Biological Control of Pythium Damping-Off of Cucumbers with *Pythium nunn*: Population Dynamics and Disease Suppression

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ABSTRACT

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Addition of *Pythium nunn* to aerated steamed soil infested with *P. ultimum* resulted in disease and pathogen suppression. Population densities of *P. ultimum* and disease incidence decreased as initial inoculum density of *P. nunn* increased. Disease suppression decreased as initial inoculum density of *P. ultimum* increased. Suppression lasted longer when inoculum of *P. nunn* was produced on 1% rolled oats (w/w) than on 0.3% ground bean leaves. Population densities of *P. nunn* were not affected by population densities of *P. ultimum* in aerated steamed soil. *P. nunn* did not increase saprophytically in raw soil unless an organic substrate was added. Cucumbers grown in aerated steamed soil infested with *P. ultimum* and *P. nunn* had greater root dry weights than plants grown in soil infested with *P. ultimum* alone. Cucumber seeds treated with *P. nunn* and planted in aerated steamed soil infested with *P. ultimum* had greater percent emergence than untreated seeds planted in the same soil. Evidence indicates that *P. nunn* requires organic substrates rather than living fungal hosts for antagonism and disease suppression to operate.

Pythium nunn Lifshitz, Stanghellini & Baker is a recently described species isolated from a grassland soil suppressive to *Pythium ultimum* (8). Scanning electron microscopy revealed that *P. nunn* is mycoparasitic on *Pythium* spp., *Phytophthora* spp., and other plant pathogenic fungi (6). *P. nunn* induced lysis of germinating sporangia of *P. ultimum* Trow enclosed in membrane filters and buried in soil infested with the mycoparasite (7). This, and other evidence (2), suggests that *P. nunn* produces antibiotic factors active against *Pythium* spp. *P. nunn* also produces β-1,3 glucanase and cellulase when grown with cell walls of *P. ultimum* and produces chitinase and β-1,3 glucanase in the presence of cell walls of *Rhizoctonia solani* Kuhn and *Sclerotium rolfsii* Sacc. (2).

Suppression of cucumber damping-off caused by *P. ultimum* was induced when inoculum of *P. nunn* was added to a raw soil amended with ground bean leaves (7). *P. nunn* was not pathogenic to cucumber (*Cucumis sativus* L. 'Straight Eight'), radish (*Raphanus sativus* L. 'Early Scarlet Globe'), pea (*Pisum sativum* L. 'Laxton Progress'), watermelon (*Citrullus lanatus* L. 'Dixie Queen'), alfalfa (*Medicago sativa* L. 'Titan'), wheat (*Triticum aestivum* L. 'Hermosillo'), or barley (*Hordeum vulgare* L. 'Steptoe') (7). Another mycoparasitic *Pythium* spp., *P. oligandrum*, also has been implicated in biological control of Pythium damping-off (1,10).

Preliminary work demonstrated the biological potential of *P. nunn* to control seedling damping-off induced by *P. ultimum* (12). The objective of this research was to study the population dynamics of *P. ultimum* and *P. nunn* in aerated steamed and raw soil, and corresponding effects on disease suppression. Protection of seeds against damping-off by use of seed treatments and reduction of root pruning by use of soil treatments with *P. nunn* were also investigated.

MATERIALS AND METHODS

Soil. Nunn sandy loam (7) was used in all experiments. The soil was air-dried, sieved through a 4-mm-mesh screen, and stored for 2–3 mo before use. The soil was moistened to approximately −0.3 bars, exposed to aerated steam at 55 C for 1 hr, and exposed to the air for 1 wk before use to eliminate indigenous *Pythium* spp. and to allow microbial populations to equilibrate.

Inoculum. Isolates of *P. nunn* (N3) and *P. ultimum* (Nl) used in previous studies (7) were maintained in culture on water agar or cornmeal agar slants. Five mycelial mats from 1-wk-old 100-ml potato-dextrose broth cultures of *P. nunn* or *P. ultimum* were added to 6 kg of twice-autoclaved Nunn sandy loam amended with 1% (w/w) ground, rolled oats or 0.3% (w/w) ground bean leaves (*Phaseolus vulgaris* L. 'Pinto'). The inoculum was incubated under aseptic conditions at 26 C in 32 × 39 × 19-cm plastic tubs covered with aluminum foil. After 3 wk, colony-forming units (cfu) of the fungi in the inoculum were determined by plate dilution on a *Pythium* selective medium (11). Soil inoculum also was examined with fluorescent microscopy (13) to determine the type of fungal propagule. The inoculum of *P. ultimum* primarily consisted of sporangia (20–30 μm diameter). The inoculum of *P. nunn* was a mixture of sporangia (15–20 μm diameter) and oospores (20–25 μm diameter). Very small amounts of viable-appearing hyphae were observed in either inocula.

