Growth Patterns and Metabolic Activity of Pseudomonads in Sugar Beet Spermospheres: Relationship to Pericarp Colonization by *Pythium ultimum*

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ABSTRACT

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Growth patterns of seed-inoculated fluorescent pseudomonads in the spermospheres of sugar beet in soil or sand maintained at −15 J/kg and 16 C were monitored over a 72-h time period. When incubated in soil, strains 33-2 (*Pseudomonas putida*), A1 (*P. fluorescens*), and ML5 (*P. fluorescens-putida*) exhibited relatively short lag phases (<4 h), with most growth occurring during the first 12 h. Strains F42 (*P. putida*) and PGS12 (*P. aureofaciens*) exhibited long lag phases (8–12 h), and their populations increased mainly between 12 and 24 h. Doubling times during the exponential growth phases were 2–3 h for all strains except *P. syringae* strain B728a, which had the longest doubling time (5–7 h). Most strains reached the stationary phase within 24 h after planting of seed. Growth rates of bacteria in spermospheres of seed planted in sand were similar, except some strains continued to grow past 24 h. When seeds were inoculated with bacteria at 10^6–10^8 cfu per seed, the incidence of pericarp colonization by *Pythium ultimum* within 48 h in soil was less in seeds treated with strains with short lag phases than in those treated with long lag phases. However, the efficaciousness of these strains was essentially negated when soil contained a high population of the fungus. The high bacterial inoculum density was important, as there was a linear relationship between inoculum densities and percentage of pericarp colonization by *P. ultimum*. To determine if bacterial cells were active or dormant (or dead), cells were incubated in a solution of 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT). The percentage of INT-active cells on seed with initial population densities of 10^7–10^8 cfu per seed ranged from about 17.2–55.9% at 0 h to 85.8% at 48 h. The percentage of INT-active cells peaked at 6–12 h for the short lag phase strains. No clear relationship between efficacy of strains in reducing pericarp colonization and the percentage of metabolically active cells was detected. On the other hand, the finding that a significant percentage of cells are metabolically active following inoculation of seed with high-density inocula explains in part why protection of the pericarp against *P. ultimum* is better even though multiplication cannot be detected. Thus, competition for nutrients and the production of secondary metabolites occurs in the absence of increasing cell number.

Fluorescent pseudomonads have been widely tested for biological control of soilborne plant pathogens because of their rapid growth rates and their ability to colonize rhizospheres and spermospheres (8,9,16,25,35). In general, the results have not yet fulfilled expectations because of such factors as lack of consistency and inability of biological agents to compete well with chemicals over a range of environmental conditions. It is becoming increasingly evident from numerous reports that effective use of biological control agents will depend on the development of techniques to enhance their activities after their application to target sites (26,27). The use of selective nutrients is one proposed method (2). However, the development of methods to improve the efficacy of biological control agents requires a much greater basic understanding of the mechanisms of interaction between pathogens and biocontrol agents, and the factors that influence the growth of microorganisms in natural ecosystems.

Colonization of sugar beet (*Beta vulgaris* L.) pericarps by *Pythium ultimum* Trow var. *ultimum* and bacteria provides an ideal system for the study of the interactions between bacteria and a pathogen. The monogerm sugar beet seed consists of a true seed surrounded by a pericarp composed of dead, corky perianth tissue (24). Since the pericarp is composed of dead tissue, the reduction of pericarp colonization by *P. ultimum* is the result of direct interaction between bacterial antagonists and *P. ultimum* without the induction of any host resistance processes. In addition, exudates released by the seed during imbibition of water support rapid growth of bacteria and *P. ultimum* in the spermosphere (21,22,28,31). The interaction between bacteria and *P. ultimum* occurs immediately after planting of the seed. Thus, this system enables quick assessment of the effectiveness of bacterial antagonists in controlling *P. ultimum*.

Sporangia of *P. ultimum* germinate within 90 min in response to water-soluble nutrients (14,31) and volatile substances (19) released from seeds. Hyphae colonize pericarps of sugar beet seed within 3.5 h after planting in soil (22). Thus, it would seem that bacterial antagonists need short lag phases and doubling times to effectively protect the pericarp from invasion by *P. ultimum*. This has not been studied in detail, nor have the relationships between growth rates and inoculum density; doubling time is usually thought to be longer at higher population densities (23). Although doubling time is an important parameter for estimating growth during the exponential growth phase, a measurement of total metabolic activity would appear to be important when studying microbial interactions when population growth is not evident. Metabolic activity is not necessarily related to cell counts. Colbert et al (2) reported the lack of relationship between cell count and metabolic activity. High metabolic activity and saturation of infection sites is probably the reason for better control of *P. ultimum* when using inoculum with a high density of cells (22,23).

It is difficult to determine if bacterial cells are multiplying or metabolically active after inoculation at high population density or after a population reaches the stationary phase. Reduction of an artificial electron acceptor, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (*p*-iodonitrotetrazolium violet or INT), by an active electron transport system has been employed to measure metabolic (respiratory) activities of bacteria in soil (17,20,29) and in aquatic environments (18,33,37). A func-

tional electron transport system capable of oxidizing NADH or NADPH is one of the essential components of metabolic activity (17). Intracellular deposits of INT formazan crystals formed by reduction of INT can be seen under a light microscope and would be a useful indicator of the metabolic activity of bacterial antagonists in spermospheres.

This paper reports on the growth of various fluorescent pseudomonads in the spermosphere of sugar beet and how bacterial growth and activities relate to suppression of *P. ultimum*. The studies of bacterial growth consider both population increase and metabolic activity as determined by the method of INT reduction. Preliminary reports were published elsewhere (3,4).

