Interaction Between Strains of Pseudomonads in Sugar Beet Spermospheres and Their Relationship to Pericarp Colonization by *Pythium ultimum* in Soil

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Accepted for publication 18 August 1994.

ABSTRACT

Fukui, R., Schroth, M. N., Hendson, M., and Hancock, J. G. 1994. Interaction between strains of pseudomonads in sugar beet spermospheres and their relationship to pericarp colonization by *Pythium ultimum* in soil. Phytopathology 84:1322-1330.

The growth and interaction of coinoculated strains of *Pseudomonas* spp. on sugar beet seed were monitored for 48 h after planting in natural soil maintained at −15 J/kg and 16 C. There was no interaction between and among strains in the spermosphere when they were inoculated together at approximately 10^4 cfu per seed. Strains capable of producing antibiotics and siderophores did not inhibit sensitive strains in the spermosphere. However, antagonism between strains often occurred when seeds were coinoculated with one strain at a high inoculum density (approximately 10^7 cfu per seed) and the other at a low density (approximately 10^4 cfu per seed). For example, growth of *Pseudomonas fluorescens-putida* ML5 was inhibited in the spermosphere when coinoculated with high densities of *P. fluorescens* A1 or *P. putida* GR12-2. In contrast, *P. putida* 33-2 and A1 were less affected under similar conditions. Although ML5 was inhibited by strains A1 and GR12-2 on King's medium B, the addition of 100 μM $FeCl_3$ or Fe-EDTA into soil did not affect growth of ML5 in the spermosphere. This suggested that siderophore production was not a mechanism that affected inhibition of ML5 in the spermosphere. Growth of rifampicin-resistant strains in the spermosphere was inhibited in the presence of near-isogenic wild type strains on seed when the two were inoculated at low and high inoculum densities, respectively. However, inhibition did not occur when the plasmid NAH7 was introduced into the low-inoculum rifampicin-resistant strain and sodium salicylate was added to the soil. The plasmid enabled the bacterium to use the substrate. A reduction to complete nullification of growth inhibition by addition of sodium salicylate in soil also was observed when the low-inoculum strain harboring the plasmid was coinoculated with different (nonisogenic) strains at high densities. These results suggest that competition for carbon was the primary factor affecting antagonism in the spermosphere. The effectiveness of ML5 in reducing pericarp colonization by *Pythium ultimum* was significantly reduced in dual strain inoculations with A1. Other combinations of strains did not suppress pericarp colonization by *P. ultimum* more effectively than did single strain inoculations.

It is generally thought that combinations of antagonistic microorganisms would be more effective in controlling soilborne pathogens than single microorganisms. The rationale is that multiple antagonists would offer more versatility in mechanisms of action against pathogens and also increase the probability of antagonists interacting with pathogens over a large range of microclimates. This should also broaden the spectrum of disease control in the field when two or more diseases are operating in tandem. However, multiple biocontrol agents will not be effective if they are antagonistic to one another. Thus, it is important to know the mechanisms by which biocontrol agents inhibit pathogens and whether or not they are inhibitory to each other.

Antagonistic interactions in vitro are usually caused by the production of secondary metabolites such as antibiotics (9,11,32) and siderophores (25,26,35). Some of these substances may affect the efficacy of biological control agents (6,15–18,29,31,34,37). However, expression of antagonism by a microorganism toward a pathogen in culture media cannot be considered evidence that the microorganism will have a functional role in controlling the pathogen in the field. In many cases, microorganisms that display in vitro antibiosis against plant pathogens were not effective in inhibiting them in greenhouse or field trials (2,23,30,31). The nutritional environment and many other factors that affect growth and survival of biocontrol agents in nature are considerably different from those in nutrient-rich culture media. This is why observed interactions of antagonists in culture media often bear little relationship to their activities in nature (e.g., when they are coinoculated onto target sites).

Damping-off of sugar beet (*Beta vulgaris* L.) offers an ideal model disease system for studying the spatial and temporal relationships between different putative biological control agents and the sugar beet seed pathogen *Pythium ultimum* Trow var. *ultimum*. Bacterial antagonists can be delivered directly and uniformly to the infection court by inoculation of seeds. Seed exudates released during imbibition of the seed support rapid growth of these bacteria and *P. ultimum* (27). Thus, this system allows quick assessment of microbial interactions and the effectiveness of antagonists in reducing pericarp colonization. Moreover, since the pericarp of sugar beet seed is dead tissue (28), it is possible to study microbial interactions without interference from host resistance factors.

This study investigates the growth of various combinations of pseudomonads in sugar beet spermospheres and relates the growth to their inhibitory behaviors on culture media. These strains are tested for their ability to suppress pericarp colonization by *P. ultimum*. A preliminary report was published elsewhere (7).

MATERIALS AND METHODS

Seed. Sugar beet seed cv. USH11 (Holly Sugar Co., Colorado Springs, CO), size 8–9, was used in all experiments. Most of the outer corky perianth had been removed from the pericarp of the seed. The word seed in this study refers to the entire structure of true seed and pericarp.

Soil. Oceano loamy sand (pH 6.9–7.0) collected from a farm field near Moss Landing, California, was used in all experiments. Soil samples taken from at least four locations in the field were mixed, sieved through a 5-mm-mesh screen, and stored in plastic bags at room temperature without being air-dried. Most experi-

ments were done with fresh, moist soil from the field. In some experiments, the soil was air-dried before use. Stocks of soil were replaced every 3 mo.

Bacterial strains. All strains used in these tests are reported to have potential as biological control agents of plant pathogens. Four strains, *Pseudomonas putida* 33-2 (22), *P. putida* F42 (21,22), *P. putida* GR12-2 (21,22), and *P. fluorescens* X (14), were obtained from Esso Ag. Biologicals, Saskatoon, Canada (formerly Allelix Inc., Ontario, Canada), courtesy of J. W. Kloepper. Strain PGS12 of *P. aureofaciens* (3) was from Plant Genetic Systems, Gent, Belgium. Four other strains, *P. fluorescens* A1 (19,20), *P. fluorescens* CR30, *P. fluorescens-putida* ML5 (27), and *P. putida* R20 (27), were isolated from potato (*Solanum tuberosum* L.) periderm, field soil, sugar beet (*B. vulgaris*) spermosphere, and lima bean (*Phaseolus lunatus* L.) rhizosphere in California, respectively. A transconjugant of R20 harboring a plasmid NAH7 (R20(pNAH7)) was obtained from S. F. Colbert (5).

To facilitate the enumeration of strains from a suspension with mixed strains, strain-specific detection media were prepared by incorporating specific antibiotics or chemicals into King's medium B (KB) (13) according to the resistance of the strain (Table 1). Recovery efficiencies greater than 95% were observed on all strain-specific detection media when compared with unamended KB. Each strain had a distinctive colony morphology on its strain-specific medium, which was used as an additional criterion to differentiate the target strain from other strains used in a mixture or from indigenous soil bacteria that were resistant to the specific antibiotics or chemicals. Strains A1, F42, GR12-2, R20, and X were spontaneous mutants resistant to rifampicin. Strains 33-2, CR30, ML5, and PGS12 were naturally resistant to the antibiotics or chemicals used for strain-specific media. For some experiments, rifampicin-resistant mutants of ML5 and 33-2 (referred to as $ML5^{Rif}$ and $33\text{-}2^{Rif}$, respectively) were selected on KB containing rifampicin at 100 $\mu g\ ml^{-1}$. Growth characteristics of these mutants in culture broth were the same as those of the respective wild types.

