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Enhancement of Population Densities of *Pseudomonas putida* PpG7 in Agricultural Ecosystems by Selective Feeding with the Carbon Source Salicylate

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Sodium salicylate (1,000 μg/ml) was delivered through a drip irrigation system to agricultural field soils planted to tomato and infested with *Pseudomonas putida* PpG7, the host of the salicylate catabolic plasmid NAH7. In nonfumigated soils infested with approximately 10^3 CFU of PpG7 per g in the top 30 cm, population densities were increased up to 112-fold within 14 days of the initial application of salicylate compared with the densities in the respective nonamended soils. Mean season-long population densities of PpG7 in the top 30 cm of soil were significantly increased ($P < 0.01$) from 216 CFU/g in nonamended soils to 1,370 CFU/g in salicylate-amended soils. In the respective rhizosphere soils, mean population densities of PpG7 were significantly increased ($P < 0.01$) from 92 to 2,066 CFU/cm of root. Soil fumigation interacted ($P < 0.01$) with salicylate amendment and further increased the mean population densities of PpG7 in nonrhizosphere soil by an additional 5,689 CFU/g of soil. This fumigation effect was not detected in rhizosphere soils. The effect of salicylate in increasing population densities of PpG7 in soil also was affected by inoculum level, field site, and soil depth. Proportionate differences were greater in soils infested with approximately 10^3 CFU of PpG7 per g than in comparable soils infested with 10^5 CFU/g. In low-inoculum soils, increases from salicylate amendments were 26- and 29-fold in rhizosphere and nonrhizosphere soils, respectively, and in high-inoculum soils, the respective increases were 5.6- and 5-fold. No increases of fungi able to utilize salicylate were detected in soils amended with salicylate. However, soil fumigation with metham-sodium significantly reduced ($P < 0.01$) population densities of fungal salicylate utilizers in rhizosphere and nonrhizosphere soils.

The activities and population densities of bacteria generally decline with time after their application to soil. Consequently, beneficial responses such as plant growth promotion, suppression of plant pathogens, and bioremediation of recalcitrant compounds are usually of short duration (12, 16, 17). Therefore, it is important that methods be developed to prolong the persistence and impact of introduced bacteria. Research published elsewhere showed that the addition of a selective carbon source to soil enhanced respiration and growth of *Pseudomonas putida* PpG7 in microcosms containing agricultural field soils but that there was not a good correlation between activity and population density (6). Activity measured by evolution of CO_2 peaked within 18 to 24 h and decreased rapidly, whereas the peak population density was maintained. The catabolic plasmid NAH7 (8) enabled *P. putida* PpG7 to utilize salicylate as a sole source of carbon in soil, whereas the indigenous bacteria could not. Indigenous fungi utilized salicylate, but strain PpG7 competed well for the substrate in the presence of these fungi. For example, in four soils representing a range of textures and organic matter contents, population densities of PpG7 increased from approximately 10^4 to approximately 10^8 CFU/g of soil when salicylate was added by using a different system. Ogunseitan (14) reported that as little as 16 μg of salicylate caused a significant increase in the amount of *nah*-related genes of bacteria in naphthalene-polluted soil.

Since laboratory microcosms reflect only a subset of environmental factors found in nature (17), experiments were undertaken to validate the response of PpG7 to salicylate addition under field conditions. The purpose of this study was to determine the efficacy of this strategy in natural and fumigated soils planted to tomato and infested with low and high population densities of the target bacterium PpG7.

MATERIALS AND METHODS

Bacterial strain and inoculum preparation. *P. putida* PpG7 (ATCC 17485) (8, 19) was used in this study. Culture and inoculum preparation were the same as reported elsewhere (6). King's medium B (10) and Ayers' minimal medium (2) were used to isolate bacteria.

Experimental design of the population study. Experiments were done at the University of California South Coast (SC) and West Side (WS) Research and Extension Centers near Irvine and Huron, Calif., respectively. Soil types were described by Colbert et al. (6). Investigations were done during the summers of 1990 (SC) and 1991 (WS). Metham-sodium, sodium salicylate, and bacterial inocula were applied through a drip irrigation system to five replicate one-row plots arranged in a randomized complete block design (4, 5, 20). Drip lines with emitters spaced at 30-cm intervals were buried to a depth of approximately 2.5 cm in the middle of planting beds and delivered irrigation water at a rate of 2 liters per emitter per h. Beds were 3.04 by 0.55 m on 1-m centers at SC and 4.57 by 0.6 m on 1.68-m centers at WS. Slow-release Osmocote fertilizer (14-14-14 formulation; 3- to 4-month formulation; Sierra Chemical Company, Milpitas, Calif.) was applied in two bands, 10 cm deep and 10 cm off-center, at a rate of 300 g/m. All plots at SC and some at WS were preplant fumigated with metham-sodium in the irrigation water at a rate of 74.8 ml of metham-sodium per m^2 (80 gal/acre). Three weeks after fumigation, approximately 2-month-old tomato seedlings (UC 82-L) were transplanted

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next to each emitter. Plants were fertilized (15-30-15 formulation; 12.4 g/m) during the first irrigation after transplanting.

