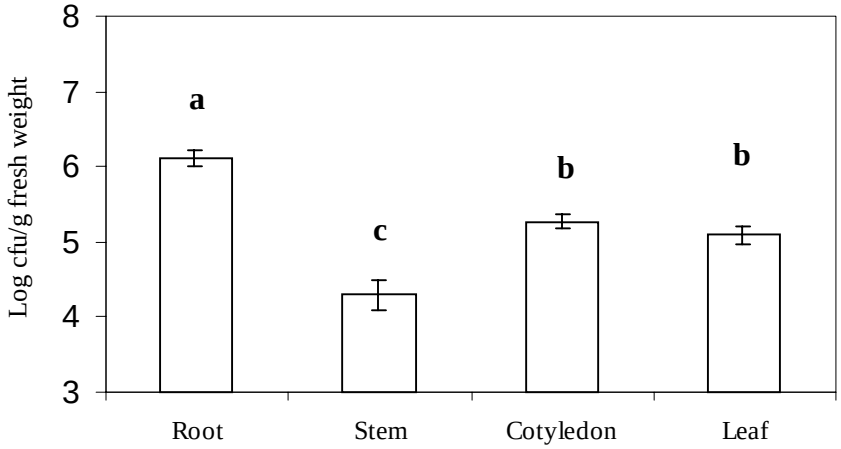

Fig. 7. Colonization of tomato plant by PGPR strain SE34r inoculated into soil-less mix 42 days after planting.

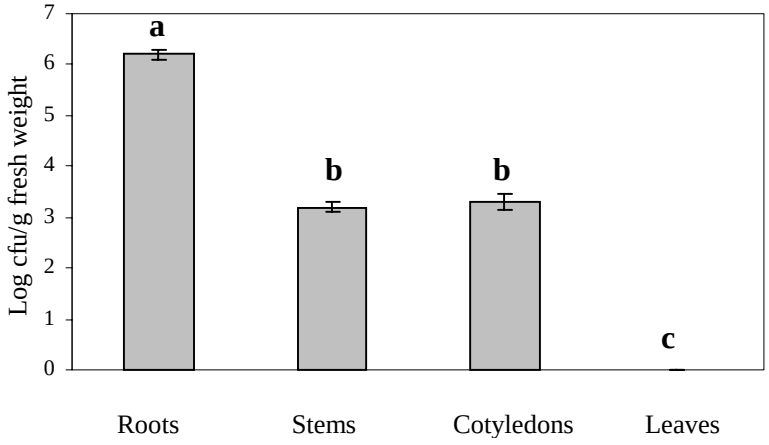

Fig. 8. Colonization of tomato plant by PGPR strain 89B61r inoculated into soil-less mix 42 days after planting.

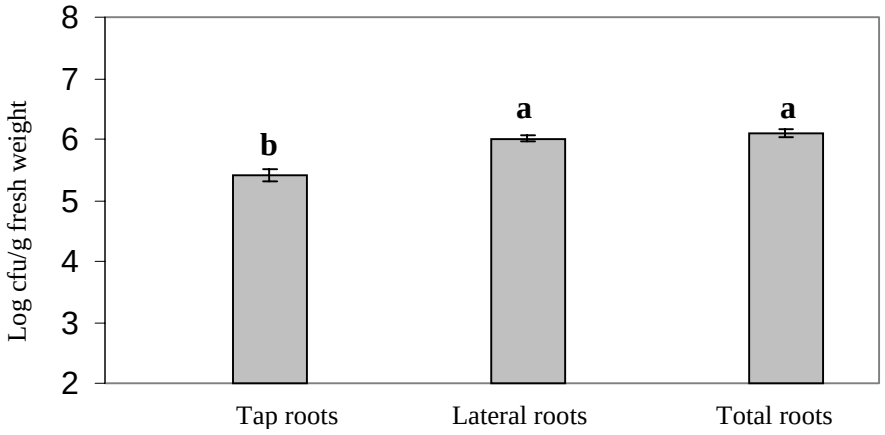

Fig. 9. Colonization of tomato roots by PGPR strain SE34r 6 weeks after planting.

