Population Dynamics of *Pseudomonas cepacia* in the Pea Spermosphere in Relation to Biocontrol of *Pythium*

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ABSTRACT

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Pseudomonas cepacia strain AMMD applied to pea seeds reduced preemergence damping-off caused by *Pythium ultimum* and *P. sylvaticum* by 47% in growth chamber experiments. Protection by the biocontrol agent was as effective as seed treatment with metalaxyl at all temperatures tested (16, 20, 24, and 28 C). Under controlled soil matric potential conditions (−6 kPa) at 24 C, *Pythium* began to infect nontreated seeds within 12 hr, and 100% of the seeds were infected 30 hr after planting. Incidence of seed infection during the first 48 hr was reduced 44–60% by seed treatment with metalaxyl or *P. cepacia* strain AMMDR1, resistant to rifampicin. No significant difference was found between the effectiveness of the fungicide and the biocontrol agent. The incidence of seed infection by *Pythium*, and doubling time of the introduced strain in the pea spermosphere, were inversely proportional to the initial population density of *P. cepacia* strain AMMDR1 on the seeds. When applied at log 4.9 colony-forming units per seed, the doubling time of this strain during the first 24 hr after planting was 3.1 hr. The introduced strain represented an increasing proportion of the population of bacteria associated with the seed during this period, an indication of its competitive success in the spermosphere.

Additional keywords: Pisum sativum, damping-off, biological control, seed colonization.

Pythium damping-off of pea (*Pisum sativum* L.) is a common and important disease that occurs wherever the crop is cultivated (3). Seeds may rot before they germinate, or seedlings may be killed before or shortly after emergence. Certain fungicides are very effective in controlling seed rots and damping-off, but some of these are likely to be withdrawn from use because of risks to the environment and to human health. In addition, the exclusive use of certain chemical seed treatments may lead to the development of pathogen populations resistant to these fungicides.

Seed rots and damping-off are well suited to biocontrol because the period of host susceptibility is very short, in some cases lasting only a few hours or days (6,13). Seeds are ideal for delivery of biological agents that control seed rots and damping-off because the microorganisms are placed directly on the infection court, and extensive root colonization is not necessary for successful disease control. Thus, many problems associated with the failure of introduced microorganisms to colonize roots and persist in the rhizosphere can be avoided (14,25). Seeds also release exudates including sugars and amino acids during imbibation and germination (23). These organic substrates are potentially a rich source of nutrients for biocontrol agents in the spermosphere. For these reasons, biological control of seed rots and damping-off has been an active area of research (5,10,13).

One problem associated with biocontrol is that biological agents, unlike fungicides, may not be active over the same range of environmental conditions that are conducive to disease. Another problem that may contribute to failure is that the initial density of biocontrol agents is insufficient, and their rate of growth too slow, to provide adequate protection against pathogens that infect rapidly. Biocontrol agents must also compete successfully with the indigenous microflora if they are to benefit from the nutrients released from seeds.

Pea seed treatment with *Pseudomonas cepacia* strain AMMD resulted in increased seedling emergence, reduced severity of Aphanomyces root rot, and increased pea yield of plants grown in a field naturally infested with *Aphanomyces euteiches* Drechsler f. sp. *pisi* and *Pythium* spp. (J. L. Parke, *unpublished*). *A. euteiches* normally does not cause seed rot or preemergence damping-off. The increased emergence suggested that suppression of other pathogens, such as *Pythium*, may be involved, because emergence was not affected by seed treatment in pasteurized soil.

The objectives of this research were to examine the interaction between *P. cepacia* and peas germinating in naturally infested soil and to determine the population dynamics of *P. cepacia* in the spermosphere in relation to the indigenous microflora and to seed infection by *Pythium*.

MATERIALS AND METHODS

Soil. Soil used in all experiments was Plano silt loam (pH 6.4) from the Arlington Agricultural Experiment Station near Arlington, WI. This soil is naturally infested with *Pythium* (429 propagules per gram [ppg] of soil) and *A. euteiches* (7.3 ppg) as assessed by soil dilution plating (22) and the most probable number method (17), respectively. Soil was air-dried, sieved through a 4.75-mm mesh screen, thoroughly mixed, and stored under cover at room temperature until used for experiments in the growth chamber. Soil was sieved through a 2-mm screen for all experiments conducted in Buchner funnels.

