Dynamics of Sugar Beet Seed Colonization by *Pythium ultimum* and *Pseudomonas* Species: Effects on Seed Rot and Damping-off

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Accepted for publication 10 February 1989 (submitted for electronic processing).

ABSTRACT

Osburn, R. M., Schroth, M. N., Hancock, J. G., and Hendson, M. 1989. Dynamics of sugar beet seed colonization by *Pythium ultimum* and *Pseudomonas* species: Effects on seed rot and damping-off. Phytopathology 79:709-716.

Pythium ultimum colonized the seed pericarps of sugar beet within 4 hr under favorable conditions in soil. Strain ML5 of *Pseudomonas fluorescens-putida* and strain R20 of *P. putida*, when inoculated onto seed, resulted in a markedly lower incidence of colonization by *P. ultimum*. The incidence of fungal colonization of seeds treated with ML5 or R20 was 6.7 and 36.7%, respectively, compared with 90% of untreated seeds 24 hr after planting. The tripartite interaction continued even when the fungus invaded the pericarp because the bacteria also colonized the tissues. Interaction between R20 and *P. ultimum* resulted in a reduction of active or viable fungal mycelium in the pericarp over time. ML5 inhibited both mycelial growth and sporangial germination, whereas R20 inhibited only mycelial growth. Only a small percentage of true seed became infected by *P. ultimum* regardless of the incidence of pericarp colonization. The amount of damping-off was related directly to the incidence of pericarp colonization ($r^2 = 0.98$). The effectiveness of R20 and ML5 in limiting pericarp colonization by *P. ultimum* was dosage dependent and independent, respectively. Both attained population densities in the endospermosphere similar to those in the ectospermosphere. Sugar beet seed supported relatively similar ectospermosphere and endospermosphere population densities of R20, ML5, or bacteria in general (10^6–10^7 colony-forming units/seed). Spermosphere population sizes of R20 and ML5 were similar regardless of the size of the initial inoculum dosage. The doubling time of ML5 in the spermosphere was 3 hr. Species of *Pseudomonas* comprised 12% of the detectable ectospermosphere bacteria and 71% of those that colonized the pericarp, showing the affinity of this group of organisms for the pericarp. Both ML5 and R20 were comparable to fungicides in suppressing damping-off by *P. ultimum* in greenhouse experiments.

Additional keywords: biological control, soilborne pathogens.

Seed rot and damping-off caused by *Pythium ultimum* Trow are important diseases of sugar beets (*Beta vulgaris* L.) in the western United States. Although fungicides are available that can protect seeds and seedlings against *Pythium* diseases, potential governmental regulatory activities are prompting the development of alternative control methods. Seed rot and damping-off by *P. ultimum* are ideal diseases to control with biocontrol agents because the susceptible period for the host is relatively short and high populations of antagonists are not required for extended periods of time (14). The spatial-temporal relationships among the host, pathogen, and biocontrol agent are critically important because *P. ultimum* responds to seed exudates by rapid germination and growth (9,16). Accordingly, the efficacy of antagonists will depend on a number of factors, such as the density and location of propagules of *Pythium* in the soil in relation to the seed, activity of the fungus, time necessary for colonization of the seed by antagonists after activation by seed exudate, and the mechanism by which they affect pathogenesis by *Pythium*.

Because of the structure of sugar beet seed, protection against *Pythium* by biocontrol agents involves different considerations than with seeds of most other crops. A monogerm sugar beet seed is termed a fruit: It consists of a true seed contained within a thick-walled pericarp. The pericarp is composed of dead perianth tissue with loosely aggregated corky cells on the outside grading into a dense, impervious inner layer of sclerenchyma cells (12). A thinner fruit cap of similar composition encloses the true seed within the pericarp. This is lifted off by the elongating radicle as the seed germinates. Traversing the pericarp is a basal pore, which is the route for water and oxygen uptake by the true seed, and, presumably, nutrient efflux from the seed. Because of this difference in structure, the spermosphere of sugar beet seed can be divided into the ectospermosphere, extending outward from the surface of the pericarp, and the endospermosphere, extending inward from the surface of the pericarp to the true seed. The endospermosphere provides an additional site for microbial colonization and for interaction between a biocontrol agent and a colonizing pathogen.

We investigated the time sequence and development of seed colonization by *P. ultimum* and subsequent seed rot and damping-off of sugar beets as affected by strains of *Pseudomonas fluorescens-putida* and *P. putida* with different antagonistic potentials.

MATERIALS AND METHODS

Seed and soil. The combined structure of the true seed and pericarp is referred to in this study as seed rather than fruit. True seed and pericarp will be referred to as such when appropriate. Sugar beet seed used was cultivar USH11, size 8-9, from Union Sugar Co., Santa Maria, CA. Seed was decorticated by a commercial mechanical process that removes most of the outer corky tissue from the pericarp.

Soils used in experiments were an Oceano loamy sand (pH 7.2) and Vernalis loam (pH 6.8) from Moss Landing, CA, and Patterson, CA, respectively. They were collected from the upper 15 cm of the soil profile and stored in polyethylene bags at 21 ± 2 C. For greenhouse experiments, soils were air dried, sieved through a 5-mm-mesh screen, and mixed in a portable cement mixer. The protocol was similar for growth chamber experiments, except that soil was sieved through a 1-mm-mesh screen. Soil inoculum densities of *P. ultimum* were determined by the soil drop assay method (15) and were expressed as germinable propagules per gram (p g^{-1}) of soil.

Bacterial strains and inoculum preparation. Strain ML5 of *P. fluorescens-putida* and strain R20 of *P. putida* were selected from the culture collection of M. N. Schroth on the basis of preliminary results showing in vitro antibiosis toward *P. ultimum* and effectiveness in control of seed colonization or damping-off by the pathogen in growth chamber or greenhouse studies. Strains were identified by the methods of Sands et al (13). ML5 had characteristics of both *P. fluorescens* and *P. putida* and, therefore,

is referred to as *P. fluorescens-putida* pending further study. Strain ML5 was isolated from the pericarp of sugar beet seed planted in Oceano field soil, and strain R20 was isolated from the rhizosphere of lima bean (*Phaseolus lunatus* L.) seedlings grown in Vernalis field soil. A spontaneous mutant of strain R20, selected on the basis of stable resistance to rifampicin (Sigma Chemical Company, St. Louis, MO) at 100 μg ml^{-1}, was used in these studies, whereas the ML5 strain was naturally resistant to streptomycin sulfate (Sigma Chemical Company) at 25 μg ml^{-1}.

