Bacterial Population Dynamics and Interactions with *Pythium aphanidermatum* in Intact Rhizosphere Soil

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ABSTRACT

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In tripartite rhizosphere (host, fungus, and bacteria) interactions, bacterial antagonism was found to be responsible for the general suppression of pathogen activity at low soil temperature. Host colonization by *Pythium aphanidermatum* was less than 25% at 20 C, in contrast to greater than 90% colonization at 27 C after 72 h. When bacterial activity was reduced or inhibited by the addition of vancomycin to soil, host colonization by the fungus at 20 C increased to 83%. Generation time of bacteria in intact rhizosphere soil was estimated to be about 8 h at both 20 and 27 C. Bacterial antagonism was shown to occur, however, before any significant increase in bacterial populations. Oospores germinated within 4 h in rhizosphere soil at 27 C but required 8–12 h at 20 C. Percent oospore germination increased in soils amended with asparagine and/or vancomycin at 20 C. These results indicated that competition for nutrients may be involved in suppression of *P. aphanidermatum* at low soil temperatures.

The rhizosphere, a zone of soil immediately adjacent to the root surface, is inhabited by diverse heterotrophic microorganisms, which influence, either directly or indirectly, the growth and development of plants (2,5,11). Root exudates presumably constitute the primary source of nutrition (carbon and nitrogen) for these microorganisms, and competition for substrate utilization is intense. Successful utilization by any one population is governed primarily by the relative competitive ability of the microorganisms and the prevailing environmental conditions (2,5).

This study was initiated to evaluate rhizosphere interactions between the resident bacterial flora, a root-infecting fungus (*Pythium aphanidermatum* (Edson) Fitzp.), and its host (sugarbeet, *Beta vulgaris* L.). This host-pathogen combination was specifically chosen as a model system for this study for two reasons. First, *P. aphanidermatum* is regarded as an opportunistic, root-infecting fungus (3,12). Previous studies (15,20) have shown that penetration and infection of the taproot of mature sugarbeets by this fungus coincides with the onset of specific soil temperatures (>27 C for 12 consecutive hours per day at the 10-cm soil depth), which occur about 8–9 mo after planting. High populations of exogenously dormant oospores, the primary source of inoculum, occur in naturally infested rhizosphere soil (within 1 mm of the sugarbeet rhizoplane) throughout the entire growing season (18,19). Thus, lack of infection and disease before the occurrence of conducive soil temperatures was not related to a lack of pathogen inoculum in the rhizosphere. Additionally, laboratory studies showed that oospores were capable of germination, as well as vegetative growth, over a temperature range of 10–43 C (1,17). These results suggested that some factors, in addition to specific soil temperature effects on the fungus alone, influence the specific timing of the onset of root infection under field conditions.

Second, as a result of extensive lateral expansion of the taproot during growth and the resultant compression of adjacent soil, intact sections of rhizosphere soil (each measuring up to 100 cm^2 and bearing the impression of the sugarbeet taproot) can be collected from field-grown sugarbeets. These intact sections of rhizosphere soil facilitate studies of microbial interactions in the rhizosphere (18).

The specific objectives of our study were to determine the influence of temperature on rate of oospore germination and generation time of bacteria in rhizosphere soil and to determine the influence of bacterial competition, mediated by soil temperature, on host colonization by *P. aphanidermatum*.

MATERIALS AND METHODS

Soil samples. Rhizosphere soil samples were obtained from 6- to 8-mo-old sugarbeets grown in a furrow-irrigated field plot located at the University of Arizona Campbell Avenue Farm in Tucson. Soil type at this location is an Aqua loam, pH 7.25 (51.9% sand, 33.0% silt, and 15.1% clay). The crop was irrigated every 14 days and, unless otherwise specified, all rhizosphere soil samples were collected immediately before irrigation. Soil moisture content (10 cm soil depth) at the time of collection was approximately 7% (−1.4 MPa).

Intact sections of rhizosphere soil bearing the impression of the root-soil interface were carefully extracted from the field by spading (18). Rhizosphere sections of soil were transported to the laboratory and processed within 1 h after collection.

