

## Possible Role of Competition for Nutrients in Biocontrol of *Pythium* Damping-Off by Bacteria

Yigal Elad and Ilan Chet

Researcher and professor, Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot 76100, Israel. Present address of first author: Department of Plant Pathology, ARO, Volcani Center, Bet Dagan, 50250 Israel.

First author was supported by a grant from the Levy Eshkol Foundation of the National Council for Research and Development, Israeli Ministry of Science and Development.

We acknowledge the technical assistance of Naomi Teler.

This research was supported by a grant from the Agency for International Development (AID), USA.

Accepted for publication 30 May 1986.

### ABSTRACT

Elad, Y., and Chet, I. 1987. Possible role of competition for nutrients in biocontrol of *Pythium* damping-off by bacteria. *Phytopathology* 77:190-195.

Of the 130 bacteria isolated from the rhizospheres of plants infested with *Pythium* spp., six were found to be efficient biocontrol agents of this pathogen under greenhouse conditions. No lytic enzymes were involved in *in vitro* interactions between the bacteria and *P. aphanidermatum*. Substances inhibiting growth of the fungus were produced by both the biocontrol agents and other rhizobacteria. On the other hand, competition for nutrients between germinating oospores of *P. aphanidermatum* and bacteria, which was unique to the biocontrol agents, significantly correlated with suppression of disease in the greenhouse. Oospore germination in rhizospheres of wheat, tomato, cucumber, melon, bean, and cotton plants was decreased in the presence of these bacteria. Broadcast

application or seed coating with these bacteria reduced disease incidence in cucumbers planted in peat-vermiculite mixture or in soil by 60–75% compared with the untreated control. Damping-off caused by *P. ultimum* in cucumbers was reduced by up to 94%. Combining low levels of *Pseudomonas putida* or *P. cepacia* with the fungicide prothiocard resulted in an additive effect. Bacteria applied to cucumber seeds became established along the roots. Populations were 100-fold higher at root tips than at their upper parts. On the other hand, except for the root tips, the total population of bacteria was decreased in the presence of the beneficial ones. Disease control was achieved in bean, pepper, melon, tomato, and cotton plants as well.

Rhizobacteria, by their interactions with various pathogens, play a major role in the biological equilibrium among microorganisms in the rhizosphere (1,23). *Pythium* spp. have a broad host range and cause preemergence and postemergence damping-off and root rots followed by considerable yield losses in many important crops.

Biological control of this pathogen with bacteria is not well established. Mitchell and Hurlvitz (15) protected tomato seedlings against damping-off caused by *P. debaryanum* Hesse with a lytic strain of *Arthrobacter*, and Hadar et al (7) protected germinating cucumber seeds with *Enterobacter cloacae* (Jordan) Harm & Edwar from rots caused by *Pythium* spp. Howell and Stipanovic (9) isolated a strain of *Pseudomonas fluorescens* Migula that produces an antibiotic inhibiting growth of *Pythium ultimum* Trow on seeds.

Rhizobacteria have been the subject of several reports (11,12,16). It was suggested that the antibiotics or siderophores produced by these bacteria displace deleterious microorganisms, presumably minor pathogens (11,12). In such a case, competition with the native microflora should also be considered. The objectives of the present work were to isolate potential antagonistic rhizobacteria, apply them against *Pythium* damping-off, and investigate their antagonistic mechanism.

### MATERIALS AND METHODS

**Isolation and characterization of bacteria.** One hundred thirty bacteria were isolated from roots of bean, cotton, radish, cucumber, and melon plants grown in soils infested with *Pythium* spp. or in peat-vermiculite growth media (PVM). Isolations were made on nutrient agar (NA) (Becton-Dickinson Corp., Cockeysville, MO) or King's medium B (KB) (10). Bacteria were identified by the Analytical Profile Index (API) system (La Balme

les Grottes 38390, Montalieu, Vercieu, France). Identification was verified by *Bergey's Manual of Determinative Bacteriology*. The following strains were used: *Pseudomonas putida* (Trev.) Migula (805 and 310), *P. cepacia* Burk (808, 814, and Y11), *Alcaligenes* Cast. & Chal. sp. (Rh2), and *Pseudomonas* (Migula) Doud & Pall. sp. (AA4, SS3, and 806). Cultures were maintained on NA for daily use. Pure cultures were grown on NA for 24 hr, washed from the agar surface with 10% skimmed milk, and freeze-dried.

**Density of bacterial populations.** Strains of the biocontrol agents resistant to rifampicin [3-(4-methylpiperazinyl-minomerhyl) rifamycin 5v] and nalidixic acid (Sigma) were selected from parent cultures by planting cell suspensions on NA containing 150 µg/ml rifampicin [3-(4-methylpiperazinyl-iminomethyl) rifamycin] and nalidixic acid and incubating them for 48 hr at 30 C. The antibiotic-resistant strains were similar to their parent cultures.

**Antagonistic properties of selected bacteria.** The cell wall-degrading enzymes—Exo-1,3-β-D-glucosidase (β-1,3-glucanase, EC 3.2.1.58); Exo-1,4-β-D-glucosidase (Cellulase, EC 3.2.1.74); and β-N-acetyl-D-glucosaminidase (Chitinase, EC 3.2.1.30)—were assayed for filtrates of bacteria grown on either laminarin (Sigma), chitin (Sigma), or cell walls or mycelium of *Pythium aphanidermatum* (Edson) Fitzp. as a sole carbon source. All enzymatic tests and preparation of fungal cell walls or mycelium were carried out according to previous publications (4,5).