Culture conditions. *Growth room.* All growth-room experiments were conducted in 6.5-cm-square plastic pots, six replicate pots per treatment. Ten cucumber seeds (*C. sativus* 'Straight Eight') were planted in each pot after soil was moistened. Plants were grown in a constant-temperature growth room at 26 C, with a 12-hr light/dark cycle (fluorescent and incandescent lighting). Plants were watered twice daily with distilled water. Soil matric potential was maintained at >-0.1 bars at all times. Matric potential was determined by measuring percent moisture between waterings in a preliminary experiment. Percent moisture was converted to matric potential by use of a moisture retention curve. At no time did percent moisture fall below 10% (−0.1 bar). Percent emergence was recorded 7 days after planting. All pots were replanted at 10, 20, and 30 days. Population densities of *P. ultimum* and *P. nunn* were determined at 1, 3, 7, 14, 21, 28, and 56 days by dilution plating on a *Pythium* selective medium (11). *P. ultimum* and *P. nunn* could be distinguished from each other by differences in colony morphology and growth rate (7).

Greenhouse. In all greenhouse experiments, 10 cucumber seeds were planted per 10-cm-diameter pot, and seedlings were thinned

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to five seedlings per pot after 1 wk. Plants were grown for 30 days in the greenhouse (22–28 C day, 11–22 C night) with no additional lighting and watered daily with nutrient solution (Peters general purpose 20-20-20, 1:200, W. R. Grace & Co., Fogelsville, PA). After 30 days, shoots and roots were removed and oven-dried at 60 C for 24 hr, and dry weights measured. Pots were sampled for population densities of *P. ultimum* and *P. nunn* at 1, 7, 14, and 21 days.

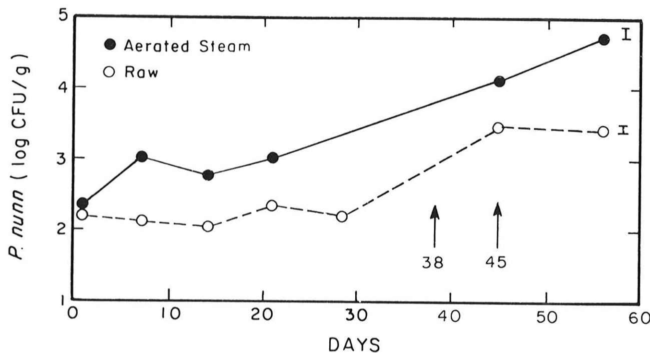


Fig. 1. Population densities of *Pythium nunn* over time in raw soil naturally infested with *P. ultimum* (20 colony-forming units [cfu] per gram) and aerated steamed soil. Initial inoculum of *P. nunn*= 300 cfu/g. Ground bean leaves (0.3% w/w) were added at 38 and 45 days. Bars represent standard errors.

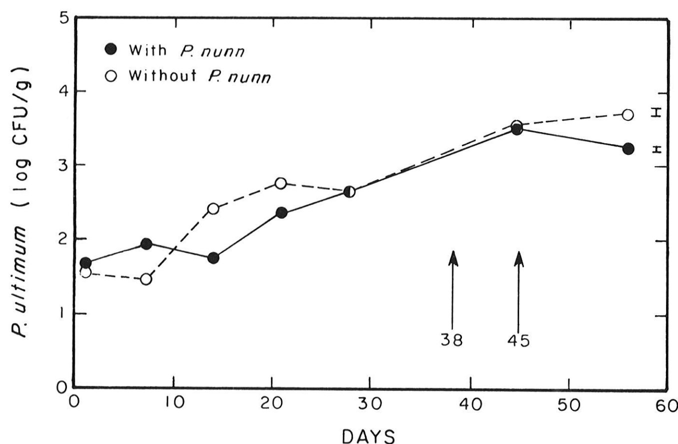


Fig. 2. Population densities of *Pythium ultimum* in naturally infested raw soil, with and without *P. nunn*. Initial inoculum density of *P. nunn* = 300 colony-forming units (cfu) per gram, *P. ultimum* = 20 cfu/g. Ground bean leaves (0.3% w/w) were added at 38 and 45 days. Bars represent standard errors.

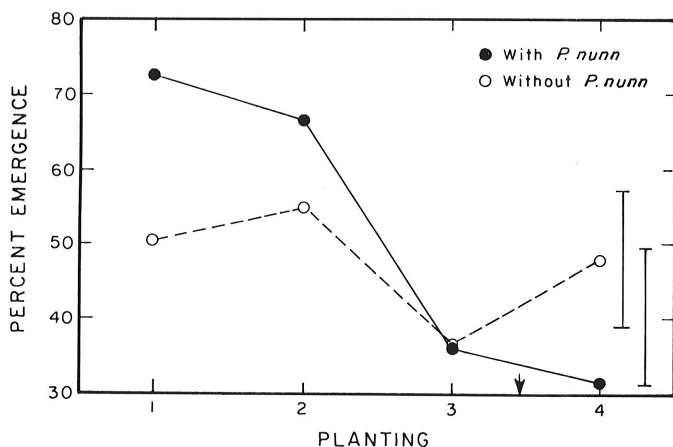


Fig. 3. Percent emergence of successive plantings of cucumbers in naturally infested raw soil, with and without *Pythium nunn*. Initial inoculum density of *P. nunn* = 300 colony-forming units (cfu) per gram, *P. ultimum* = 20 cfu/g. Two amendments of ground bean leaves (0.3%, w/w) were added between the third and fourth plantings.

Seed treatment. *Soil inoculum slurry.* For seed treatment with a slurry of inoculum, 1 g of inoculum of *P. nunn* (1% rolled oats) was mixed with 9 ml of 1% (w/v) Pelgel solution (The Nitragin Co, Milwaukee, WI). The soil inoculum slurry was poured over 60 cucumber seeds in a 12× 12× 2-cm weighing dish. Untreated seeds were coated with 10% (w/v) slurry of aerated steamed soil in 1% Pelgel solution. All seeds were air-dried for 24 hr before planting.