A single application of PpG7 was made 1 week after transplanting at SC and immediately after transplanting at WS. The inoculum was removed from bacterial lawns on King's medium B (20 petri dishes [150 by 25 mm]) by washing with 1 liter of 10 mM phosphate buffer (PB; approximately 5×10^9 CFU/ml). The stock 1 liter of inoculum was then delivered to the field at full strength (high inoculum) or diluted 100-fold (low inoculum).

Sodium salicylate was applied to soils during the final 1 h of a routine irrigation at a rate of 1,000 μg/ml. Salicylate was applied five times at SC and seven times at WS, initially 1 week after bacterial introduction and approximately every other week thereafter. Treatments at SC consisted of applications of high inoculum into fumigated soil, with and without salicylate. These treatments were repeated at WS. Treatments at WS also included applications of low inoculum with and without salicylate in both fumigated and nonfumigated soils. Fumigated and nonfumigated plots with salicylate, but without the addition of PpG7, were included as controls.

Population levels of PpG7 in rhizosphere and nonrhizosphere soil. PpG7 was sampled from tomato roots and nonrhizosphere soils prior to applications of salicylate (1 week after bacterial application, approximately every other week thereafter). One plant was randomly selected from each of five replicate plots per treatment, and the root system was removed from one side of the drip line with a spade (30-cm-diameter by 30-cm-deep volume of soil). Most of the soil was shaken loose, and the root-and-soil mass was placed into a plastic bag. Soil samples were taken in sterile glass or plastic vials at depths of 5, 15, and 30 cm below the drip line emitter in the hole from which the roots were lifted. Soil and root samples were transported in an iced plastic cooler and stored at 5°C or processed immediately. Root samples from SC were processed on the day of sampling, and soil samples were processed within 2 days. Root samples from WS were processed the same or next day, and soil samples were processed within 2 days.

Root samples were shaken free of loose soil, and up to 10 secondary roots were excised from the taproot. One-centimeter segments were removed from each secondary root at the taproot proximal end, the middle, and the taproot distal end. The root segments were bulked by segment site (proximal, median, terminal) in 10 ml of PB and agitated with a vortex mixer for 1 min, and the washings were serially diluted in 9-ml PB blanks. Two 50-μl subsamples from each dilution were then spotted onto separate plates of Ayers' minimal medium (2) containing 10 mM sodium salicylate (MMsal) in duplicate. Spots diffused to a diameter of approximately 2 cm. Plates were incubated on the benchtop (25 ± 5°C) or in an incubator (28°C). Colonies were counted between 2 and 5 days of incubation. Only spots which contained 1 to 200 colonies were counted. Authentication of strain PpG7 was done by removing colonies from the spot plates, streaking the colonies on MMsal, and observing colony growth and morphology as reported elsewhere (6). More than 850 colonies, putatively identified as PpG7, were further tested for their phenotypic profiles in conjunction with those isolated in the experiments of Colbert et al. (6). These included isolates from all field plots, including control soils amended only with salicylate. This also was done to determine if the plasmid might have been transferred to other pseudomonads.

Soil samples were processed by placing about 1 g (fresh weight) from each sample depth (5, 15, and 30 cm) into a 9-ml PB blank. The samples were agitated with a vortex mixer for 1 min, serially diluted in PB, plated, and incubated as described previously. Leftover soil was weighed and dried at 110°C, and the percent moisture was determined for each sample.

Data analysis. Population densities of PpG7 at SC and PpG7 and fungal utilizers of salicylate at WS were estimated from dilutions with counts ranging between 1 and 100 colonies. Bacterial and fungal counts were expressed as CFU per centimeter of root or gram of oven-dried soil and as the logarithm (base 10) of these values. Data sets were analyzed for normality with the Kolmogorov-Smirnov test for goodness-of-fit (CoStat Statistical Software, Cohort Software, Berkeley, Calif.) (18) and found to conform more closely to lognormal rather than normal distributions. Therefore, all data sets were $\log_{10}$ transformed prior to statistical analysis.