Bacteria. *P. cepacia* strain AMMD (ATCC 52796) was isolated from the rhizosphere of pea grown in the Aphanomyces root rot nursery at the Arlington Agricultural Experiment Station. A spontaneous mutant strain, AMMDR1, resistant to rifampicin (at 100 μg/ml), was used in some of the studies. Stock cultures of the wild-type and mutant strains were stored in 5% dimethyl sulfoxide at −80 C.

Seed treatment. AMMD or AMMDR1 was grown in nutrient broth yeast extract (NBY) (24) shake culture. After 48 hr, 2.5 ml of the turbid suspension was plated on NBY agar and incubated for 24 hr at room temperature. The bacterial lawn scraped from each plate was thoroughly mixed with 25 pea seeds (*P. sativum* 'Perfection 8221'). Control seeds were nontreated or were treated with metalaxyl (Apron 25W, Ciba-Geigy, Greensboro, NC) at the rate of 6.25 mg a.i./100 seeds. Seeds treated with bacteria or metalaxyl were dried at room temperature for 24 hr in a sterile cabinet before being sown. In each experiment, five or 10 seeds

per treatment were assayed for populations of bacteria at the time of planting. Seeds were placed in 20 ml of sterile distilled water, sonicated for 20 sec, then dilution-plated onto NBY agar, trypan blue tetracycline agar (TBT) medium selective for *P. cepacia* (4), or TBT supplemented with rifampicin (100 μg/ml) (TBTR). Plates were incubated at room temperature for 48 hr (NBY) or for 5 days (TBT or TBTR), at which time colonies were counted.

Biocontrol of preemergence damping off. Nontreated seeds and seeds treated with AMMD, AMMDR1, or metalaxyl were compared for their effects on pea seedling emergence in a growth chamber experiment conducted at 16, 20, 24, and 28 C. Seeds were sown 2 cm deep in 30- × 30- × 5-cm plant propagation flats (Jiffy Corp., W. Chicago, IL) filled with soil to a depth of 4 cm. The experiment was a split plot design with flats as the whole plot unit, temperature as the whole plot treatment (three flats per temperature), and seed treatments within flats as the subplot treatment. Each flat (= one replicate) contained 20 seeds of each seed treatment. Flats were watered daily to maintain soil moisture conditions adequate for germination. The number of emerged seedlings was recorded daily during days 3–7 after planting. Percentage of emerged seedlings was converted with the arcsin square-root transformation. Because the response to temperature was nonlinear, the data were analyzed with analysis of variance. Analysis was performed on plant stand for each day separately, so a Bonferroni correction factor was applied to P values and Fisher's protected LSD values to compensate for nonindependence of stand counts over time (18). The experiment was conducted three times, and results from a single representative experiment were chosen for presentation.

At the conclusion of each experiment, a subsample of nonemerged seeds and seedlings from the nontreated controls from each of the temperatures was plated to isolate the pathogen(s) involved in seed rot and damping-off. Isolations were also made from seeds 48 hr after planting in a fourth test. Roots or seeds were surface-disinfested in 0.05% sodium hypochlorite for 1 min, rinsed, blotted, and plated onto water agar and metalaxyl-benomyl-vancomycin (MBV) medium (15). Fungal colonies were transferred to MBV or cornmeal agar plates for identification.

Dynamics of *Pythium* seed infection in relation to seed treatments. Three seed treatments (nontreated, metalaxyl, and AMMDR1) were compared for their effects on pea seed infection by *Pythium* during the first 48 hr after planting. The population density of aerobic bacteria and of AMMDR1 associated with the seeds was also determined. This experiment was conducted in a 24 C growth chamber with a 12-hr photoperiod.