Inocula of the two bacteria were prepared by growing them on King's medium B (KB) (5) at 28 C for 48 hr, scraping from the medium, then suspending in equal volumes of 0.1 M $MgSO_4$ and 1% methylcellulose (Mallinkrodt, Inc., St. Louis, MO) to produce a bacterial density of approximately 10^9 colony-forming units (cfu) ml^{-1}. Seeds were inoculated by placing them in a plastic bag and shaking them with just enough inoculum to wet them. Seeds then were either coated with talc and air dried at ambient temperature overnight or air dried without application of talc. The resultant population density of R20 or ML5 on seed was approximately 10^8 and 10^7 cfu per seed, respectively, unless indicated otherwise.

Dynamics of seed colonization, seed rot, and damping-off. The time sequence of pericarp and true seed coat colonization by *P. ultimum* was determined at intervals over the first 48 hr after planting. Seeds were planted 1-cm deep in 5.5-cm-diameter × 4.5-cm-deep brass rings containing Oceano soil naturally infested with 17 p g^{-1} of *P. ultimum*. Ten seeds were planted per ring, with three replicates per time interval. Rings were placed on a −50 kPa ceramic pressure plate, and soil matric potential was adjusted to −15 kPa by a pressure plate extraction system. Rings were transferred onto plastic petri dish lids and covered with polyethylene plastic held in place by a rubber band to maintain a constant soil moisture level. They were incubated in a growth chamber at a daytime temperature of 21 C and a nighttime temperature of 16 C, with a 12-hr diurnal fluorescent light cycle.

At each time interval, seeds were recovered by wet sieving, surface disinfested in 0.5% sodium hypochlorite for 1 min, and rinsed with tap water. The true seeds were dissected aseptically from the pericarps, and true seed and pericarp were plated on 2% water agar containing 15 μg ml^{-1} of benomyl (WAB). They were examined for incidence of colonization by *P. ultimum* after incubation at ambient temperatures for 18–24 hr.

The same experiment was repeated two more times but over a 6-day period, with the seed and seedlings plated on WAB medium; three replicates were assayed each day. The ungerminated seeds were recovered and surface disinfested. After true seeds were aseptically removed from the pericarp of ungerminated seeds, each was plated on WAB. Sites of seedling infection by *P. ultimum* were discerned by tracing origins of growth on the medium with a dissecting microscope. At the end of 6 days, all remaining ungerminated true seeds colonized by *P. ultimum* were considered to be rotted.

Greenhouse assay of biocontrol agents. Seed was treated with R20 or ML5 and planted in Oceano and Vernalis soils as previously described to determine the effectiveness of the bacteria in reducing the incidence of seed rot and damping-off. Disease control was compared with seed treatment with fenaminosulf or metalaxyl. Fenaminosulf and metalaxyl were applied to seed at a rate of 1.31 and 0.31 g a.i. kg^{-1} seed, respectively. Ten seeds were planted 1-cm deep in 10-cm-diameter ceramic pots containing either Oceano or Vernalis soil infested with 75–150 p g^{-1} of *P. ultimum*. There were five or six replicates per treatment in a completely randomized experimental design. Pots were incubated on a greenhouse bench at a daytime temperature of 20–27 C and nighttime minimum of about 16 C and were watered as needed to maintain a moist soil surface. Disease was assessed by recording the percent healthy seedlings periodically over 3 or 4 wk. Damped-off seedlings were collected and assayed on WAB to verify that *P. ultimum* was the cause of disease.

Population dynamics of biocontrol agents, *Pythium* seed colonization, and disease control. The effect of R20 or ML5 on the dynamics of pericarp colonization by *P. ultimum* over a 48-hr period after planting of seed was examined in growth chamber studies by comparing bacterially treated seed with untreated seed, seed treated with 0.1 M $MgSO_4$ and 1% methylcellulose only, and metalaxyl-treated seed. Seeds were planted in brass rings containing Oceano soil as previously described, with 10 seeds per ring and three replicates of each treatment per time interval in a completely randomized experimental design. Other details of the methods for the experiment were identical to those previously described for growth chamber experiments. Seeds were recovered at various intervals, surface disinfested, plated on WAB, then observed for incidence of pericarp colonization after 18–24 hr as previously described.

The effect of R20 or ML5 on incidence of pericarp colonization by *P. ultimum* and subsequent seed rot and damping-off was examined in growth chamber studies. Incidence of pericarp colonization was determined after 1, 3, and 5 days, whereas seed rot and damping-off were evaluated 5 days after planting. Other seed treatments and the experimental design and methods for the experiment were similar to those described above, with 10 seeds planted per brass ring and three replicates of each treatment per time interval. At 1 and 3 days after planting, the ungerminated seeds and pericarps from germinated seeds were recovered, surface disinfested, and plated on WAB to determine incidence of pericarp colonization. After 5 days, seeds, pericarps, and seedlings were recovered and surface disinfested. True seeds were removed aseptically from the pericarps of ungerminated seeds, and the pericarps and true seeds were plated on WAB. Intact seedlings also were plated on WAB to verify that *P. ultimum* was the cause of damping-off.

The population dynamics of R20 and ML5 on treated seeds were determined at 24-hr intervals over the 5-day period after planting in a separate experiment from that described above. Ten seeds were planted in Oceano soil in each brass ring as described above, with five replicates of each treatment per time interval in a completely randomized experimental design. Other details of the methods for the experiment were similar to those previously described. At each 24-hr interval, the ungerminated seeds or pericarps and fruit caps from germinated seeds were recovered and shaken to remove loosely adhering soil. The 10 seeds from each replicate were pooled, placed in 10 ml of 10 mM phosphate buffer, and vigorously agitated for 1 min, and dilution series were prepared.

