Survival of *Pseudomonas putida*, a Biological Control Agent, in Soil

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ABSTRACT

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When cells of *Pseudomonas putida* were added to soil, suppressiveness to Fusarium wilt of flax was induced. Survival characteristics of this bacterium were determined. A rifampin-resistant mutant of the bacterium was used to monitor population densities in soil. Recovery of *P. putida* as reflected by the number of colony-forming units (cfu) detected immediately after the addition of *P. putida* to soil was greatest at high matric potential. When added at −100 bars, no *P. putida* were detected; however, when soils were dried slowly to −100 bars, the bacterium survived. When different initial concentrations of *P. putida* were added to nonsterile soil, subsequent increase of bacteria was greater when soil received the lower inoculum level. *P. putida* survived through winter under ambient environmental conditions in two Colorado soils, with population densities increasing after January. Rhizosphere colonization of radish plants by *P. putida* was lower in soils that were biologically active (incubated 1 wk at near −0.3 bars before the introduction of *P. putida*) compared to soils that were previously air-dry. Population densities of the bacterium in the rhizospheres of cucumber and radish plants remained high throughout field trials.

In the Salinas Valley in California, Fusarium wilt-suppressive soil has been identified (24) and, despite continuous cropping of susceptible cultivars, disease does not develop. Previous work indicated that antagonistic *Pseudomonas* spp., isolated from this soil (21,22) or other soils (15), can suppress Fusarium wilt incidence when added to conducive soil infested with the pathogen.

Effective induction and maintenance of disease suppression by introduced microbial antagonists may be largely dependent on the survival of these agents. In some cases (18), the amount of suppressiveness induced was directly related to the population density of the antagonist in the root zone. Thus, establishment and domination of these microorganisms in the infection court could be a prerequisite to biological control.

The objective of the present investigation was to study the survival, in nonsterile soil, of a *Pseudomonas* capable of inducing suppressiveness to Fusarium wilt. A preliminary report was published (10).

MATERIALS AND METHODS

Analyses of the three soils used in these investigations are given in Table 1. Two of the soils, Fort Collins clay loam (FCCL) and Nunn sandy loam (NSL), were conducive to Fusarium wilt diseases (21,22). Both soils were air-dried, sieved to pass a 4-mm screen, and stored in metal cans. *Fusarium*-suppressive soil, collected from the Salinas Valley in California (24), was kept moist until microbial isolations were made. Matric potential curves of the three soils, generated by the method of Fawcett and Collis-George (11), are presented in Fig. 1. Soil moisture content was determined by oven-drying soil samples at 105 C for 24 hr. Unless otherwise noted, NSL was used in experiments.

Isolations of fluorescent *Pseudomonas* spp., and assessment of possible biological control of Fusarium wilt of flax induced by *Fusarium oxysporum* Schlecht. emend Snyd. & Hans. f.s. *lini* (Bolley) Snyd. & Hans. were performed as described by Scher and Baker (22).

Selection and enumeration of rifampin-resistant mutants. Stable spontaneous mutants of *Pseudomonas* spp. resistant to rifampin were selected by streaking wild-type isolates on King's Medium B (KB) agar (13) amended with 100 μg rifampin per milliliter (Calbiochem-Behring Corp., La Jolla, CA 92112).

Rifampin-resistant mutants (Rfm), when added to soil, were enumerated by placing soil samples in dilution blanks containing 0.1 M $MgSO_4$. Tenfold dilutions were made, and 0.5 ml of the final dilutions were spread on KB agar amended with 100 μg of rifampin per milliliter, 100 μg active ingredient (a.i.) of cycloheximide (Calbiochem-Behring Corp., La Jolla, CA 92112) per milliliter, and 30 μg a.i. of benomyl (E. R. Dupont Co., Wilmington, DE 19898) per milliliter. Triplicate plates were made of each dilution, and the number of colony-forming units (cfu) were enumerated after 48 hr.

Survival of *Pseudomonas* in fallow soil. In laboratory studies of bacterial survival in fallow soil, 100 g of air-dry soil was added to half-pint mason jars. In experiments requiring sterile soil, the soil-filled jars were autoclaved for 1 hr on each of 3 consecutive days. By this procedure, no microorganisms were detected when this soil was transferred to KB. Bacterial suspensions in 0.1 M $MgSO_4$ (1 ml), with an appropriate amount of sterile distilled water (to achieve desired matric potential), were added to each jar. Soil within the jars was evenly mixed and packed approximately to bulk density (1.3 g/cm^3). The jars were incubated in a growth chamber at 27 C in completely randomized blocks. Bacterial counts were made at various time intervals by destructively sampling four replicate jars per treatment.