Mean population densities of PpG7 and salicylate-utilizing fungi in nonamended and salicylate-amended soils were analyzed as factorial randomized complete-blocks analyses of variance (CoStat Statistical Software) (13). Analyses included comparisons of the following: (i) mean population densities of PpG7 in low-inoculum soils (± metham-sodium, ± salicylate) at WS; (ii) mean population densities of PpG7 in high-inoculum fumigated soils (± salicylate) at SC and WS; (iii) mean population densities of PpG7 in fumigated soils receiving low or high inoculum (± salicylate) at WS; and (iv) mean population densities of fungal salicylate utilizers in fumigated and nonfumigated soils with or without salicylate. Where significant differences were detected, the Student-Newman-Keuls multiple-comparison test was used to separate the means.

Mean population densities presented in the text are averages of transformed data pooled from all sample dates and soil depths or root segments unless otherwise noted. Results presented in figures are season-long or individual sample date averages of transformed data for each sample site ± standard errors of the means. The means for treatments with low inoculum were from WS data, and those with high inoculum were from SC and WS data. There were five replicates per sample, three sample positions, and five and eight sample dates at SC and WS, respectively.

RESULTS

Authentication of strain PpG7. The phenotypes of 1,000 soil isolates of putative PpG7 including those from Colbert et al. (6) were combined and tested for carbon and nitrogen utilization patterns (9). There was complete correlation between presumptive identification of PpG7 on MMsal and identification by phenotypic tests. There was no indication of plasmid transfer to other pseudomonads. Isolation of other naphthalene-utilizing bacteria was negligible. In a few isolations, including those from plots amended with salicylate without PpG7, other naphthalene-utilizing bacteria were found. These had entirely different colony morphologies and were easily distinguished from PpG7.

Effect of salicylate on population densities of PpG7 in low-inoculum soils. The population densities of strain PpG7 in low-inoculum nonrhizosphere soils at WS 1 week after introduction through drip lines were approximately 11,000, 450, and 900 CFU/g soil at 5-, 15-, and 30-cm depths, respectively. Population densities of PpG7 were increased up to 207- and 112-fold in fumigated and nonfumigated soils,

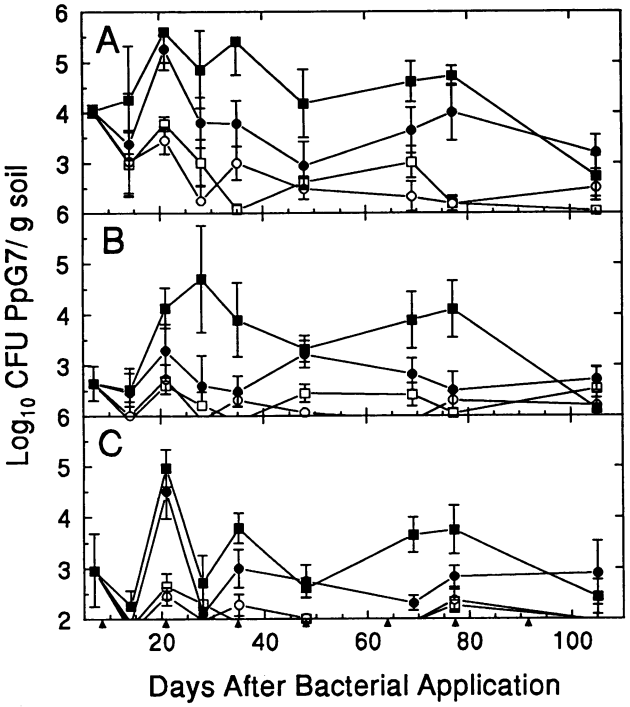

FIG. 1. Population dynamics of PpG7 in nonfumigated and metham-sodium-fumigated soils at WS at 5 (A), 15 (B), and 30 (C) cm below the drip line. Values are the mean population densities (± standard errors) at each sample date in salicylate-amended and fumigated (■), salicylate-amended and nonfumigated (●), nonamended and fumigated (□), and nonamended and nonfumigated (○) plots. Sodium salicylate was added at 1,000 μg/ml in the irrigation water on dates indicated by triangular markers along the x axis. The detection limit of PpG7 in soil was approximately 100 CFU/g.