Oospores. Oospores for seed treatments were produced by growing the fungus for 2 wk at 20 C on rolled oat and water agar medium (3) supplemented with dry bean leaves. Oospores were separated from bean leaves by grinding in a Waring Blendor for 30 sec and by centrifugation at 2,500 *g* for 5 min. The pellet was resuspended in distilled water, and oospore concentration was measured with a hemocytometer. One milliliter of oospore suspension was also dilution-plated on *Pythium* selective medium to determine the concentration of germinable oospores. Pelgel (1%) was added to the oospore suspension. Sixty cucumber seeds were treated with 4 ml of oospore-Pelgel suspension according to the methods described above. Seeds not treated with the fungus were coated with 1% Pelgel solution.

The following three treatments were used: seeds planted in aerated steamed soil, seeds planted in aerated steamed soil infested with 30 cfu/g of inoculum of *P. ultimum* (1% rolled oats), and seeds treated with *P. nunn* planted in soil infested with *P. ultimum*. Experiments were conducted in a constant-temperature growth room under the conditions described. Percent emergence was recorded 7 days after planting.

Statistical analyses. Pots were arranged in the growth room and greenhouse benches in a randomized complete block design. All experiments were done twice, with similar results. Data from the first trials are presented. Percent emergence data were adjusted to percent of uninoculated control. The adjusted disease incidence was transformed to probits. In the analysis, 100% disease incidence was converted to 99.9% and 0% disease was converted to 0.1% before probit transformation. Two-way analyses of variance were performed on the transformed data, and mean separations were made with Duncan's multiple range tests. Disease suppression was measured as difference in percent emergence (probits) between the treatments with and without *P. nunn*. Linear regression lines were constructed for inoculum density/disease incidence (ID/DI) data and compared to determine statistical difference in regression coefficients.

RESULTS

Population dynamics in raw unamended soil. Zero or 300 cfu/g of *P. nunn* (1% rolled oats) were added to raw Nunn sandy loam naturally infested with 20 cfu/g of *P. ultimum* and to aerated steamed soil. After 38 and 45 days, all soils were amended with 0.3% ground bean leaves. Experiments were conducted in the growth room.

No saprophytic increase of *P. nunn* was detected in raw soil over a 30-day period (Fig. 1). In aerated steamed soil, population densities of *P. nunn* increased from 300 to 1,000 cfu/g during the first 7 days. When 0.3% ground bean leaves were added at 38 days, population densities in both raw and pasteurized soils increased approximately 10-fold during the next 7 days.

No reduction in populations of *P. ultimum* was observed in raw soils infested with *P. nunn* (Fig. 2). Population densities of *P. ultimum* increased from 30 to 300 cfu/g during the first 28 days but increased more rapidly after the addition of 0.3% bean leaves. However, no statistical reductions in populations of *P. ultimum* among treatments with and without *P. nunn* were detected.

Disease suppression at the first planting was significant in raw soil naturally infested with *P. ultimum* and amended with 300 cfu/g of *P. nunn* (Fig. 3). However, no suppression was evident in the second and third planting or in the fourth planting after amendments with bean leaves.

Effects of initial population density of *P. ultimum*. In the first experiment, inocula (1% oats) were mixed with aerated steamed to achieve population densities of 0, 100, 300, or 1,000 cfu/g of *P. ultimum*, together with either 0 or 300 cfu/g of *P. nunn*. Another

experiment was similar to the first, except that inoculum of *P. nunn* was grown on 0.3% bean leaves. Autoclaved formulation of *P. nunn* also was added as a treatment in the second experiment. Both experiments were done in a growth room.

In the experiment with *P. nunn* grown on a 1% rolled oats formulation, disease suppression was significant at all inoculum levels of *P. ultimum* in all three replantings. Significant differences were observed between all ID/DI regression lines of treatments with and without *P. nunn*. However, consistently less disease suppression was observed as the initial inoculum levels of *P. ultimum* increased. Suppression of seedling damping-off also decreased with each successive planting. In the experiment with *P. nunn* grown on 0.3% bean leaves, disease suppression was significant in the first planting at all inoculum levels of *P. ultimum* except 1,000 cfu/g. However, suppression was lost in the second, third, and fourth plantings at all inoculum levels of *P. ultimum* except 10 cfu/g. No suppression was found in treatments when the autoclaved formulation of *P. nunn* was added.