To control soil matric potential while the seeds were imbibing and germinating, this experiment was conducted with a Büchner funnel hanging water column apparatus (1). Each 600-ml funnel, fitted with a fine-grade fritted glass plate (Kimax 28400-600F), was connected to a length of plastic tubing filled with degassed water under vacuum. The open end of the tube was placed in a water reservoir that was raised or lowered to establish the desired matric potential. Each funnel was filled with sieved soil to a depth of 2 cm. On the day before planting, the soil was first saturated to +1 kPa and then allowed to drain to −60 kPa (1 kPa = 10 mbar) by lowering the reservoir to 60 cm below the fritted plate.

The experiment was a completely randomized design with three seed treatments × three replicates per sampling period × nine sampling periods. A replicate consisted of 10 seeds that were planted in a single funnel and harvested together. Seeds were gently pressed into the soil and a thin layer (3 mm) of additional soil was spread over them. Funnels were covered with foil to reduce evaporative water loss. Every 6 hr, the 10 seeds from each funnel were removed from the soil, placed together in 20 ml of sterile distilled water, sonicated for 20 sec, and vortexed for 20 sec. A 1-ml aliquot of this suspension was removed for dilution plating on 10% tryptic soy agar (11) amended with cycloheximide (100 μg/ml) (TSAC) and TBTR. Seed coats were removed; then seeds were rinsed in tap water, surface disinfested in 0.05% sodium hypochlorite for 30 sec, rinsed in sterile water, and blotted on paper towels. Seeds were separated into two halves, and each was placed face down on water agar (two halves per plate). Seeds were incubated at room temperature for 4 days and examined daily for *Pythium* (Fig. 1). Representative cultures were saved on cornmeal agar for species identification.

Percentage of infected seeds at each sampling period was transformed with the arcsin square-root transformation, and data were analyzed with one-way analysis of variance. Significant differences between means at each sampling period were determined with Fisher's protected LSD ($P = 0.05$). The experiment was repeated three times, and results from a single representative experiment were chosen for presentation.

Relationship between population density of AMMDR1 and incidence of *Pythium* infection. Three population densities of AMMDR1 applied to seeds were compared for their effects on the incidence of seed infection by *Pythium*. Seeds were treated with a high, medium, or low population density of AMMDR1 roughly corresponding to log 8.5, 7.0, or 6.0 colony-forming units (cfu) per seed and planted in naturally infested soil in the funnel apparatus described previously. Three replicate funnels, each containing 10 seeds, were used for each of the three population density treatments. Population densities of AMMDR1 and of total aerobes were assessed for each group of 10 seeds per funnel at time of planting and at harvest 24 hr later by dilution plating the wash suspension on TBTR and TSAC. Pea seeds were then surface-disinfested, plated on water agar, and scored for *Pythium* infection as described previously. The experiment was conducted three times. Regression analysis was performed with the multiple general linear hypothesis model of Systat 3.2 (Systat, Inc., Evanston, IL).

Doubling time of AMMDR1 and total aerobes in the pea spermosphere. Pea seeds treated with approximately log 5.0 cfu of AMMDR1 per seed were planted in naturally infested soil in the funnel apparatus. Seeds were harvested 4, 8, 12, 16, and 24 hr after planting to determine the growth rate of AMMDR1 in comparison to that of the indigenous aerobic bacteria in the pea spermosphere. Three replicate funnels, each containing 10 seeds, were harvested at each sampling period, and the wash suspension was dilution-plated on TBTR and TSAC. The experiment was conducted three times. Because no significant differences were found among experiments, the data from all three experiments were pooled for regression analysis. Doubling time (t_d) was calculated according to the formula: $(\log N_t - \log N_o)/0.301t = 1/t_d$, where N_o = original cfu, N_t = final cfu, t = time in hours.