MATERIALS AND METHODS

Seed. Sugar beet seed of cv. USH11 (Holly Sugar Co., Colorado Springs, CO) size 8–9 was used in all experiments. The seed was processed to remove most of the outer corky perianth materials from the pericarp. The whole structure, composed of a true seed and surrounding dead perianth tissue (or pericarp), is referred to as a seed. Seeds were not disinfested for most of the experiments. For certain experiments, seeds were surface-disinfested with 2.0% sodium hypochlorite for 2 min, washed thoroughly under running tap water, and air-dried overnight on a sterile air bench.

Soil. The soil was an Oceano loamy sand (pH 6.9–7.0) collected from a farm field near Moss Landing, California, unless otherwise noted. 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. Stocks of soil samples were replaced every 3 mo.

Bacterial strains and detection media. Two strains of *Pseudomonas putida,* 33-2 (13) and F42 (12,13), were obtained from Esso Ag. Biologicals, Saskatoon, Canada (formerly Allelix, Inc., Ontario, Canada), courtesy of J. W. Kloepper. *Pseudomonas aureofaciens* PGS12 (1) was from Plant Genetic Systems, Gent, Belgium. *Pseudomonas syringae* B728A (15,36) was from S. E. Lindow. *Pseudomonas fluorescens* A1 (10,11) and *P. fluorescens-putida* ML5 (22) were originally isolated from potato (*Solanum tuberosum* L.) periderm and the spermosphere of sugar beet (*Beta vulgaris* L.), respectively, in California.

Enumerations of inoculated bacteria from soil suspensions were made on King's medium B (KB) (7) containing cycloheximide at 100 μg ml^{-1} and antibiotics according to the resistance of the strain. Strains A1, B728a, and F42 were spontaneous rifampicin-resistant mutants (50 μg ml^{-1}), strains ML5 and PGS12 were naturally resistant to streptomycin sulfate (100 μg ml^{-1}) and $CuSO_4$ (800 μg ml^{-1}), and strain 33-2 was naturally resistant to spectinomycin dihydrochloride (100 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}). All antibiotics were obtained from Sigma Chemical Corp., St. Louis, MO.

Inoculum preparation and seed inoculation with bacteria. Each strain was grown for 48 h at 28 C on four KB agar plates, and the resulting lawns were resuspended in a mixture of 1.5 ml of 1.0% hydroxypropyl methylcellulose (Methocel HG, Dow Chemical Company, Midland, MI) and 0.1 M $MgSO_4$ (22). A bacterial suspension (1.5 ml) and 5 g of sugar beet seeds were mixed in a small plastic bag. The inoculated seeds were air-dried at room temperature (22–26 C) overnight before planting in soil. The resultant density of the bacterial population on the seed ranged from 10^6 to 10^8 cfu per seed, depending on the strain. A dilution of the methylcellulose-$MgSO_4$ mixture made from the densest initial bacterial suspension was used for inoculating seeds to produce progressively lower bacterial population densities on the seed. Noninoculated seed was treated only with the carrier.

Adjusting water potential in soil and monitoring bacterial population densities in spermospheres. The growth of bacterial strains on inoculated seeds was examined by the method of Osburn et al (22). Briefly, approximately 80 g of moist soil was placed in 5.5-cm-diameter brass rings on a −50 J/kg ceramic pressure plate. Occasionally, sand (Monterey sand, size 60, RMC Lonestar, Pleasanton, CA) was used instead of soil. Ten to 14 seeds per ring were planted approximately 1 cm deep, and the soil was wetted to saturation with distilled water precooled to 16 C and adjusted to −15 J/kg soil matric potential using a pressure plate extraction system. After equilibration for 3–4 h, the rings with soil were transferred onto the lids of plastic petri dishes, covered with plastic bags to maintain constant soil moisture, and incubated in a growth chamber at 16 C. The experimental design was usually a randomized complete block (RCB) or a randomized block (RB).

Population densities of bacteria were determined at time intervals of interest by randomly removing five of 10 seeds from a ring, tapping gently to remove loosely adhering soil, pooling in 5 ml of 10 mM sterile phosphate buffer (pH 6.9), and vortexing for 2 min. The population densities of the different bacterial strains then were determined by dilution plate counting on appropriate antibiotic-containing media. For a 0-h reading, soil or sand was adjusted to −15 J/kg, and inoculated seeds were sown and recovered after 10 min.

Growth patterns and doubling times of pseudomonads in the spermosphere. The growth patterns and doubling times of six pseudomonad strains were examined in sugar beet spermospheres. Preliminary studies indicated that there were substantial differences among them in growth rates. The methods were as described above. Each of the six strains was inoculated onto seed at two inoculum densities, the lowest at about 10^3 and the highest at about 10^5 cfu per seed. The bacterial population densities in the spermosphere were determined over a 72-h period at 4-h intervals for the first 24 h. An RB design was used, with four replicates per strain per time interval (a total of 20 seeds was taken from four rings), each replicate consisting of five seeds. The experiment was done separately for each strain and soil or sand combination. The population density of total aerobic bacteria also was determined from the same bacterial suspension by plating on half-strength tryptic soy agar (TSA) containing cycloheximide at 100 μg ml^{-1}. To determine growth rates in the spermosphere, doubling time (T_D) was estimated from population densities in the first 24-h period by the formula: $(\log N_H - \log N_L)/0.301T = 1/T_D$, where N_L and N_H are the lowest and the highest population densities, respectively, and T is the time required for the population increase from N_L to N_H. The experiment was done twice.

Estimation and adjustment of inoculum density of *P. ultimum* 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 (30). To pasteurize soil, 500-ml beakers, each containing 400 g of moist soil, were covered with aluminum foil or plastic wrap and warmed in a heated water bath at 55–57 C for 15 min (34). No hyphae of *P. ultimum* growing out of soil drops were detected after soil was pasteurized. To adjust the inoculum density of *P. ultimum* in soil, a soil naturally infested with a known inoculum density of *P. ultimum* was diluted with a predetermined amount of the same pasteurized soil and mixed thoroughly in a twin-shell blender or a portable cement mixer for at least 30 min. The inoculum density of *P. ultimum* in the blend then was confirmed by the soil drop assay method.