The ectospermosphere population densities of R20 and ML5 were determined by plating 100-μl samples of each dilution on KB medium containing rifampicin (KBrif) at 100 μg ml^{-1} or streptomycin (KBstrep) at 25 μg ml^{-1}, respectively. Plates were incubated at 28 C for 24–48 hr, and the resultant bacterial counts were expressed as colony-forming units per seed. The endospermosphere population densities of R20 and ML5 from the same seeds were determined by rinsing the seeds under running tap water for 1 min, treating them in 0.5% sodium hypochlorite for 1 min, rinsing again with tap water for 30 sec, triturating in 10 mM phosphate buffer, and dilution plating on KBrif and KBstrep. Resultant bacterial counts were expressed as colony-forming units per seed.

Inocula density and doubling times. The importance of inoculum density of R20 and ML5 in limiting the incidence of pericarp colonization by *P. ultimum* was investigated. Strains R20 and ML5 were inoculated onto seed at densities ranging from 10^6–10^8 and 10^5–10^7 cfu/seed, respectively. Seed treatments, experimental design, and methods for the experiment were similar to those described previously, with 10 seeds planted per brass ring and three replicates of each treatment per time interval. Seeds were recovered at 24 and 48 hr, surface disinfested, and plated on WAB to assess incidence of pericarp colonization.

The effect of inoculum density on subsequent spermosphere population densities of R20 and ML5 was determined by inoculating seeds at concentrations ranging from 10^4 to 10^8 and 10^3 to 10^6 cfu/seed, respectively. Measurements were made at 24 and 48 hr after planting; experimental design and methods were similar to those described above, with 10 seeds planted per brass ring and three replicates of each treatment per time interval. At 24

and 48 hr, seeds from each replicate were recovered, pooled, and placed in 10 ml of 10 mM phosphate buffer. The total ectospermosphere and endospermosphere population densities of R20 and ML5 were determined by triturating the seeds, agitating for 1 min, then dilution plating on KBrif and KBstrep, respectively, as previously described. Resultant bacterial counts were expressed as colony-forming units per seed.

The doubling time of ML5 when applied to sugar beet seed at log 5.65 was determined according to previously described methods with six replicates of 10 seeds per time interval. Population densities were determined at 4-hr intervals from 0 to 16 hr and then at 24 hr. Seeds were agitated for 90 sec in phosphate buffer and plated.

Ectospermosphere and endospermosphere population densities of soil bacteria. Colonization of the ectospermospheres and endospermospheres of untreated seed by soil bacteria was assayed in growth chamber studies at 24 and 48 hr. Population densities were expressed as total detectable aerobic bacteria and fluorescent pseudomonads. Ten seeds were planted in each brass ring containing Oceano soil as described above, with five replicates at each time interval. Other details of the methods for the experiment were similar to those previously described. At 24 and 48 hr, seeds were recovered and shaken to remove loosely adhering soil. The 10 seeds from each replicate were pooled, placed in 10 ml of 10mM phosphate buffer, and vigorously agitated for 1 min, and then dilution series were prepared.

The population density of aerobic bacteria in the ectospermosphere was determined by plating 100-μl samples of each dilution with tenth-strength tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI). Plates were incubated at 28 C for 24–48 hr, and resultant bacterial counts were expressed as colony-forming units per seed. The population density of fluorescent pseudomonads in the ectospermosphere was determined by plating on KB medium, incubating at 28 C for 24–28 hr, then enumerating the fluorescent bacterial colonies under ultraviolet irradiation. Population densities of aerobic bacteria and fluorescent pseudomonads in the endospermosphere were determined from the same seeds by rinsing them under tap water 1 min, treating in sodium hypochlorite for 1 min, rinsing again with tap water for 30 sec, triturating in 10 mM phosphate buffer, and dilution plating on TSA and KB as described above.

In vitro antibiosis against *P. ultimum*. The inhibitory effect of R20 and ML5 on sporangial germination of *P. ultimum* was assayed on KB, TSA, potato-dextrose agar (PDA), Luria agar (LA), and nutrient agar (NA). Fifty-microliter samples of a 10^9 cfu ml^{-1} suspension of the bacteria were transferred to the center of each plate and incubated at ambient temperature for 48 hr. Inoculum of isolate 78-11 of *P. ultimum* was prepared by growing the fungus at ambient temperature on oatmeal agar-water slants (4) and harvesting the mycelium, sporangia, and oospores from the surface of the water after 10 days. Propagules were separated from the mycelium by homogenizing in a blender at low speed for 2 min, then filtering twice through cheesecloth. The sporangia-containing filtrate was sprayed on plates containing the bacteria, and plates were incubated overnight at ambient temperature. Plates were observed for sporangial germination and compared with control plates similarly sprayed with sporangial inocula but lacking bacteria.

Inhibition of mycelial growth of *P. ultimum* was assayed on KB and NA media by transferring the bacteria to the center of each plate as described above and incubating at ambient temperature for 48 hr. Mycelial plugs of *P. ultimum*, grown on 2% water agar for 24 hr, then were placed at opposite edges of each plate. Plates were incubated at ambient temperature and observed after 24, 48, and 72 hr for mycelial growth and compared with control plates containing the fungus but lacking bacteria.

The effect of iron on the inhibition of sporangial germination of *P. ultimum* and mycelial growth by R20 and ML5 also was determined in the assays described above by supplementing the media with $FeCl_3$ at 10^{-4} M.

Analysis of data. All data were subjected to analysis of variance and, when appropriate, means were separated with Duncan's multiple range test. Percent data were transformed to arcsins for statistical analyses, but actual percentages are given in tables. Regression analyses were performed when appropriate. In Table 1, regression analysis for colonization of pericarps was done with time sequences of 24–96 hr only because the seeds had germinated at later times, the cotyledons were above ground, and dryness inhibited further interaction among organisms.