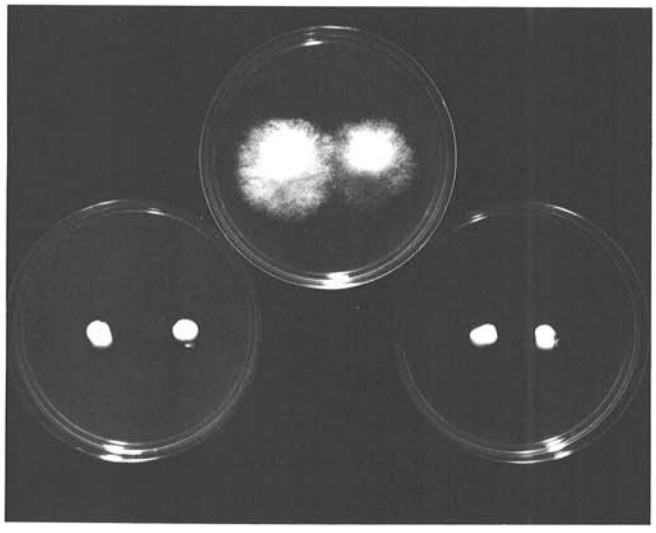

Fig. 1. Assay for pea seed infection by *Pythium* spp. Top: Nontreated seed infected with *P. ultimum*. Bottom left: Seed treated with *Pseudomonas cepacia* strain AMMDR1 at log 8.0 cfu/seed. Bottom right: Seed treated with metalaxyl.

RESULTS

Biocontrol of preemergence damping-off. Temperature did not have a significant effect on emergence ($P < 0.085$) except on day 3 ($P < 0.025$). The interaction between temperature and seed treatment was not significant for any day. Therefore, stand count data from all four temperatures were combined in comparing seed treatment effects. Seed treatment had a highly significant effect ($P < 0.005$) on pea seedling stand for days 4–7 (Fig. 2). Emergence of seeds treated with AMMD, AMMDR1, and metalaxyl were all significantly greater than emergence of the nontreated seeds on days 5–7 after planting, and no significant differences were found among these three treatments. Seedling stands among the fungicide or bacterial treatments at the end of the experiment averaged 47% greater than for the nontreated controls.

All of the fungi isolated from seeds and nonemerged seedlings were identified as species of *Pythium*. More than 90% of the isolates from each of the temperatures were *P. ultimum* Trow, and approximately 10% were *P. sylvaticum* Campbell and Hendrix.

Dynamics of *Pythium* seed infection in relation to seed treatments. In all three experiments, *Pythium* began to infect seeds within 12 hr after planting, and 100% of the nontreated seeds were infected by 30 hr (Fig. 3). Disease incidence was significantly reduced ($P < 0.028$) by seed treatment with both AMMDR1 and metalaxyl as compared to the nontreated control, but no significant difference was found between the effectiveness of the biocontrol agent and the fungicide. Seven days after planting, disease incidence was reduced by 44 or 60%, compared to the nontreated control, by seed treatment with AMMDR1 or metalaxyl, respectively.

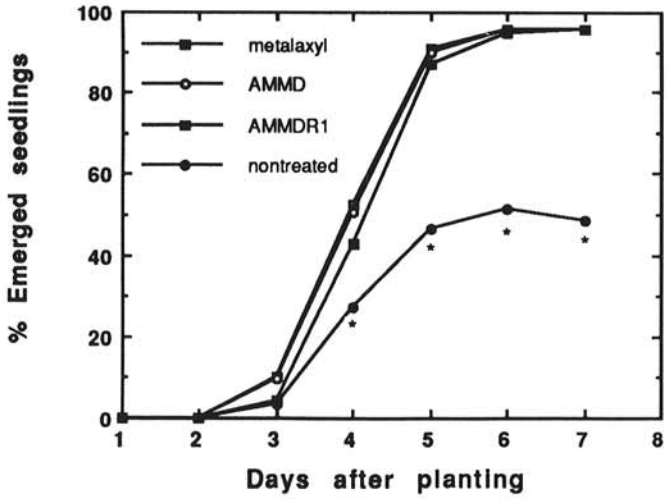

Fig. 2. Time course of pea seedling emergence in naturally infested soil. Seeds were treated with metalaxyl, *Pseudomonas cepacia* strain AMMD, or *P. cepacia* strain AMMDR1 (resistant to rifampicin) or were nontreated. For each day, significant differences among treatments are indicated by asterisks.