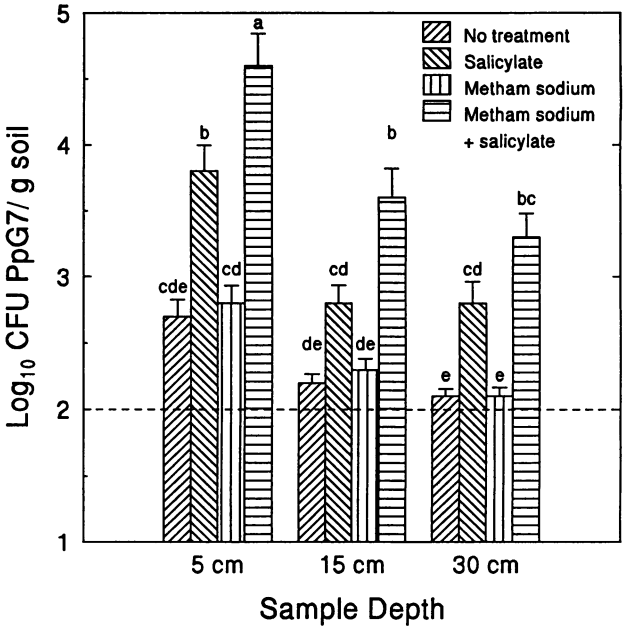

FIG. 2. Season-long mean population densities of PpG7 in metham-sodium-fumigated and nonfumigated soils amended with salicylate at WS. Mean season-long population densities (± standard errors) represented by bars below the same letter are not significantly different at $P = 0.05$. Soils were infested with approximately 10^3 CFU of PpG7 per g. Sodium salicylate was added at 1,000 μg/ml in the irrigation water. The dashed line indicates the theoretical detection limit.

respectively, within 14 days of the first application of salicylate (Fig. 1). Comparison of season-long means derived from data pooled from all sample dates and depths revealed that amendments of salicylate resulted in significantly greater ($P < 0.01$) mean population sizes of PpG7 in both fumigated and nonfumigated soils. Season-long means were 7,059 and 243 CFU/g of soil in salicylate-amended and nonamended fumigated plots, respectively, and 1,370 and 216 CFU/g of soil in the respective amended and nonamended nonfumigated plots. Multiple comparisons ($P = 0.05$) of season-long population means indicated that amendments of salicylate significantly increased population densities of PpG7 at all sample depths except at 15 cm in nonfumigated soil (Fig. 2). However, a significant interaction ($P = 0.02$) indicated that the population increase caused by salicylate amendment was affected by sample depth. As shown in Fig. 2, a greater increase in population density occurred at a 5-cm depth than at a 15- or 30-cm depth in soils amended with salicylate. Analysis of the data in Fig. 2 also indicated a significant interaction ($P < 0.01$) between fumigation and salicylate treatments. The mean population densities of PpG7 in salicylate-amended soils increased more in fumigated plots (6,815 CFU/g) than in nonfumigated plots (1,154 CFU/g).

The effect of salicylate in increasing population densities of PpG7 was also evident in rhizosphere soils. Population densities in tomato rhizospheres in both fumigated and nonfumigated plots amended with salicylate were increased up to approximately 37-fold compared with population densities in nonamended plots within 7 to 14 days of the initial application of salicylate (Fig. 3). An analysis of season-long means derived from data pooled from all sample dates and root segments (Fig. 4) indicated that amendments of salicylate to soil resulted in significantly greater ($P < 0.01$) population densities of PpG7 throughout the season. Mean season-long population densities of PpG7 were 2,413 and 94 CFU/cm of root in fumigated soils amended and not amended with salicylate, respectively. In the respective nonfumigated rhizosphere soils, the population densities were 2,066 and 92 CFU/cm of root. The data in Fig. 4 also show that neither fumigation nor proximity of sampled root segments to the taproot appreciably affected results.

Effect of salicylate amendment on population densities of PpG7 in high-inoculum soils. The initial population densities of PpG7 after introduction to fumigated soils at 5-, 15-, and 30-cm depths were approximately 2.6×10^5, 1.7×10^4, and 1.25×10^4 CFU/g of soil at WS and 3.3×10^5, 1.8×10^5, and 1.7×10^5 CFU/g of soil at SC. Data showing the effects of salicylate amendments on population dynamics during the season were similar to those previously presented for low-inoculum soils at WS for both rhizosphere and nonrhizosphere soils. Mean season-long population densities of PpG7, derived from pooling data from all soil depths at WS and SC, were significantly greater ($P < 0.01$) in both nonrhizosphere and rhizosphere soils amended with salicylate than in nonamended soils. Mean season-long population densities in amended and nonamended nonrhizosphere soils were 63,567 and 16,574 CFU/g of soil, respectively. In rhizosphere soils, mean population densities derived from all