To study survival under the ambient environmental conditions in Colorado, population densities of Rfm were monitored in the NSL and the FCCL at three depths. Air-dry portions of the soils were mixed with a suspension of Rfm and distilled water to bring soil water content to −0.3 bars matric potential in both the NSL and the FCCL. Soil was packed at approximately bulk density in rigid plastic (asbestos butylene styrene) tubes of 4 cm i.d. and 5 cm long. Nylon netting with 3-mm^2 holes was secured with strapping tape to the ends of each tube. The soil-filled tubes were buried at 5-, 10-, and 25-cm depths in adjacent miniplots containing appropriate soil types. At monthly intervals, a random sample of four replicates per treatment was retrieved from each depth. The center core of soil from each tube was removed for soil moisture determination and counts of Rfm.

Rhizosphere colonization by *Pseudomonas*. To study populations in the rhizosphere, roots were vigorously shaken to

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remove loose soil. Roots plus tightly adhering soil were weighed, added to dilution blanks, and placed on a rotary shaker for 20 min. Washed roots were removed, blotted to remove excess moisture, and weighed to determine amount of soil removed. Serial dilutions of soil suspensions remaining in the blanks were made.

To determine if biologically active soils influence initial colonization of the rhizosphere by Rfm, soil was incubated in covered trays at 27 C either air-dry or at approximately water-holding capacity for 1 wk. After incubation, moisture level of the air-dry soil was increased to that of the wetted soil. These soils were each divided into two portions and suspensions of Rfm were added to give population densities of either 10^5 or 10^7 cfu/g dry soil. Initial counts of population densities were made, treatments divided into four replicates of 300-g portions, added to pots, and planted with 15 radish seeds (*Raphanus sativus* L. 'Scarlet Globe'). After 3 days, plants were thinned to 10 per pot. Pots were placed in completely randomized blocks, watered daily with nutrient solution, and numbers of cfu from the rhizosphere were determined after 1 wk.

Survival of *Pseudomonas* in the rhizosphere of cucumber plants in the field was determined. The Rfm was added to FCCL at an initial population density of 10^7 cfu/g dry soil. Portions (600 g) of bacteria-amended soil were added to pots and planted with four cucumber seeds (*Cucumis sativus* L. 'Straight Eight') per pot. Cucumber seeds were germinated in a growth room at 27 C and, 10 days after sowing, seedlings and pot soil were transferred to hills and grown in FCCL in the field with irrigation as required.

Rfm colonization of radish roots from plants grown under field conditions was evaluated. Two methods of bacterial application were used: a xanthan gum-talc seed coating method described elsewhere (25), and a furrow application (25 ml of a 10^9 cfu/ml suspension per 3-m row). Rhizosphere counts were made 5 wk after planting. The experiment was repeated later in the growing season.

Data were subjected to an analysis of variance and mean separations were performed with Fisher's least significant difference procedure ($P = 0.05$).

RESULTS

Isolation of fluorescent *Pseudomonas* spp. and assessment of their biological control potential. Fluorescent *Pseudomonas* spp. isolates were obtained from mycelial mats (22) on soil suppressive to *Fusarium*. Several were capable of significantly reducing flax wilt incidence when added at 10^7 cfu/g dry soil infested with the pathogen. One isolate, designated N-1, identified as *Pseudomonas putida* (Trevisan) Migula, was chosen for further study based on its potential as a biological control agent (Fig. 2). The incidence of Fusarium wilt (DI) was significantly different at 32 days, with 48% DI in the control where no bacteria were added, and 14% with isolate N-1.

Selection and enumeration of rifampin-resistant mutants. A Rfm of *P. putida* isolate N-1, capable of growing on agar amended with 100 μg rifampin per milliliter, was obtained; it was designated N-1R. When added to nonsterile soil, enumeration of the mutant (N-1R) was possible because few soil organisms other than N-1R were able to grow on the modified KB agar.

Survival of the wild-type versus rifampin-resistant mutant. To compare cell survival, bacterial suspensions containing either wild-type N-1 or the Rfm N-1R were added at 10^7 cfu/g dry soil to jars containing sterile soil. Two moisture levels were chosen corresponding initially to −0.3 and −15 bars matric potential. There were no significant differences in survival characteristics between the isolates at either matric potential.

Effect of matric potential on *Pseudomonas* in fallow soil. Isolate N-1R was added to nonsterile soil (10^7 cfu/g dry soil) at four moisture levels corresponding to −0.3, −2, −15, and −100 bars matric potential. The number of viable cells immediately after the