RESULTS

Dynamics of seed colonization, seed rot, and damping-off. Colonization of the sugar beet seed pericarp and true seed coat by *P. ultimum* in the first test was detected by 4 and 12 hr, respectively, after planting in naturally infested field soil (Fig. 1). The incidence of pericarp colonization was 73% by 12 hr and 93% by 36 hr. The incidence of colonization of true seed coat was 30% by 24 hr and 57% by 48 hr (Fig. 1). A subsequent experiment revealed that, although *P. ultimum* rapidly colonized pericarps by 24 hr, colonization then began to die out as indicated

TABLE 1. Time sequence and incidence of seed colonization, seed rot, and damping-off of sugar beet caused by *Pythium ultimum*[x]

Time (days)	Seed germination[y] (%)	Incidence of colonization[y] Pericarp (%)	Incidence of colonization[y] True seed coat (%)	Seed rot and damping-off[z] (%)
1	0.0	95.0	22.2	0.0
2	0.0	89.6	46.7	0.0
3	66.7	73.3	···	6.7
4	86.7	73.3	···	20.0
5	86.7	73.3	···	46.7
6	90.0	70.0	···	76.6

[x]Seeds were planted in Oceano loamy sand naturally infested with 17 propagules of *P. ultimum* per gram of soil maintained at a matric potential of −15 kPa, day temperature of 21 C, and night temperature of 16 C.
[y]Values are the mean of three replicates, 10 seeds per replicate. The true seed was germinated by day 3.
[z]Seed rot and damping-off data through day 5 reflect only the percentage of infected seedlings regardless of colonization of the true seed coat by *P. ultimum*. Data for day 6 assume that all ungerminated true seeds colonized by *P. ultimum* are rotted.

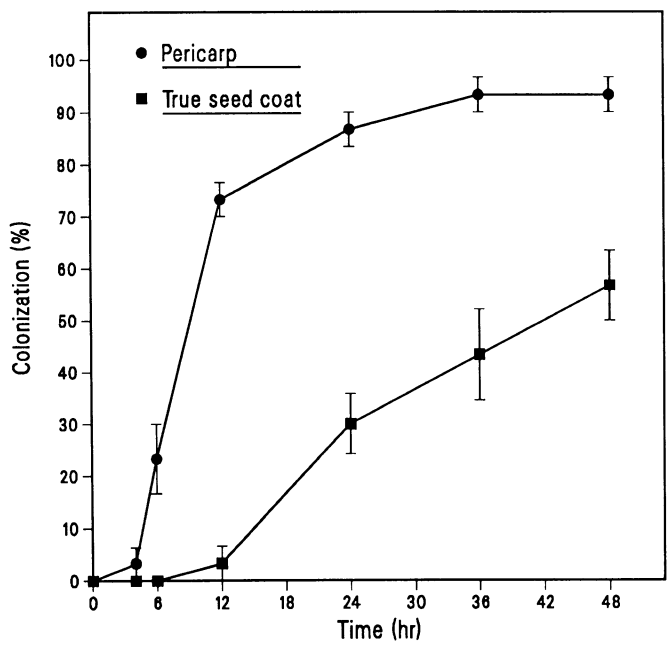

Fig. 1. Rate and incidence of colonization of the pericarp and true seed coat of sugar beet seed by *Pythium ultimum*. Vertical bars indicate standard error.

by a significant regression ($P < 0.02$) with time—95% at 24 hr to 73% at 96 hr ($Y = 87.3 - 7.57X$, $r^2 = 0.43$) (Table 1). Percentages of colonization and time sequences varied slightly in other experiments as influenced by factors such as temperature and moisture.

Despite the high percentage of pericarps and true seed coats that were colonized by *P. ultimum* by 48 hr after planting, 67% of the seed germinated by 3 days, and the incidence of damping-off was about 7% (Table 1). However, disease incidence increased progressively and was 77% after 6 days. Disease occurred as seed rot and as both preemergence and postemergence damping-off. The amount of seed rot and damping-off after 6 days was significantly correlated with the incidence of pericarp colonization after the same period of time ($r^2 = 0.99$, $P < 0.01$) (the value of r^2 was determined from the combined results of the two 6-day identical experiments). Preemergence damping-off resulted from infection of the radicle or cotyledons by *P. ultimum* at the sites of contact with the previously colonized pericarp. Postemergence damping-off also usually resulted from infection of the hypocotyl where it contacted the previously colonized pericarp. The pericarp usually remained contiguous with the hypocotyl of the developing seedling after seed germination. Both experiments described above were repeated at least twice with similar results.

Greenhouse assay of biocontrol agents. The incidence of damping-off by *P. ultimum* was approximately 50 and 56% less in response to R20 and ML5 seed treatments, respectively, compared with untreated seed when planted in either Oceano or Vernalis field soils (Table 2). Suppression of disease incidence by R20 and ML5 was comparable to metalaxyl- or fenaminosulf-treated seed. Disease suppression with R20 and ML5 seed treatments resulted primarily from a reduction of the incidence of seed disease and preemergence damping-off by *P. ultimum*, whereas disease suppression with fungicide-treated seed resulted from control of both preemergence and postemergence damping-off. Both bacterial strains were tested at least twice in each soil with similar results.

Population dynamics of biocontrol agents and *P. ultimum*, and disease suppression. Pericarp colonization by *P. ultimum* was delayed and limited by R20 and ML5 seed treatments compared with untreated seed (Fig. 2). Fungal colonization of seed treated with R20 and ML5 was not detected until 12 and 24 hr, respectively, whereas colonization of untreated seed was detected by 4 hr. *P. ultimum* colonized 90% of the untreated seed by 24 hr. The incidence of colonization of R20- and ML5-treated seed was significantly inhibited to 37 and 7%, respectively, after 24 hr, and the differences were maintained through 48 hr. The reduced incidence of colonization of ML5-treated seed was not significantly different from metalaxyl-treated seed, which were not colonized by *P. ultimum* through the 48-hr assay period.