In vitro antibiosis among bacterial strains. In vitro antibiosis was determined for nine *Pseudomonas* strains by streaking various combinations on KB and potato-dextrose agar (PDA) with or without amendments of 100 μM $FeCl_3$. The first bacterial strain was streaked onto agar media and incubated at 16 C for 48 h; then the cells were scraped off the surface of the media. The remaining cells were killed with chloroform vapor. The second bacterial strain then was inoculated onto culture media by streaking vertically to the streak of the first strain and incubated at 16 C. Growth of the second strain near the streak of the first strain was examined over a 72-h period. The tests were done twice.

Inoculum preparation and seed inoculation with single and dual strains. Each bacterial strain was grown for 48 h at 28 C on four agar plates of KB. The resulting lawns were suspended in equal volumes (1.5 ml each) of 1.0% hydroxypropyl methylcellulose (Methocel HG, Dow Chemical Company, Midland, MI) and 0.1 M $MgSO_4$ (27). For single-strain inoculations, 1.5 ml of a bacterial suspension and 5 g of sugar beet seeds were mixed in a small plastic bag. For dual strain inoculations, an equal volume of cell suspensions of two strains were mixed to make 1.5 ml of bacterial suspension. An approximately 400-fold dilution of the methylcellulose-$MgSO_4$ mixture made from the initial bacterial suspension was used to obtain lower bacterial population densities on the seed. To prepare an inoculum with two strains at different inoculum densities, 3.0 ml of diluted suspension containing one strain was used to suspend cells of another strain from four KB plates, and 1.5 ml of this mixture was then applied to 5 g of seeds. The inoculated seeds were air-dried at room temperature (22–26 C) overnight before planting in soil.

TABLE 1. Strain-specific medium used for each strain

Strain	Antibiotics and chemicals added to King's medium B (ml^{-1})[z]
33-2	Spectinomycin dihydrochloride (100 μg), Chloramphenicol (25 μg), KCl (20 mg)
$33\text{-}2^{Rif}$	Rifampicin (20 μg)
A1	Rifampicin (50 μg)
CR30	Ampicillin (100 μg), Rifampicin (5 μg), Kanamycin (2 μg)
F42	Rifampicin (50 μg), Chloramphenicol (50 μg)
GR12-2	Rifampicin (50 μg)
ML5	Streptomycin sulfate (100 μg), $CuSO_4$ (800 μg)
$ML5^{Rif}$	Rifampicin (20 μg)
PGS12	Streptomycin sulfate (100 μg), $CuSO_4$ (800 μg)
R20	Rifampicin (50 μg)
R20(pNAH7)	Rifampicin (50 μg)
X	Rifampicin (10 μg), Kanamycin (2 μg), 8-hydroxyquinoline (20 μg)

[z] All antibiotics and chemicals were purchased from Sigma Chemical Corp., St. Louis, MO. All strain-specific detection media contained cycloheximide at 100 $\mu g\ ml^{-1}$ to suppress fungal growth on the media.

Adjustment of soil environmental conditions and monitoring bacterial population density in the spermosphere. The pressure plate extraction system (27) was used to adjust soil matric potential. Approximately 80 g of moist soil was placed in each of 5.5-cm-diameter brass rings on a −50 J/kg ceramic pressure plate. Ten seeds per ring were planted approximately 1 cm deep in the soil, which was then wetted to saturation with distilled water precooled at 16 C and adjusted to −15 J/kg soil matric potential using the pressure plate extraction system. After equilibration for 3–4 h, the rings with soil were transferred onto lids of plastic petri dishes, covered with plastic bags to maintain constant soil moisture, and incubated at 16 C.

At various time intervals, five of 10 seeds were randomly recovered from each ring (usually four replicate rings per time interval) and suspended in 5 ml of 10 mM sterile phosphate buffer (pH 6.9). Separate rings were used for each time interval. The population density of bacteria on the seed then was determined by dilution plate counting on a strain-specific medium. Data are usually expressed as an average of four replicates (five seeds per replicate, 20 seeds in total) per time interval.

Growth of bacterial strains in dual strain inoculation in the spermosphere. Two strains from the group A1, GR12-2, PGS12, and R20 in all combinations were coinoculated onto seed at low densities, 10^4–10^5 cfu per seed. Growth of the strains in the spermosphere were determined at various time intervals over a 48-h period. The experiment was done twice.

Growth of strains in the spermosphere also was monitored after inoculating two strains at different inoculum densities. Strain 33-2, A1, or ML5 was inoculated onto seed at approximately 10^4 cfu per seed in dual strain inoculations with several other strains at 10^6–10^8 cfu per seed. Population densities in the spermosphere were determined after 24 and 48 h. The experiments were in a randomized complete block design (RCB) with four blocks. At each time interval, five seeds from each of two rings per block were sampled.

In a separate trial, $33\text{-}2^{Rif}$ and $ML5^{Rif}$ were inoculated onto seed singly at lower densities and in combination with their respective wild type strains at higher densities. Population densities of the mutant and the wild type in the spermosphere then were determined with four replicates, each consisting of five seeds, at various time intervals over 48 h. The experiment was performed separately for $33\text{-}2^{Rif}$ and $ML5^{Rif}$, and was done twice for each strain pair.

To determine if siderophore production played a role in growth inhibition, the experiments were done with soil amended with $FeCl_3$ or Fe-EDTA. Soil was air-dried and saturated with an aqueous solution of 100 μM $FeCl_3$ or Fe-EDTA. The soil matric potential was adjusted to −15 J/kg, as described above. For the control, soil was moistened with distilled water. Seed treated with ML5 singly or in combination with A1 or GR12-2 at a greater density was planted in soil. Population densities of ML5 in the spermospheres were determined after 24 and 48 h, with four replicates per sampling time, each replicate consisting of five seeds. The experiment was done twice as a two-factor experiment in a completely randomized design with split plots (CRD split plot). Strain pairs were the main plots, and the different solutions were

the subplots.

Effect of carbon availability on growth of dual strains. To determine if competition for carbon is a primary factor affecting the growth of dual strains, growth of R20 and R20(pNAH7) were examined by monitoring population densities in the spermosphere of sugar beet in soil amended with sodium salicylate. R20(pNAH7) utilizes salicylate as a carbon source (5). Strain R20(pNAH7) was inoculated onto seed at a lower density singly or in combination with the wild type R20 (not resistant to rifampicin) at a higher density. Sodium salicylate (Fisher Chemical, Fisher Scientific, Inc., Fairlawn, NJ) was added to air-dried soil at concentrations of 0, 100, and 300 $\mu g\ g^{-1}$ of soil and mixed thoroughly in a twin-shell soil blender. Seeds were planted in soils, and bacterial populations were monitored in each soil, adjusted to −15 J/kg and 16 C, at various time intervals over a 60-h period. Population densities of wild type R20 and R20(pNAH7) were determined with four replicates per time interval, each replicate consisting of five seeds. The total population density (both R20 and R20(pNAH7)) was enumerated by plating on KB containing cycloheximide at 100 $\mu g\ ml^{-1}$. To determine the number of R20(pNAH7) colonies in the population, colonies which grew on KB containing cycloheximide were replica plated onto minimal medium containing 10 mM sodium salicylate (5) and KB containing rifampicin (50 $\mu g\ ml^{-1}$). The population density of wild type R20 was estimated by subtracting the number of colonies that grew on minimal medium containing 10 mM sodium salicylate or KB containing rifampicin (R20(pNAH7)) from the number of colonies that grew on KB containing cycloheximide. The experiment was done twice and designed as a factorial (two-factor) experiment arranged in CRD split plot. Strain pairs were the main plots, and concentrations of sodium salicylate were the subplots.