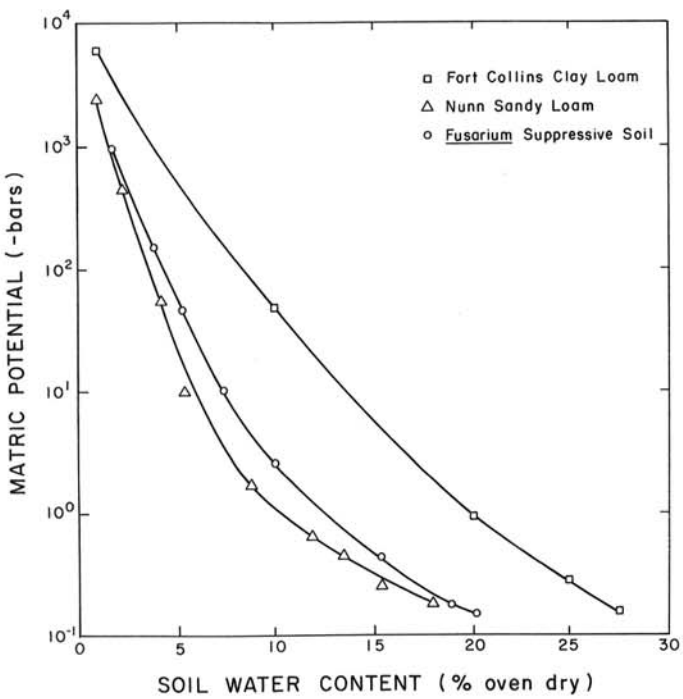

Fig. 1. Matric potential curves of the three soils used in these investigations.

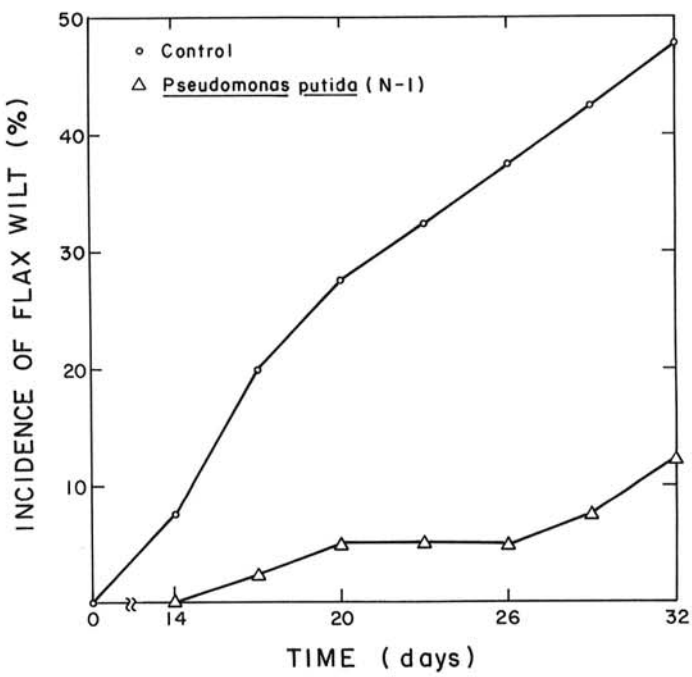

Fig. 2. Effect of addition of *Pseudomonas putida* (isolate N-1R) at 10^7 cfu/g of soil on flax wilt incidence. At the end of the experiment wilt incidence differed significantly ($P = 0.05$) from the control.

TABLE 1. Soil analysis

Soil name	pH	Conductivity (mmhos/cm)	Lime	Organic matter (%)	NO_3^-N (μg/g)	P (μg/g)	K (μg/g)	Zn (μg/g)	Fe (μg/g)	Texture
Nunn	7.3	0.4	Low	1.1	1	1	198	0.5	3.2	Sandy loam
Fort Collins	7.4	0.4	High	1.9	4	10	139	16.5	2.5	Clay loam
(*Fusarium*-suppressive)	7.4	4.5	Low	1.4	100	65	460	14.3	16.4	Sandy loam

addition of the bacteria varied with soil moisture, with significantly greater survival at high matric potential (Fig. 3). When bacteria were added at −100 bars, no cfu were detected by the procedures used. Populations of N-1R increased the first week in soil at the three highest moisture levels and then slowly declined. Comparison of survival curves showed significant differences in survival due to moisture level. By the fifth week, matric potentials had decreased to −2, −13, and −100 bars for the three highest moisture levels. However, even when matric potential of soil initially at −15 bars had decreased to −100 bars, over 5×10^5 cfu/g dry soil were recovered.

Effect of initial population density and matric potential on the survival of *Pseudomonas*. Recovery of isolate N-1R was found to be dependent on initial population density and matric potential in nonsterile soil. Bacteria were added at either 10^5 or 10^7 cfu/g dry soil at moisture levels adjusted initially to −0.3, −2, and −15 bars matric potential (Fig. 4). Relative increase in number of cells was greater when the initial population density was 10^5 than at 10^7 cfu/g dry soil. At 3 days, population densities had increased an average of 20× at the lower inoculum level as compared with less than 4× when the cells were added at 10^7 cfu/g dry soil. In addition, maximum densities were significantly higher in soils receiving the larger population densities regardless of matric potential.