TABLE 2. Effect of bacterial treatments on incidence of damping-off of sugar beet caused by *Pythium ultimum*

Soil[u]	Treatment	Healthy seedlings[v,w] (%)
Vernalis loam	Untreated	34 b
	Pelleted[x]	28 b
	Metalaxyl[y]	78 a
	R20[z]	66 a
	ML5[z]	74 a
Oceano loamy sand	Untreated	44 b
	Pelleted	40 b
	Fenaminosulf[x]	84 a
	R20	72 a
Oceano loamy sand	Untreated	34 b
	Pelleted	26 b
	Metalaxyl	82 a
	ML5	68 a

[u] Each soil was naturally infested with 75–150 propagules of *P. ultimum* per gram of soil.
[v] Values are the mean of five replicates, 10 seeds per replicate, 3 or 4 wk after planting.
[w] Means followed within soil groups by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[x] Pelleted seeds were treated with a mixture of 0.1 M $MgSO_4$ and 1% methylcellulose, then either coated with talc and air dried overnight or air dried without application of talc.
[y] Metalaxyl 25WP and fenaminosulf 35WP were applied at a rate of 0.31 and 1.31 g a.i. kg^{-1} seed, respectively.
[z] R20 and ML5 are strains of *Pseudomonas putida* and *P. fluorescens-putida*, respectively, and were inoculated on seed in a mixture of $MgSO_4$ and methylcellulose (described above) at a rate of 10^8 and 10^7 colony-forming units per seed, respectively.

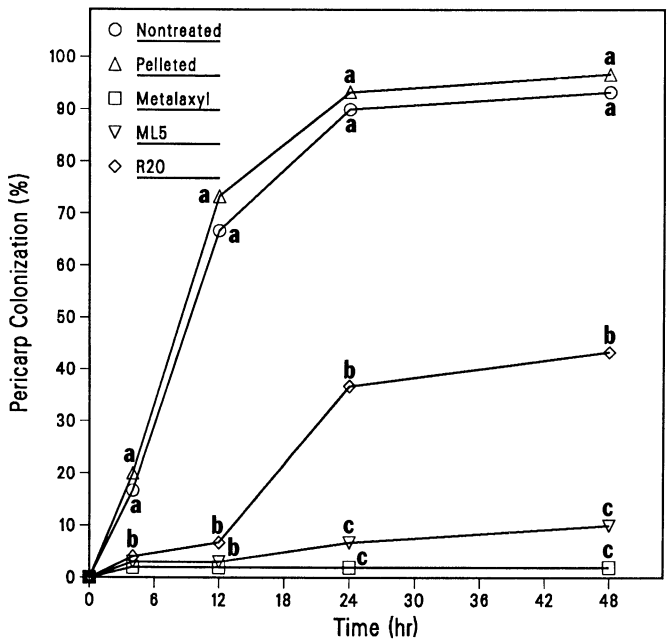

Fig. 2. Effect of bacterial biocontrol agents on colonization of sugar beet pericarp by *Pythium ultimum*. Pelleted seeds were treated with a mixture of 0.1 M $MgSO_4$ and 1% methylcellulose. ML5 and R20 are strains of *Pseudomonas fluorescens-putida* and *P. putida*, respectively, and were inoculated on seed in $MgSO_4$ and methylcellulose at a rate of 10^7 and 10^8 colony-forming units/seed, respectively. Means at a given sampling time with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 3. Effect of bacterial biocontrol agents on incidence of pericarp colonization, seed rot, and damping-off of sugar beet caused by *Pythium ultimum*[t]

Treatment	Pericarp colonization[u] Day 1	Day 3	Day 5	Seed rot and damping-off[u,v]
Untreated	100.0 a[w]	86.7 a	79.0 a	81.5 a
Pelleted[x]	96.7 a	86.7 a	76.7 a	75.0 a
Metalaxyl[y]	0.0 c	0.0 c	0.0 c	0.0 c
ML5[z]	0.0 c	3.3 c	10.0 bc	13.3 bc
R20[z]	50.0 b	36.7 b	23.3 b	20.0 b

[t] Seeds were planted in Oceano loamy sand naturally infested with 17 propagules of *P. ultimum* per gram of soil maintained at a matric potential of −15 kPa, day temperature of 21 C, and night temperature of 16 C.
[u] Values are the mean of three replicates, 10 seeds per replicate.
[v] Seed rot and damping -off data assessed at 5 days after planting.
[w] Means within columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[x] Pelleted seeds were treated with a mixture of 0.1 M $MgSO_4$ and 1% methylcellulose.
[y] Metalaxyl 25WP was applied at a rate of 0.31 g a.i. kg^{-1} seed.
[z] R20 and ML5 are strains of *Pseudomonas putida* and *P. fluorescens-putida*, respectively, and were inoculated on seed in a mixture of 0.1 M $MgSO_4$ and 1% methylcellulose at a rate of 10^8 and 10^7 colony-forming units per seed, respectively.

Experiments comparing the efficacy of strains ML5 and R20 with metalaxyl in limiting both pericarp colonization by *P. ultimum* and disease incidence yielded results complementary to those presented previously (Table 3). Both ML5 and R20 significantly suppressed disease compared with the control; ML5 was similar to the metalaxyl treatment in suppressing disease and in completely inhibiting pericarp colonization within the first 24 hr. Pericarps of controls were 95–100% colonized. The dying out of *P. ultimum* in the colonized pericarps after initial invasion again was detected with the two controls and R20 (untreated, $Y = 94 - 6.7X$, $r^2 = -0.78$, $P < 0.001$; pelleted control, $Y = 88.3 - 5.6X$, $r^2 = -0.70$, $P < 0.004$; R20, $Y = 49.15 - 4.05X$, $r^2 = -0.77$, $P < 0.002$).