Determination of the incidence of pericarp invasion by *P. ultimum* in soil. The general method used in the experiments was to plant 14 seeds per ring in soil, which then was adjusted to −15 J/kg and incubated at 16 C as described above. The extent of pericarp colonization by *P. ultimum* was determined after 1 or 2 days by recovering seed from sand by wet sieving, cleaning with a strong jet of water, surface-sterilizing in 0.5% sodium hypochlorite for 1 min, rinsing thoroughly under running tap water, and plating each seed on 2% water agar (WA) containing benomyl (Benlate 50 WP) at 15 μg ml^{-1}. Hyphae of *Pythium* spp. (mostly *P. ultimum* and occasionally other species) growing out of a pericarp were verified with a dissecting microscope after incubation at room temperature for 24 or 48 h. Fast-growing, coenocytic hyphae (1–3 cm long at 24 h) were verified as *P. ultimum,* and slow-growing, coenocytic hyphae (<2 cm at 48 h) were verified as other *Pythium* spp.

Effect of bacteria on colonization of sugar beet pericarp by *P. ultimum*. To examine the efficacy of different bacterial strains in preventing colonization of the sugar beet pericarp by *P. ultimum,* each of six *Pseudomonas* strains was inoculated onto

seeds at inoculum densities of about 10^6–10^8 cfu per seed. These six strains were selected because they were representative of strains that had short and long lag phases following inoculation of seed. Some seeds were treated with metalaxyl (Apron 25W) at the rate of 0.3 mg a.i./g of seed as a comparison to bacteria-treated seeds. Other control seeds were treated only with a mixture of 1.0% methylcellulose and 0.1 M $MgSO_4$. Seeds of all treatments (a total of eight) were planted in soil containing approximately 25 or 50 propagules of *P. ultimum* per gram (p/g) of soil. Soil was adjusted to −15 J/kg and incubated at 16 C, as described above. The incidence of pericarp colonization was determined after 48 h. The experiment was done twice in RCB split plot with five blocks. Seed treatments with different bacterial strains were the main plot treatments, and inoculum densities of *P. ultimum* were the subplot treatments. Each block consisted of 32 rings, two rings per treatment, and 14 seeds in each ring. All seeds were sampled for colonization of the pericarps as described above. The population densities of bacteria after 48 h were determined with previously described methods.

To examine the effect of bacterial inoculum density on the colonization of *P. ultimum,* three fluorescent *Pseudomonas* strains (33-2, A1, and ML5) were inoculated individually onto seed at three different inoculum densities (high, medium, and low), as described above. Medium and low inoculum densities were 1:10 and 1:100 dilutions of the highest density. The seeds were planted in soil containing approximately 10 p/g of soil. The experiment was done twice in an RCB design with four blocks, two rings per treatment, 14 seed per ring. At various time intervals, seeds were assayed for *P. ultimum* colonization and the population density of the bacterial strains, as described above.

Determination of proportion of bacterial cells actively respiring on seed. Since enumeration of actively respiring cells requires visual counting of bacteria with light microscopy, the experiment was conducted under gnotobiotic conditions using autoclaved Monterey sand instead of natural soil to avoid contaminating bacteria. Experimental procedures for determining actively respiring bacterial cells were adapted from the method of Norton and Firestone (20) using INT (Sigma Chemical Co., St Louis, MO).

Surface-sterilized seed was inoculated at approximately 10^7–10^8 cfu per seed with each of six selected *Pseudomonas* strains and then planted in autoclaved sand adjusted at −15 J/kg and incubated at 16 C. Brass rings and pressure plates used to adjust matric potential were sterilized in 70% ethanol overnight before use. At various time intervals (0, 6, 12, 24, and 48 h), five of 10 seeds in each ring were randomly taken from the sand and vortexed in 3.0 ml of 50 mM sterile Tris buffer (pH 7.5). This bacterial suspension (0.1 ml) was used to determine bacterial population density on the seed, while the other 2 ml was mixed with an equal volume of INT solution (0.5 mg ml^{-1} in 50 mM Tris buffer at pH 7.5 sterilized by filtration) and incubated on a slow-rotating shaker at 28 C for 1 h in the dark. Generally, longer incubation times resulted in greater numbers of bacterial cells stained by INT (17,20). However, incubation times longer than 1 h caused large clumps of bacterial cells to occur in the solution or adsorption of bacterial cells to the surface of the glass test tubes. Consequently, this significantly reduced the number of cells counted in a microscopic field. At the end of the 1-h incubation, the reaction was stopped by adding 1 ml of 18.5% formaldehyde to the solution. This was stored overnight at 5 C to maximize the formation of visible INT-formazan crystal inclusions inside the cells (32).

To facilitate enumeration of bacteria by light microscopy, the fixed solution stored overnight was sonicated for 2 min to disperse cells and to achieve a more uniform distribution for counting. Several fourfold dilutions were prepared from this solution, and two drops (50 μl) of each dilution were placed on thin-layer WA and dried on a sterile aerated bench. Agar plugs taken from the region where the bacterial suspension was placed were transferred onto microscopic slides and observed with a phase contrast microscope (1,000×). Twenty to 100 bacterial cells per microscopic field were counted on an agar plug. Cells with visible INT-formazan crystal inclusions were enumerated using phase contrast and transmitted light alternatively. At least 400 individual cells per agar plug were counted from microscopic fields lying horizontally or vertically across the agar plug. The proportion of cells containing INT-formazan crystals to total cells was determined with four replications (total of more than 1,600 cells) at each incubation time. The factorial experiment, examining the factors strain and sampling time, were done twice for each bacterial strain.