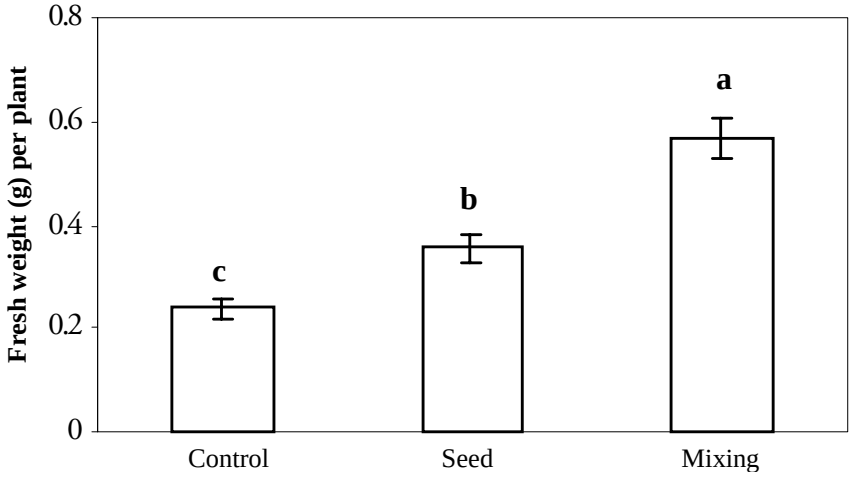

Fig. 10. Tomato seedling growth promotion by seed application and mixing into soil-less media of SE34. Control: Non-treated control; Seed: dropping 100ul of SE34 (10^9 cfu/ml) onto one seed; Mixing: Mixing SE34 into "Speedling" media at 10^8 cfu/g mix.

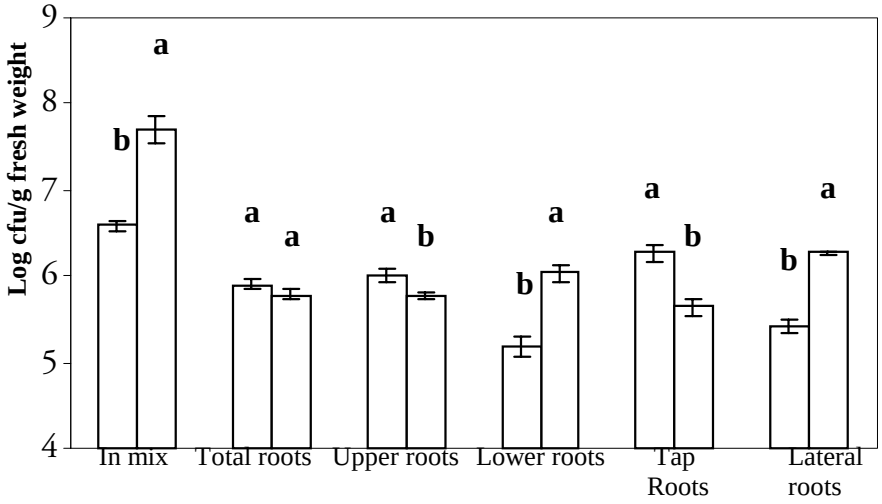

Fig. 11. Colonization of tomato roots by PGPR strain SE34r with two application methods 6 weeks after planting. In mix: populations in mix around roots; Total roots: population in whole root system; Upper root &lower roots: upper half & lower half of the whole roots; Taproots: roots removing the entire lateral root; Lateral roots: roots without tap roots. Columns with light background stood for seed application, dark columns represented mixing application. See text for description of the two application methods.

Discussion

The results presented here demonstrated a dose-dependent relationship of tomato plant growth promotion and ISR against tomato late blight. The thresholds of inoculum densities for tomato plant growth and ISR with two different PGPR strains were determined. Correlation analysis indicated a strong relationship between plant growth promotion and ISR against late blight in tomato plants by the select two PGPR strains. Persistence of two select PGPR strains in soil-less media and tomato plants showed that *B. pumilus* strain SE34r survived in soil-less media better than did *P. fluorescens* strain 89B61r. Colonization of whole tomato plants with the two strains was apparently different. Two application methods which were generally used to delivery PGPR for plant growth promotion and ISR showed different effects on growth promotion and different colonization patterns.