Bacterial population dynamics. Generation time of resident bacteria per gram of rhizosphere soil was assessed as follows. Intact sections of rhizosphere soil were placed (rhizosphere-side up) on a bed of vermiculite in plastic trays, and sufficient tap water was then added to the vermiculite to saturate the rhizosphere sections. This procedure simulated movement of irrigation water into the rhizosphere. Trays containing the rhizosphere sections were then incubated at 20 and 27 C. After various intervals of time, which ranged from 0 to 24 h, three soil samples, each measuring 1 $cm^2 \times 0.1$ cm deep and weighing 0.4 g, were scrapped from each of three rhizosphere soil sections, individually placed in 200 ml of sterile distilled water (SDW) in a 250-ml flask containing glass beads, and mixed for 15 min. Serial 10-fold dilutions were made, and 0.1-ml aliquots of appropriate dilutions were plated in triplicate on King's medium B (7). Colony counts were made after 72 h of incubation at 27 C. Resultant bacterial colonies were expressed as colony forming units (cfu) per gram of soil. The experiment was conducted three times and results from a single representative experiment were chosen for presentation.

Additionally, to simulate the influence of root exudates on the population dynamics of rhizobacteria, intact sections of rhizosphere soil were collected in the field, brought to the laboratory, and amended as follows. Immediately before a simulated irrigation, four 1-$cm^2 \times 0.3$-mm thick pieces of freshly cut potato tuber tissue (i.e., bait) were placed on the surface of each of three rhizosphere soil sections. Soil immediately under each piece of bait, to a depth of 1 mm, was collected and assayed as described above at various time intervals. The experiment was conducted

three times, and results from a single experiment were chosen for presentation.

Oospore germination. Effects of incubation temperature on the rate of oospore germination and germ tube growth in intact rhizosphere soil were assessed as follows. Intact sections of rhizosphere soil were collected as previously described, placed on a bed of vermiculite, and incubated at 20 or 27 C. After 2 h, the rhizosphere soil sections were irrigated as previously described. Immediately after wetting, 0.5 ml of a suspension of exogenously dormant oospores (containing 2×10^4 oospores per milliliter) (17) were dispensed onto marked sites on the surface of the rhizosphere sections. Preliminary studies showed that 88% of the oospores were germinable. Artificially infested rhizospheres were then replaced at their respective incubation temperatures. Direct observation of oospore behavior in rhizosphere soil at various time intervals was accomplished by microscopic examination of stained soil smears (13). Percent oospore germination and germ tube lengths were expressed as the average of three observations, 100 oospores per observation at each incubation temperature. The experiment was conducted three times.

Oospore germination in the above studies occurred in the presence of the resident rhizobacterial population and a limited supply of nutrients (i.e., no influx of root exudates). Additional studies were therefore conducted to determine if percentage oospore germination could be influenced by the addition of an exogenous source of nutrients (i.e., simulating root exudates moving into the rhizosphere) and/or inhibition of bacterial competition for these nutrients. Intact sections of rhizosphere soil, artificially infested with oospores, were drenched with 1% asparagine (which served as a exogenous source of nutrition), drenched with 1% asparagine containing 2,000 μg/ml vancomycin, or drenched with sterile distilled water containing 2,000 μg/ml vancomycin. Vancomycin, an antibiotic that inhibits cell wall synthesis, is primarily effective against gram-positive bacteria and exhibits no fungal activity. Preliminary data (authors, *unpublished data*) indicated that *Bacillus* spp. were the dominant bacteria isolated from rhizosphere soil and were the dominant bacteria isolated from the rhizoplane of mature sugarbeet roots (16). Amended rhizosphere soil sections were then incubated at 20 and 27 C. Oospore germination was assayed as described above. The experiment was repeated two times and results from a single representative experiment were chosen for presentation. Data were analyzed using SAS two-way ANOVA. Treatment means at each time interval were compared by calculating LSD at $P < 0.05$.