Data analysis. Prior to statistical analysis, data pertaining to the incidence of sugar beet colonization by *P. ultimum* were transformed by the arcsine-transformation. In the study of the effect of different bacterial inoculum densities on pericarp colonization by *P. ultimum,* percentages of pericarp colonization were transformed by the multiple-infection transformation method (5) to examine the relationship between inoculum density and the estimated number of pericarp invasions by *Pythium* hyphae.

Data in repeated trials were analyzed first by preliminary analysis of variance (ANOVA) using trials as a qualitative independent variable. Prior to the pooling of data from two separate trials, a significance of trial by treatment(s) interaction was determined by preliminary ANOVA, and homogeneity of variances between trials was verified (6). Pooled data were analyzed by ANOVA to test main effects of treatments and treatment-by-treatment interactions. The significance of differences between pairs of treatment means was done with Fisher's protected least significant difference test (protected LSD). Linear regression was used to compare dose-responses in the relationships between inoculum density of bacteria on seed and the number of pericarp invasions by *P. ultimum.*

RESULTS

Growth patterns and doubling times of pseudomonads in the spermosphere. Three different patterns of bacterial growth were observed in the spermosphere of sugar beet seed in Oceano loamy sand. Strains 33-2, A1, and ML5 exhibited relatively short (<4 h) lag phases, so short in the case of 33-2 that it was not detected; their populations increased markedly during the first 12 h of incubation (Fig. 1A–C). Strains F42 and PGS12 had relatively long lag phases (8–12 h), and their population densities increased primarily after 12 h (Fig. 1D and E). Strain B728a grew relatively slowly during the entire 24-h time period and exhibited the smallest increase in population density (<0.60 log cfu per seed) of all tested strains. (Fig. 1F). The stationary phase was reached within 24 h for all strains. Strain B728a had the longest doubling time, 5–7 h. Doubling times for other strains were about 2–3 h and 3–4 h when initial inoculum densities were below and above 10^5 cfu per seed, respectively. Doubling times were not necessarily related to the length of the lag phase.

Growth patterns of the six strains in the spermosphere of seed planted in sand were about the same as those in soil. Doubling times averaged 2–3 h for all strains. In the spermosphere in sand, the population densities of F42, PGS12, and B728a continued to increase after 24 h (Fig. 1D–F), whereas no marked population increase after 24 h was observed with 33-2, A1, or ML5 (Fig. 1A–C). Doubling times in the second trial were similar to the first in both sand and soil.

The detected population size of total aerobic bacteria on the spermoplane after planting noninoculated seed in soil for 10 min was about 2×10^5 cfu per seed. The density increased to nearly 10^7 cfu per seed by 72 h. Estimated average doubling time of total aerobic bacteria was 7.1 h during the exponential growth stage (Fig. 2).

Relationship between bacterial lag phases and pericarp colonization by *P. ultimum*. The effects of strain and inoculum density on *P. ultimum* colonization of pericarps were both significant ($P = 0.05$) (Table 1). All *Pseudomonas* strains, when inoculated onto seed at high inoculum densities, caused statistically significant reductions in the incidence of pericarp colonization by *P. ultimum* (in soil, 28 p/g of soil) compared to nontreated seed,

although the effect was not great in many cases. None of the strains reduced the frequency of colonization as low as did the metalaxyl treatment (0.7%). The strains with short lag phases (33-2, A1, and ML5) were more effective than strains with long lag phases. Strain ML5 was the most effective (24.4% colonization) of all strains. However, strain 33-2 and A1 were only partially effective in reducing pericarp colonization (65.9 and 73.7%, respectively) compared to ML5. The incidence of pericarp colonization was more than 80% in seeds treated with the other three strains. A high population of *P. ultimum* in soil essentially nullified the efficaciousness of the pseudomonad strains.

Relationship between inoculum density of bacteria on seed and number of hyphal invasions of pericarps by *P. ultimum*. Analysis of the data indicated a significant linear relationship ($P < 0.05$) between inoculum density of pseudomonad strains and both the percentage of seed colonized and the number of invasions per seed by *P. ultimum* (transformed data) (Fig. 3). The analysis indicated the importance of a high initial inoculum, since efficaciousness was substantially lower in the low-inoculum tests, despite the fact that the strains attained population densities within 24 h that were similar to those in the high-inoculum tests (Table 2). The population size of the pseudomonads in the high-inoculum tests changed only slightly during the experiments.

The slope values for colonization of pericarps by *P. ultimum* were surprisingly not significantly different whether the experiments were carried out for 24 or 48 h (t test, $P = 0.05$) (Fig. 3). However, the comparison of the slope values when examining the efficaciousness of the different strains indicated that ML5 was significantly better than strains 33-2 and A1 (t test, $P = 0.05$). Strain A1 was the least effective of the tested strains and had the lowest coefficients of correlation.