Survival and retention of biocontrol ability of *Pseudomonas* in fallow soil under ambient environmental conditions. Similar survival curves, generated over a period of 10 mo, were obtained when isolate N-1R was added to either FCCL (Fig. 5A) or NSL (Fig. 5B) and buried in the field.

Soil type affected the number of viable cells counted, with lower recovery from FCCL. When equivalent bacterial suspensions were added to both soils, initial counts of 1.2×10^7 and 3.7×10^7 cfu/g dry soil were obtained for the FCCL and NSL, respectively. After burial in October, population densities declined in both soils, reaching minima in January. Bacterial numbers increased over the next 2-mo period. Maximum numbers of cfu were observed in March and were over 8.3×10^4 and 1.3×10^6 for FCCL and NSL, respectively, at the 25-cm depth. Subsequent sampling revealed a steady decline of survivors for the duration of the experiment.

Survival in the NSL was favored at the lower depths where matric potential was higher; however, survival did not appear to be related to depth in the FCCL.

To test whether bacterial survivors had retained their biological control potential, random colonies formed on dilution plates were selected as test reisolates. In a typical experiment (Fig. 6), flax wilt incidence was significantly reduced when either the original isolate N-1R (stored lyophilized until use), or N-1R reisolates (A and B) were added to infested soil at 10^7 cfu/g dry soil as compared with the control where no bacteria were added.

Influence of biologically active soils on colonization of *Pseudomonas* in the rhizosphere. Soil was incubated 1 wk at near −0.3 bars to stimulate biological activity. This treatment (NAD), before the introduction of N-1R, decreased subsequent rhizosphere colonization of radish plants by N-1R compared with soil which was air-dry at the beginning of the experiment (AD) (Fig. 7). In AD soil, population densities of N-1R in the rhizosphere were 26× higher after 1 wk than the initial population density of 10^5 cfu/g soil. In contrast, densities were less than 2× higher after 1 wk when bacteria were added to NAD soil. When N-1R was added at 10^7 cfu/g soil, population densities in the rhizosphere had increased 4× in AD soil, while they had less than doubled in NAD soil; however, these differences were not statistically significant.

Colonization of *Pseudomonas* in the rhizosphere of plants grown under field conditions. Initial population density of N-1R in the rhizosphere of cucumber seedlings was 4.3×10^6 cfu/g soil at the time of transfer to the field (Fig. 8). Subsequent sampling revealed a steady decline in the number of viable cells surviving in the rhizosphere soil. However, population densities 9 wk after transplanting were over 1.1×10^5 cfu/g soil.

In the field plots containing radish, population densities of N-1R at harvest (5 wk) were 8.2×10^6 and 3.2×10^5 cfu/g soil for the