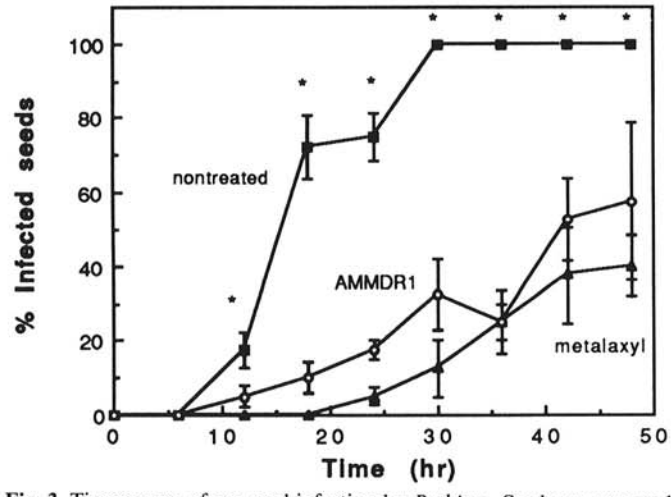

Fig. 3. Time course of pea seed infection by *Pythium*. Seeds were treated with metalaxyl or with *Pseudomonas cepacia* strain AMMDR1 or were nontreated. Values plotted are means ± the standard error. For each day, significant differences among treatments are indicated by asterisks.

When seeds were coated with the biocontrol agent at log 8.0 cfu/seed, the population of *P. cepacia* strain AMMDR1 increased gradually to log 8.7 cfu/seed during the 48-hr experiment (Fig. 4). Plate counts on TBTR and TSAC were not significantly different for this treatment, indicating that all of the bacteria recovered from seeds were *P. cepacia* strain AMMDR1. Populations of aerobic bacteria associated with nontreated seeds or seeds treated with metalaxyl increased rapidly from the first sampling period at 6 hr until the end of log phase at 18 hr. For this 12-hr period, the doubling time for bacteria associated with nontreated seeds was 2.4 hr, and for bacteria from the metalaxyl-treated seeds it was 3.1 hr. At the 6-hr sampling period, the population of bacteria associated with metalaxyl-treated seeds was consistently about 1 log unit greater than for nontreated seeds. Maximum populations of approximately log 7.5 cfu/seed were attained in both treatments at 36 hr. No colonies were recovered on TBTR from nontreated seeds or seeds treated with metalaxyl.

Relationship between population density of AMMDR1 and incidence of seed infection by *Pythium*. High initial population densities of bacteria were more effective in suppressing seed infection by *Pythium* than were lower inoculum densities (Table 1). The actual number of bacteria applied to seeds in each population density treatment differed among the three experiments. Regression analysis combining all three experiments showed that the relationship of initial population density to disease incidence was significant ($P < 0.001$) and that the slopes among the experiments were not significantly different ($P > 0.10$). However, there was a significant effect of differing intercepts among experiments ($P < 0.001$). The relationship between initial population density and disease incidence is described by the model $Y = 110.7 - 7.75X$ ($R^2 = 0.64$). Regression of disease incidence against inoculum density at 24 hr was also significant ($P = 0.012$) ($R^2 = 0.44$). When inoculum density at 24 hr was added to the model, the R^2 increased to 0.68, but this did not improve the model significantly ($P = 0.125$). The relatively low R^2 value indicates

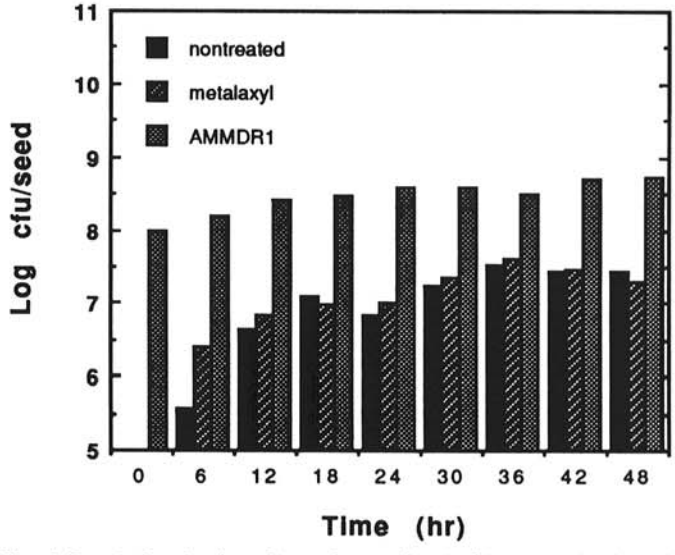

Fig. 4. Population density of bacteria associated with pea seeds planted in naturally infested soil. Seeds were treated with *Pseudomonas cepacia* strain AMMDR1 or with metalaxyl or were nontreated. All of the colony-forming units recovered from the AMMDR1 treatment were resistant to rifampicin.