Respiratory activity of bacteria in spermosphere. The percentage of cells on inoculated seed at the time of planting that contained intracellular INT-formazan deposits (INT-active) was greatest for B728a (55.9%) and ML5 (54.0%), and lowest for F42 (32.0%) and PGS12 (17.2%) (Fig. 4). This difference was significant at $P = 0.05$. The percentage of INT-active cells continued to differ significantly ($P = 0.05$) among the different strains at various sampling times. With 33-2, A1, and ML5, the percentage of INT-active cells was maximal within 12 h, 62.3, 65.9, and 83.1%, respectively, whereas the percentage of INT-active cells for strains F42 and PGS12 did not markedly increase after planting

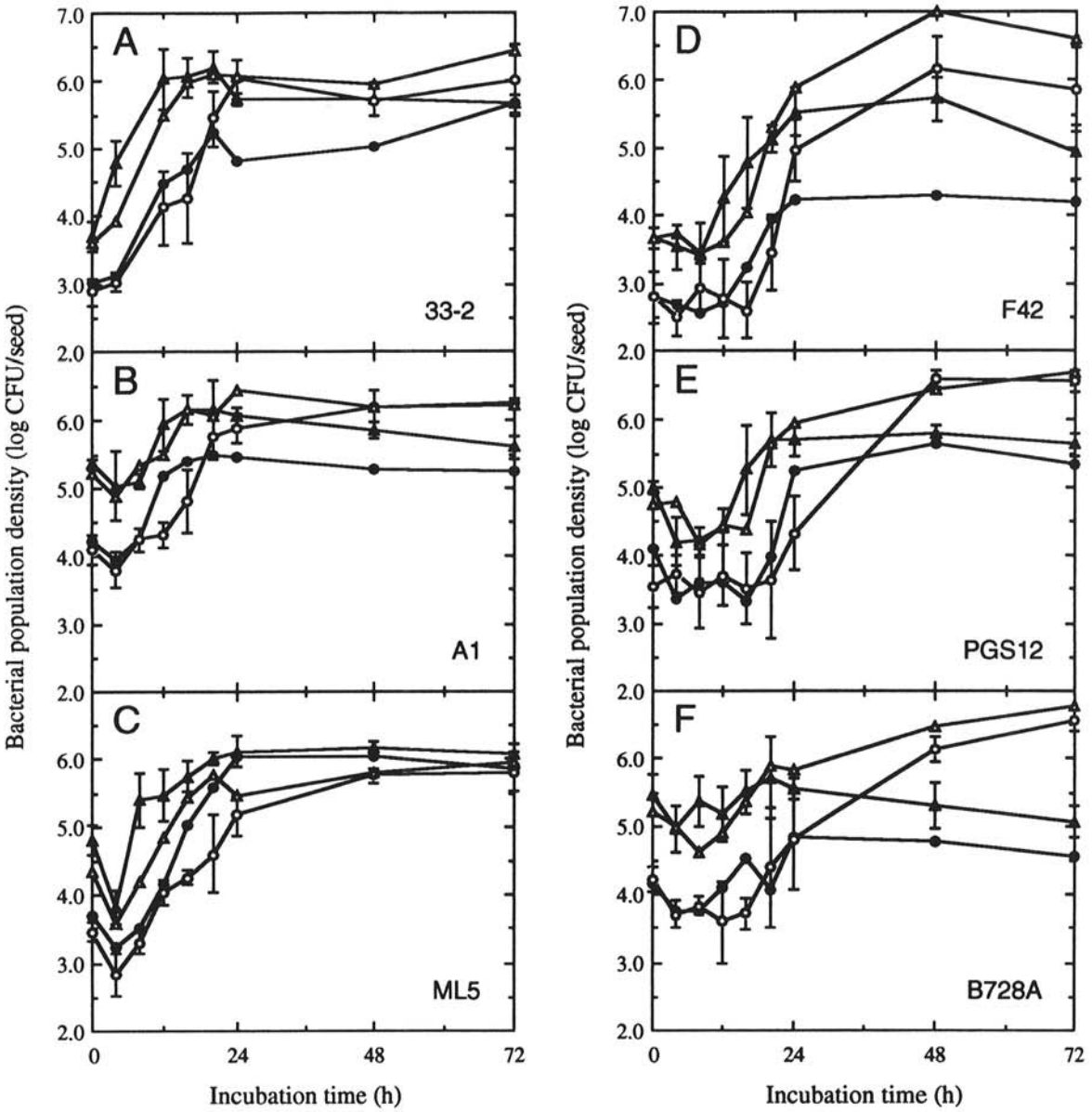

Fig. 1. Growth of *Pseudomonas* strains in the spermospheres of sugar beet from two different inoculum densities (circle and triangle) in Oceano loamy sand (●, ▲) and in sand (○, △) maintained at −15 J/kg and 16 C. Each data point represents the mean of four replicates, with each replicate consisting of a pool of five seeds. Vertical bars indicating standard deviations are shown. **A,** *Pseudomonas putida* 33-2; **B,** *P. fluorescens* A1; **C,** *P. fluorescens-putida* ML5; **D,** *P. putida* F42; **E,** *P. aureofaciens* PGS12; and **F,** *P. syringae* B728a.

and were substantially lower than other strains during the 48-h incubation period (Fig. 4D and E). The maximum percentages reached during incubation for F42 and PGS12 were 41.9% (12 h) and 31.8% (6 h), respectively. With B728a, the percentage of INT-active cells gradually increased over time and was 85.8% by 48 h, the highest among all strains (Fig. 4F). High population densities (10^7–10^8 cfu per seed) of bacterial strains were maintained in the spermosphere during the 48-h period of incubation.

DISCUSSION

The effectiveness of *Pseudomonas* strains in reducing pericarp colonization by *P. ultimum* was in part related to the length of their lag phases, metabolic state, and inoculum size. The most efficacious strains (ML5, A1, and 33-2) had the shortest lag phases in the spermosphere in both soil and sand. Unquestionably, efficacy is also dependent on other characteristics, such as the ability to compete for substrates and the production of secondary metabolites. However, when protecting seeds or other infection courts from fast-acting fungi such as *Pythium* spp., key requisites for efficacy must be a high population size, a high metabolic state, and the ability to attain such states rapidly. Certain species of *Pythium* can germinate, invade the pericarp, and infect the true seed of sugar beet before most bacteria have attained exponential growth. This explains why a slow-growing bacterium like *P. aureofaciens* PGS12 is a poor protectant of seeds.