Host colonization by *P. aphanidermatum*. A quantitative bioassay (14), which permits evaluation of the absolute inoculum potential of *P. aphanidermatum* and simulates microbial interactions occurring in the rhizosphere, was used to determine the influence of soil temperature and bacterial antagonism on host colonization by *P. aphanidermatum*. A naturally infested, air-dried field soil, containing 16 ± 2 oospores per gram of soil (4), was placed in petri dishes (150 × 50 mm). Petri dishes were then placed at 20 or 27 C. After 2 h incubation time, soil in the dishes was brought to saturation with either SDW or SDW containing 2,000 μg/ml vancomycin. Immediately after the soil samples were wetted, 20 pieces of fresh potato tuber tissue, 1-cm^2 × 0.3-cm thick, were placed 1 cm apart on the surface of the soil in each dish. Baited samples were then replaced at their respective incubation temperatures. After various time intervals, which ranged from 1 to 72 h, baits were removed, washed in running tap water for 5 min, blotted dry, plated on a selective medium (4), and incubated at 37 C. Percentage of baits colonized by *P. aphanidermatum* was determined after 24 h. The experiment was conducted three times. Data from the three experiments were combined after homogeneity of error variance was proven by Bartlett's test and were analyzed using SAS two-way ANOVA. Treatment means at each time interval were compared by calculating LSD at $P < 0.05$.

In addition to determining percentage of baits colonized by *P. aphanidermatum*, estimates were also made regarding the effects of vancomycin on the population dynamics of the resident bacterial flora in the soil at 20 C. Population densities of aerobic bacteria were determined in soil samples collected before baiting (0 time) and at various time intervals (4, 8, 12, and 24 h) after baiting. A 0.4-g sample of soil (1 $cm^2 \times 0.1$ cm deep) was collected directly underneath each bait (equivalent to rhizosphere soil) and 1 cm away from the baits (equivalent to nonrhizosphere soil) and individually placed in 200 ml of SDW in a 250-ml flask