that approximately 35% of the variation in disease incidence remained unaccounted for, suggesting that other factors, such as genetic variation or nicks in the seed coat (20,21), may have affected susceptibility of seeds to infection by *Pythium*.

For population densities between log 5.0 and log 8.0 cfu/seed, the effect of initial population density on the doubling time of *P. cepacia* strain AMMDR1 was significant ($P = 0.01$). Doubling time was less when the initial population density was low and greater when a higher population density was applied. The model for this relationship is $Y = -12.2 + 2.91X$ ($R^2 = 0.54$). Populations on seeds treated with log 8.0–8.3 cfu/seed increased very slowly to an average of log 8.4 cfu/seed (doubling time = 87 hr). The density of bacteria on seeds treated with less than log 8.5 declined to log 8.4 cfu/seed (data not shown). These data suggest that log 8.4 cfu/seed represents the "carrying capacity" of *P. cepacia* strain AMMDR1 in the pea spermosphere 24 hr after planting.

Doubling time of AMMDR1 and total aerobes in the pea spermosphere. The population of *P. cepacia* strain AMMDR1 increased from an original density of log 4.88 to log 7.17 cfu/seed at the end of log phase 24 hr later (Fig. 5). This represents a doubling time of 3.1 hr. The doubling time of the indigenous aerobic bacteria associated with the treated seeds for the same time period was 30.9 hr. Strain AMMDR1 made up 18% of the total population of bacteria associated with the seeds 4 hr after planting, but by 24 hr this proportion had increased to 40%.

DISCUSSION

The increase in pea seedling emergence by *P. cepacia* strain AMMD appears to result from biocontrol of seed infection by *P. ultimum* and *P. sylvaticum*. Although the soil was heavily infested also with *A. euteiches* f. sp. *pisi*, *Aphanomyces* was never isolated from seeds or seedlings sampled during the first 7 days after planting. This observation is consistent with previous reports on the epidemiology of Aphanomyces root rot on peas grown in the field (16).

One of the potential limitations to the use of biological agents for disease control is that they may not function over the same spectrum of environmental conditions as do chemical fungicides (6). In this study, *Pythium* preemergence damping-off occurred over a broad range of temperatures (16–28 C), and seed treatment with bacteria was as effective as metalaxyl at all temperatures. There was no difference in the effectiveness of the rifampicin-resistant strain AMMDR1 as compared to the wild-type strain. Soil temperatures in Wisconsin, however, are frequently lower than 16 C at the time peas are planted. The efficacy of these biological seed treatments at cooler temperatures has not been tested.

Doubling time differed according to the initial population density on the seed, as observed previously (14). Doubling time was greater when low population densities were applied to the seed, and application of bacteria in excess of the carrying capacity (log 8.4 cfu/seed) resulted in a population decline. Strain AMMDR1 has the potential for rapid growth in the pea spermosphere (doubling time = 3.1 hr), as do the indigenous bacteria when nontreated seeds are planted (doubling time = 2.4 hr). However, seed treatment with strain AMMDR1 drastically reduced both the population density and the growth rate of indigenous bacteria associated with the seeds.

The rapid infection of pea seeds by *Pythium* observed in this study, which began within 12 hr after planting, is similar to that in previous reports (10,21). *P. ultimum* sporangia germinate within 90 min in response to water-soluble nutrients (10,19) and volatiles (12) released from imbibing seeds. For effective protection against seed infection by *P. ultimum*, the biocontrol agent must be active within this period. In spite of the rapid doubling time of *P. cepacia* strain AMMDR1, protection against *Pythium* was reduced when the initial population density of bacteria was less than log 8.0 cfu/seed. Seed treatment with log 7.0 cfu resulted in a population of log 8.0 cfu/seed 24 hr later, but apparently this was too late to effectively control seed infection by *Pythium*. Initial application of a high population density of bacteria to seeds appears necessary to ensure that a "critical mass" of bacteria is present for protection within the first 12 hr.