Most studies on population dynamics of bacteria in the rhizosphere are essentially measuring the survival of quiescent cells after inoculation (2). Thus, the metabolic state of cells is a highly important factor to consider when studying microbial interactions. The use of INT to evaluate the state of metabolism in bacterial cells explained in part why high-density inoculants are generally much more effective than low-density inoculants, despite the fact that little to no multiplication is detected during incubation of inoculated seeds. The INT tests revealed that a significant proportion of the cells was metabolically active, ranging from 20 to 85% over a 24-h period. Thus, an apparent stationary population size could in fact be the result of relatively equal multiplication and death of cells. It also may reflect a largely nondividing but still metabolically active population of cells. Bacteria could be quite antagonistic to *P. ultimum* while not actively dividing. Comparisons of INT-activities did not show any major differences between various *Pseudomonas* strains. However, the three strains with short lag phases (33-2, A1, and ML5) had a higher percentage of INT-active cells than two of the three long lag phase strains (F42 and PGS12). Moreover, INT-activity reached the maximum within 12 h in all short lag phase strains. This indicates that stationary phase was reached by 12 h in these strains even when they were inoculated onto seed initially at high-population densities, which agrees with their growth characteristics from low-inoculum densities. Thus, early production of secondary metabolites inhibitory to *P. ultimum* can be expected for these strains. On the other hand, INT-activity of B728A still increased after 24 h. It appears that the population of B728A did not reach the stationary phase until after 48 h. This may explain why B728A was not effective in controlling pericarp invasion by *P. ultimum*, despite high metabolic activity throughout the 48-h time period.

A comparison of the slopes of the different bacterial strains indicated that strain ML5 was the most efficacious in reducing pericarp colonization by *P. ultimum* (Fig. 3). For example, data indicated that every 10-fold increase in inoculum density resulted in about 40 fewer pericarp invasions per 100 seeds. Although we expect that this particular relationship would vary considerably depending on the experimental conditions, the data nevertheless helps to explain why biological control agents seldom match pesticides when there is heavy disease pressure. It is not possible to inoculate seeds with a high enough density of cells to protect against all of the possible invasion sites by the pathogen because of the size of the seed and its carrying capacity.

The significance of the linear relationships between inoculum density and efficacy of bacterial strains in reducing *P. ultimum* colonization of pericarps should be considered cautiously for

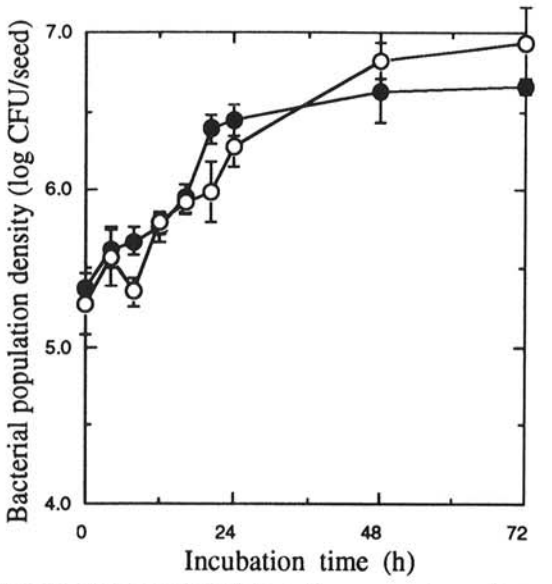

Fig. 2. Growth of total soil bacteria in the spermosphere of sugar beet in Oceano loamy sand adjusted to −15 J/kg and maintained at 16 C. The results of two identical experiments are indicated by closed and open circles. Each data point represents the mean of four replicates, with each replicate consisting of a pool of five seeds. Vertical bars indicate standard deviations.

TABLE 1. Comparison of strains of *Pseudomonas* with short and long lag phases on reducing colonization of sugar beet pericarp by *Pythium ultimum* in soil

Treatment/strain[t]	Population density of strain on seed (log cfu/seed)[u] At planting[v]	After 48 h	Pericarp colonization by *P. ultimum*[w] (%)
28 Propagules *P. ultimum* per gram soil			
Metalaxyl[x]			0.7 a[y]
ML5 (SLP)	5.97 ± 0.41	6.47 ± 0.06	24.4 b
33-2 (SLP)	7.06 ± 0.14	7.01 ± 0.13	65.9 c
A1 (SLP)	7.28 ± 0.09	7.03 ± 0.12	73.7 d
F42 (LLP)	7.54 ± 0.16	6.77 ± 0.46	80.9 e
B728A (LLP)	7.63 ± 0.14	7.33 ± 0.24	83.3 e
PGS12 (LLP)	6.68 ± 0.44	7.00 ± 0.14	83.9 e
Control			93.6 f
54 Propagules *P. ultimum* per gram soil			
Metalaxyl[x]			14.8 A
A1 (SLP)	7.36 ± 0.21	ND[z]	94.2 B
ML5 (SLP)	6.53 ± 0.15	ND	96.0 BC
33-2 (SLP)	7.32 ± 0.12	ND	98.2 C
PGS12 (LLP)	7.27 ± 0.23	ND	98.6 C
F42 (LLP)	7.58 ± 0.37	ND	98.6 C
B728A (LLP)	7.93 ± 0.06	ND	100.0 C
Control			99.6 C

[t] SLP = short log phase; LLP = long lag phase.
[u] Determined from two trials with four pools of seven seeds per trial.
[v] Bacterial population density was determined before planting seeds in soil.
[w] Incidence of pericarp colonization after 48 h in soil was determined from two trials with five replicates per trial, 28 seeds per replicate. Trial-by-treatment(s) interactions were not significant according to the preliminary analysis of variance, and variances between two trials were homogeneous. The effects of strain and inoculum density of *P. ultimum* were both significant ($P = 0.05$), and there was a significant interaction between the two effects.
[x] Applied at the rate of 0.3 mg a.i./g of seed.
[y] Numbers followed by the same letters were not significantly different according to Fisher's protected LSD ($P = 0.05$). For each seed treatment, the average incidence was significantly different between the two soils containing different populations of *P. ultimum*.
[z] Not determined.