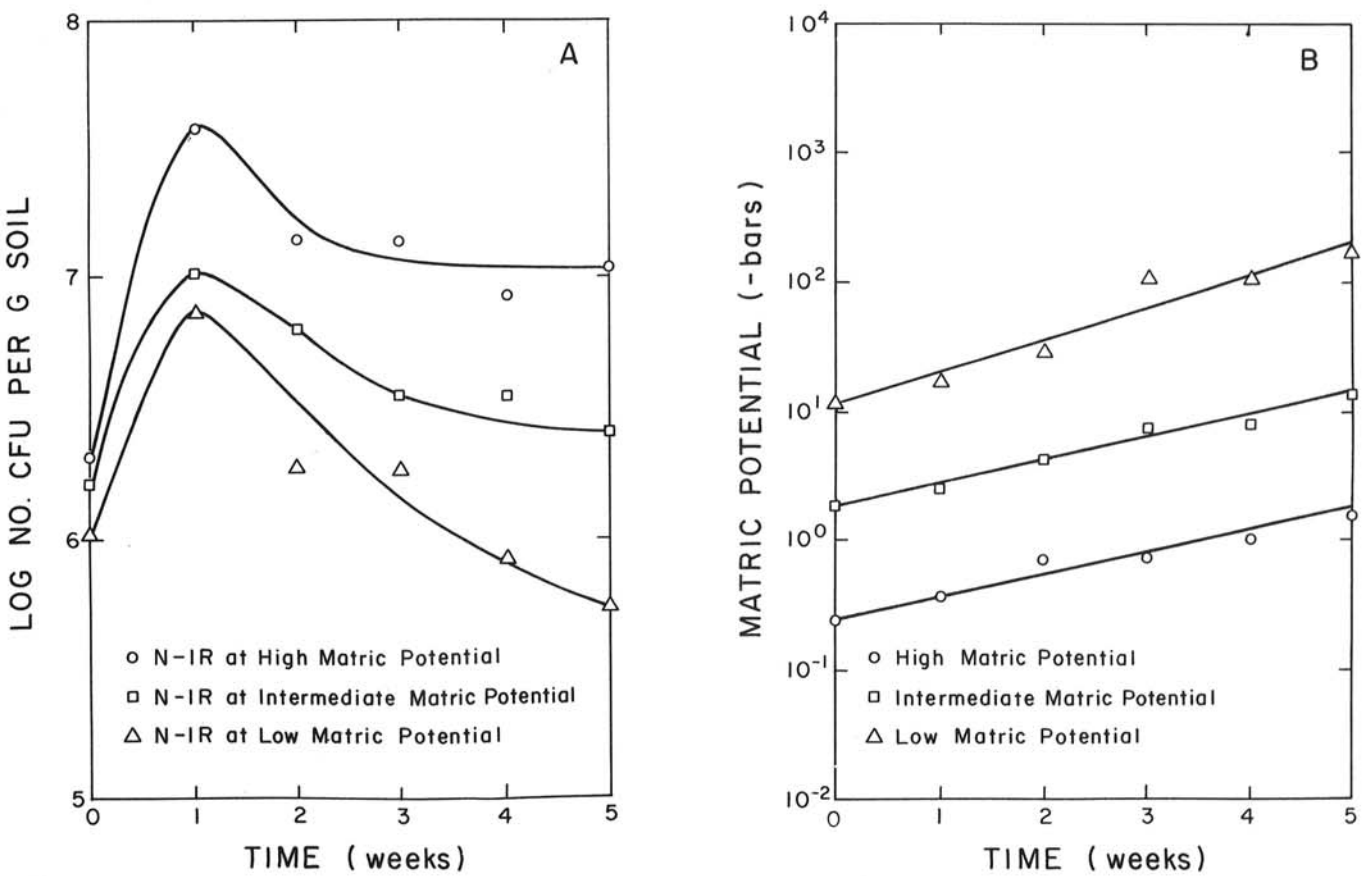

Fig. 3. A, Survival curves of *Pseudomonas putida* (isolate N-1R) added to fallow, nonsterile soil (10^7 cfu/g dry soil). **B,** Moisture levels during the experiment. Initial levels corresponded to −0.3, −2, and −15 bars matric potential.

furrow application and seed application, respectively. When the experiment was repeated, similar colonization occurred with counts of 3.3×10^6 and 2.3×10^4 cfu/g soil obtained at 5 wk.

DISCUSSION

In this study, survival of an antagonistic *P. putida*, capable of significantly reducing Fusarium wilt incidence (Fig. 2), was examined as affected by various factors in nonsterile soil. Addition of *P. putida* (isolate N-1R) to soil at favorable matric potentials did not result in rapid death as often occurs when bacteria are added to soil. For example, the population density of *Agrobacterium tumefaciens* added to nonsterile soil decreased almost four log units in 8 wk (9). *Micrococcus luteus* rapidly lost viability, with less than 1% of the initial population surviving 2 wk after addition to soil (3). When *Escherichia coli* was added to soil, numbers decreased six log units in 24 days (14). In contrast, rhizobia added to soil survived well, with numbers decreasing only one log unit in 8 wk (5). In the present study, population densities of *P. putida* increased after addition to soil and, after 5 wk, numbers were often higher than the initial population densities (Figs. 3 and 4).

The number of viable cells recovered immediately after addition of *P. putida* to soil was favored by high matric potential (Fig. 3). Rapid death of the bacterium occurred when added to soil at −100 bars matric potential. Burr et al (2) reported similar results when potato seedpieces, surface-soaked with *Pseudomonas* spp., were placed in soils with low soil water potential. Population densities of *Pseudomonas* spp. differed by nearly three log units 96 hr after they were planted in soils with water potentials of −16.1 and −1.7 bars. *Pseudomonas* spp. are considered to be drought susceptible, with rapid loss of viability when exposed to desiccated soils (4). Labeda et al (17) postulated that, in sterile soil, water is the controlling factor in the death of *Pseudomonas* spp. However, when *P. putida* was added to soil, which was allowed to dry slowly over a 5-wk period to −100 bars, over 5×10^5 cfu/g soil could be recovered (Fig. 3).

The ability of *P. putida* to survive low soil matric potentials might have been due to an increase in internal osmotic tension of the cells. Data presented by Chen and Alexander (4) suggest that there is a relationship between the ability to persist throughout prolonged drought and high internal osmotic tension. When the internal osmotic potential of bacterial cells of drought-susceptible species was raised by growth on media with low water activity, the isolates were more tolerant to dry soil conditions. Perhaps, *Pseudomonas* spp., existing in soils undergoing slow drying, may be able to increase cell osmotic tension, and thus, become more resistant to desiccation. In addition, it is possible that clay colloids could form favorable microenvironments protecting bacteria in dry soil. In their work with *Rhizobium*, Danso and Alexander (5) found that the root-nodule bacteria were able to withstand drying in soils. In dry sand, however, the bacteria died readily, perhaps due to the lack of such microenvironments.