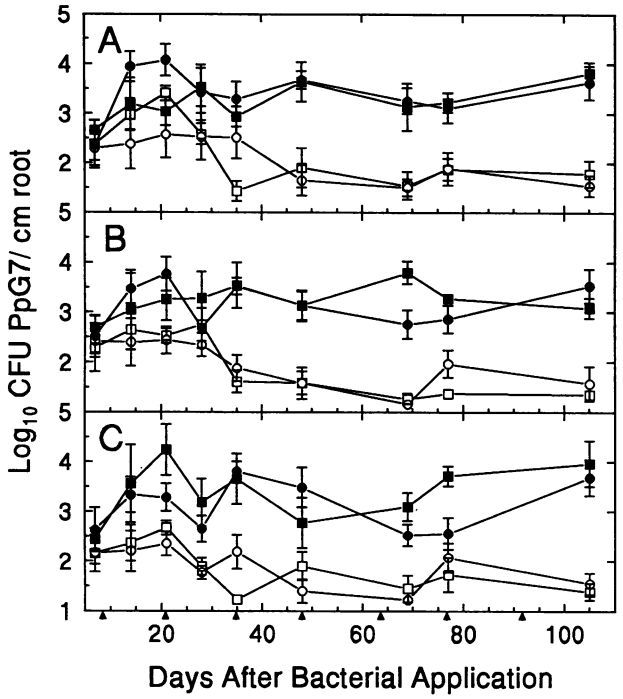

FIG. 3. Population dynamics of PpG7 in rhizosphere soil from 1-cm secondary root segments in metham-sodium-fumigated and nonfumigated soils at WS. Segments were proximal (A), median (B), and terminal (C) to the taproot. Values are mean population densities (± standard errors) at each sample date in salicylate-amended and fumigated (■), salicylate-amended and nonfumigated (●), nonamended and fumigated (□), and nonamended and nonfumigated (○) plots. Sodium salicylate was added at 1,000 μg/ml in the irrigation water on the dates indicated by triangular markers along the lower *x* axis.

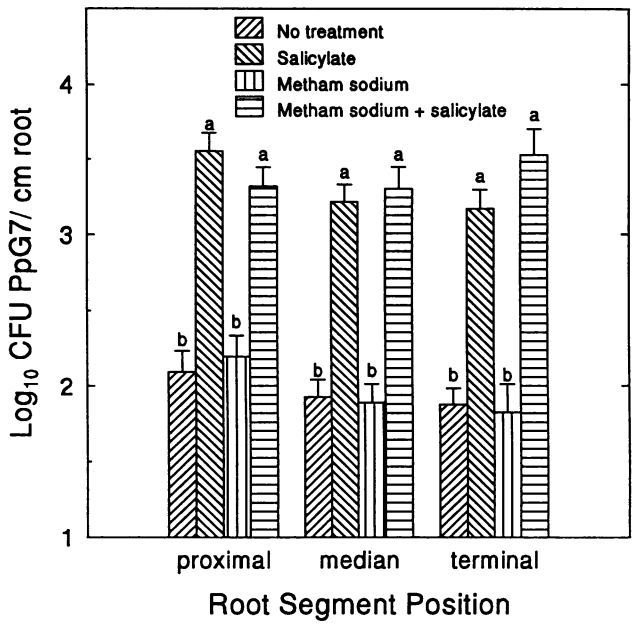

FIG. 4. Season-long mean population densities of PpG7 in metham-sodium-fumigated and nonfumigated rhizosphere soils amended with salicylate at WS. Mean season-long population densities (± standard errors) represented by bars below the same letter are not significantly different at $P = 0.01$. Soils were infested with approximately 10^3 CFU of PpG7 per g. Sodium salicylate was added at 1,000 μg/ml in the irrigation water.

root segments were 16,477 and 5,141 CFU/cm in amended and nonamended soils, respectively.

Although the response of PpG7 to salicylate amendment at WS was similar in high-inoculum soil to that shown in Fig. 1 for low-inoculum soil, proportionate increases were greater in low-inoculum soils. However, the overall increase in CFU was much greater in high-inoculum soils. The mean differences in amended and nonamended rhizosphere soils (pooled root segments) were 1.41 and 0.75 $\log_{10}$ CFU/cm of root in low-inoculum (26-fold increase) and high-inoculum (5.6-fold increase) soils, respectively. The respective differences in nonrhizosphere soils were 1.46 and 0.70 $\log_{10}$ CFU/g in the low-inoculum (29-fold increase) and high-inoculum (5-fold increase) plots. These proportionate increases in population densities of PpG7 in low-inoculum plots were significantly greater ($P < 0.01$) in both rhizosphere and nonrhizosphere soils than in high-inoculum plots.