In a separate trial, R20(pNAH7) was inoculated onto seed at a lower density in dual strain inoculations with several other strains at higher densities. As the controls, some seeds also were inoculated with R20(pNAH7) singly and in combination with the wild type R20. Bacterial population densities in the spermosphere were determined at 36 and 60 h after planting seeds in soil, with or without addition of sodium salicylate (at 100 $\mu g\ g^{-1}$ of soil), adjusted to −15 J/kg, and incubated at 16 C. The experiment was done twice with the design described above.

Adjustment of inoculum density of *P. ultimum* and measurement of incidence of pericarp invasion by *Pythium* spp. in soil. The inoculum density of *P. ultimum* in soil was determined on water agar by the soil drop assay method of Stanghellini and Hancock (33). To pasteurize soil, 500-ml beakers, each containing 400 g of moist soil, were covered with aluminum foil and warmed in a heated water bath at 55–57 C for 15 min (36). No hyphae of *P. ultimum* growing in soil drops were detected after the soil was pasteurized. The inoculum density of *P. ultimum* in naturally infested soil was adjusted to desired densities by diluting with predetermined amounts of the same soil (after pasteurization) mixed thoroughly in a twin-shell blender or a portable cement mixer for at least 30 min. The resultant inoculum density in the blend was confirmed by the soil drop assay method.

Fourteen seeds per ring were planted in the soil, which was adjusted to −15 J/kg using the pressure plate extraction system as described above and incubated at 16 C. After 48 h, the seeds were recovered by wet sieving, cleaned with a strong jet of water, surface-sterilized in 0.5% sodium hypochlorite for 1 min, rinsed thoroughly under running tap water, and plated onto 2% water agar (WA) containing benomyl (Benlate 50 WP) at 15 $\mu g\ ml^{-1}$ (27). Hyphae of *Pythium* spp. (mostly *P. ultimum* but occasionally other species) growing out of pericarps were enumerated after incubation at room temperature for 48 h. The incidence of pericarp invasion by *Pythium* spp. was determined with 28 seeds pooled from two rings per replicate and expressed as an average of four replicates.

Effect of seed inoculation with dual bacterial strains on reducing pericarp colonization by *P. ultimum* in soil. Strains 33-2, A1, GR12-2, and ML5 were used in the experiments, since they were among the most effective strains in reducing pericarp colonization by *P. ultimum* in preliminary experiments. Strain ML5 was inoculated singly or in combination with 33-2, A1, or GR12-2 onto seed, both at high inoculum densities. In a separate trial, seeds were inoculated singly with 33-2, A1, or GR12-2, and in all combinations of these three strains (including triple strains in combination) at high inoculum densities. All bacteria-treated seeds were planted in soil containing approximately 25 propagules of *P. ultimum* per gram of soil, and the incidence of pericarp invasion was determined after 48 h as previously described. Control seeds were treated with a mixture of 1.0% methylcellulose and 0.1 M

TABLE 2. In vitro antibiosis between *Pseudomonas* strains on agar media

Strains inoculated second	Strains inoculated first on King's medium B																	
	Nonamended									Amended with 100 μM $FeCl_3$								
	33-2	A1	CR30	F42	GR12-2	ML5	PGS12	R20	X	33-2	A1	CR30	F42	GR12-2	ML5	PGS12	R20	X
33-2	+[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CR30	−	−	+	+	+	±	+	±	+	+	+	+	+	+	+	+	+	+
F42	−	−	−	+	−	−	+	−	±	+	+	+	+	+	+	+	+	+
GR12-2	−	±	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ML5	+	−	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+
PGS12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R20	−	−	−	±	−	±	+	+	−	+	+	+	+	+	+	+	+	+
X	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Strains inoculated first on potato-dextrose agar																	
	Nonamended									Amended with 100 μM $FeCl_3$								
	33-2	A1	CR30	F42	GR12-2	ML5	PGS12	R20	X	33-2	A1	CR30	F42	GR12-2	ML5	PGS12	R20	X
33-2	−	−	−	−	+	±	+	−	+	±	±	−	−	+	±	+	−	±
A1	−	−	−	−	±	±	−	−	−	−	−	−	−	±	±	−	−	±
CR30	±	−	−	−	±	±	+	±	+	±	−	−	±	±	±	+	±	±
F42	−	−	−	−	±	±	±	−	+	±	−	−	−	±	−	±	−	±
GR12-2	±	−	±	±	+	+	+	+	+	+	−	+	+	+	±	±	+	+
ML5	±	−	−	−	+	+	+	+	+	+	−	−	−	±	+	+	±	+
PGS12	−	−	−	−	+	−	+	+	+	+	±	−	−	+	+	+	−	+
R20	−	−	−	−	±	±	±	−	±	±	−	−	−	±	±	±	−	−
X	−	−	−	−	+	+	+	+	+	+	−	+	+	+	−	+	+	+

[a] Growth of strain inoculated second: + = normal growth, equivalent to the growth in single inoculation; ± = reduced growth, compared to the growth in single inoculation; and − = no growth after 24 h, but slight growth occasionally observed after 72 h.

$MgSO_4$. Both experiments were done twice with treatments arranged in RCB with four replicates.

Data analysis. All data in repeated trials were first analyzed by preliminary analyses of variance (ANOVA) using trials as a qualitative independent variable. Prior to pooling data from two separate trials, significance of the trial-by-treatment(s) interaction was determined by preliminary ANOVA, and homogeneity of variances between trials was verified (10). Incidence data for pericarp colonization by *P. ultimum* were transformed by arcsine-transformation prior to statistical analyses. Pooled data were analyzed by ANOVA to test effects of treatments (the main plot and subplot factors) and treatment-by-treatment interactions. Mean separation on qualitative independent variables was performed by Fisher's least significant difference test (protected LSD) and/or Duncan's multiple range test. Main effects of quantitative variables (concentrations of sodium salicylate) were analyzed by repeated measures ANOVA. All statistical analyses were conducted by Microsoft Excel Version 4.0 (Microsoft Corporation, Redmond, WA), except for repeated measures ANOVA, which was done with SAS (Statistical Analysis System, SAS Institute, Cary, NC).