Effect of inoculum density. The incidence of pericarp colonization by *P. ultimum* of ML5-treated seed 24 and 48 hr after treatment was significantly less compared with untreated seed at all three inoculum dosages tested (Table 4) and was not significantly different from metalaxyl-treated seed regardless of inoculum dosage. The incidence of colonization of R20-treated seed also was significantly less than that of untreated seed at all three inoculum dosages. However, in contrast to ML5, the effectiveness of the bacterium in inhibiting *P. ultimum* at 48 hr

TABLE 4. Effect of bacterial biocontrol agents on incidence of colonization of the sugar beet pericarp by *Pythium ultimum* when inoculated on seed at different inoculum densities[u]

Treatment	Inoculum density (log cfu/seed)	Pericarp colonization by *Pythium ultimum*[v] (%) 24 hr	48 hr
Untreated	···	76.7 a[w]	90.0 a
Pelleted[x]	···	80.0 a	83.3 a
Metalaxyl[y]	···	0.0 d	0.0 e
ML5[z]	7.29	6.7 d	13.3 de
ML5	6.35	13.3 cd	16.7 de
ML5	5.55	16.7 cd	13.3 de
R20[z]	8.21	26.7 c	23.3 cd
R20	7.46	46.7 b	36.7 bc
R20	6.32	53.3 b	50.0 b

[u]Seeds were planted in Oceano loamy sand naturally infested with 17 propagules of *P. ultimum* per gram of soil maintained at a matric potential of −15 kPa, day temperature of 21 C, and night temperature of 16 C.
[v]Values are the mean of three replicates, 10 seeds per replicate.
[w]Means within columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[x]Pelleted seeds were treated with a mixture of 0.1 M $MgSO_4$ and 1% methylcellulose.
[y]Metalaxyl 25WP was applied at a rate of 0.31 g a.i. kg^{-1} seed.
[z]R20 and ML5 are strains of *Pseudomonas putida* and *P. fluorescens-putida*, respectively, and were inoculated on seed in a mixture of 0.1 M $MgSO_4$ and 1% methylcellulose at the rates indicated.

TABLE 5. Spermosphere population densities of bacterial biocontrol agents on and in sugar beet seeds when inoculated on seed at different inoculum densities[x]

Treatment	Log cfu/seed[y] 0 hr	24 hr	47 hr
ML5[z]	6.69 ± 0.22	7.17 ± 0.03	7.21 ± 0.01
	5.97 ± 0.11	6.79 ± 0.03	7.11 ± 0.05
	4.88 ± 0.15	6.53 ± 0.11	6.71 ± 0.26
	4.25 ± 0.06	6.32 ± 0.07	6.31 ± 0.01
	3.82 ± 0.20	6.25 ± 0.04	6.19 ± 0.13
R20[z]	8.11 ± 0.08	7.32 ± 0.07	7.13 ± 0.03
	7.16 ± 0.01	7.20 ± 0.02	6.73 ± 0.07
	6.21 ± 0.05	6.85 ± 0.01	6.41 ± 0.09
	5.12 ± 0.01	6.21 ± 0.04	6.19 ± 0.02
	4.85 ± 0.08	6.14 ± 0.11	6.17 ± 0.06

[x]Seeds were planted in Oceano loamy sand maintained at a matric potential of −15 kPa, day temperature of 21 C, and night temperature of 16 C.
[y]Values are the mean of three replicates, 10 seeds per replicate, followed by the standard error. Total population densities in the ectospermosphere and endospermosphere were determined by recovering the seeds, shaking them to remove loosely adhering soil, triturating each 10-seed sample in 10 mM phosphate buffer, then dilution plating them on King's medium B.
[z]R20 and ML5 are strains of *Pseudomonas putida* and *P. fluorescens-putida*, respectively, and were inoculated on seed at the rates indicated at time 0.

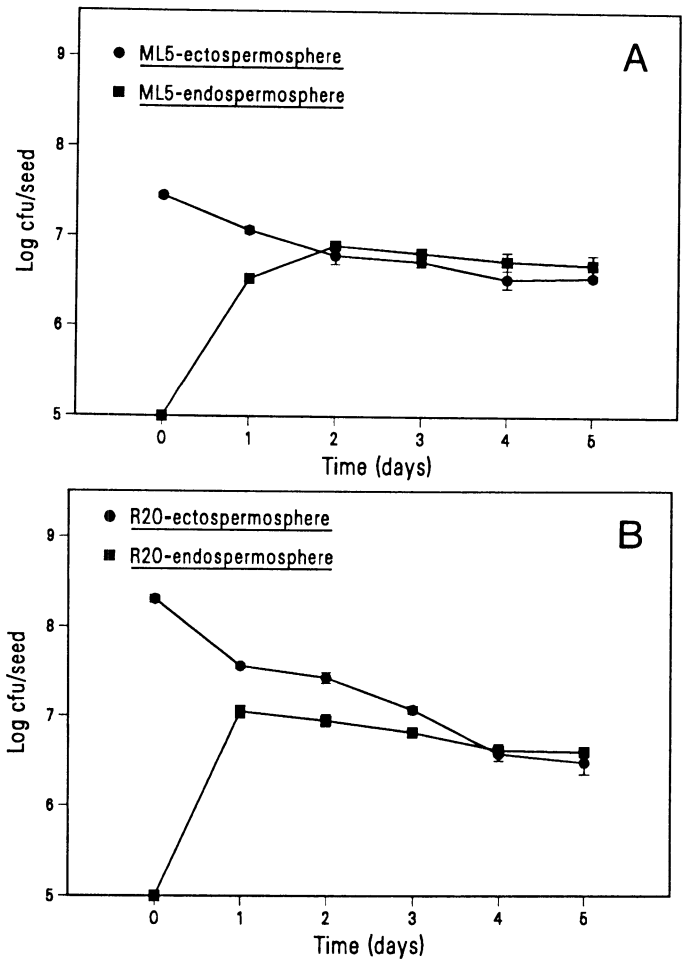

Fig. 3. Ectospermosphere and endospermosphere population densities of **A,** strain ML5 of *Pseudomonas fluorescens-putida*, and **B,** strain R20 of *P. putida* on and in sugar beet seed over a 5-day period after planting in Oceano loamy sand soil. Vertical bars indicate standard error. The bars do not appear at some time periods because of the small sizes of the errors.