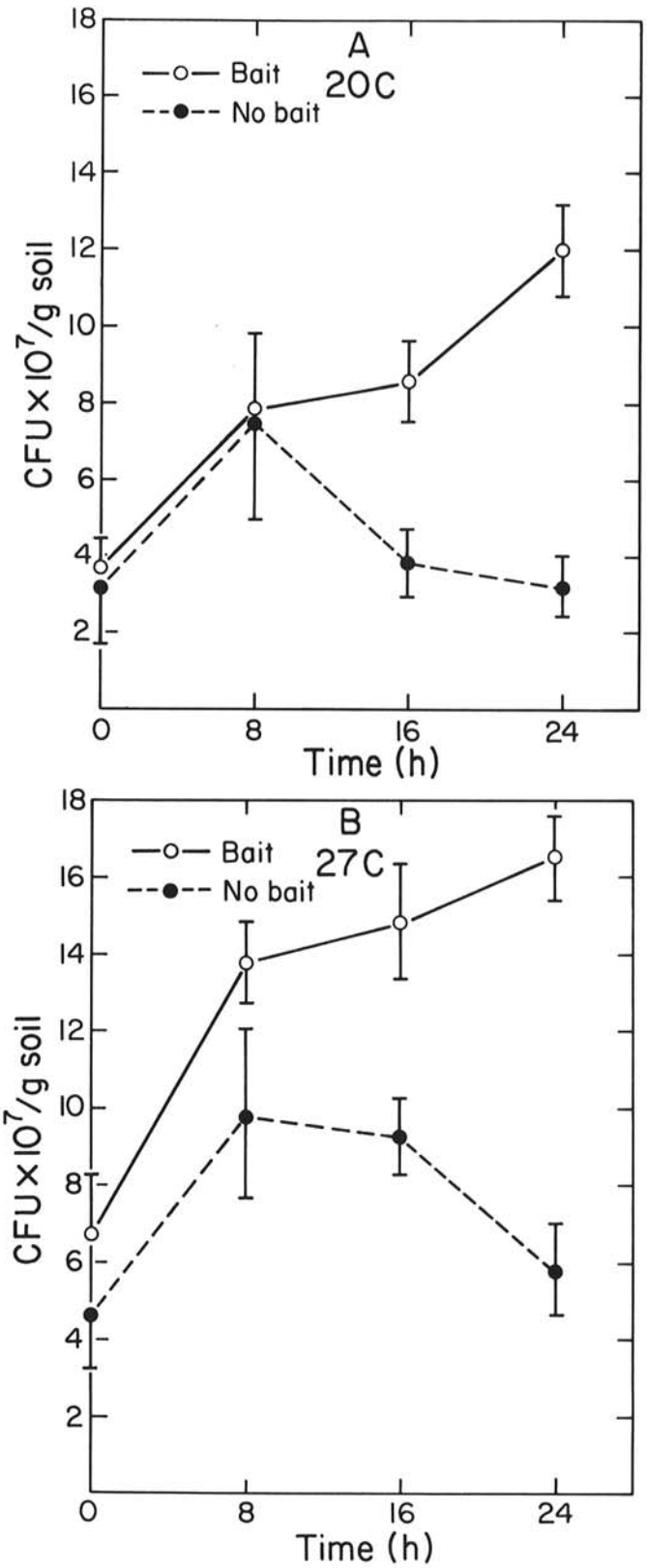

Fig. 1. Population density of bacteria in intact rhizosphere soil at various times (h) following an irrigation. Data are given for bacterial population densities in soil directly under potato tuber tissue (bait) and in the absence of bait (no bait) at **A,** 20 C, and **B,** 27 C. Standard errors are shown.

containing glass beads and mixed for 15 min. Serial 10-fold dilutions were made, and 0.1-ml aliquots of appropriate dilutions were plated in triplicate on King's medium B. Resultant bacterial colonies were expressed as cfu/g of soil. The experiment was conducted two times and results from a single representative experiment was chosen for presentation.

RESULTS

Bacterial population dynamics. The population dynamics of bacteria were estimated in intact rhizosphere soil samples before and after simulated irrigation in the laboratory for treatments where potato tuber tissue (bait) was present and absent (no bait). For each treatment, samples were maintained at either 20 or 27 C. For both treatments across both temperatures, bacterial populations were found to double in the first 8 h following the application of water (Fig. 1A and B). Growth rates for all populations diminished over the next 16 h, becoming negative for no-bait treatments but remaining positive for bait treatments.

Oospore germination. Direct observation showed that oospore germination occurred in unamended (control treatment) rhizosphere soil sections within 4 h and progressively increased to a maximum of 53% after 12 h incubation at 27 C (Fig. 2A). At 20 C, however, germinated oospores were observed in unamended rhizosphere sections only after 8 h of incubation and reached a maximum of 11% after 12 h (Fig. 2B). Germ tube growth rates of *P. aphanidermatum* were also influenced by temperature. Germ tube growth rates of 146 ± 30 and 636 ± 53 μm/h were recorded at 20 and 27 C, respectively. Significant increases in percentage germination were recorded in rhizosphere soils amended with asparagine, vancomycin, or vancomycin plus asparagine at 20 C (Fig. 2B), whereas at 27 C significant increases in percentage germination were recorded only in rhizosphere soils amended with vancomycin plus asparagine (Fig. 2A).

Host colonization by *P. aphanidermatum*. In the absence of vancomycin (control), host colonization by *P. aphanidermatum* was significantly greater ($P < 0.05$) at 27 C than it was at 20 C for all time intervals measured. At 27 C, there was no significant difference in host colonization by *P. aphanidermatum* between vancomycin and unamended (control) soil treatments (Fig. 3B). However, at 20 C, host colonization by *P. aphanidermatum* was significantly higher in soils treated with vancomycin compared to unamended (control) soils (Fig. 3A).

Effects of vancomycin on bacterial population densities in soil are presented in Fig. 4A and B. Bacterial populations in rhizosphere as well as nonrhizosphere unamended (control) soil doubled within 8 h but required more than 12 h to double in vancomycin-amended soils.