RESULTS

In vitro antibiosis among strains of pseudomonads. Patterns of inhibition among test strains on KB are shown in Table 2. Strains F42 and R20 were inhibited by the greatest numbers of strains, whereas strains 33-2, A1, and PGS12 were not inhibited

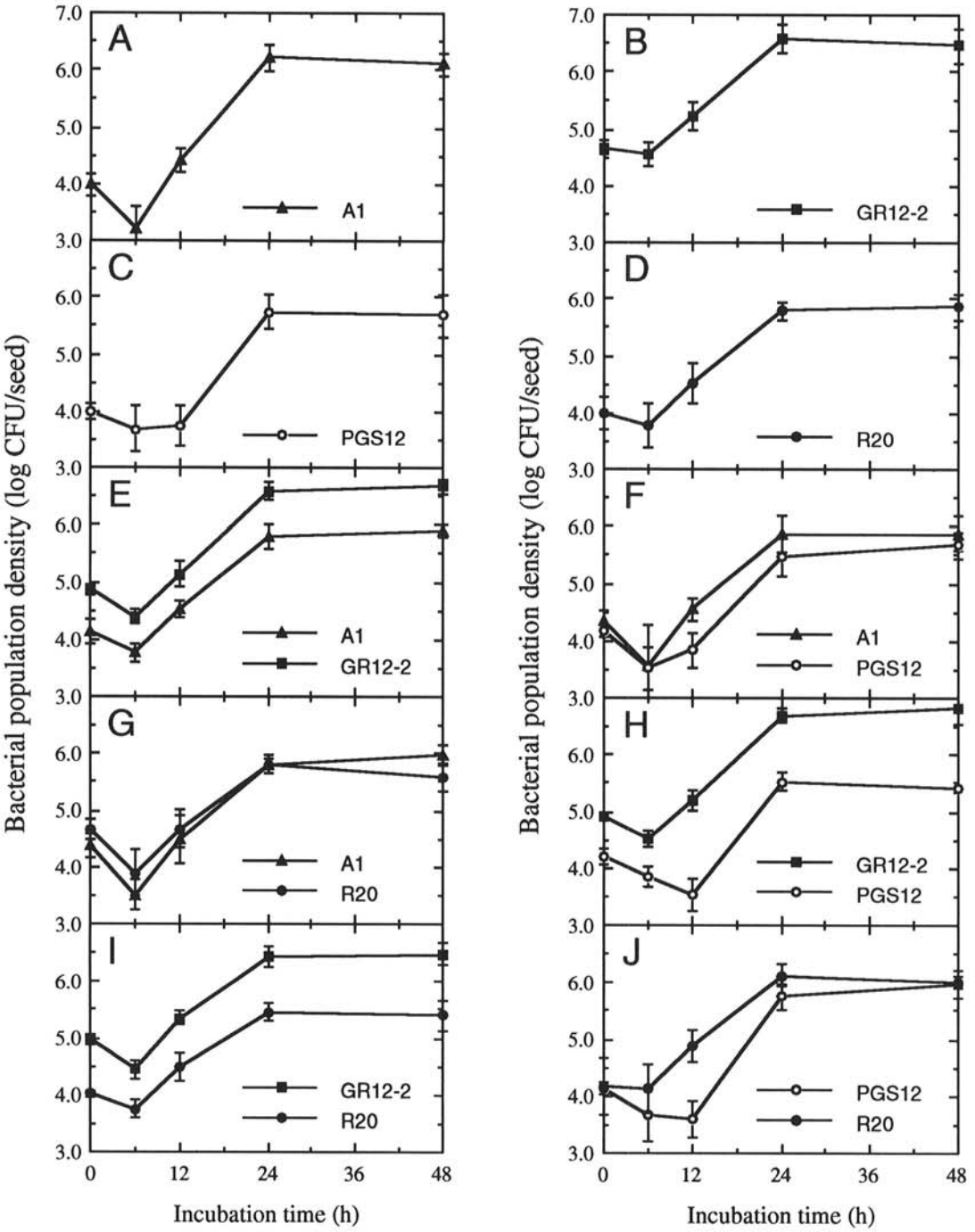

Fig. 1. Population growth of pseudomonads in dual strain inoculations in the spermosphere of sugar beet in soil adjusted to −15 J/kg and incubated at 16 C. Strain A1 of *Pseudomonas fluorescens,* GR12-2 of *P. putida,* PGS12 of *P. aureofaciens,* and R20 of *P. putida* were used for single- and dual-strain inoculations. Each data point is the mean value of two trials, each with four replicates, a pool of five seeds per replicate. Bars represent standard errors. **A,** A1 alone, **B,** GR12-2 alone, **C,** PGS12 alone, **D,** R20 alone, **E,** A1 and GR12-2, **F,** A1 and PGS12, **G,** A1 and R20, **H,** GR12-2 and PGS12, **I,** GR12-2 and R20, and **J,** PGS12 and R20.

by any strains. Strains 33-2 and A1 suppressed the growth of many strains, whereas F42 and PGS12 did not inhibit any. All inhibitory effects on KB were nullified when KB was amended with 100 μM $FeCl_3$. Patterns of inhibition on PDA are presented in Table 2. Strains 33-2, A1, CR30, F42, and R20 were self-inhibitory. All or nearly all strains were inhibited by A1, CR30, and F42; whereas none were inhibited by GR12-2. Strain GR12-2 was inhibited by the least number of strains. The addition of $FeCl_3$ to PDA altered the patterns of inhibition; some disappeared and a few intensified.

Growth and interaction of *Pseudomonas* strains in the spermosphere. Contrary to results in vitro, there was no apparent interaction among any of the test strains in the spermosphere when they were coinoculated onto seed at inoculum levels of approximately 10^4 cfu per seed (Fig. 1). The general growth patterns of each strain in dual inoculations were surprisingly similar to those obtained with single-strain inoculation, considering the expected variation that occurs among experiments and the difficulty in obtaining the identical initial population densities on seed among experiments. All growth curves exhibited similar lag, exponential, and stationary phases. Although the population densities of A1 and R20 coinoculated with GR12-2 were slightly less at 24 and 48 h compared to those when inoculated alone (Fig. 1A vs. 1E and 1D vs. 1I), the initial population densities of GR12-2 in these combinations were about 10 times higher than that of A1 or R20. The population density of R20 in combination with A1 also was slightly less than the density in single inoculation by 48 h, but not during the exponential phase (Fig. 1D vs. 1G). There also were no interactions when five strains (33-2, A1, F42, ML5, and PGS12) were coinoculated onto seed (data not shown). Regardless of the number of strains inoculated onto seed, each strain reached the stationary phase at approximately the same time as when inoculated alone.

Some interaction usually occurred when two strains were coinoculated onto seed with one at a high inoculum density, but the extent of the interaction varied greatly depending on the strains. Growth of ML5 was strongly inhibited when mixed with high densities of some strains, such as A1, GR12-2, and X, but only slightly by R20, F42, and 33-2 (Table 3). The extent of the interaction varied somewhat when comparing incubation periods of 24 and 48 h. Strain 33-2 was similarly affected by high inoculum densities of other strains (Table 3), but it was less sensitive to the effects of the other strains than ML5. Strain A1 was not inhibited at 24 h when coinoculated with three other strains (Table 3). The population size of the strains inoculated at high densities remained approximately the same throughout the test period.