For production of inhibitory substances, bacteria were grown for 40 hr in a potato-dextrose and nutrient broth (each 15 g/L) medium (PDNB). Bacterial cells were separated from the medium by centrifugation at 3,000 g, then the supernatant was sieved through a 0.45-µm pore size filter (Millipore Corp., Bedford, MA). The cellfree medium, diluted with a fresh, double concentration of PDNB, was seeded with a mycelial disk taken from a 48-hr-old culture of *P. aphanidermatum*. After 48 hr of incubation at 28 C, the mycelium was dried for 4 days at 60 C and weighed. Presence of antibiotics was also tested in solid NA.

Mats of mycelium removed from cultures of *P. aphanidermatum* grown for 48 hr in petri plates containing liquid PDNB were transferred into a fresh medium to check their growth in the presence of bacteria. Dry weight gained by the fungus during the

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interaction period was measured 48 hr later and compared with the average dry weight of the fungus before incubation with the bacteria.

**Enrichment of soil with oospores.** One hundred milliliters of carrot medium, in Roux bottles (18), was seeded with mycelial disks taken from the margin of a 48-hr culture of *P. aphanidermatum* and incubated for 2 mo at 30 C. Mycelial mats containing oospores were placed on four layers of cheesecloth, washed with running distilled water for 5 min, suspended in tap water, and homogenized in a Waring Blendor for 5 min. Oospores were separated on a 40- $\mu$ m-mesh Nitex nylon screen (Tetko Inc., Elmsford, NY), resuspended in 100 ml of water, and added to 1 kg of air-dried, well-mixed loamy sand soil (pH 7.6) whose characteristics have been described previously (6). The soil was placed in plastic bags, incubated for 2 wk at 30 C, sieved through a 2-mm-mesh screen, and stored at 4 C until needed. This soil contained an average of  $4.5 \times 10^5$  colony-forming units (cfu) of *P. aphanidermatum* per gram, as determined on a selective medium according to Schmitthenner (22). Microscopic observations revealed that colonies originated predominantly from oospores.

**Oospore germination tests in soil.** Bacterial strains that were grown on NA plates for 24 hr were collected, washed twice, and resuspended in distilled water to the desired concentration. Oospore-enriched soil samples (5 g each) were placed in a 10-ml test tube. Aliquots (0.1 ml) of glucose and asparagine solutions (to induce germination [19]) and bacterial suspensions were added to give the desired concentrations at 15% dry weight (-0.3 bar) water content. Soil was mixed thoroughly and incubated at 30 C for 24 hr. Three 0.5-g subsamples of each treatment were transferred to test tubes for processing, staining with Calcofluor New M2R (American Cyanamide Company, Bound Brook NJ) (20), and viewing at  $\times 160$  under a UV light microscope for fluorescence. One hundred oospores were counted for each of the three replicates per treatment.

Oospore germination in the rhizosphere was tested using the method developed by Elad and Baker (3). Oospore-enriched soil was mixed with bacteria and placed between two glass slides along with roots of pregerminated plants. Test plants were cucumber (*Cucumis sativus* L. 'Shimshon'), tomato (*Lycopersicon esculentum* L. 'Marmand Rehovot'), pepper (*Capsicum annuum* L. 'Maor'), melon (*Cucumis melo* L. 'Galia'), bean (*Phaseolus vulgaris* L. 'Brittlewax'), wheat (*Triticum aestivum* L. 'Scout'), and cotton (*Gossypium herbaceum* L. 'Pima U. SJ2'). Root exudates induced germination of oospores. Rhizosphere soil was separated from the roots after 48 hr of incubation, and oospore germination was assessed according to the procedure mentioned earlier.

**Infestation of greenhouse growth media.** *P. aphanidermatum* or *P. ultimum* were isolated from diseased cucumber seedlings on a selective medium for *Pythium* spp. (22) and identified according to Middleton (14) and Waterhouse (25). Growth medium contained 75% Canadian peat and 25% vermiculite no. 2. Oospores of *P. aphanidermatum* were added to PVM, after adjusting their concentration with a hemacytometer, by diluting the suspension with water to the desired level. The pH of PVM was 6.0.

In most experiments with both *Pythium* spp., infested peat was used as inoculum. Three or four repeated plantings with cucumbers, when 90–100% of the plants had died from damping-off, were used to increase inoculum. Inoculum was suspended in 500 ml of water to allow complete mix with the growth medium. The population of *Pythium*, as counted on Schmitthenner's selective medium (22), ranged from  $5 \times 10^3$  to  $5 \times 10^4$  cfu/g of dried infested medium.

Bacteria were applied either as water suspensions mixed with the total growth medium or by dipping seeds into a suspension of the candidate strain, drying at room temperature, and planting in the greenhouse.

Polypropylene boxes (7  $\times$  19  $\times$  14 cm) were filled with the infested media, planted with 10 seeds each, irrigated once a day, and the number of diseased seedlings recorded. All experiments were conducted under greenhouse conditions at 28–32 C and consisted of six replicates. Experiments were repeated at least twice. Severity of disease symptoms in cotton was calculated

according to an index ranging from 0 for healthy, symptomless plants to 4 for plants covered with *Pythium* lesions over more than 80% of their hypocotyl. Preemergence damping-off, which occurred in each treatment, was calculated by deducting the rate of emerged plants in the pots of this treatment from the average emergence in the medium free of *Pythium*.