When different initial population densities of *P. putida* were

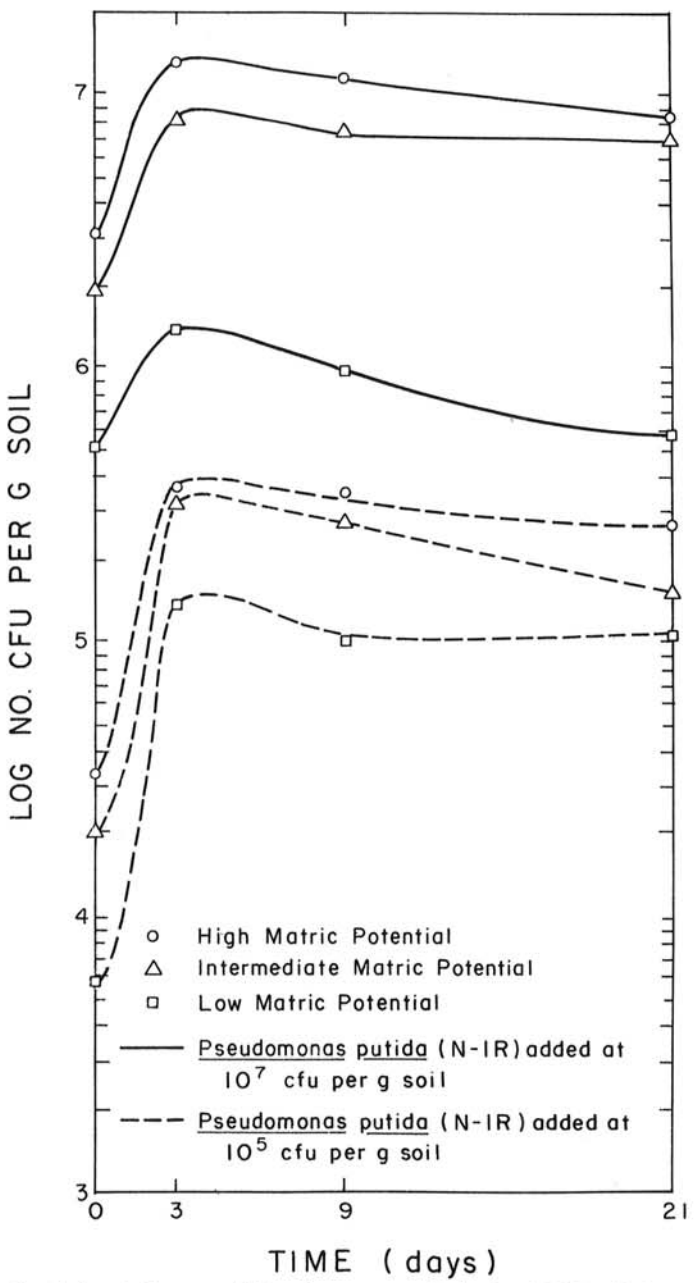

Fig. 4. Survival curves of *Pseudomonas putida* (isolate N-1R) added at either 10^5 or 10^7 cfu/g dry soil at moisture levels initially at −0.3, −2, and −15 bars matric potential.

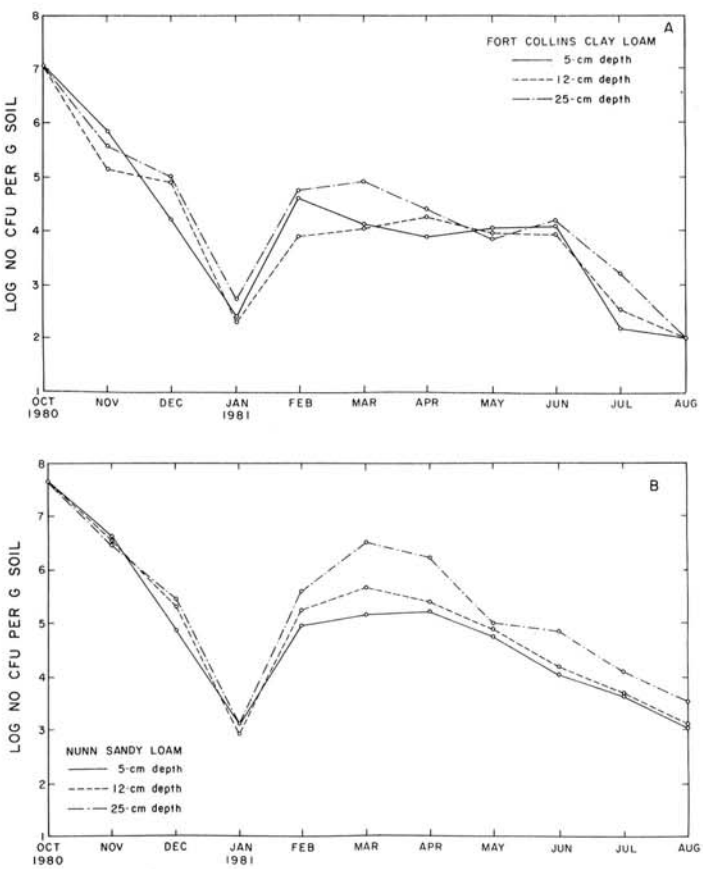

Fig. 5. Survival of *Pseudomonas putida* (isolate N-1R) under ambient environmental conditions in fallow **A,** Fort Collins clay loam or **B,** Nunn sandy loam, at three depths.