Analysis of effect of field site on population dynamics of PpG7. An analysis of the effect of field site on population dynamics of PpG7 was made by comparing the results of identical high-inoculum fumigated plots at WS and SC (Fig. 5). When data from all soil depths were pooled, an analysis indicated that population densities were significantly less ($P < 0.01$) at WS than at SC in both amended and nonamended soils. However, pooling of data masked a significant interaction ($P < 0.01$) between field site and sample depth. At the 5-cm depth, there was no difference in season-long means

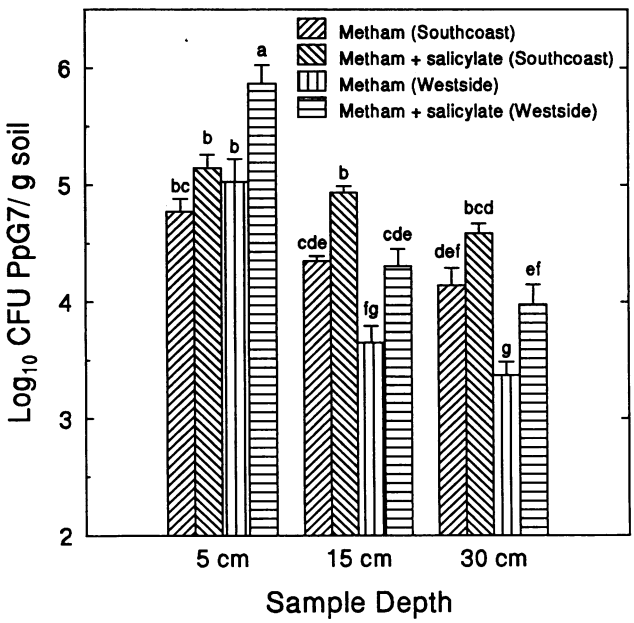

FIG. 5. Season-long mean population densities of PpG7 in metham-sodium-fumigated soils amended with salicylate at WS and SC. Mean season-long population densities (± standard errors) represented by bars below the same letter are not significantly different at $P = 0.01$. Soils were infested with approximately 10^5 CFU of PpG7 per g. Sodium salicylate was added at 1,000 μg/ml in the irrigation water.

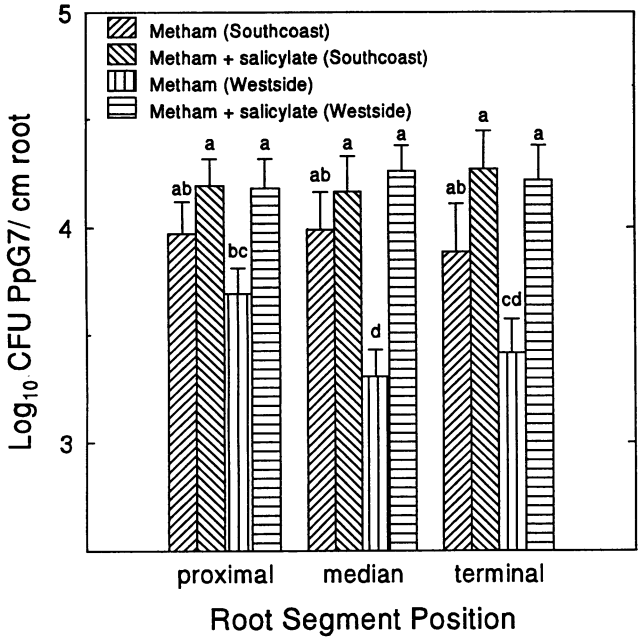

FIG. 6. Season-long mean population densities of PpG7 in metham-sodium-fumigated rhizosphere soils amended with salicylate at WS and SC. Mean season-long population densities (± standard errors) represented by bars below the same letter are not significantly different at $P = 0.01$. Soils were infested with approximately 10^5 CFU of PpG7 per g. Sodium salicylate was added at 1,000 μg/ml in the irrigation water.