The fungicide prothiocarb (Previcur 50%) was mixed with PVM alone or combined with bacterial suspensions to control disease caused by *P. aphanidermatum* in cucumbers.

Population dynamics along cucumber roots were tested by the rhizosphere competence technique of Scher et al (21) as modified by Ahmed and Baker (*personal communication*). Two longitudinal halves of a 50-ml conical polypropylene tube (Falcon Div., Becton-Dickinson, Oxnard, CA) were filled with wet sandy loam soil (15%, -0.3 bar), and one seed coated with bacteria was placed between both parts, 1 cm from the top. The two parts were sealed with two rubber bands and incubated in a polyethylene plastic bag for 5 days at 30 C under artificial light without additional watering. At the end of the incubation period, the tube halves were separated and the plant gently removed to assess the rhizosphere population as mentioned earlier.

Counts of bacteria from soil or peat were carried out by plating the appropriate dilutions, using the drop plate method (17), on NA or KB medium. Results were expressed as colony-forming units per gram. Rhizosphere populations were assessed by separating the soil adhering to the specific root segments (20 mm each), in water, by shaking them in a rotary shaker for 30 min at 100 rpm. Average dry weight of rhizosphere soil was calculated by measuring the weight of 20 samples of root segment that could be separated according to the distance from the base of the stem.

## RESULTS

**Isolation of potentially antagonistic bacteria.** Bacteria were isolated from roots of bean, cotton, radish, cucumber, and melon plants. Selected plants were uprooted from soils naturally infested with *P. aphanidermatum*. Of the 130 bacterial isolates tested for their ability to suppress damping-off in cucumbers, the six that were superior in reducing disease incidence by 17–67% (Fig. 1) were selected for further experiments. These strains are *Pseudomonas putida* (805 and 310), *P. cepacia* (808, 814, and Y11), and *Alcaligenes* sp. (Rh2).

**Antagonistic activity of bacteria in culture.** *Lytic enzymes.* None of the strains produced  $\beta$ -1,3-glucanase, chitinase, or cellulase when grown on laminarin, chitin, cellulose, or cell walls of *P. aphanidermatum*. Similarly, when placed in a liquid medium containing a mycelial mat of *P. aphanidermatum* as a sole carbon source, none of the bacteria degraded the mycelium.

*Inhibitory substances.* Bacteria were separated from the PDNB medium in which they were grown after 40 hr. The cellfree media, mixed with a double concentration of PDNB, were seeded with *P. aphanidermatum* and incubated for 48 hr for dry weight determination. Dry weight of mycelium grown in unamended control medium was 84.4 mg per plate. Bacterial isolates 805, 808, Rh2, and 310 inhibited growth of *P. aphanidermatum* by 87.9, 85.4, 49.8, and 39.0%, respectively, whereas isolates AA4, 806, and SS3, which did not control *Pythium*, reduced it by 76.5, 89.6, and 70.4%, respectively, compared with the control (dry weight 84.4 mg per plate) grown in unamended medium. Growth of *P. aphanidermatum* was inhibited by only 5.7% when grown in a medium that previously supported growth of the same fungus. No significant inhibition of growth of mycelium was observed on solid medium seeded with the test bacteria.

*Competition.* Competitive ability of both the bacteria and mycelium of *Pythium* was tested in dual cultures. *P. aphanidermatum* was grown in liquid PDNB for 48 hr and transferred into fresh medium along with different bacterial isolates. Strains 805, 808, Y11, Rh2, 310, and 814 inhibited fungal growth by 50, 45, 60, 98, 14, and 62%, respectively, compared with the control culture (dry weight 63.5 mg per plate) grown in bacteria-free medium. However, other bacteria, when grown together in liquid culture, also inhibited growth of *P.*

*aphanidermatum*.

**Inhibition of oospore germination by bacteria.** Bacteria were introduced into raw soil enriched with oospores of *P. aphanidermatum*, and glucose and asparagine were added. Oospore germination, determined after 24 hr of incubation, was inhibited by up to 57% by the biocontrol agents ( $2 \times 10^8$  cfu/g of soil) compared with only 13–20% inhibition by bacteria not effective in disease control under greenhouse conditions (Fig. 2). Similarly, when the initial population level of the bacteria was reduced to  $2 \times 10^7$  cfu/g of soil, germinability of oospores was inhibited by 20–50%.

A significant correlation at  $P = 0.05$  or  $0.01$  was found between the ability of the various bacteria (applied by broadcast or seed coating) to reduce *Pythium* damping-off in cucumbers grown under greenhouse conditions and their ability to inhibit oospore germination in soil enriched with 150 or 250  $\mu\text{g/g}$  glucose or PVM.

TABLE 1. Influence of bacteria of germination of oospores of *Pythium aphanidermatum* rhizospheres of various crops<sup>y</sup>

Bacterial isolate	Oospore germination (%)						
	Wheat	Tomato	Melon	Cucumber	Bean	Cotton	Av.
805	7 a <sup>z</sup>	10 a	8 a	18 a	21 a	15 a	13
808	31 b	11 a	12 a	19 a	19 a	12 a	17
AA4	41 c	35 b	41 b	46 b	57 b	27 b	41
Untreated control	64 d	37 b	46 b	57 b	65 b	36 b	51

<sup>y</sup>Seeds of various crops were germinated in moist bags and placed on soil enriched with oospores, between two glass slides. Germination was calculated, after 3 days of incubation, by the Calcofluor staining method (20).