added to nonsterile soil, subsequent increase of the bacteria was greater when soil received the lower inoculum level. This effect was evident when the bacteria multiplied in fallow soil (Fig. 4), or colonized the rhizosphere of radish (Fig. 7). It is possible that multiplication was restricted when *P. putida* was added initially at 10^7 cfu/g dry soil by a soil-imposed limit. Labeda et al (17) postulated that in sterile soil the maximum colonization limit of *Pseudomonas* was probably due to nutrient availability, space availability, cell migration to new colonizing sites, or a combination of those factors. However, when soil received an initial population density of 10^5 cfu/g soil, maximum numbers reached were always well below those attained in soil that had received the higher inoculum level (Fig. 4). This implies that, in nonsterile soil, there is a microbially imposed limit as well.

Population density of *P. putida* was significantly lower when air-dry soil was moistened 1 wk before planting than when moistened at the time of planting (Fig. 7). This reduction in rhizosphere colonization might reflect differences in the nutritional status of the soil, as well as the presence of competitors, predators, and other organisms detrimental to *Pseudomonas*. It is well documented that predation by indigenous protozoa was responsible for the decline of *Rhizobium* and *Xanthomonas* in nonsterile soil (6,7,12,19,20). Additionally, Darbyshire and Greaves (8) found that the addition of bacteriophagous soil amoeba to the root medium affected population density of *Pseudomonas* spp.

P. putida, which existed in soil under ambient environmental conditions in Colorado, were subject to a wide variety of interacting factors that affected their survival (Fig. 5A and B). Season of year had a pronounced effect on population densities over the course of the experiment. As was considered typical of bacteria in soils in temperate regions, numbers diminished in winter (16). Bacteria surviving in winter soils were thought to be in a state of biochemical inactivity, ready for reactivation when conditions became more favorable. Cell numbers were greatest in the spring, perhaps due to increased soil temperature and availability of organic materials (1).

Soil type affected numbers of *P. putida* recovered, with counts of the bacteria in the NSL (Fig. 5B) often 10× higher than found in the FCCL (Fig. 5A). However, this might reflect a difference in the ability to recover the bacterium rather than survival. Bacteria,

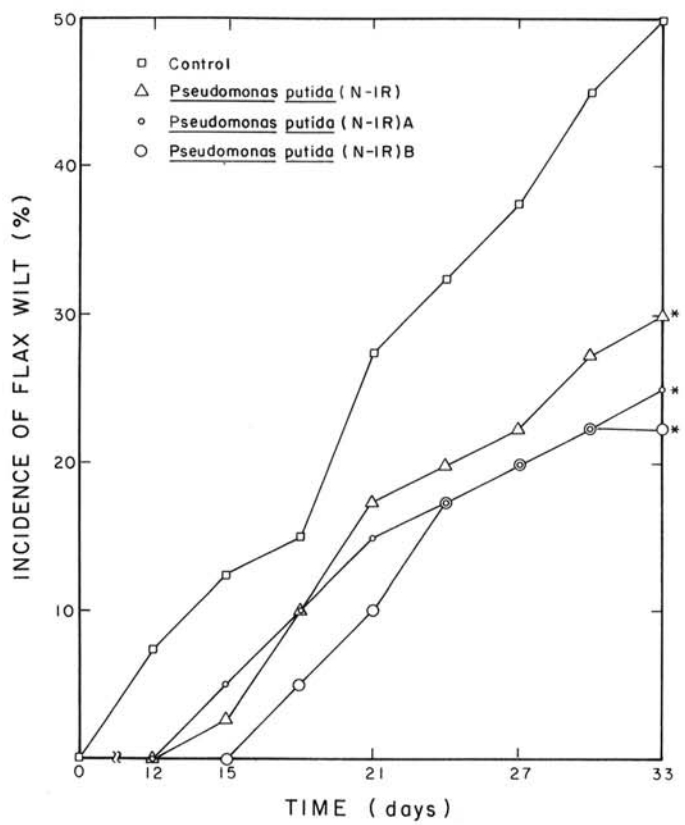

Fig. 6. Effect of addition of *Pseudomonas putida* (isolate N-1R) or test reisolates (N-1R)A and (N-1R)B at 10^7 cfu/g dry soil on flax wilt incidence. Wilt incidence in all *P. putida* treatments was significantly reduced ($P = 0.05$) compared to the control.