The incidence of disease induced by *P. ultimum* when *P. nunn* was not present was not influenced by inoculum levels of 10^1 to 10^3 cfu/g of soil (Figs. 4, 5). This trend was true for all plantings and was evidenced by regression slope values not significantly different from zero. In the 1% rolled oats experiment at the second and third plantings (Fig. 4B, C), disease incidence was above 60% in all treatments without *P. nunn*; in the 0.3% bean leaves experiment,

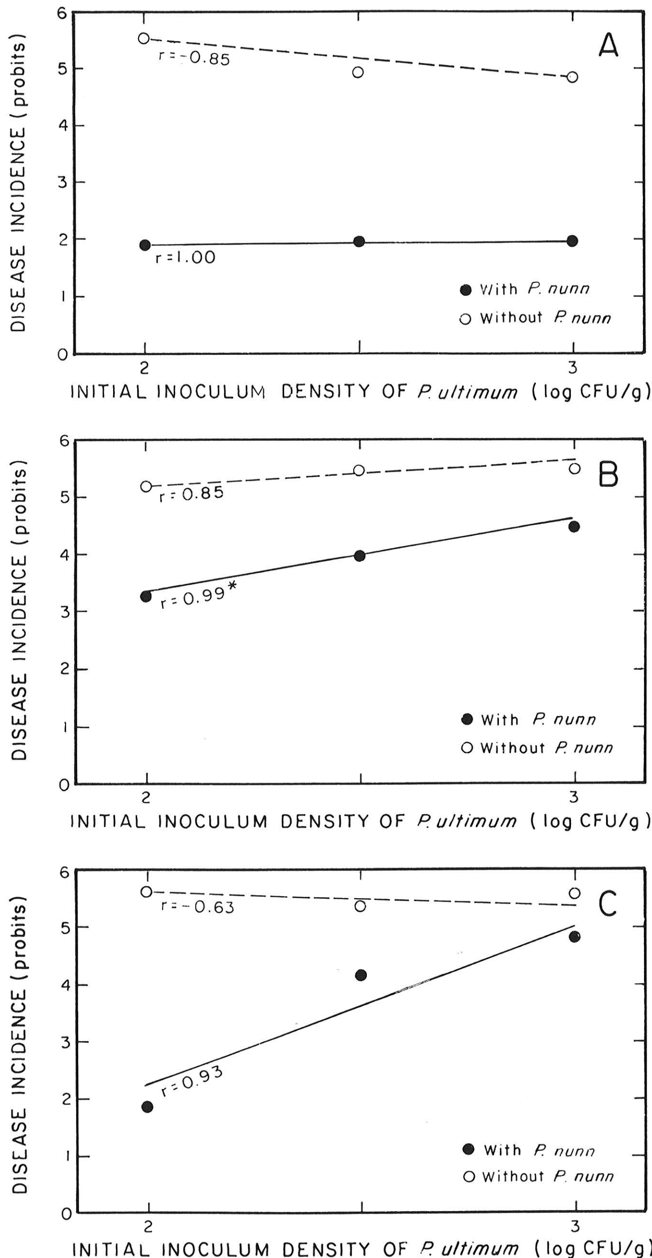


Fig. 4. Effect of initial inoculum density (ID) of *Pythium ultimum* on incidence of cucumber damping-off, with and without *P. nunn* (initial ID = 300 colony-forming units per gram). **A,** first planting; **B,** second planting; **C,** third planting. Inocula of both *P. ultimum* and *P. nunn* produced on autoclaved soil plus 1% rolled oats (w/w). Correlation coefficient significant at $P \leq 0.05$.