The interactions and lack of interactions among strains when coinoculated onto seeds (Table 3) at high and low inoculum densities were not necessarily related to patterns of antagonism on PDA and KB (Table 2). For example, strain 33-2 was inhibited by six of seven coinoculated strains (GR12-2, X, ML5, R20, A1, and F42) on seed, whereas there were no interactions on KB. On PDA, three of six aforementioned strains were inhibitory to 33-2. Similarly, the patterns of inhibition of ML5 on PDA by various strains did not correlate with findings in the coinoculation studies. The inhibition pattern of ML5 on KB suggested that the same strains that were inhibitory in vitro were inhibitory in planta, but this was not caused by the same mechanism. The addition of iron nullified inhibition of ML5 by strains A1 and GR12-2 on KB, whereas ML5 was still inhibited in the spermospheres when iron was added to soil (Table 4).

Antagonism was expressed when near-isogenic strains were coinoculated onto seed at low and high densities. Growth of strains $33\text{-}2^{Rif}$ and $ML5^{Rif}$ in the spermosphere was inhibited by their respective near-isogenic wild type pairs inoculated at higher densities (Fig. 2).

Effect of carbon availability on growth of interacting strains in the spermosphere. The same pattern of inhibition that was found with the near-isogenic pairs, 33-2 and ML5, was observed when coinoculating seed with wild type R20 (high inoculum) and R20(pNAH7) (low inoculum). Population densities of R20(pNAH7) inoculated alone on seed were significantly greater ($P = 0.05$) at 24, 36, and 60 h than when coinoculated with R20 (Fig. 3A, D). However, this inhibition of R20(pNAH7) was nullified by the addition of the carbon source salicylate to soil at all time intervals ($P = 0.05$), except at 60 h (Fig. 3B, E and C, F). Moreover, the repeated measures analysis indicated that the concentration of salicylate had a significant effect on growth of R20(pNAH7) when coinoculated with R20 (Fig. 3D, E, F). At concentrations of 100 and 300 $\mu g\ g^{-1}$ of soil, population densities of R20(pNAH7) were significantly greater ($P = 0.05$) than those in nonamended soils at 24, 36, and 60 h. However, salicylate at concentrations of 300 $\mu g\ g^{-1}$ was initially toxic and increased the length of the lag phase (as noted in Figure 3C and F and in other work [5]). When inoculated alone, the growth of R20(pNAH7) was unaffected by the addition of salicylate to soil (Fig. 3A, B, C), except for the aforementioned lengthening of the lag phase with 300 $\mu g\ g^{-1}$ of soil (Fig. 3C) and at the 60-h time interval with amendments of 100 or 300 $\mu g\ g^{-1}$ of soil.

When coinoculated with different strains at high inoculum densities, the growth of R20(pNAH7) in the spermosphere was inhibited by all strains in nonamended soil (Table 5). Growth inhibition by 33-2 was the greatest among all strains tested. Growth inhibition by PGS12 and ML5 was the weakest among the test strains, including wild type R20.

The addition of salicylate to soil resulted in a significant to complete reduction in growth inhibition of R20(pNAH7) in all

TABLE 3. Growth of ML5, 33-2, or A1 in the sugar beet spermosphere when coinoculated with other strains[w]

Strain paired with ML5 and initial inoculum density (log cfu/seed)	Population density of ML5 (log cfu/seed)[x]		
	At planting[y]	After 24 h	After 48 h
A1 (6.58 ± 0.15)	3.80 ± 0.12 a[z]	3.24 ± 0.34 a	3.98 ± 0.59 a
GR12-2 (7.35 ± 0.15)	3.86 ± 0.15 a	4.69 ± 0.28 b	4.66 ± 0.22 b
X (7.11 ± 0.22)	3.84 ± 0.33 a	4.76 ± 0.21 b	4.65 ± 0.18 b
CR30 (6.85 ± 0.23)	3.90 ± 0.14 a	4.81 ± 0.15 b	5.25 ± 0.06 c
R20 (7.30 ± 0.02)	4.12 ± 0.09 a	5.20 ± 0.09 c	5.81 ± 0.20 e
F42 (6.95 ± 0.15)	3.90 ± 0.17 a	5.64 ± 0.32 d	5.45 ± 0.38 cd
33-2 (6.74 ± 0.41)	3.97 ± 0.35 a	5.64 ± 0.17 d	5.67 ± 0.29 de
Not paired	3.80 ± 0.28 a	5.97 ± 0.31 e	5.85 ± 0.24 e
LSD	0.32	0.23	0.31
Strain paired with 33-2 and initial inoculum density (log cfu/seed)	Population density of 33-2 (log cfu/seed)[x]		
	At planting[y]	After 24 h	After 48 h
GR12-2 (7.28 ± 0.12)	4.20 ± 0.35 b[z]	5.03 ± 0.14 a	5.14 ± 0.29 ab
X (7.24 ± 0.11)	3.84 ± 0.20 b	5.10 ± 0.19 ab	5.12 ± 0.40 a
ML5 (6.05 ± 0.49)	4.06 ± 0.45 b	5.13 ± 0.22 ab	5.41 ± 0.42 c
R20 (7.21 ± 0.14)	4.40 ± 0.15 b	5.22 ± 0.12 ab	5.32 ± 0.31 abc
A1 (6.84 ± 0.33)	4.00 ± 0.33 b	5.27 ± 0.42 b	5.14 ± 0.15 ab
F42 (6.74 ± 0.12)	3.40 ± 0.09 a	5.51 ± 0.18 c	5.38 ± 0.20 bc
PGS12 (6.24 ± 0.32)	4.26 ± 0.34 b	5.86 ± 0.20 d	5.91 ± 0.24 d
Not paired	4.02 ± 0.43 b	6.11 ± 0.14 e	6.05 ± 0.35 c
LSD	0.43	0.23	0.24
Strain paired with A1 and initial inoculum density (log cfu/seed)	Population density of A1 (log cfu/seed)[x]		
	At planting[y]	After 24 h	After 48 h
33-2 (6.92 ± 0.15)	4.15 ± 0.14 b[z]	5.36 ± 0.08 a	5.12 ± 0.18 a
PGS12 (6.80 ± 0.17)	4.35 ± 0.13 b	5.55 ± 0.36 a	5.42 ± 0.27 b
ML5 (6.67 ± 0.21)	4.21 ± 0.21 b	5.61 ± 0.31 a	5.54 ± 0.34 bc
Not paired	3.76 ± 0.39 a	5.55 ± 0.41 a	5.72 ± 0.26 c
LSD	0.32	0.34	0.29

[w] Seeds were planted in soil adjusted to −15 J/kg and incubated at 16 C.
[x] Bacterial population density on seed was determined in four blocks, 10 seeds per block, and is indicated by an average value and the standard deviation.
[y] Population densities at planting were determined with seeds before planting in soil, with four replicates, five seeds per replicate.
[z] Mean values of population densities of ML5, 33-2, or A1 were compared among treatments (paired strains in columns) at each sampling time by Fisher's protected LSD. Numbers indicated by the same letters in each column were not significantly different at $P = 0.05$.

test combinations at 36 h (Table 5). The population density of R20(pNAH7) coinoculated with PGS12 and ML5 was not significantly different than R20(pNAH7) inoculated alone. Although population densities of R20(pNAH7) in pair combinations decreased slightly at 60 h compared to those at 36 h, the densities were still significantly greater in salicylate-amended soil than nonamended soil.