<sup>z</sup>Numbers in each column followed by the same letters are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

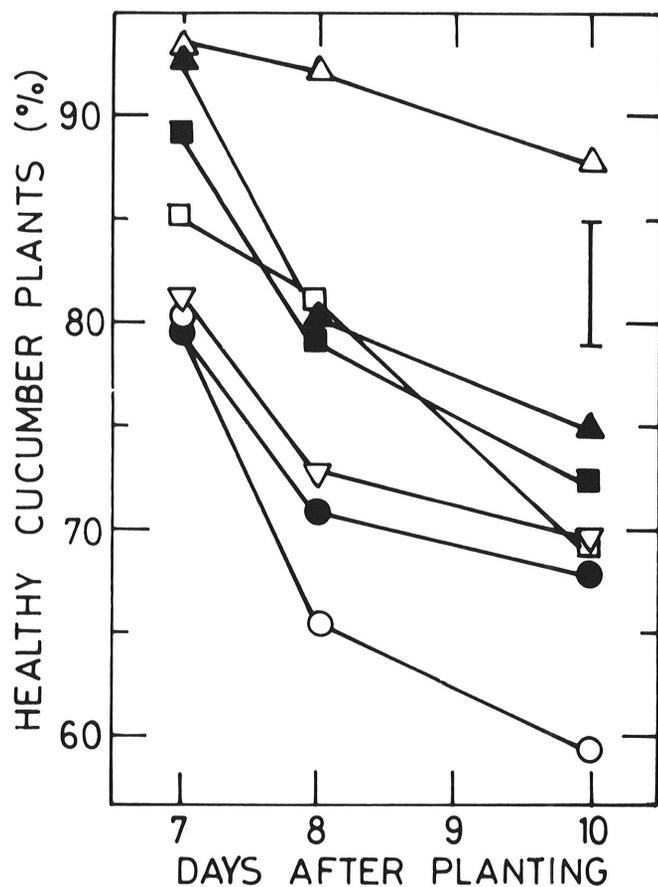


Fig. 1. Influence of bacterial suspensions on the percentage of healthy cucumber plants planted in peat-vermiculite medium infested with *Pythium aphanidermatum*. Treatments were control (o) and bacterial isolates 805 (▲), 808 (△), 310 (▽), Rh2 (●), Y11 (■), and 814 (□). Bar =  $\text{LSD}_{0.05}$ .

To determine whether the bacteria produce inhibitory substances affecting oospore germination, cellfree supernatants of 24-hr-old cultures of the different bacteria were added to the oospore-enriched soil. No significant reduction in oospore germinability was observed unless bacterial cells were present in the soil.

Germination of oospores in rhizospheres of wheat, tomato, melon, cucumber, bean, or cotton in the presence of bacteria was compared with their germination in the untreated control (Table 1). Average inhibitions of germination in all plant crops by isolates 805 and 808 were 72.3 and 66.4%, whereas isolate AA4, which is not capable of controlling *Pythium* in the greenhouse, inhibited germination by only 5–36%.

**Biological control of damping-off in cucumbers.** *Effects of bacterial concentrations.* Bacteria were suspended in water and mixed with PVM to give two initial population levels of  $10^7$  and  $10^8$  cfu/cm<sup>3</sup>. Isolates 805 and 808 were the most effective in control of *Pythium* when applied in concentrations of  $10^8$  cfu/cm<sup>3</sup> (Table 2). Relatively high percentages of healthy cucumber plants (up to 62% reduction of disease) were obtained when bacteria were applied in concentrations of  $10^7$  cfu/cm<sup>3</sup>. Cells of bacterial isolates 805 and 808 were suspended in three low concentrations to give final counts of  $4 \times 10^7$ ,  $8 \times 10^6$ , and  $2 \times 10^6$  cfu/cm<sup>3</sup> of PVM. The concentration of cells of isolate 808 influenced the efficacy of disease control, whereas differences between concentrations of applied isolate 805 were not reflected in marked differences in disease control (Fig. 3). Preemergence damping-off in untreated control was 71.6%, whereas it was reduced to 21.1–26.3 and 2.1–10.5% by treatments with isolates 808 and 805, respectively.