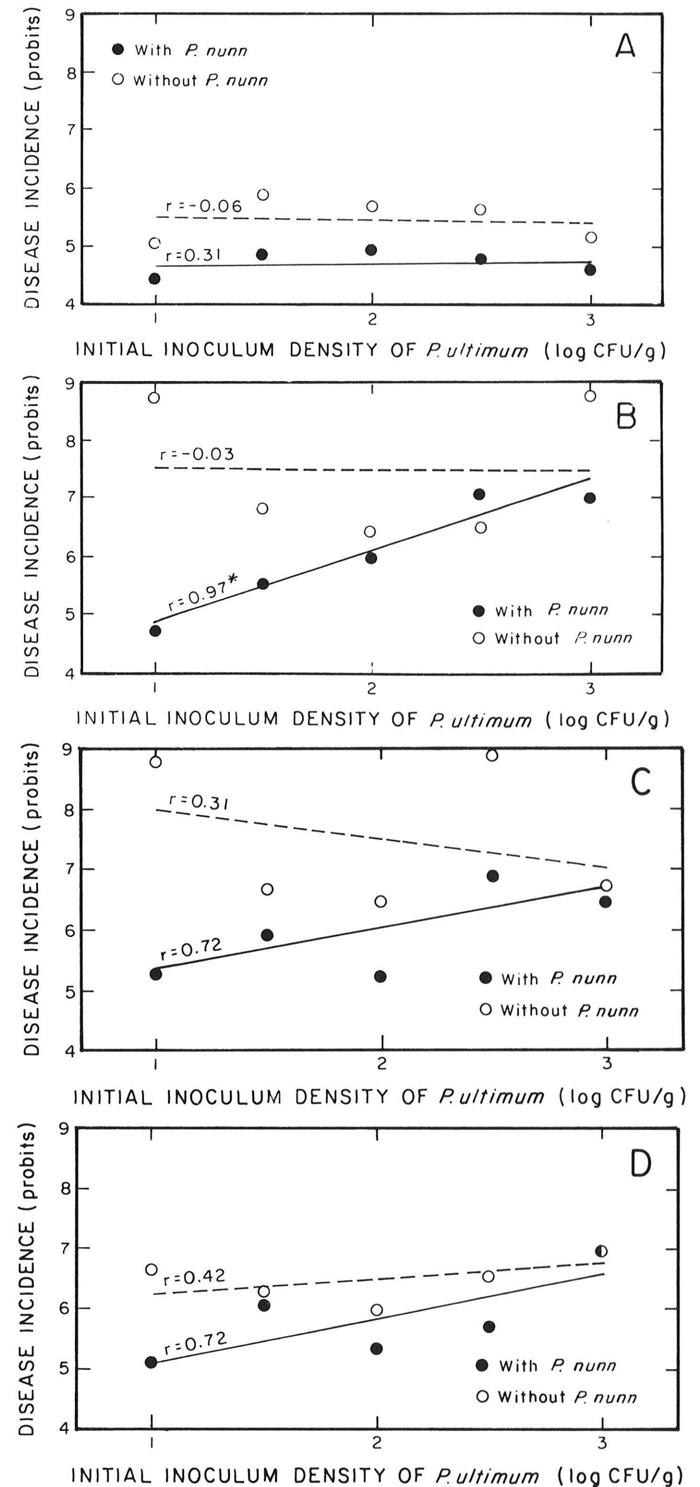


Fig. 5. Effect of initial inoculum density (ID) of *Pythium ultimum* on incidence of cucumber damping-off, with and without *P. nunn* (initial ID = 300 colony-forming units per gram). **A,** first planting; **B,** second planting; **C,** third planting, **D,** fourth planting. Inoculum of *P. ultimum* was produced on autoclaved soil plus 1% rolled oats; inoculum of *P. nunn* was produced on autoclaved soil plus 0.3% ground bean leaves. Correlation coefficient significant at $P \leq 0.05$.

disease incidence was above 90% in these same treatments (Fig. 5B–D).

In treatments with *P. nunn*, the slope value of the ID/DI curve was not significantly different from zero at the first planting (Figs. 4A, 5A), and disease incidence was less than in the treatments without *P. nunn*. In successive plantings, however, disease incidence was affected by initial inoculum density of *P. ultimum*, as evidenced by slope values significantly greater than zero (Figs. 4B–C, 5B–D). Less disease was measured in treatments with lower levels of initial inoculum of *P. ultimum*. With each successive planting, the disease incidence increased, but the slope of the ID/DI lines always remained positive in treatments with *P. nunn*.

In both experiments, population densities of *P. ultimum* were significantly less in the treatments with *P. nunn* added (Figs. 6, 7). After 7 days, no statistical difference in population densities were observed among treatments with different initial inoculum densities of *P. ultimum*, so population densities were averaged at each time. Populations of *P. ultimum* were reduced 1–2 log units in the 1% rolled oats experiment, but only 0.5–1 log unit in the 0.3% bean leaves experiment. By 30 days in the 0.3% bean leaves experiment, population densities of *P. ultimum* in the treatments with and without *P. nunn* were not statistically different.

Because no statistical differences were seen in population densities of *P. nunn* among treatments, population densities were averaged over treatment at each time. Population densities of *P. nunn* increased more rapidly where the inoculum was grown on a 1% rolled oats formulation (Fig. 8). The logarithmic increase in population was delayed by 7 days in the experiment where *P. nunn* was grown on 0.3% bean leaves.

Effects of initial population density of *P. nunn*. Inoculum (1% rolled oats) of *P. ultimum* and *P. nunn* were added to aerated steamed soil to achieve population densities of 0 or 30 cfu/g of *P. ultimum* and 0, 30, 100, 300, 1,000 or 3,000 cfu/g of *P. nunn*. Experiments were conducted in a growth room.

The population density of *P. ultimum* was inversely correlated with the initial inoculum density of *P. nunn* added to the soil at the start of the experiment (Fig. 9). Overall population densities of *P. ultimum* increased over time, but increasing levels of *P. nunn* still resulted in decreased populations of *P. ultimum*. Increasing levels of *P. nunn* also decreased disease incidence (Fig. 10). All regression lines (Figs. 9, 10) had slope values significantly less than zero. At every planting, the addition of 3,000 cfu/g of *P. nunn* to soil infested with *P. ultimum* resulted in disease incidences not statistically different from treatments where *P. ultimum* was not added.

Effect of *P. nunn* on root pruning caused by *P. ultimum*. Zero or 100 cfu/g of *P. ultimum* and 0 or 300 cfu/g of *P. nunn* (1% rolled oat inoculum) were added to aerated steamed soil, and placed in

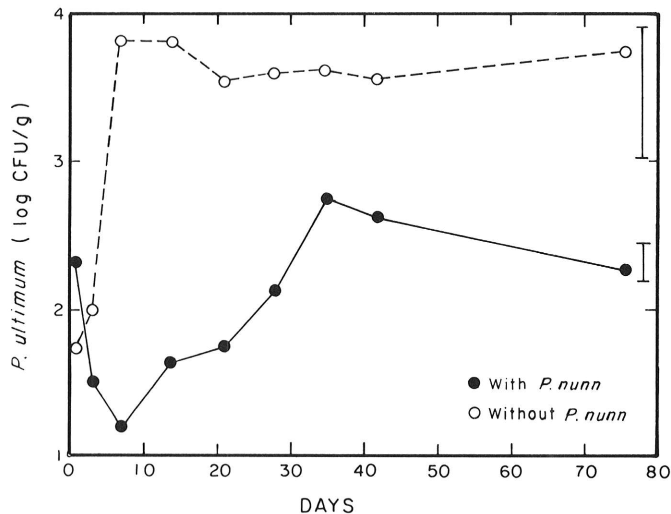


Fig. 6. Population densities of *Pythium ultimum* in aerated steamed soil over time, with and without *P. nunn*. Initial inoculum density (ID) of *P. nunn* = 300 colony-forming units per gram. Population densities of *P. ultimum* were averaged among initial ID treatments. Bars represent standard errors. Inocula of *P. ultimum* and *P. nunn* was produced on autoclaved soil plus 1% rolled oats.