Effect of dual strain inoculation on controlling pericarp colonization by *Pythium* in soil. None of the combinations of strains reduced pericarp colonization by *P. ultimum* more effectively than

TABLE 4. Effect of $FeCl_3$ or Fe-EDTA added to soil on growth of ML5 in single- and dual-strain inoculations in the sugar beet spermosphere

Strain(s) inoculated	Inoculum density at planting (log cfu/seed)[x]	Solution used to moisten soil	Population density of ML5[y] (log cfu/seed)	
			After 24 h	After 48 h
ML5	3.30 ± 0.28	Water	5.80 ± 0.24 c[z]	5.86 ± 0.12 c
		100 μM Fe-EDTA	5.67 ± 0.30 c	5.76 ± 0.22 c
		100 μM $FeCl_3$	5.90 ± 0.23 c	6.04 ± 0.12 c
ML5 and A1	3.58 ± 0.12	Water	3.37 ± 0.53 a	3.65 ± 0.15 a
		100 μM Fe-EDTA	3.59 ± 0.35 a	3.68 ± 0.36 a
	6.40 ± 0.20	100 μM $FeCl_3$	3.48 ± 0.38 a	3.63 ± 0.48 a
ML5 and GR12-2	4.02 ± 0.30	Water	5.28 ± 0.10 b	5.07 ± 0.19 b
		100 μM Fe-EDTA	5.34 ± 0.19 b	5.27 ± 0.13 b
	6.70 ± 0.23	100 μM $FeCl_3$	5.24 ± 0.13 b	5.32 ± 0.07 b
LSD			0.32	0.28

[x] Population densities at planting were determined with seeds before planting.

[y] Bacterial population density and standard deviation of the mean on seed was determined from two trials, four replicates per experiment, five seeds per replicate.

[z] Data from two trials were pooled and analyzed at each sampling time by ANOVA, since there was no significant interaction between trial and treatment(s) by preliminary ANOVA and variances in two trials were homogeneous. There was a significant difference in mean population densities of ML5 among strain pairs (the main plot factor) at both sampling times, but there was no significant difference among solutions (the subplot factor) or significant interaction between strain pair and solution. Numbers indicated by the same letters in each column were not significantly different according to Fisher's protected LSD ($P = 0.05$).

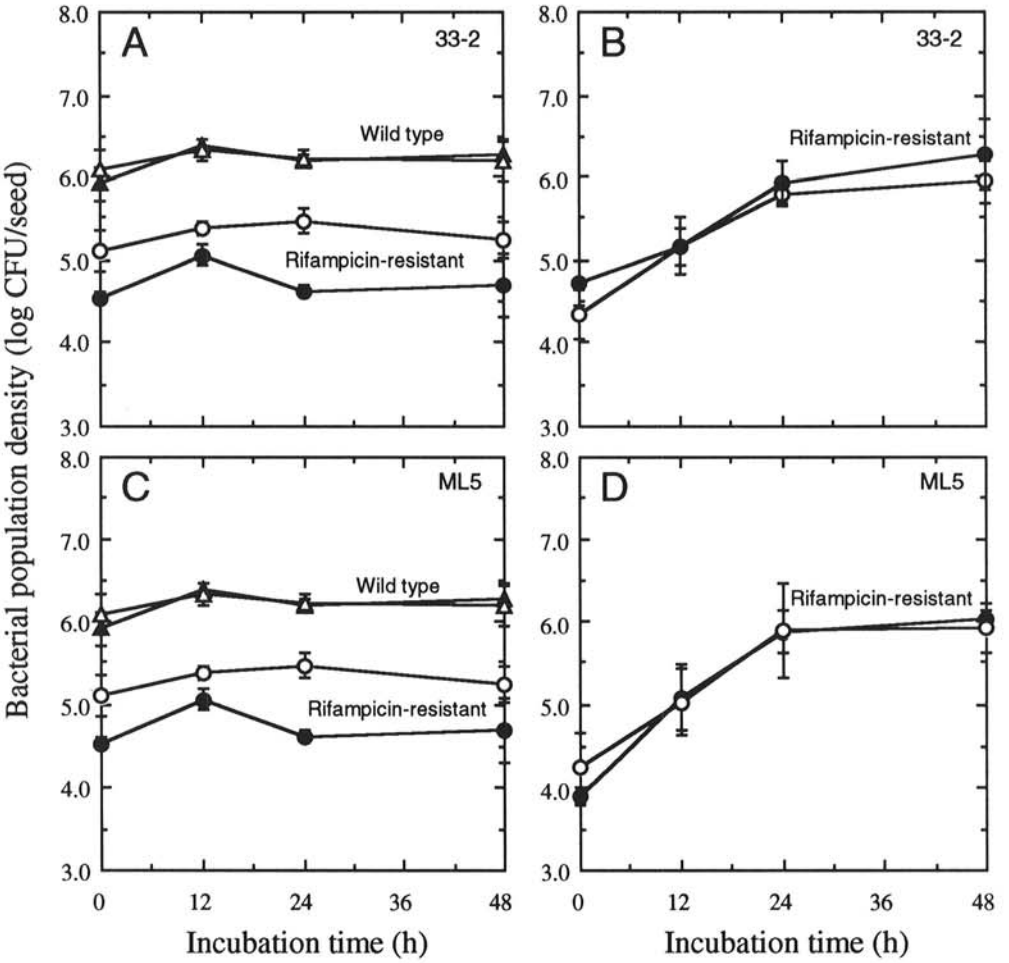

Fig. 2. Antagonism between near-isogenic strains coinoculated onto sugar beet seed in the spermosphere in soil adjusted to −15 J/kg and incubated at 16 C. The population densities of rifampicin-resistant spontaneous mutants and wild type strains are indicated by circles and triangles, respectively. The open data points represent the results from the second trial. Each data point is the mean value of four replicates, each consisting of a pool of five seeds. Bars represent standard errors. **A,** 33-2^{Rif} coinoculated with wild type strain 33-2 of *Pseudomonas putida,* **B,** 33-2^{Rif} inoculated alone, **C,** $ML5^{Rif}$ coinoculated with wild type strain ML5 of *P. fluorescens-putida,* and **D,** $ML5^{Rif}$ inoculated alone.

single-strain inoculations. Strain ML5 reduced the colonization frequency to 40.2%, compared with 90.1% for the control seeds (Table 6). However, ML5 coinoculated with A1 was less effective in preventing pericarp colonization by *P. ultimum*. Although the same amounts of inocula of ML5 and A1 were used in the combination treatment, the initial population density of ML5 (after drying seeds overnight) was less than 10^4 cfu per seed, whereas that of A1 was 6.39 log cfu per seed. In contrast, in dual strain inoculations with 33-2 or GR12-2, ML5 had nearly the same effect on reducing pericarp colonization as when used alone. In these combinations, the initial population density of ML5 was about 10^6 cfu per seed. In a separate experiment, strain A1 significantly reduced the effectiveness of GR12-2 when inoculated in combination (Table 6). The effect of GR12-2 also was reduced when coinoculated with 33-2, although colonization frequencies were not significantly different.