several reasons. For example, if one were to imprudently extrapolate and extend the regression line of ML5 (Fig. 3E) past the range of observations, one might conclude that an inoculum density of $>10^7$ cfu per seed would totally eliminate *P. ultimum* colonization. This has never been attained by us in any experiment regardless of the experimental conditions (22; unpublished data). Furthermore, increasing dosages of a bacterial inoculant and measuring the response is not the same as working with a pesticide, because many biotic and abiotic factors affect the inoculum and subsequent dynamics of organismal growth. Although we do not question the necessity of having large inoculum densities to reduce disease, we are not convinced that the linear relationships as found in our relatively simple experiments would necessarily hold in other bacterial–fungal relationships, or if we had used different soils with a range of *P. ultimum* inocula. In fact, in a different set of experiments (22), the relationship between inoculum density and disease control was not clear. Moreover, the efficacy of ML5 was much better than that reported herein, even though the same strain, soil moisture, and conditions of incubation were used. Although the same soil was used in both studies (collected from a vegetable field), the biotic and abiotic properties probably differed, since crops were rotated and management practices may have varied among seasons. In addition, Osburn et al (22) used air-dried soil that was usually stored for 1 mo or longer prior to use. This slight alteration in experimental procedure also made a great difference, in that the density of ML5 was fivefold greater in the air-dried soil than in the fresh moist soil (R. Fukui, *unpublished*). This exemplifies the problem that different laboratories have in validating the efficacies of reported biological control strains since the experimental conditions are seldom the same.

Although colonization of the pericarp by *P. ultimum* was relatively high in these experiments, it should be recognized that percentage infection of the true seed is considerably lower (19,20). Colonization of the pericarp does not necessarily lead to infection, and in some cases the fungus dies in the pericarp, presumably because of interaction with inoculated antagonists that also colonize these tissues (19,20).

Effective seed-protecting biocontrol strains may have a number of characteristics in common. Short lag phases, rapid doubling times, and the ability to respond to a variety of nutrients seem