These results agree with previous studies that showed strong correlations between inoculum densities or initial populations on seeds/seed pieces and biological control capacity and that inoculum thresholds of PGPR are required for biological control and growth promotion (Suslow, 1982; Bull et al. 1991; Iswandi et al 1987). With regard to ISR, Raijmakers et al (1995) demonstrated significant a non-linear asymptotic relation between the rhizosphere population density of *P. putida* WCS358 or *P. fluorescens* WCS374 and the level of disease suppression of Fusarium wilt of radish and threshold of 10^5 cfu/g roots was required for ISR. For growth promotion, Iswandi et al (1987) demonstrated with *Pseudomonas* sp. strain 7NSK2 at least 10^6 cfu/seed to enhance maize growth significantly.

In our study, regression analysis of growth promotion and disease reduction demonstrated a strong correlation between growth increases and ISR activity by the two select PGPR strains. Although the relations between dose of inoculum and growth, and between dose and biological control have been reported, the relation between growth promotion and PGPR-mediated ISR has not been demonstrated. We reported here significant strong correlations between plant growth and ISR indicated as Y=0.89 X +7.7 for SE34 and Y=1.2X + 3.4 for 89B61, suggesting PGPR-mediated plant growth promotion and ISR are inter-related for the tested PGPR strains. This could lead to the hypothesis that the main effect of such PGPR is not induced disease resistance, but rather induced physiological changes which are manifested in multiple ways, primarily as growth promotion and secondarily as increase of some defense compounds.

Persistence of strains SE34r and 89B61r in soil-less media differed since populations of SE34r did not decrease until 4 weeks after planting while 89B61r decreased, indicating that spore-forming Bacilli strains survive better in growing media than Pseudomonad strains (Fig.5). Colonization dynamics studies showed that populations of strain SE34r at day 3 after planting increased sharply at least one log unit, and strain 89B61 at 5 day after planting increased about two log units, indicating that populations of both strains in spermosphere increased very quickly (Fig.6). This can be explained by the immediate nutrients releasing from tomato seeds which support bacteria for proliferation in spermosphere. The difference of population increasing in spermosphere suggested that pseudomonad bacteria (89B61r) might multiply quickly than bacilli bacteria. Since the populations of both strains declined as tomato roots grew, the early stage of colonization could be considered to be more important for growth promotion and ISR, as suggested previously by van Loon et al.1998.

One critical criterion to differentiate ISR from other modes of action of biological control by PGPR is the spatial separation of PGPR from target pathogens. We sampled for both strains SE34 and 89B61 in tomato roots, stems, cotyledons, and leaves. The results showed that there were no 89B61r detected on tomato leaves, while SE34r moved up to leaves with the populations about 10^5cfu/g when inoculated into soil-less media (Fig.7 & 8). These findings demonstrated that the systemic disease reduction by 89B61 was attributed to ISR. The mechanisms of systemic protection by SE34 seemed to be complicated. SE34 was found to induce the hypersensitive reaction and some defense-related chemical compounds in tomato. Therefore, ISR was apparently involved in late blight reduction by SE34. Since SE34 exhibits inhibition to *P.infestans in vitro* (data not shown), the persistence of SE34 on tomato leaves in disease reduction needs to be studied.

To ensure the consistent performance of PGPR on growth promotion and ISR activities, the application methods are often considered to be an important aspect. Seed application is a very commonly used method to improve plant growth for row crops, but incorporation of PGPR into soil-less media is an alternative for the vegetable transplant industry. Mixing SE34r into soil-less media significantly improved tomato seedling growth, compared to seed application (Fig. 10). Colonization of tomato plants by SE34r with the two methods was apparently different. Seed application resulted in significantly higher populations in upper roots and in taproots while mixing into media was exactly opposite (Fig. 11). A separate experiment conducted to detect the differences of colonization in lateral roots, taproots and total roots when mixing SE34 into soil-less

media indicated that populations of SE34r in lateral roots were significantly higher than in taproots (Fig.10). As the root grows, mixing application continues to provide additional inocula. In contrast, bacteria by seed application must disperse along the growing roots from a single local source of inoculum (Parke, 1991). With respect to distribution of PGPR along the roots, seed application generally results in a lognormal pattern along the roots with highest population near the seeds and the lower population near the root tips (Loper et al. 1984). These results agree with the points made by Schroth and Becker (1990) that population sizes of PGPR are greater on roots when inoculum is incorporated into soil than when applied as seed treatment, and that transplant systems offer ideal delivery methods for use of PGPR. Taken together, we conclude that mixing application into soil-less media provide better performance in growth promotion than seed application and this was attributed to the uniform colonization of the whole root system.

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