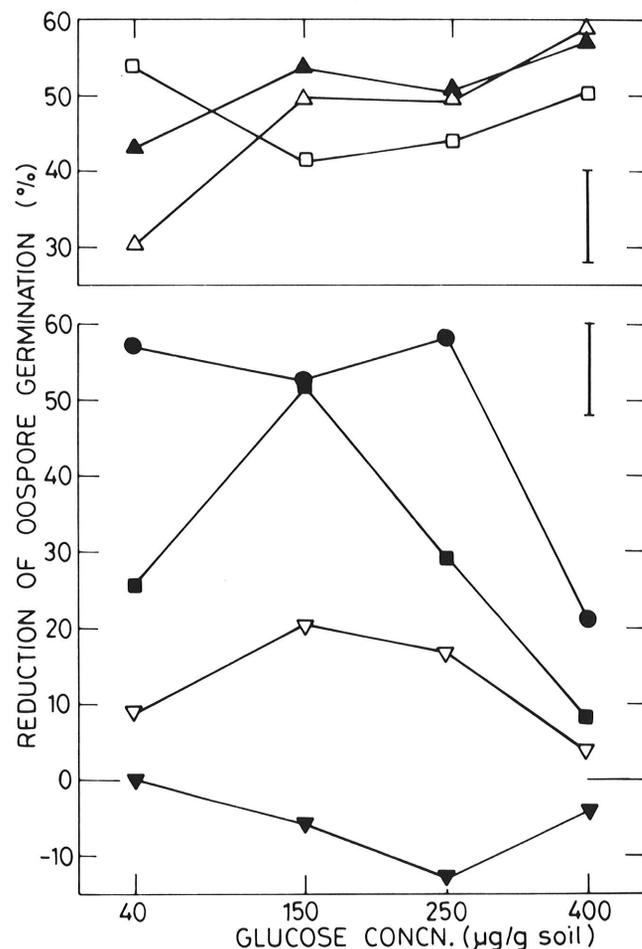


Fig. 2. Effects of suspensions of bacterial biocontrol agents 805 (▲), 808 (△), 814 (□), Rh2 (●), and Y11 (■) and other isolates, AA4 (▽) and SS3 (▼) on the percentage of reduction of oospore germination in soil amended with glucose and asparagine (5:1). Bar =  $\text{LSD}_{0.05}$ . Reduction was calculated according to the following formula:  $[1 - (A/B)] \times 100$ , where  $B$  = percentage of germination in the control without bacteria and  $A$  = percentage of germination in bacterial treatments.

**Effects of bacterial seed coating and integrated control.** Cucumber seeds were dipped in bacterial suspensions ( $8 \times 10^9$  cfu/ml) and planted in PVM. Damping-off caused by *P. aphanidermatum* was recorded during 12 days after sowing. The most efficient isolates were 805, 808, and Rh2, which reduced disease incidence by 57–67%, compared with isolates 814 and 310, which reduced disease incidence by 38–42% (Fig. 4). Preemergence damping-off, 27.5% in the untreated control, was reduced to 0–12.5% by the different bacterial isolates.

Bacterial isolates, at the low rate of  $3 \times 10^6$  cfu/cm<sup>3</sup> of PVM, were combined with prothiocarb at the rate of 0.25 ml/L of PVM. Incidence of damping-off caused by *P. aphanidermatum* in the untreated control was 89%. Isolates 805 and 808, alone, decreased *Pythium* damping-off by only 9–15%, because the bacterial level was low. A significant decrease in disease incidence, 34%, was obtained by the chemical treatment. Combination of prothiocarb with strain 805 resulted in 46% disease reduction; however, combination of prothiocarb with strain 808 was significantly superior, yielding an 80% reduction in disease incidence.

**Effects of bacteria on disease buildup during successive replanting.** Cucumber seeds were planted five times at 10-day intervals in PVM mixed with suspensions of isolates 805 or 808, and the percentages of healthy plants were recorded at the end of each growth cycle. In the untreated control, disease incidence of *Pythium*, which was 93% at the end of the first cycle, stabilized at 50% between the third and fifth growth cycles. Disease incidence in bacteria-treated media was 12–45% during the five successive plantings and was 12 and 27% at the end of the fifth cycle in treatments of strains 808 and 805, respectively.

**Control of damping-off caused by *P. ultimum* in cucumbers.** Control of *P. ultimum* in cucumbers was tested in PVM amended with each of the bacterial isolates. Disease incidence was drastically reduced by all isolates in the first growth cycle (Fig. 5).

TABLE 2. Influence of bacterial concentration<sup>1</sup> in peat-vermiculite medium on ability of strains to control *Pythium aphanidermatum* in cucumber

Bacterial isolate	Emergence (%) 7 days after planting		Healthy plants (%) 11 days after planting	
	$10^7$ cfu/cm <sup>3</sup>	$10^8$ cfu/cm <sup>3</sup>	$10^7$ cfu/cm <sup>3</sup>	$10^8$ cfu/cm <sup>3</sup>
Rh2	18 a <sup>2</sup>	42 b	12 a	27 b
805	60 bc	83 d	48 c	70 d
Y11	37 ab	48 b	18 c	37 bc
808	77 cd	72 c	60 d	68 d
814	37 ab	51 b	10 a	32 b
310	42 b	53 b	19 ab	41 bc

<sup>1</sup>Suspensions of 24-hr-old bacteria were mixed with artificially infested peat-vermiculite container medium to give  $10^7$  or  $10^8$  cfu/cm<sup>3</sup>.