The mechanism by which *P. cepacia* strain AMMD protects against seed infection by *Pythium* is not known. The bacterium inhibits mycelial growth of *Pythium* species and lyses zoospores of *Pythium aphanidermatum* (Edson) Fitzp. in vitro (J. L. Parke and E. B. King, *unpublished data*). Other strains of *P. cepacia* produce antibiotics implicated in biocontrol, including pyrollnitrin and altericidins (7–9). Production of antibiotics by *P. cepacia* strain AMMDR1 is currently under investigation. Another possible mechanism of biocontrol is competition for nutrients. The rapid growth of *P. cepacia* in the pea spermosphere may represent a significant nutrient sink for pea seed exudates, thereby reducing the stimulus required for sporangia germination by *P. ultimum*. Competition for rhizosphere nutrients was implicated in biocontrol of *Pythium aphanidermatum* by *P. cepacia* (2). An understanding of the dynamics of seed colonization by the biocontrol agent and the pathogen is important to success in biological control (14). In the work reported here, interactions between the introduced bacterium and the pathogen that were critical for biocontrol occurred within the first 24 hr after planting. A high initial population density of the biocontrol agent contributed greatly to protection from rapid infection by *Pythium*. The capacity for rapid growth and the potential for sustaining high populations in the spermosphere may also have contributed to successful competition of the introduced strain with the indigenous microflora.

TABLE 1. Population dynamics of *Pseudomonas cepacia* strain AMMDR1 on pea seeds and the incidence of seed infection by *Pythium* 24 hr after planting

Population density (log cfu/seed) at		Doubling time (hr)[a]	Disease incidence at 24 hr	AMMDR1 at 24 hr as percent of total aerobes
0 hr	24 hr			
8.42[b] (0.06)	8.45 (0.49)	240.8	40 (3.8)	95
6.96 (0.15)	8.02 (0.07)	6.8	63 (4.2)	68
6.07 (0.12)	7.42 (0.10)	5.3	63 (4.3)	60

[a] Based on the change in population density from 0 to 24 hr after planting.
[b] Values are means of nine replicates of 10 seeds each (± standard error) from pooled data from three experiments. Because the actual population density of bacteria differed among the three experiments, data were analyzed with regression analysis (see text).

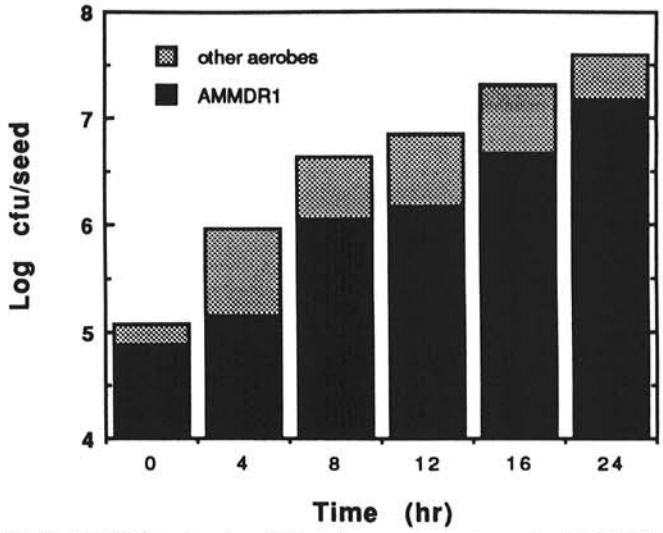

Fig. 5. Population density of *Pseudomonas cepacia* strain AMMDR1 and other aerobic bacteria recovered from pea seeds during the first 24 hr after planting in naturally infested soil. Seeds were treated with *P. cepacia* strain AMMDR1 at an inoculum density of log 4.88 cfu/seed.

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