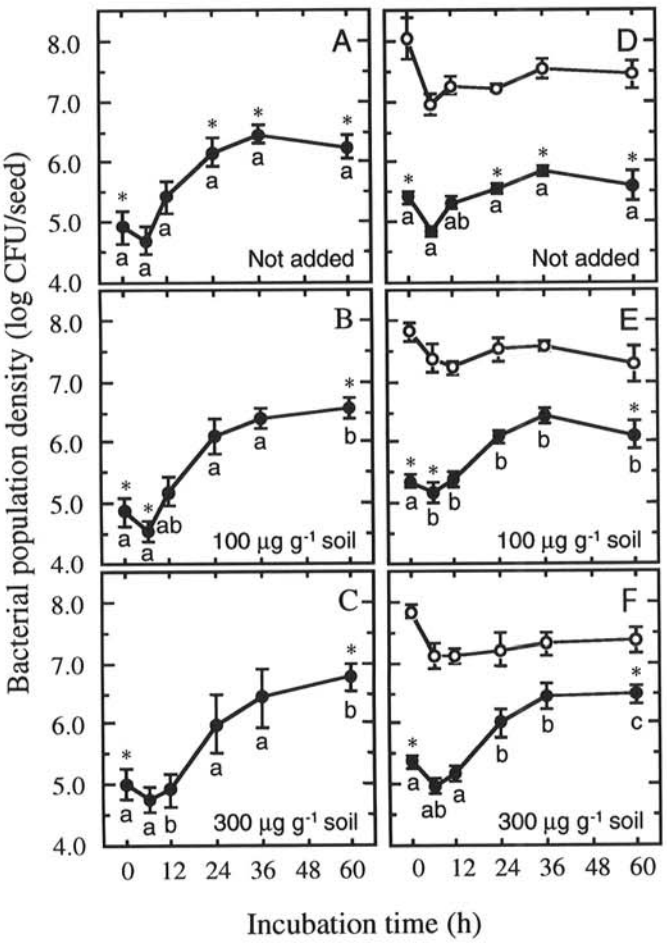

Fig. 3. Effect of sodium salicylate on growth of R20(pNAH7) in the spermosphere of sugar beet seed when inoculated singly (A–C) and in combination with the wild type strain R20 (D–F). **A,** R20(pNAH7) inoculated alone in soil not amended with sodium salicylate, **B,** R20(pNAH7) inoculated alone in soil amended with sodium salicylate at 100 $\mu g\ g^{-1}$, **C,** R20(pNAH7) inoculated alone in soil amended with sodium salicylate at 300 $\mu g\ g^{-1}$, **D,** R20 coinoculated with R20(pNAH7) in soil not amended, **E,** R20 coinoculated with R20(pNAH7) in soil amended with sodium salicylate at 100 $\mu g\ g^{-1}$, and **F,** R20 coinoculated with R20(pNAH7) in soil amended with sodium salicylate at 300 $\mu g\ g^{-1}$. Strains R20 and R20(pNAH7) are represented by open and closed circles, respectively. Each data point is the mean value of eight replicates in two trials, with a pool of five seeds per replicate. Bars represent mean standard errors in two trials. The effect of salicylate on nullifying antagonism of R20 to R20(pNAH7) was determined by comparing the population densities of R20(pNAH7) inoculated singly or in combination with R20 at different time intervals in soil not amended or soil amended with salicylate at 100 or 300 $\mu g\ g^{-1}$ (A vs. D, B vs. E, and C vs. F) by repeated measures ANOVA. An asterisk indicates a pair of mean values of R20(pNAH7) that are significantly different from each other ($P = 0.05$). The effect of concentrations of salicylate on growth of R20(pNAH7) inoculated singly and in combination with R20 was compared at each time interval by repeated measures ANOVA (A vs. B vs. C and D vs. E vs. F). Population densities at the same time intervals with the same letters are not significantly different ($P = 0.05$).

DISCUSSION

There was no evidence of any significant interaction among *Pseudomonas* strains in the spermospheres of sugar beet seeds when coinoculated at approximately 10^4 cfu per seed. Growth patterns of the strains during the exponential growth phase were similar to those of strains inoculated alone. Moreover, bacterial strains that were highly inhibitory to each other in vitro coexisted with no observable inhibitory effects when coinoculated on seed. These findings suggest that there is much less direct interaction among microorganisms (biocontrol agents and pathogens, for example) in the spermospheres and rhizospheres than is usually postulated. With respect to antibiosis occurring in the spermosphere or rhizosphere, investigators generally make the assumption that when a biocontrol agent reduces infection by another organism, it is due to a direct interaction. However, there is little data to substantiate this, and other mechanisms cannot be ruled out, such as induced resistance or a change in the community structure caused by the biocontrol agent, which in turn affects the population of the pathogen.

It is well-known that patterns of antagonism among organisms in vitro do not necessarily occur in vivo, although the reasons are not clear. We suggest the following reasons for the lack of relationship in our studies, and why antibiotic and siderophore production played no apparent role in affecting bacterial populations in the spermosphere. Aside from the nutritional environment being very different between a rich culture medium and a germinating seed, the dynamics of bacterial growth and spatial relationships greatly differ. On culture media, the producer bacterial strain was grown first and had reached the stationary phase before the second strain was seeded onto the media. On seed, both strains were inoculated simultaneously, and the stationary phase was reached at approximately the same time. Since the stationary phase is the time when secondary metabolites are usually produced, it is not likely that antibiosis is important at the early stages of colonization. With respect to spatial relationships, two bacterial strains are located contiguously on culture media; but on seed, the density of bacteria may be too low for interaction to occur among developing microcolonies. Scanning electron micrographs (8) support this supposition. During the first 24 h after the incubation of sugar beet seed inoculated at 10^4 cfu per seed, the growing colonies were so spatially separated that competition for nutrients would likely be minimal.

Although interactions among the coinoculated *Pseudomonas* strains often occurred when two strains were inoculated at high and low inoculum densities, respectively, there again was no consistent pattern between antagonism in culture media and growth patterns in planta. Growth inhibition also occurred with combinations of near-isogenic strains. Some strains, such as 33-2 and A1, were less sensitive or not affected, respectively, by challenge strains, even though their initial inoculum densities were much less than those of the challengers. It is noteworthy that strains 33-2 and A1 were inhibited by a number of strains on PDA but by none on KB. Perhaps they were able to utilize siderophores from a range of bacterial strains. Certain *Pseudomonas* strains utilize siderophores produced by different strains (4,12). On the other hand, growth of ML5 in the spermosphere was inhibited by strains A1, GR12-2, and X, but not by others. These strains also inhibited growth of ML5 or R20 on KB, suggesting that competition for iron might be the mechanism, since the addition of iron to KB nullified the inhibitions (siderophore production). However, this would not account for growth inhibition in the spermosphere, since the addition of iron to soil did not change the inhibitory effects.