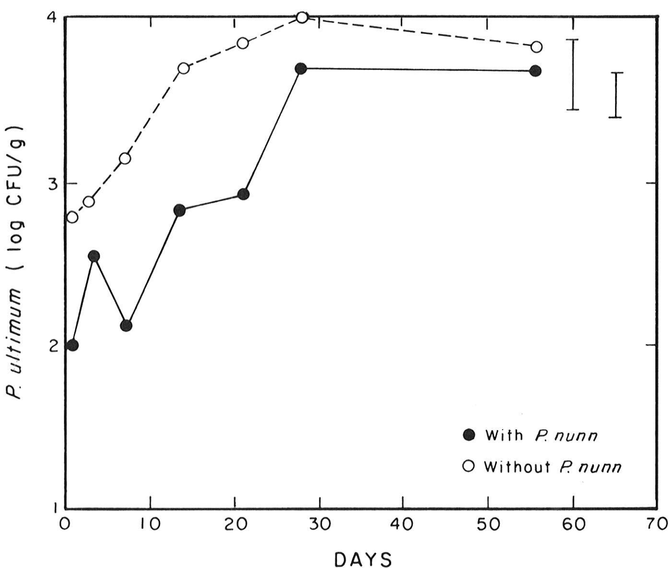


Fig. 7. Population densities of *Pythium ultimum* in aerated steamed soil over time, with and without *P. nunn*. Initial inoculum density (ID) of *P. nunn* = 300 colony-forming units per gram. Population densities of *P. ultimum* were averaged among initial ID treatments. Bars represent standard errors. Inoculum of *P. nunn* was produced on autoclaved soil plus 0.3% ground bean leaves.

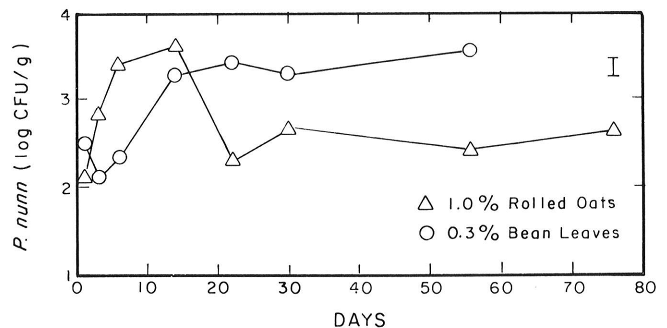


Fig. 8. Population densities of *Pythium nunn* in aerated steamed soil over time. Initial inoculum density (ID) of *P. nunn* = 300 colony-forming units per gram. Population densities of *P. ultimum* were averaged among initial ID treatments. Bar represents standard error.

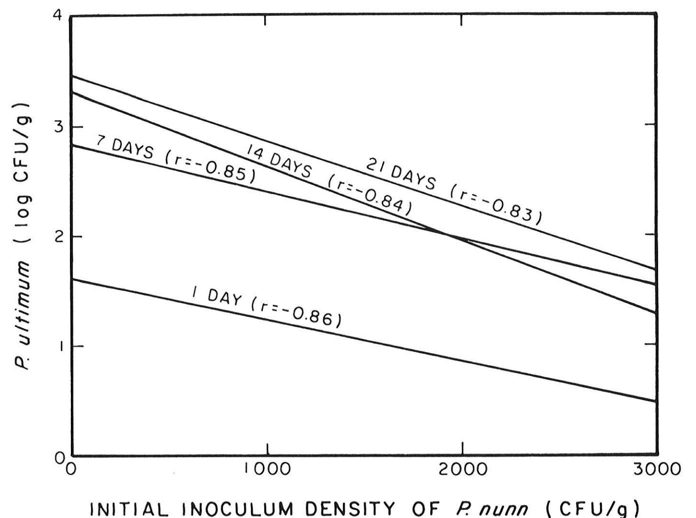


Fig. 9. Effect of initial inoculum density (ID) of *Pythium nunn* on population densities of *P. ultimum*. Initial ID of *P. ultimum* = 30 colony-forming units per gram. All correlation coefficients significant at $P \leq 0.05$.

the bottom half of the pots. The top half was filled with aerated steamed soil and planted as described above.

P. ultimum reduced shoot dry weights, compared to uninfested controls (Fig. 11). However, addition of *P. nunn* to soil infested with *P. ultimum* did not significantly increase shoot dry weights.

P. ultimum also significantly reduced the root dry weights of cucumbers, compared to uninoculated controls (Fig. 12). Root dry weights were significantly higher in the treatments where *P. nunn* was added to aerated steamed soil infested with *P. ultimum* than in treatments with *P. ultimum* alone. Significant reductions in population densities of *P. ultimum* were also seen in the treatments where *P. nunn* was added.