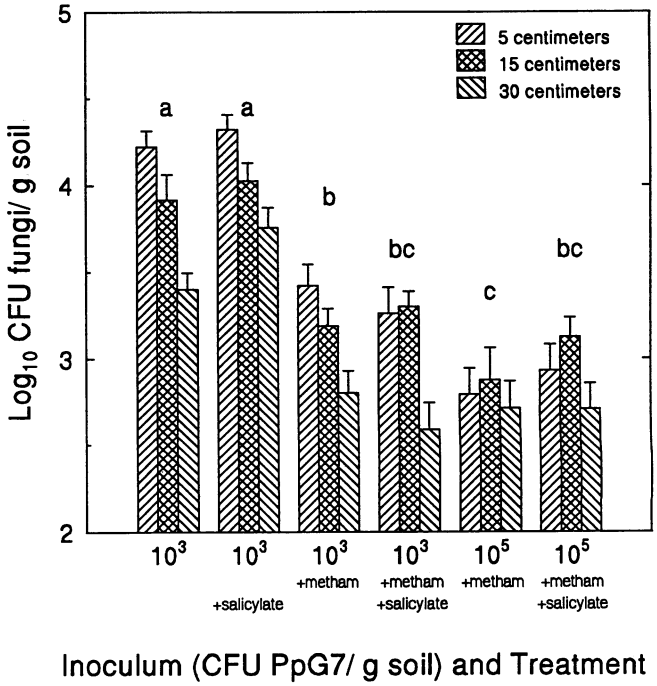

FIG. 7. Season-long mean population densities (± standard errors) of fungal salicylate utilizers at 5-, 15-, and 30-cm depths in metham-sodium-fumigated and nonfumigated WS soils amended with salicylate. Mean population densities of treatments (pooled depths) below the same letter are not significantly different at $P = 0.01$. Sodium salicylate was added at 1,000 μg/ml in the irrigation water.

between the population densities at WS (18% sand) and SC (75.5% sand) for nonamended soils, whereas at the 15- and 30-cm depths, population densities at WS were significantly lower ($P = 0.01$) than those at SC. Results in salicylate-amended soils were similar, except that the population density was significantly greater at the 5-cm depth at WS than at SC.

Field site also had a significant effect on rhizosphere populations. Pooling of data from all root segments indicated that population densities of PpG7 were significantly less ($P < 0.01$) at WS than at SC in nonamended soils. However, there was a significant interaction ($P < 0.01$) between salicylate treatment and field site (Fig. 6). Whereas mean season-long population densities of PpG7 in nonamended rhizosphere soils were less at WS than at SC, populations of PpG7 at WS and SC were statistically the same when salicylate was added to the soil.

Effect of salicylate amendment and soil fumigation on population densities of salicylate-utilizing fungi. Analysis of pooled data from all soil depths and root segments revealed that the addition of salicylate did not increase season-long mean population densities of fungal salicylate utilizers in soil (Fig. 7) or on roots (Fig. 8) at WS. However, mean population densities of salicylate-utilizing fungi in soil and on roots were significantly reduced ($P < 0.01$) by fumigation with metham-sodium. Season-long mean fungal population densities from data pooled from fumigated soils were 180 and 1,000 CFU/g in rhizosphere and nonrhizosphere soils, respectively. The respective values from nonfumigated soils were 630 and 7,940 CFU/cm of root in rhizosphere and nonrhizosphere soils.

DISCUSSION

Field data collected over 2 years from different locations have shown that application of a specific carbon source enhanced the population densities of an introduced bacterial strain in soil and on plant roots. This extends the results of other laboratory studies indicating that salicylate amendment selectively increased the metabolic activity and population growth of *P. putida* PpG7 in soil microcosms (6, 14). Although the metabolic status of PpG7 was not directly measured in the field, population increases in salicylate-amended soils presumably reflected metabolic activity. Ogunseitan et al. (14) isolated *nah* mRNA transcripts from salicylate-degrading strains 30 days after inoculation. This indicated that bacteria were still metabolically active. This is important because enhancement of active processes, such as colonization and production of antibiotic compounds, will lead to more effective use of beneficial organisms such as plant growth promoters and bioremediation agents.

Ecological niches can support only a finite amount of biomass, and increasing the level of a specific substrate ultimately reaches the point where there is no effect because of other limiting factors (11). Thus, the seeding of soil or inoculation of plant parts with high-density inocula prevents accurate determinations of the ability of a strain to grow. Thus, if the environmental parameters are equal, a small initial population of a bacterium will increase relatively more than a large initial population. Indeed, the mean population densities of PpG7 in salicylate-amended soils at WS were increased 29-fold in low-inoculum plots compared with 5.6-fold in high-inoculum plots. This indicates great potential for