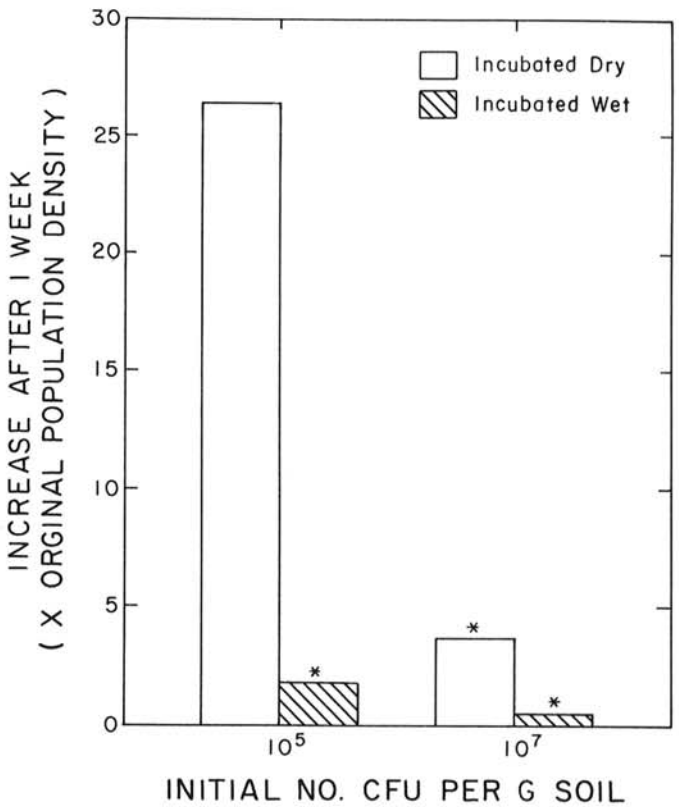

Fig. 7. Influence of biologically active soils on colonization of *Pseudomonas putida* (isolate N-1R) in the radish rhizosphere. Soil was incubated 1 wk at near −0.3 bars to stimulate biological activity. This treatment, before the introduction of N-1R (10^5 cfu/g soil), decreased subsequent rhizosphere colonization compared to soil which was air-dry at the beginning of the experiment. A similar reduction in colonization occurred when bacteria were added at 10^7 cfu/g soil.

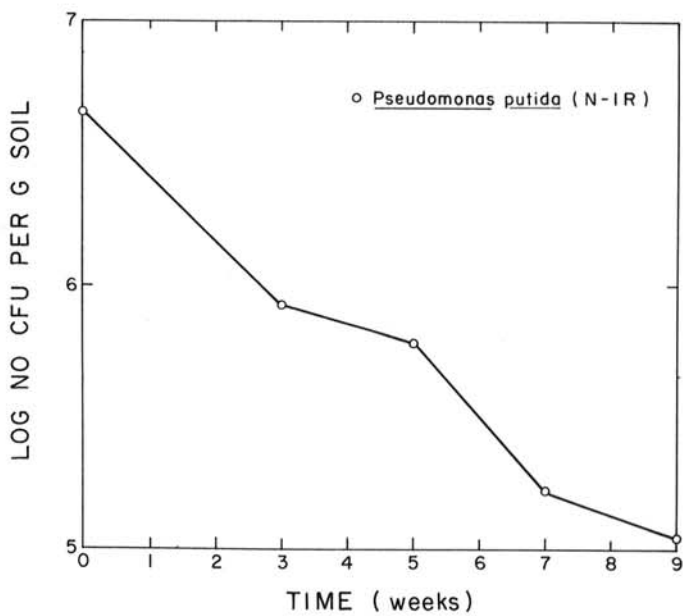

Fig. 8. Number of cfu of *Pseudomonas putida* (N-1R) in the rhizosphere of cucumber plants grown under field conditions in a 9-wk trial.

which existed in the clay loam, may have been more tightly adsorbed by soil colloids, thus lowering plate counts.

Survival of *P. putida* in the NSL was greater at a 25-cm depth than at 5- or 10-cm (Fig. 5B). This possibly reflected a more favorable matric potential at the lower depth. However, survival of the bacterium in FCCL could not be correlated similarly (Fig. 5A).

In the colonization of cucumber and radish plant roots, *P. putida* persisted throughout the field trials. Colonization and persistence of *Pseudomonas* in the rhizosphere has been demonstrated (2,25,26), reflecting its competitive abilities as a rhizosphere-competent organism (23).

The ability of certain *Pseudomonas* spp. to induce suppressiveness to Fusarium wilts may be largely dependent on their root-colonizing ability. Therefore, an understanding of how alterations in the soil environment affect the survival and activity of *Pseudomonas* spp. could lead to more successful applications of the biological control agent to soil.

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