Seed treatments with *P. nunn*. Cucumber seeds treated with either the soil formulation or oospores (220 germinable oospores per seed) of *P. nunn* emerged significantly better than untreated seeds planted in aerated steamed soil infested with *P. ultimum* (Fig. 13).

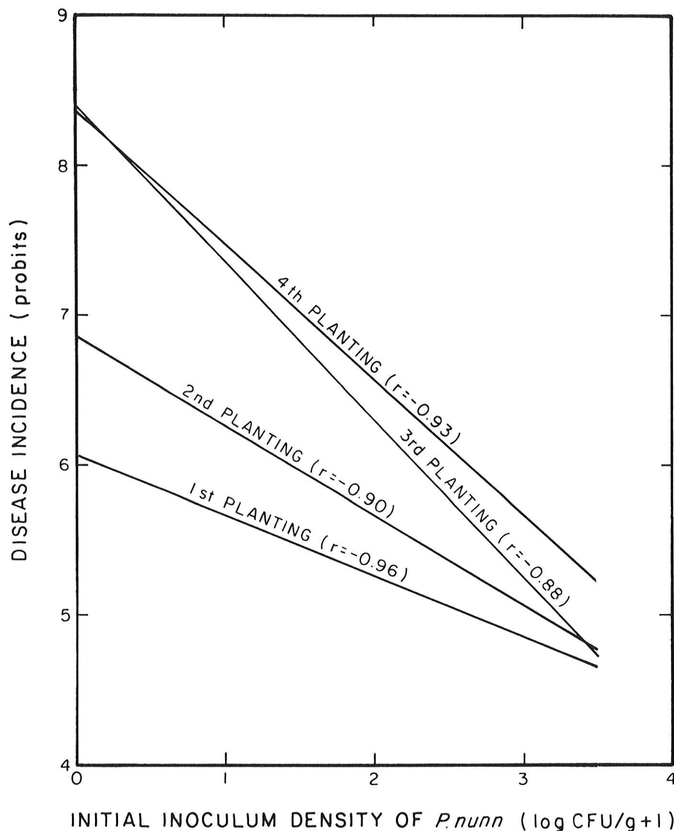


Fig. 10. Effect of initial inoculum density (ID) of *Pythium nunn* on incidence of cucumber damping-off caused by *P. ultimum*. Initial ID of *P. ultimum* = 30 colony-forming units per gram. All correlation coefficients significant at $P \leqslant 0.05$.

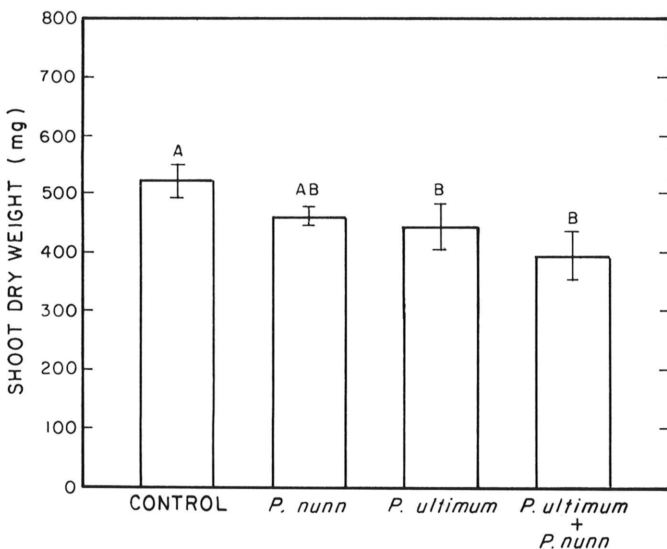


Fig. 11. Shoot dry weights of 30-day-old cucumbers planted in pots with two soil layers. The top layer contained aerated steamed soil and the bottom layer contained aerated steamed soil infested with *Pythium ultimum* and/or *P. nunn*. Initial inoculum density of *P. ultimum* = 100 colony-forming units (cfu) per gram, *P. nunn* = 300 cfu/g. Bars represent standard deviations. Treatments with the same letters are not statistically different (P = 0.05) according to Duncan's multiple range test.

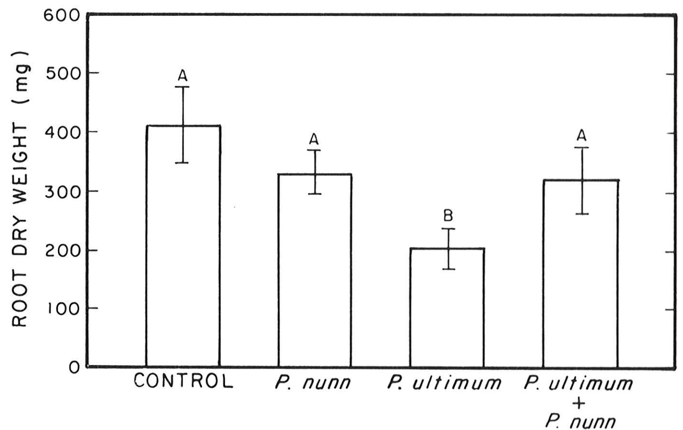
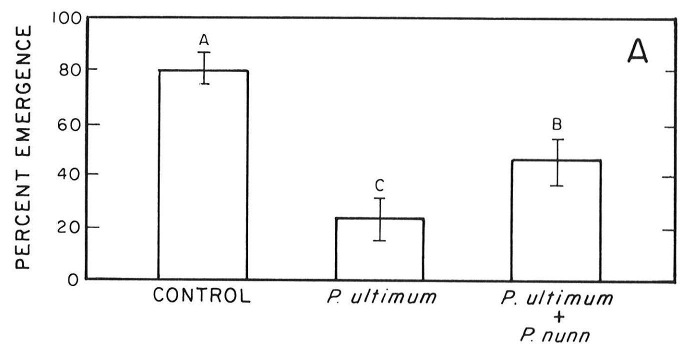