TABLE 6. Ectospermosphere and endospermosphere colonization of sugar beet seed by soil bacteria[w]

Time (hr)	Ectospermosphere[x] Log cfu/seed[y]	Percent fluorescent pseudomonads[z]	Endospermosphere[x] Log cfu/seed	Percent fluorescent pseudomonads
24	6.14 ± 0.09	8.0 ± 1.2	4.78 ± 0.11	50.2 ± 6.7
48	6.29 ± 0.03	11.6 ± 1.9	5.77 ± 0.06	71.2 ± 3.0

[w]Seeds were planted in Oceano loamy sand maintained at a matric potential of −15 kPa, day temperature of 21 C, and night temperature of 16 C.
[x]Ectospermosphere and endospermosphere population densities expressed in terms of total detectable aerobic bacteria and fluorescent pseudomonads.
[y]Values are the mean of five replicates, 10 seeds per replicate, followed by the standard error.
[z]Values are the proportion of fluorescent pseudomonads to total aerobic bacteria (expressed as a percentage) followed by the standard error.

after planting was dosage dependent ($r^2 = -0.50$, $P = 0.02$).

Ectospermosphere and endospermosphere population densities of soil bacteria. The total detectable ectospermosphere and endospermosphere population densities of R20 and ML5, when inoculated onto seed at densities ranging from 10^3 to 10^6 cfu/seed, increased in a 48-hr time period to densities of 10^6 to 10^7 cfu/seed depending upon the initial inoculum concentration (Table 5). Magnitude of the increase was inversely proportional to the inoculum density on the seed. The doubling time of ML5 at log 5.65 per seed was 3.0 hr, determined at 4-hr intervals. Doubling times on a 24-hr basis in most cases ranged from 3 to 20 hr depending upon the concentration of bacteria applied to seed. When R20 was applied to seed at a higher dosage (10^8 cfu/seed), the population density declined approximately 10-fold to 10^7 cfu/seed over 48 hr.

The ectospermosphere population density of strain R20 on treated seed declined from an initial density of 2.1×10^8 to 3.7×10^7 cfu/seed over the first 24 hr after planting (Fig. 3). However, during the same 24-hr period, R20 colonized the pericarps internally to attain an endospermosphere population density of 1.2×10^7 cfu/seed. The population density in the ectospermosphere continued to decline with time and was 3.5×10^6 cfu/

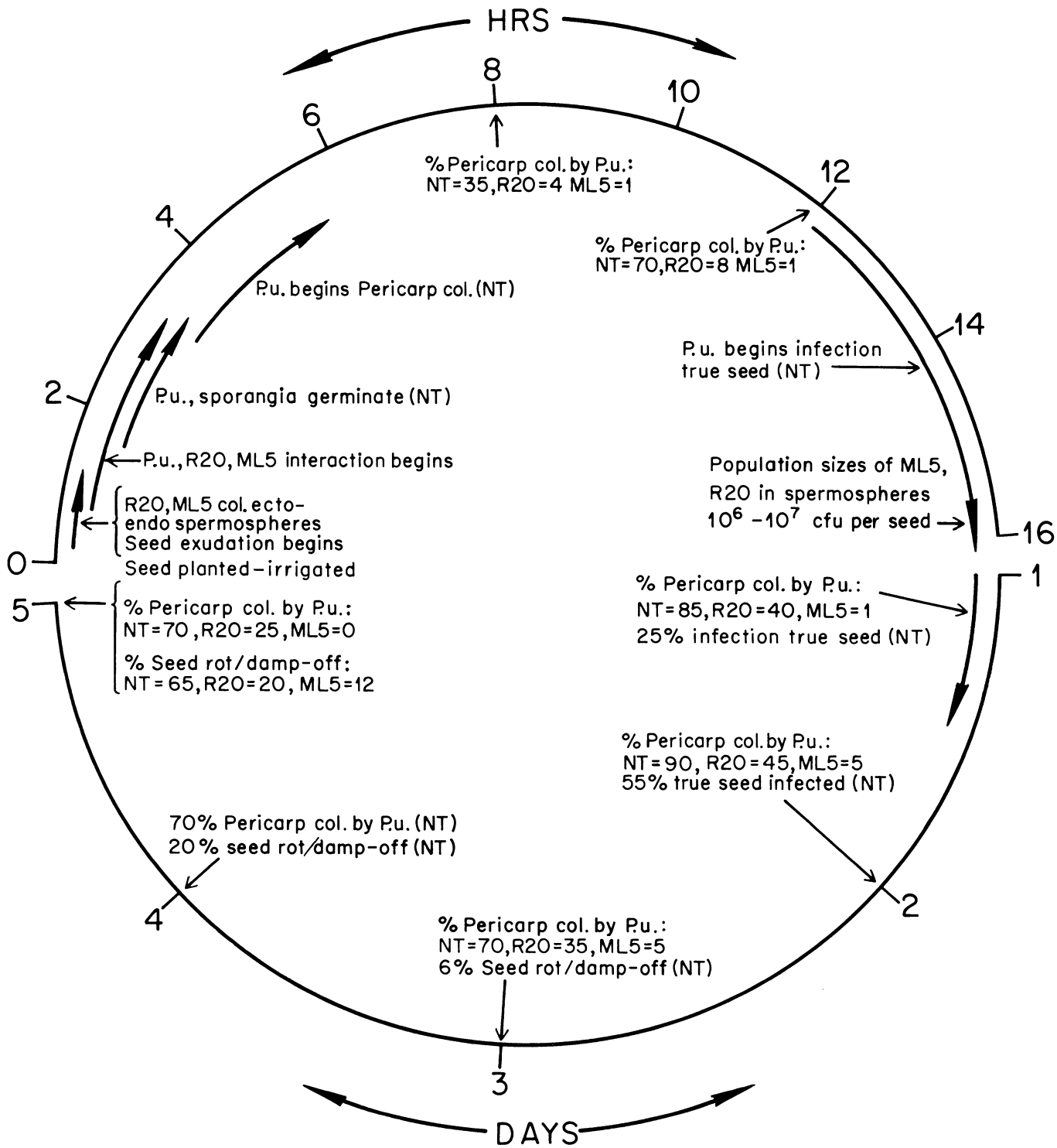

Fig. 4. Stylized scenario showing the dynamics and sequence of events in the tripartite interactions among the host, *Pythium ultimum*, and two bacterial biocontrol agents: strain ML5 of *Pseudomonas fluorescens-putida* and strain R20 of *P. putida*. P. u. = *P. ultimum*, and NT = untreated control seeds (all other seeds inoculated with ML5 or R20).

seed at 4 days. The population density in the endospermosphere also declined over the next 4 days. The population density of ML5 in the ectospermosphere declined from an initial density of 2.9×10^7 to 6.8×10^6 cfu/seed over the first 48 hr after planting, whereas the endospermosphere population density increased over 48 hr to 8.0×10^6 cfu/seed (Fig. 3). Over the next 3 days, the population densities of ML5 in the ectospermosphere and endospermosphere remained virtually unchanged.