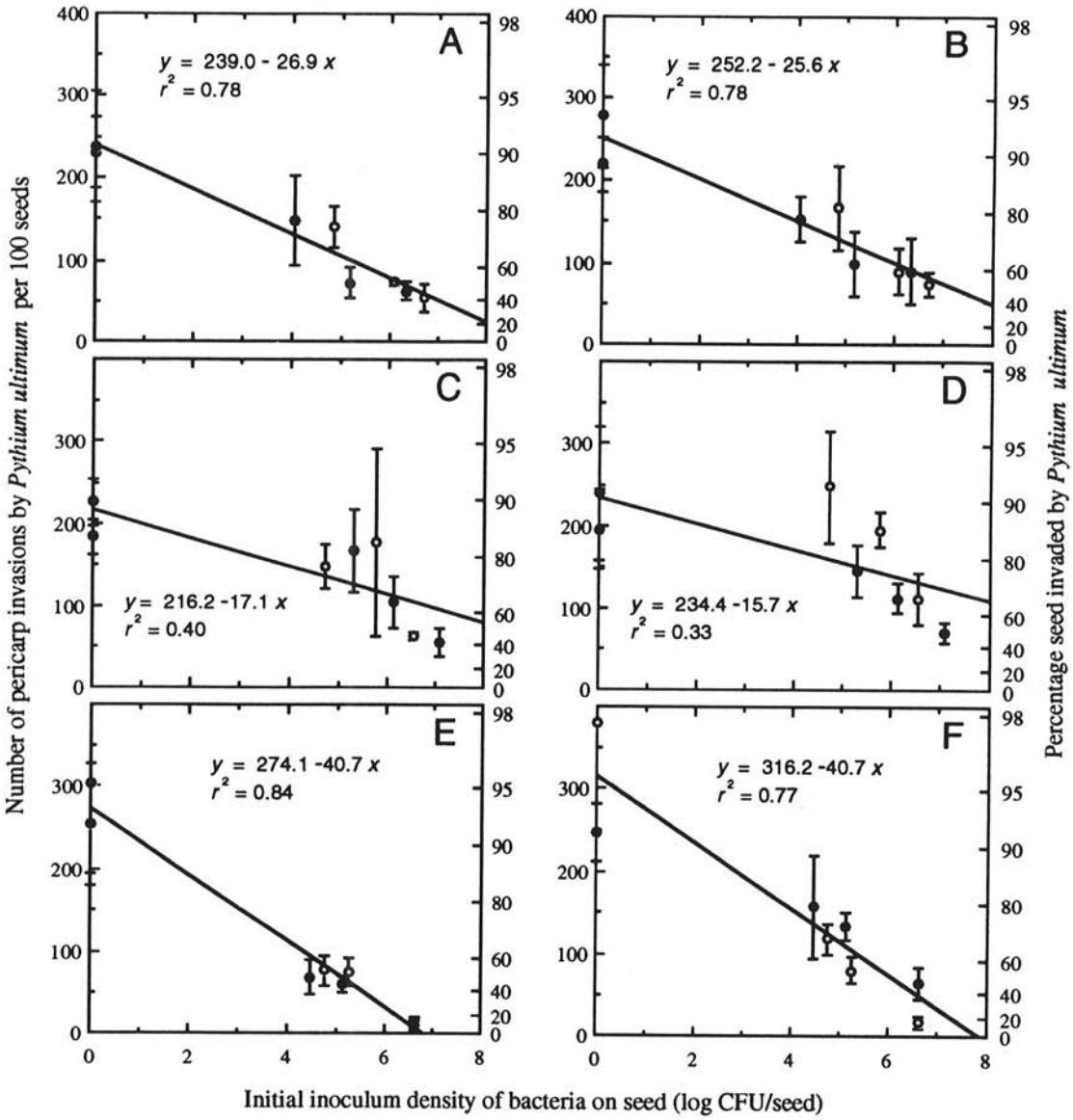

Fig. 3. Relationships between initial inoculum density of three *Pseudomonas* strains on sugar beet seed and the number of hyphal invasions of pericarps by *Pythium ultimum*. **A and B,** *Pseudomonas putida* 33-2; **C and D,** *P. fluorescens* A1; and **E and F,** *P. fluorescens-putida* ML5. Incidence of pericarp colonization (right scale) was determined at 24 h (A, C, and E) and 48 h (B, D, and F) after planting, with four replicates, 28 seeds per replicate. The experiment was done twice and the data were pooled since variances between trials were homogeneous. Vertical bars represent standard deviations (after transformation). All regressions were significant ($P = 0.05$), and r^2 is the coefficient of determination. The slope values and y intercepts are indicated with respective 95% confidence intervals.

TABLE 2. Population densities at 24 and 48 h of *Pseudomonas* strains 33-2, A1, and ML5 inoculated on sugar beet seed at three different densities

Strain	Population density (log cfu/seed)[y]		
	At planting[z]	24 h	48 h
33-2	6.52 ± 0.16	6.84 ± 0.18	7.19 ± 0.14
	5.61 ± 0.36	6.43 ± 0.14	6.40 ± 0.10
	4.44 ± 0.53	6.11 ± 0.17	6.10 ± 0.19
	Untreated	...	...
A1	6.82 ± 0.20	6.72 ± 0.22	6.38 ± 0.27
	5.94 ± 0.18	6.41 ± 0.10	6.42 ± 0.11
	4.99 ± 0.32	6.02 ± 0.21	6.59 ± 0.07
	Untreated	...	...
ML5	6.63 ± 0.16	6.53 ± 0.22	6.64 ± 0.14
	5.18 ± 0.42	6.19 ± 0.16	6.46 ± 0.10
	4.60 ± 0.19	6.28 ± 0.15	6.38 ± 0.16
	Untreated	...	...

[y] Bacterial population density was determined from two trials with four pools of seven seeds per trial.
[z] Bacterial population was determined before planting seeds in soil.

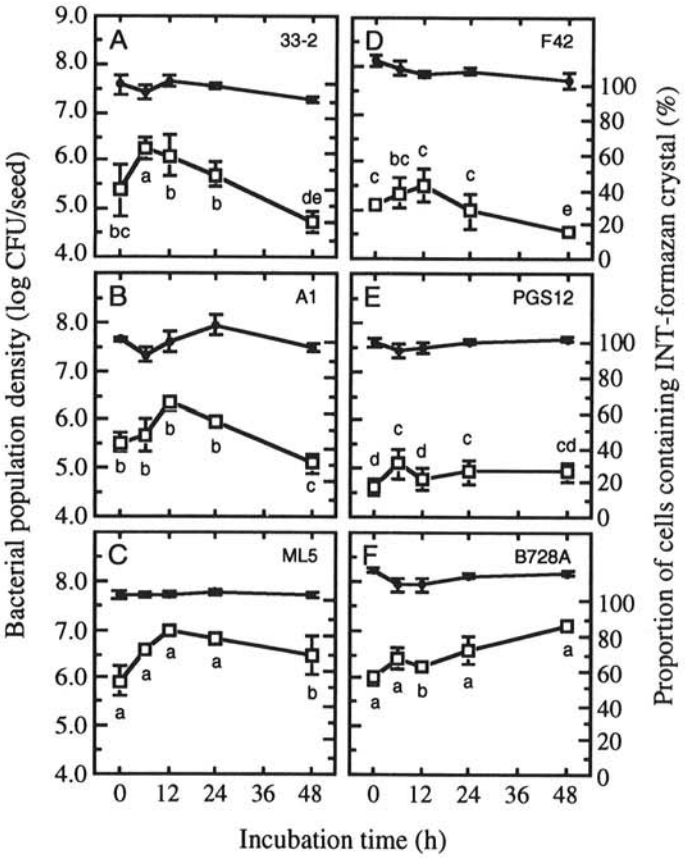

Fig. 4. Population densities and proportion of cells that reduced 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) in the spermospheres of sugar beet in sand. **A,** *Pseudomonas putida* 33-2; **B,** *P. fluorescens* A1; **C,** *P. fluorescens-putida* ML5; **D,** *P. putida* F42; **E,** *P. aureofaciens* PGS12; and **F,** *P. syringae* B728a. Solid circles indicate bacterial population densities in the spermosphere (left scale), and open squares represent proportion of cells containing INT-formazan crystals (INT-active) after incubation in a solution of INT at 25 μg ml^{-1} for 1 h at 28 C in the dark (right scale). Each data point for bacterial population density represents the mean of four replicates, with each replicate consisting of a pool of five seeds. The proportion of INT-active cells is the mean of four measurements, each determined by counting at least 400 cells. Vertical bars indicate standard deviations. According to analysis of variance, the main (strain) and subplot (incubation time) effects were significant at ($P = 0.05$) and there was a significant interaction between strain and incubation time. Percentages of INT-active cells indicated by the same letter are not significantly different ($P = 0.05$) at each incubation time by Fisher's protected LSD.

to be key factors. With respect to inhibitory secondary metabolites, any number of them could play an important role. However, successful seed colonization would seem to be the first and most important criterion for an efficacious strain in control of *Pythium* preemergence damping-off.

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