Fig. 12. Root dry weights of 30-day-old cucumbers planted in pots with two soil layers. The top layer contained aerated steamed soil, and the bottom layer contained aerated steamed soil infested with *Pythium ultimum* and/or *P. nunn*. Initial inoculum density of *P. ultimum* = 100 colony-forming units (cfu) per gram, *P. nunn* = 300 cfu/g. Bars represent standard deviations. Treatments with the same letters are not statistically different (P = 0.05) according to Duncan's multiple range test.



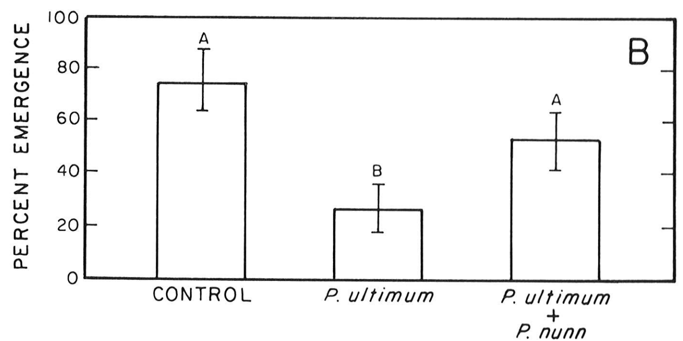


Fig. 13. Percent emergence of cucumber seeds untreated or treated with *Pythium nunn* planted in aerated steamed soil infested with *P. ultimum*. Initial inoculum density of *P. ultimum* = 30 colony-forming units per gram. Control treatments were untreated seeds planted in aerated steamed soil. **A,** *P. nunn* applied as oospore suspension. **B,** *P. nunn* applied as a 10% (w/w) soil inoculum slurry with 1% Pelgel (w/v). Bars represent standard deviations. Treatments with the same letters are not statistically different (P = 0.05) according to Duncan's multiple range test.

DISCUSSION

The addition of *P. nunn* to aerated steamed soil resulted in suppression of cucumber damping-off induced by *P. ultimum* (Figs. 4, 5, 10). The inoculum density of *P. ultimum* used in all these experiments was above the ED_{50}, and the inoculum density of *P. nunn* used (300 cfu/g) was the lowest effective dosage that gave biological control. The attainment of disease suppression under these rigorous conditions shows the promising potential of *P. nunn* as a biological agent. Disease suppression in aerated steamed soil was influenced by the initial inoculum density of *P. ultimum* (Figs. 4, 5) and population density of *P. nunn* (Fig. 10). *P. nunn* also reduced the inoculum density of *P. ultimum* by 0.5–2.0 log units (Figs. 6, 7, 9). Pathogen suppression, as evidenced by reduced inoculum density of *P. ultimum*, appears to be the primary cause of disease suppression. But *P. nunn* also could reduce the inoculum potential of the pathogen, which would not be detected by dilution-plating techniques.

Results of these experiments indicated that organic substrate available to *P. nunn* was important for the biological control of *P. ultimum*. Three separate lines of evidence support this contention. First, in the experiment with *P. nunn* grown on a 1% rolled oat formulation, suppression persisted for three plantings. In the second experiment, with the 0.3% bean-leaf formulation, disease suppression was lost after the first planting, except in treatments in which initial inoculum density was 10 cfu/g of *P. ultimum*. Second, population densities of *P. nunn* were not affected by the population densities of *P. ultimum* initially added to the soil. This supports the hypothesis that *P. nunn* is primarily dependent on organic substrates for population increases, not on the population densities of its fungal host *P. ultimum*. This biological control system is clearly unlike the well-studied *Trichoderma-Rhizoctonia* system (4,5,9), where the mycoparasite *T. harzianum* used thalli of *R. solani* as a food base to increase its population density. Third, in an unamended raw soil, saprophytic increase of *P. nunn* was not detected (Fig. 1), and *P. nunn* did not reduce the inoculum density of *P. ultimum* (Fig. 2). In contrast, addition of organic substrate to a raw soil after 38 days resulted in a 10-fold increase in population densities of *P. nunn*; however, disease suppression did not result, perhaps because the inoculum density of *P. ultimum* already was high (570 cfu/g) when the amendment was added. This suggests that rapid saprophytic increases of *P. nunn*, when population densities of *P. ultimum* are relatively low, are favorable for disease suppression. This also could explain the lack of long-lasting suppression in the 0.3% bean leaves experiment where saprophytic increase of *P. nunn* was delayed (Fig. 8).

Therefore, manipulation of organic amendments rather than providing fungal hosts of *P. nunn* constitute the substrates required for antagonism leading to disease suppression. This explains why suppressiveness was induced when Lifshitz et al (7) repeatedly added bean leaf meal to a soil containing a low initial population density of *P. nunn*.

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