DISCUSSION

The overall objective of our study was to determine the mechanisms associated with lack of host colonization by *P. aphanidermatum* at soil temperatures below 27 C. Bacterial antagonism

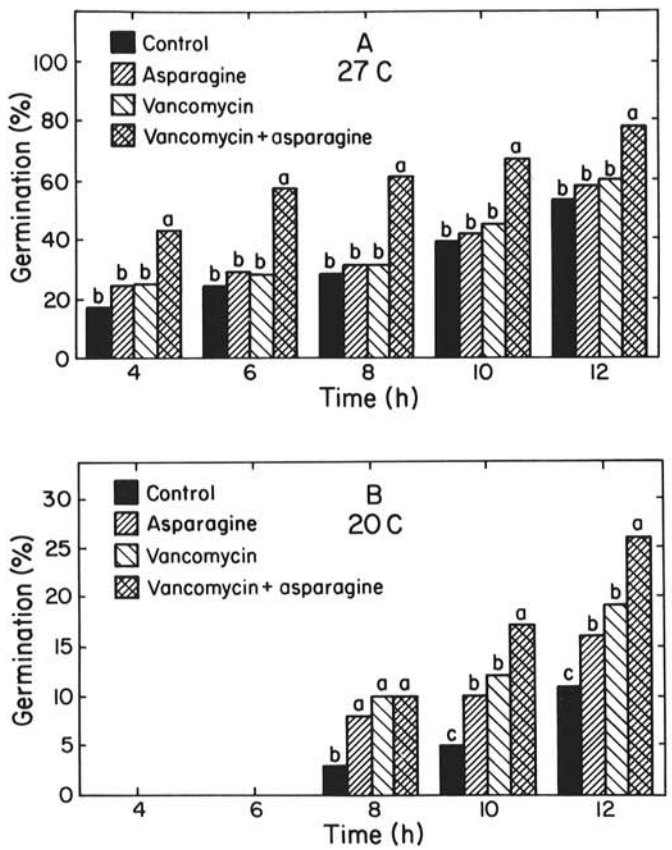

Fig. 2. Percent germination of oospores of *Pythium aphanidermatum* in intact rhizosphere soil at **A,** 27 C, and **B,** 20 C. Data are given for unamended soil (control), and soils amended with a food base (asparagine), an antibiotic (vancomycin), and a food base plus an antibiotic (asparagine plus vancomycin). Differences ($P < 0.05$) among treatments at each time interval are indicated.