<sup>2</sup>Numbers in each parameter followed by the same letters are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

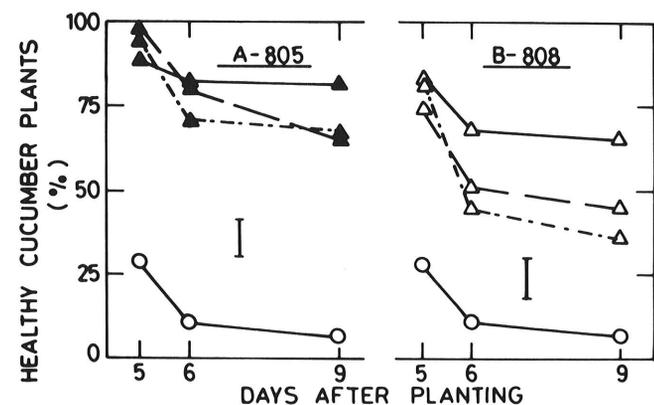


Fig. 3. Effects of isolates A, 805 and B, 808 applied at concentrations of  $2 \times 10^6$  (---),  $8 \times 10^6$  (—), and  $4 \times 10^7$  (—) cfu/cm<sup>3</sup> (o = untreated control) on percentage of healthy cucumber plants planted in peat-vermiculite infested with *Pythium aphanidermatum*. Bars =  $LSD_{0.05}$ .

However, after 2 wk of the second growth cycle, there were 7, 18, 51, 42, 4, 71, and 17% healthy plants in the untreated control and treatments with isolates 808, 805, 310, 814, Rh2, and Y11, respectively.

**Biological control of *Pythium* damping-off in pepper, melon, bean, tomato, and cotton.** Isolates 805 and 808 were mixed with PVM, and their influence on disease caused by *Pythium* in several crops was examined in the greenhouse. Both isolates reduced disease incidence in melons, tomatoes, peppers, and beans (Fig. 6).

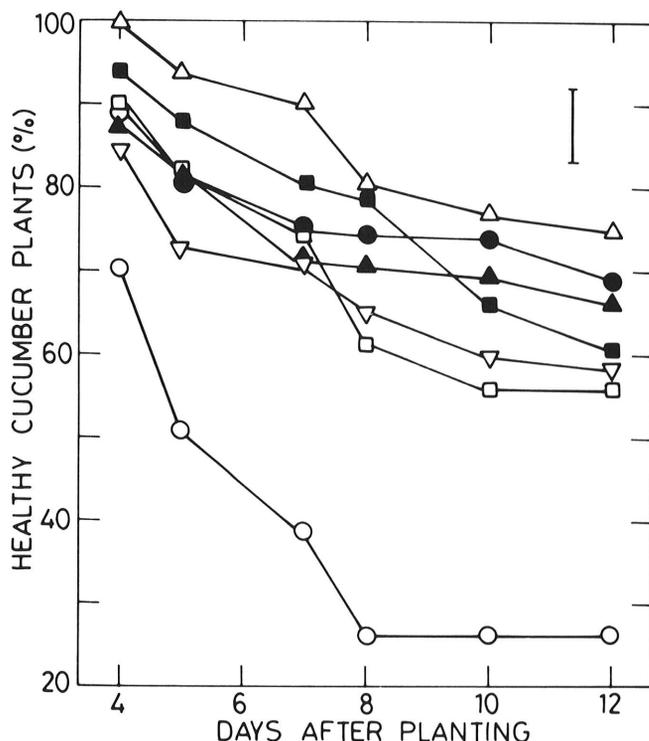


Fig. 4. Influence of seed coating with bacterial isolates 805 (▲), 808 (△), Rh2 (●), Y11 (■), 310 (▽), and 814 (□) (o = untreated control) on the percentage of healthy cucumber seedlings planted in peat-vermiculite medium infested with *Pythium aphanidermatum*. Bars =  $LSD_{0.05}$ .

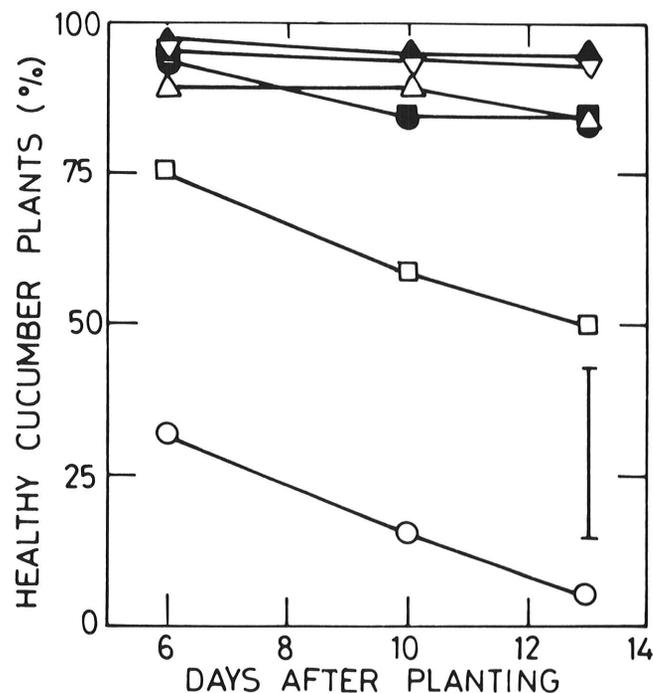


Fig. 5. Stand of cucumber plants in peat-vermiculite medium infested with *Pythium ultimum* and treated with isolates 805 (▲), 808 (△), Rh2 (●), Y11 (■), 310 (▽), 814 (□), or untreated (o).

Disease indexes in cotton, calculated according to root coverage by *Pythium* lesions, were 2.17, 0.83, and 1.0, respectively, in the untreated control and in treatments with isolates 805 and 808. The respective percentages of disease incidence were 92, 50, and 62. Disease incidence was decreased by 19–53% when pepper, bean, and tomato plants were replanted in the original growth mixture.