The total bacterial population density detectable in the ectospermosphere on untreated seed was 1.5×10^6 cfu/seed at 24 hr and increased only slightly by 48 hr; fluorescent pseudomonads made up 8.0 and 11.6% of the total, respectively (Table 6). The bacterial population density in the endospermosphere at 24 hr was 6.8×10^4 cfu/seed and increased almost 10-fold by 48 hr to 6.2×10^5 cfu/seed; fluorescent pseudomonads made up 50.2% of the total population density at 24 hr and 71.2% by 48 hr.

In vitro antibiosis of biocontrol agents against *P. ultimum*. Both R20 and ML5 inhibited mycelial growth of *P. ultimum* when tested in vitro on KB and NA media. ML5 also inhibited sporangial germination of *P. ultimum* on KB, NA, PDA, and LA media. The only exception was on TSA media, where ML5 inhibited mycelial growth but had no effect on sporangial germination. However, unlike ML5, R20 had no apparent effect on sporangial germination on any media used. The effect of R20 and ML5 on mycelial growth or sporangial germination was unaffected by addition of Fe^{+3} to media.

DISCUSSION

Sugar beet seed is an ideal focal point for studying plant-microbe interactions in the soil environment and for developing a biological control model. Seed rot and damping-off caused by *P. ultimum* are a direct consequence of the capacity of the fungus to colonize seed pericarps as mediated by the antagonistic activity of associative microflora. The critical series of events leading to disease is over by 3–5 days. If the seed does not rot because of infection of the true seed, damping-off may result later from infection of the radicle or hypocotyl where they contacted the previously colonized pericarp. Thus, protection by biocontrol agents needs to span only a few days. Although the seed and the spermosphere represent a much simpler system compared with roots and the rhizosphere, the dynamics of the interactions are nevertheless complex and interdependent. Data on the dynamics of these events coupled with other related work on *Pythium* and the spermosphere (6,8,10) allow the development of a stylized scenario on the tripartite interactions among the host, fungus, and bacterial antagonists (Fig. 4).

The first critical event in the scenario is activation of the bacterial antagonists and propagules of *Pythium* (primarily sporangia) by organic exudates diffusing from the seed upon imbibition of water (Fig. 4) (11). Stanghellini and Hancock (16) found that sporangia of *P. ultimum* could germinate within 90 min, and that hyphal growth rates exceeded 300 μm/hr after being exposed to nutrients. Accordingly, our study revealed that *P. ultimum* under ideal conditions could enter the seed pericarp within 4 hr of planting. By 8–12 hr, there was substantial colonization of the pericarp. This is consistent with reports of rapid colonization of pea and cucumber by the fungus (6,10,17). Activation of the bacterial antagonists by seed exudate occurs almost immediately, as evidenced by a 3-hr doubling time with ML5. Moreover, *P. ultimum* and bacteria interacted within 4 hr of planting because colonization of treated seed by *P. ultimum* was inhibited during this time frame. The dynamics of the interaction between the bacterial antagonists and fungus were unexpected, considering the relatively slow doubling times for bacteria in soil and in plant material. Under such conditions, doubling times reportedly range from 4 to 6 hr (1–3). The doubling times of ML5 in the spermosphere of sugar beet seed were much faster, although there was considerable variation depending upon the initial population density applied to seeds. We conclude that sufficient metabolic activity occurred within 4 hr of planting and moistening to allow an interaction with *P. ultimum*.

The critical event in the scenario, following possible inhibition of sporangial germination or mycelial growth, occurs in the seed pericarp. Although *P. ultimum* may gain ingress into the seed, apparently it continues to be affected by the bacterial antagonists, including those that naturally colonize the pericarp. Both ML5 and R20 readily colonized the pericarp. The incidence of colonization of the pericarps by *P. ultimum* greatly declined in seeds inoculated with R20. Because ML5 was so effective in preventing the initiation of colonization of the pericarp, any subsequent decline in colonization was below the limits of detection. Pericarps apparently are favorable ecological niches for species of *Pseudomonas* as shown in experiments where they readily enter the pericarp from the ectospermosphere in disproportionate numbers.

The next event after pericarp colonization by *P. ultimum* is infection of true seed. However, a smaller proportion of the true seeds became infected regardless of the incidence of pericarp colonization. This suggests that the true seed coat is somewhat resistant to penetration by the fungus. The incidence of pericarp colonization, therefore, appears to be the key factor that determines the amount of seed rot and damping-off, the last events in the disease cycle. Microscopy of infected tissues revealed that infections leading to damping-off originated from sites where hypocotyls and cotyledons contacted the fungal-colonized pericarps.

The dynamics of bacterial colonization of plant tissues will vary among bacteria as influenced by various environmental factors (7). An understanding of the ecology of potential antagonists, therefore, is essential for developing strategies for biocontrol. This is reflected by the differing characteristics of R20 and ML5. For example, the inhibition by ML5 of seed colonization by *P. ultimum* was dosage independent, whereas a critical dosage of R20 was necessary for maximum effectiveness. This occurred even though both bacteria readily multiplied in the ectospermosphere and endospermosphere and no striking differences in population dynamics were observed. Notably, there appears to be an upper limit to the population density that a sugar beet seed will support. This ranged from 10^6 to 10^7 cfu/seed, depending upon the initial inoculum dosage. With dosages greater than 10^6–10^7 cfu/seed, population densities declined to this range. Even the population densities of "total" bacteria in the spermosphere fell within this range. That R20 and ML5 obtained population densities of 10^6–10^7 cfu/seed in association with soil bacteria indicates their competitiveness and the advantage of prior site occupation resulting from seed treatment. This competitiveness accounts in part for their effectiveness in reducing the incidence of seed rot and damping-off by *P. ultimum*.

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