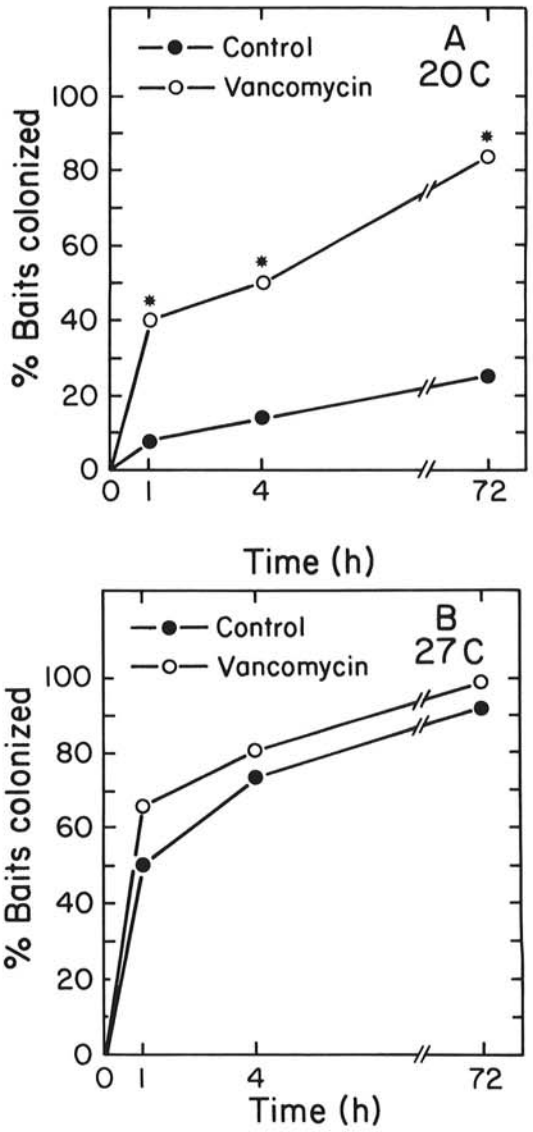

Fig. 3. Host colonization by *Pythium aphanidermatum* measured as percentage of potato tuber baits colonized at **A,** 20 C, and **B,** 27 C. Data are given for unamended soil (control) and soil amended with an antibiotic (vancomycin). Differences ($P < 0.05$) between treatments at each time interval are indicated by an asterisk.

in the rhizosphere was probably responsible for the general suppression of pathogen activity at low soil temperatures. Results showed that little oospore germination (11%) or host colonization (25%) occurred at 20 C, whereas more than 50% germination and 90% colonization occurred at 27 C. When bacterial activity was inhibited or reduced by the addition of an antibiotic (vancomycin) to the soil, however, host colonization by the fungus at 20 C increased to 83%.

Percent and rate of oospore germination, as well as germ tube growth rates, were significantly lower at 20 C than at 27 C. For example, about 20% of the oospores germinated within 4 h in rhizosphere soil at 27 C but required 8–12 h at 20 C. Germ tube growth rates at 27 C were also about 4 times faster than at 20 C. In contrast, the generation time (doubling time) of the resident bacterial population in rhizosphere soil was approximately 8 h and apparently was not affected by soil temperatures. Thus, at low soil temperatures the relative competitive advantage (i.e., the advantage of one organism over another as determined by their respective growth rates) for utilization of nutrients required for microbial growth apparently favors the rhizobacteria over the pathogen. When bacterial activity was inhibited by addition of vancomycin at 20 C, the relative competitive advantage favored the fungus. At high soil temperatures (27 C or greater), the fungus, by virtue of its speed and rate of germination, has the competitive advantage over the resident bacterial population in the rhizosphere. These results dramatically illustrate the effect(s) of temperature on the outcome of interactions between a root-infecting pathogen and the resident bacterial flora in the root-soil interface.

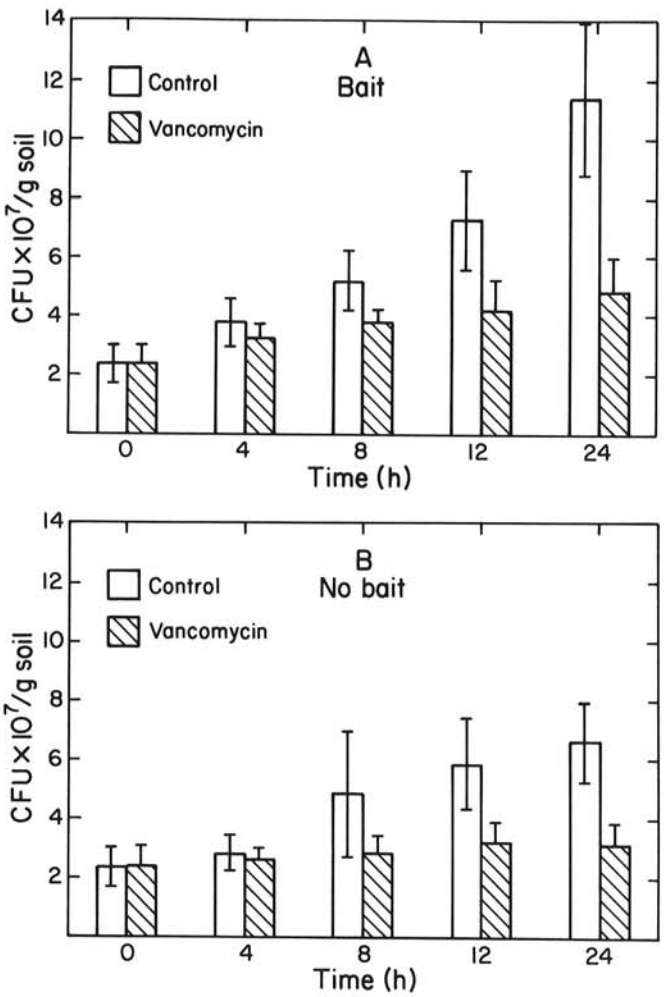

Fig. 4. Population density of bacteria in soil at various time intervals (h) following an irrigation at 20 C. Data are given for bacterial population densities in unamended soil (control) and soil amended with an antibiotic (vancomycin). **A,** soil directly under potato tuber tissue (bait), and **B,** in the absence of bait (no bait). Standard errors are shown.