The most plausible explanation for the inhibition of the low inoculum strains when coinoculated with other strains is that the high inoculum strains utilized most of the seed exudates and

TABLE 5. Growth of R20(pNAH7) inoculated in combination with other strains in sugar beet spermospheres

Strain paired with R20(pNAH7) and inoculum density (log cfu/seed)	Sodium salicylate[v]	Population density of R20(pNAH7) (log cfu/seed)[w]		
		At planting[x]	After 36 h	After 60 h
33-2 (6.95 ± 0.26)	−	5.03 ± 0.11 b[w]	4.61 ± 0.21 a[y]	3.96 ± 0.27 a[y]
	+		5.97 ± 0.15 A	5.17 ± 0.19 A
CR30 (7.87 ± 0.14)	−	5.00 ± 0.23 b	5.15 ± 0.25 b	4.56 ± 0.24 b
	+		6.09 ± 0.28 AB	4.98 ± 0.40 A
Wild type R20 (8.11 ± 0.11)	−	4.99 ± 0.15 b	5.16 ± 0.13 b	4.78 ± 0.42 b
	+		6.20 ± 0.11 BC	5.68 ± 0.21 B
PGS12 (7.39 ± 0.22)	−	5.02 ± 0.14 b	5.77 ± 0.18 c	5.46 ± 0.28 c
	+		6.34 ± 0.19 CD	5.85 ± 0.29 B
ML5 (6.95 ± 0.08)	−	4.95 ± 0.20 b	5.79 ± 0.22 c	5.85 ± 0.12 d
	+		6.32 ± 0.19 CD	6.16 ± 0.16 C
Not paired (control)	−	4.73 ± 0.23 a	6.44 ± 0.23 d*[z]	6.13 ± 0.26 d
	+		6.42 ± 0.23 D*	6.61 ± 0.30 D
LSD		0.18	0.21	0.28

[v] Sodium salicylate was added to soil at the rate of 100 $\mu g\ g^{-1}$ of soil.
[w] Bacterial population density on seed was determined from two separate trials, four replications per trial, five seeds per replicate. Bacterial population densities and standard deviations are mean values of two trials.
[x] Population densities at planting were determined with seeds before planting.
[y] Data from two trials were pooled and analyzed at each incubation time by ANOVA, since there was no significant interaction between trial and treatment(s) by preliminary ANOVA, and variances in two trials were homogeneous. There was a significant difference in mean population densities of R20(pNAH7) among strain pairs (the main plots) and between nonamended and amended soils (the subplots) at both 36 and 60 h, with a significant interaction between strain pair and soil amendment. Numbers indicated by the same letters in each column were not significantly different according to Fisher's protected LSD ($P = 0.05$).
[z] Numbers in each treatment (strain pair) indicated by asterisks were not significantly different according to Fisher's protected LSD ($P = 0.05$).

TABLE 6. Effect of seed inoculation with single and dual strains of pseudomonads on reducing colonization of sugar beet pericarp by *Pythium ultimum*[t]

Strain(s) inoculated	Initial inoculum density (log cfu/seed)[u]	Incidence of colonization (%)[v]
ML5	5.38 ± 0.37	40.2 a[w]
ML5	6.00 ± 0.22	37.5 a
and 33-2	6.56 ± 0.28	
ML5	<4.00[x]	57.1 b
and A1	6.39 ± 0.27	
ML5	5.86 ± 0.24	44.9 a
and GR12-2	7.43 ± 0.21	
Untreated[y]		90.1 c
LSD		6.35[z]
GR12-2	8.00 ± 0.12	42.4 a[w]
33-2	6.51 ± 0.29	60.5 b
A1	6.89 ± 0.30	69.2 cd
GR12-2	7.71 ± 0.24	55.8 b
and 33-2	6.26 ± 0.20	
GR12-2	7.73 ± 0.12	71.0 d
and A1	6.45 ± 0.24	
33-2	6.33 ± 0.57	70.0 cd
and A1	6.47 ± 0.39	
GR12-2	7.64 ± 0.18	62.2 bc
and 33-2	6.04 ± 0.36	
and A1	6.31 ± 0.22	
Untreated[y]		92.0 e
LSD		4.50[z]

[t] The soil contained 25 *P. ultimum* propagules per gram soil. The results from two different experiments are shown in the table.
[u] Bacterial population density was determined with seeds before planting and expressed as the average value from two trials, four replicates per trial, seven seeds per replicate.
[v] Incidence of pericarp colonization by *P. ultimum* after 2 days in soil was determined from two trials, four replicates per trial, 28 seeds per replicate. Incidence data were transformed by the arcsine-transformation prior to statistical analysis. There was no interaction between trial and treatment (strain pairs) according to the preliminary ANOVA, and variances in two trials were homogeneous.
[w] Values indicated by the same letters were not significantly different ($P = 0.05$) according to Fisher's protected LSD.
[x] An estimate of the population of ML5, since A1 drastically reduced the population of ML5 on seed prior to planting.
[y] Seeds for the control were treated with a mixture of 1.0% hydroxypropyl methylcellulose and 0.1M $MgSO_4$.
[z] An arcsine-transformed value.

occupied the favorable ecological niches. This also best explains the inhibition between near-isogenic strains when coinoculated. Strong supporting evidence for nutrient deprivation as the key mechanism for inhibition comes from the finding that the addition of the substrate salicylate to soil enabled R20(pNAH7) to grow almost as if it were independent of R20, thus nullifying inhibition. Furthermore, the addition of salicylate to soil caused a significant reduction of inhibition of R20(pNAH7) in all combinations of strains.

It was noteworthy that the growth of R20(pNAH7) when inoculated onto seeds singly did not differ in salicylate-amended and nonamended soil until after 36 h, except at 300 $\mu g\ g^{-1}$ of soil, which was initially toxic. Colbert et al (5) found that the strain does not appear to utilize salicylate efficiently in the nutrient-rich environment of the germinating seed. Therefore, the early growth response of R20(pNAH7) to salicylate when coinoculated with the high-inoculum strain R20 indicates that nutrient deprivation occurred early during seed germination.

On the other hand, if two strains had different nutritional spectra, they might be expected to grow independently of each other. It would be important to confirm this by developing detailed nutritional profiles of the competing strains. The theory of competition for carbon source and sites is consistent with the proposed mechanism of reduction of frost injury of potato caused by *P. syringae*. The mutant appeared to be similar to the wild type in growth rates (1,24).

No combination of pseudomonads was more efficacious than single strains in suppressing pericarp colonization by *P. ultimum*. In fact, coinoculation with some combinations, such as A1 and ML5, caused a considerable reduction in efficacy. ML5 was the most efficacious of all strains but also was inhibited by some bacterial strains that were coinoculated at high densities. This raises the question of the strategy of using single or multiple strains in biological control. In many cases, there may be no advantage in using multiple strains because of cost. It appears that seeds can only support a finite population of bacteria, and thus additional strains have little effect. Theoretically, compatible multiple strains might be advantageous when dealing with multiple diseases or a disease that has multiple infection sites. Also, a single strain may not grow equally well in a variety of environmental conditions.

A comparison of the results of work reported herein with results obtained by Osburn et al (27) clearly show a marked difference in the ability of ML5 to affect pericarp colonization by *P. ultimum*.

The principal reason is that the soil and the inoculum density of *P. ultimum* used in these experiments placed far greater disease pressure on the sugar beet seed than that in the previous study. This may explain why biological control agents in some experiments appear to be as good as chemical pesticides, whereas in other experiments they are less effective. It is obvious from these studies that seed protection by bacteria is inadequate when conditions are ideal for fungal infections. Thus, there is a great need for developing systems to enhance the activity of biological control agents once they have been inoculated onto plant parts or released into natural ecosystems. Consistency will depend on improving formulations, identifying more efficacious strains, and perhaps developing adjuvants to aid the activities of the biocontrol agents.

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