**Competitive root colonization by antibiotic-resistant biocontrol agents: general populations of bacteria and *Pythium*.** Isolates 805 and 808 were selected for resistance to rifampicin and nalidixic acid. Both decreased disease incidence caused by *Pythium* in cucumbers by 50% when applied to soil. Efficient recovery of the resistant bacteria was obtained on medium supplemented with both antibiotics at a rate of 150 µg/ml.

Cucumber seeds were coated with suspensions of isolates 805, 808, 814, Y11, or Rh2. Two longitudinal halves of plastic tubes were filled with soil infested with *Pythium* and sealed together with one coated seed in each tube. The roots, exposed after 5 days, were cut into segments 0–1, 2–3, 4–5, and 6–7 cm from the stem base. General populations along the untreated roots ranged between  $2.0 \times 10^6$  and  $2.2 \times 10^5$  cfu/g of soil in the segments 0–1 and 6–7 cm (Fig. 7). Application of isolates 814, Y11, and Rh2 to the seeds decreased the general bacterial populations to  $4.0 \times 10^3$ – $2.3 \times 10^5$  cfu/g of soil in segments 0–1 and 4–5 cm, whereas isolates 805 and 808 decreased bacterial numbers to  $10^2$  cfu/g in segments 2–3 and 4–5 cm. However, the general populations at the tips of treated roots did not differ from those of the untreated ones.

The specific populations of bacteria applied to seeds before incubation were  $2.2 \times 10^1$ – $2.0 \times 10^6$  cfu/g of rhizosphere soil (Fig. 7). Except for isolate Y11, bacterial counts at root tips were up to 100 times higher than at the upper root parts. There was a positive correlation between the levels of general population and those of the biocontrol agents in all root segments except the tips. Colonization of cucumber roots by *P. aphanidermatum* was reduced by 50–100% in the presence of the biocontrol agents.

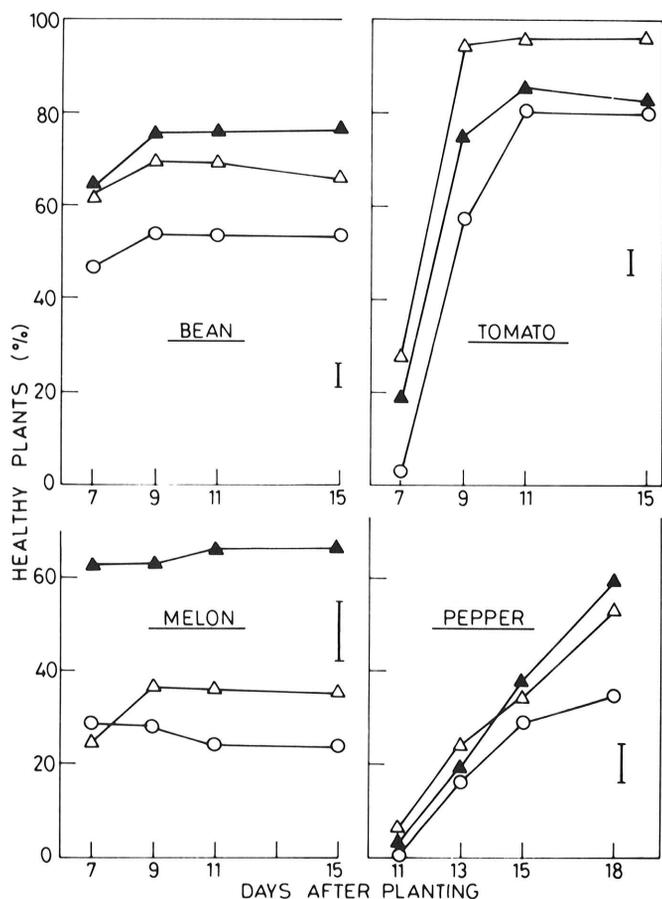


Fig. 6. Percentage of healthy plants in peat-vermiculite medium infested with *Pythium aphanidermatum* and treated with isolates 805 (▲) or 808 (△) or untreated (○).

## DISCUSSION

A major objective of this study was to isolate potential bacteria and characterize their activity in controlling *Pythium* spp. The ability of six bacteria applied to PVM or soil to reduce the incidence of damping-off in the greenhouse was tested. About 60–90% of disease reduction was obtained under growth conditions prevailing in our experiments. Although cucumber was the major test plant throughout this work, disease control was also obtained in other crops susceptible to *P. aphanidermatum*, e.g., pepper, melon, bean, tomato, and cotton (Fig. 6). The bacteria were also most effective in controlling *P. ultimum* (Fig. 6). One application of the biocontrol agents was sufficient to prevent disease buildup in replanted cucumbers. The additive control achieved by combining bacterial application, at a low rate to achieve disease control, with the fungicide prothiocarb (commonly used against *Pythium*) reduced disease incidence 80%.

Our results indicate that the presence of bacteria along roots of susceptible hosts reduced the establishment of *Pythium* along the roots, which are the potential sites for its attack.