The above scenario is based upon comparisons of the relative growth rates of competing rhizosphere microorganisms, and such comparisons have been reported for several other tripartite rhizosphere interactions (8,9,10). Generation times alone, however, may not be the only factor involved in determining the outcome of rhizosphere interactions. A high incidence of host colonization by *P. aphanidermatum*, assessed by the potato bait method (which simulates rhizosphere-rhizoplane interactions), occurred within 1–4 h at a suppressive soil temperature (i.e., 20 C) when bacterial activity was reduced or inhibited by the addition of vancomycin to the soil. These results indicated that, in the absence of vancomycin, antagonism at 20 C occurred within 1–2 h and definitely before any significant multiplication of the resident rhizosphere bacterial flora. Although the specific antagonistic mechanism involved in early tripartite interactions in soil has not been identified, a recent study by Osburn et al (9) on the dynamics of seed colonization by *P. ultimum* versus *Pseudomonas* (which was applied as a seed treatment) indicated that antibiosis may be involved (9). A similar conclusion was reported by Lifshitz et al (8), who investigated the nature of *Pythium* suppression by *Trichoderma* spp. Results of greenhouse investigations by Elad and Chet (6), however, indicated that antibiosis was not involved and that competition for nutrients (i.e., host exudates) between *P. aphanidermatum* and certain bacteria (applied either directly to the seed or soil) was correlated with suppression of root disease. Oospore germination was decreased in the presence of these bacteria. In our study, oospore germination increased in soils amended with asparagine and/or vancomycin at 20 C. These results indicated that competition for nutrients may be involved in suppression of *P. aphanidermatum* at low soil temperature. Our results also indicate that the nutrients required for bacterial multiplication or oospore germination were not dependent upon an influx of nutrients from the host (i.e., exudates). Apparently, sufficient nutrients were either present or released from dry soil upon rewetting.

Although soil temperature plays a primary role in determination of the outcome of interactions between *P. aphanidermatum* and the resident bacterial flora, soil moisture is also equally important. Soil moisture levels under field conditions can fluctuate dramatically (0 to −1.4 MPa) depending on the frequency of irrigation. Previous studies have demonstrated that oospore germination and host colonization by *P. aphanidermatum* is restricted to soil moisture levels between 0 and −0.1 MPa (13). Similarly, maximum bacterial activity also occurs at these same soil matrix potentials (5). Based upon the apparent sensitivity of *P. aphanidermatum* to bacterial antagonism, the question arises as to the precise timing of root colonization by *P. aphanidermatum* under field conditions. Our hypothesis is that oospore germination and root colonization occur primarily within a short period of time (perhaps 1–2 h) immediately following an irrigation. Pathogen activity, as well as bacterial activity, in rhizosphere soil would be minimal immediately before an irrigation. Upon irrigation, intense competition for utilization of soluble nutrients would occur. *P. aphanidermatum* would, depending on the temperature, have the competitive advantage over resident bacteria by virtue of the rapidity of oospore germination and rate of growth. Knowledge of the precise timing of root infection under field conditions may result in the development of a disease management strategy. Such studies are currently under investigation.

These results support and extend the characterization of *P. aphanidermatum* as an opportunistic root-infecting pathogen. The fungus is apparently not a competitive rhizosphere inhabitant and essentially plays a waiting game until an opportune time presents itself for activity. This opportune time apparently exists upon an irrigation in the presence of a conducive soil temperature—a brief period of time when the fungus has the competitive advantage over rhizosphere bacteria.

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