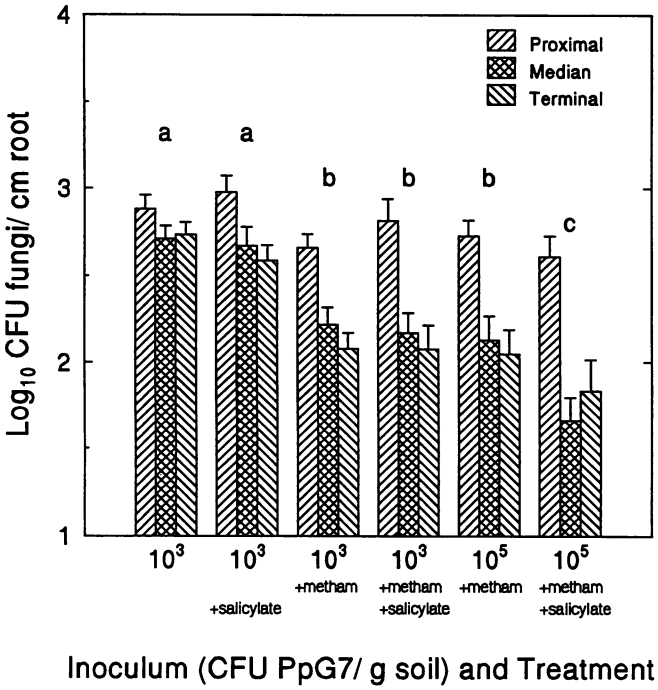

FIG. 8. Season-long mean population densities (± standard errors) of fungal salicylate utilizers in rhizospheres of secondary root segments proximal, median, and terminal to the taproot in metham-sodium-fumigated and nonfumigated WS soils amended with salicylate. Mean population densities of treatments (pooled root segments) below the same letter are not significantly different at $P = 0.01$. Sodium salicylate was added at 1,000 μg/ml in the irrigation water.

increasing target bacterial populations which have been added to soil at low numbers or which have fallen from initially high numbers. However, even small increases in population densities may have great biological significance because of increased metabolic activity and production of beneficial compounds in the soil and rhizosphere. When environmental conditions differ, as for example at two different field sites, the response of organisms to selective feeding might be expected to vary. It was found that the distribution of PpG7 in soil was affected by field site, with reduced population densities at 15- and 30-cm depths at WS compared with those at SC. This may have been caused by physical barriers to bacterial movement in the clay soil at WS. However, selective feeding with salicylate was effective at both sites.

The application of salicylate to soil also caused substantial increases of PpG7 in the rhizosphere. The increased population sizes in the rhizosphere are likely the result of several nonexclusive mechanisms. The enhanced reservoir of inoculum in amended soil should increase the probability of contact between PpG7 and a passing root. Increased population densities near the soil surface also would provide more inoculum for dissemination during irrigation (3). Most important, salicylate provided an additional carbon source for PpG7 in the rhizosphere, where competition for nutrients is intense.

The selection of an appropriate carbon source involves compromises between substrates which are highly recalcitrant (selective but support slow growth) and substrates which are less resistant to degradation (support rapid growth but are less selective). Low-molecular-weight aromatic compounds, such as salicylate, are moderately resistant to attack (12, 15). Other laboratory research with various agricultural soils indicated that native bacterial utilizers of salicylate were below detection levels (6). However, fungal utilization of salicylate was evident in all soils tested. The suppression of fungal activity in laboratory microcosms with cycloheximide allowed PpG7 to attain greater population density increases in soils amended with salicylate. Fumigation of field soil also enabled PpG7 to attain higher population densities in response to the addition of salicylate. The effect of fumigation was found only in plots receiving salicylate, suggesting that increased population sizes of PpG7 were caused by reduced competition of fungi for salicylate and not the elimination of bacterial predators (1). Although we have not detected salicylate-utilizing fungal plant pathogens, care must be exercised when adding substrates to soil since there always is the possibility that deleterious organisms may use them.

The strategy of selectively feeding beneficial organisms in the field has a precedent in the use of cover crops to increase specific populations of soil microorganisms (7). Cover crops affect microorganisms in part by the substrates released by exudation and decomposition. The use of specific substrates or groups of substrates is envisioned as a means of enhancing the competitive abilities of target organisms in an analogous manner. The growth response of PpG7 to amendments of salicylate in the rhizosphere and nonrhizosphere soils indicates great promise for the use of nutrition as a means of managing populations of organisms. However, it also is very apparent from the results of Colbert et al. (6) that activity and population density are not necessarily correlated. Thus, methods need to be developed to provide a prolonged source of nutrients to maximize activity. Although PpG7 is not an organism of interest for affecting plant growth, its plasmid or specific genes which confer utilization of salicylate could be transferred to known beneficial organisms, thereby allowing selective feeding.

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