Damping-off, induced by *Pythium* spp., has been controlled by coating seeds with a variety of antagonistic microorganisms (7–9, 13, 24). In this work, seed coating was as effective as direct application of bacteria into the soil (Fig. 4). Seed treatments are an attractive method for introducing bacteria to the soil-plant environment, because in this way, the bacteria have an opportunity of being the first colonizers of the roots. It was evident that bacteria, introduced as seed treatments, moved along the roots. Weller (26) showed the distribution of *Pseudomonas fluorescens* introduced via seeds on seminal roots of winter wheat. Similarly, we have counted higher populations at root tips. Moreover, the lower counts of indigenous bacteria obtained in wheat (26) and cucumber (Fig. 7) in the presence of the introduced strains indicate the obvious competition between them. Populations of the indigenous bacteria generally were greatest where populations of the biocontrol agents were great (Fig. 7), probably because both

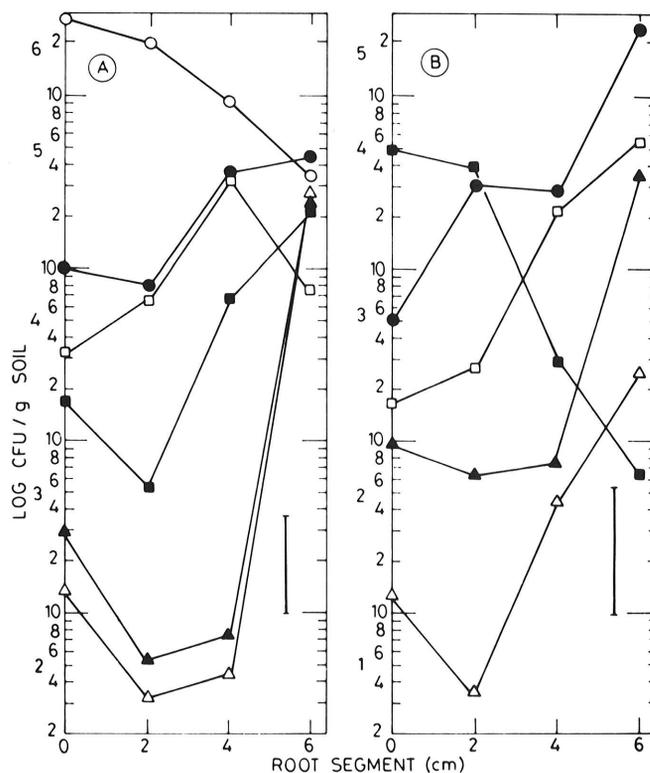


Fig. 7. Densities of A, general bacteria and B, biocontrol agents in the rhizosphere of 6-day-old cucumber plants. Isolates 805 (▲), 808 (△), Rh2 (●), Y11 (■), and 814 (□) (○ = untreated control) were applied to seeds planted between two longitudinal halves of plastic tubes filled with soil. Bar =  $LSD_{0.05}$ .

were stimulated by the same factor—rhizosphere nutrients. Intense competition for resources such as nutrients among rhizosphere bacteria is very probable. Seed coating places the bacteria where they are needed the most, at the most important site of interaction with the plant-rotting *Pythium* spp. The ability of a bacterial strain to colonize or establish a large population in the rhizosphere is a crucial factor that determines the importance of rhizobacteria as root associates.

Significant positive correlation was observed between inhibition of oospore germination and disease reduction induced by the same bacteria. Moreover, inhibition of germination of oospores in the rhizospheres of several plants was achieved by the biocontrol agents. On the other hand, there was no direct observable interaction between spores and bacteria. Siderophores may also be excluded as a possible mechanism for control of *Pythium*, because the pH of the PVM was calibrated to 6.0. In this case, iron is available in high concentration. This may not be the case in *Pythium*-infested soil where pH is about 7.6, as was shown in the interaction of *Fusarium oxysporum* and *Pseudomonas* sp. (3).

Oospore germination has already been shown to be affected by exogenous nutrients (9). Nutrients supplied by root exudates stimulate oospore germination in the rhizosphere. It therefore appears that bacteria may compete with germinating oospores for available carbon or nitrogen sources, and by eliminating these resources, the bacteria reduce the percentage of oospore germination. Similar results were obtained recently with chlamydospores of *Fusarium* spp. (3). Moreover, the reduction in the general population of bacteria along the roots caused by the biocontrol agents is probably due to competition (Fig. 7). Although lysis was suggested as a mechanism in the control of *Pythium* sp. (15) when cell wall lytic bacteria are added to soil, such heterolytic activity was not confirmed in vitro in our studies. Production of inhibitory substances may be a potential means by which the bacteria affect the plant pathogen. *Pythium* is indeed known to be sensitive to such inhibitory effects. However, our in vitro experiments have shown that bacteria, unable to induce suppressiveness in soil, effectively inhibited mycelium of the pathogen. This may suggest that inhibitory substances or antibiotics do not play a major role in our systems.

Broadbent et al (2) added *Bacillus subtilis* (Fren.) Cohn to soil treated by aerated steam. *P. ultimum* was controlled on *Antirrhinum*, possibly by decreasing infection rather than by antibiosis. Hadar et al (7) protected seedlings from disease caused by *Pythium* by application of *Enterobacter cloacae*. Howell and Stipanovic (9) concluded that a strain of *Pseudomonas fluorescens* was antagonistic to *P. ultimum* because of the production of an antibiotic (pyoluteorin) by the bacterium.

Our results demonstrate the potential of bacteria to control *Pythium*. Further research is needed to develop the formulation of bacterial biocontrol agents and exploit them in agriculture.

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