In Vitro and in Vivo Effects of *Pseudomonas* spp. on *Pythium aphanidermatum*: Zoospore Behavior in Exudates and on the Rhizosphere of Bacteria-Treated Cucumber Roots

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**ABSTRACT**


Roots of 4-day-old cucumber (*Cucumis sativus* cv. Corona) seedlings grown in hydroponic solution in test tubes were either not treated or treated with one of five strains of *Pseudomonas corrugata* and *P. fluorescens*. After 24 h, one-half of the plants were transferred to tubes with fresh nutrient solution (changed treatment) and one-half were left in the original solution with the bacteria (no-change treatment). After 48 h, the nutrient solution containing root exudates was filtered-sterilized and tested for ability to attract zoospores of *Pythium aphanidermatum*, using a capillary tube bioassay. Fewer zoospores were attracted to root exudates from seedlings treated with any bacterial strain (no-change treatment) than to root exudates from seedlings not treated with bacteria; the distance of zoospore travel in the capillary tubes was also reduced. Germination of zoospores in root exudates from bacteria-treated roots was also significantly reduced by four of the five strains, in both the changed and no-change treatments. Three-day-old seedlings treated or not treated with bacteria were inoculated with a zoospore suspension of *P. aphanidermatum*. Two hours after inoculation, the roots were fixed, stained with malachite green, counterstained with acriflavine orange, and observed with epifluorescence microscopy. Observations of 0.3-mm sections from the first 20 mm of root tip from each plant were recorded with video microscopy, and the number of encysted zoospores and percent zoospore germination were determined. Frequency of sections with no zoospores was higher in roots treated with bacterial strains than in those not treated, and the frequency of sections with >30 zoospores was reduced. One of the strains also increased the frequency of sections with no zoospore germination, whereas another strain decreased the frequency of sections with 51–75% germination. These bacteria reduced the attraction, encystment, and germination of zoospores of *P. aphanidermatum* both in situ and in root exudates from treated cucumber seedlings, possibly by utilizing carbon and nitrogen compounds in the root exudates that are required by *P. aphanidermatum* prior to infection.

*Pythium aphanidermatum* (Edson) Fitzp, reproduces asexually and is dispersed by biflagellate zoospores. Zoospores in aquatic media, such as soil water or plant nutrient solution in hydroponic systems, respond to chemoattractants, particularly amino acids produced by the host. They swim to and accumulate on the root surface, where they are induced to encyst by chemoattractants or by root surface polysaccharides that may interact with surface receptors on the zoospore (*6,*9,10,12,14,15,23). The zoospore cysts germinate rapidly in response to certain amino acids (10). A suitable inductive surface also may be needed for germination of the cysts (30). Germ tubes from the cysts penetrate plant tissue, resulting in stem or root infection. It is obvious that zoospore motility, encystment on the plant surface, and germination of the cysts are important in the infection process. Thus, one of the strategies to control *Pythium* diseases is to target zoospores.

Application of different species of *Pseudomonas*, originally isolated from the rhizosphere of cucumber plants, to plant culture media such as nutrient solution or rock wool reduced root and stem rot of cucumber in both laboratory and greenhouse experiments (18,19). Previous studies showed that some of these bacteria also promoted the growth of cucumbers and induced resistance to the pathogen (18,31). These bacteria also may affect the pathogen, especially the zoospores at the prepenetration stages, since bacteria and zoospores may occupy the same ecological niche on the root surface, which may be the sites of root exudation (21). Root exudates, which contain many organic and inorganic substances, are important in the establishment and maintenance of populations of rhizosphere microorganisms (1,20,24). Root exudates also provide an external source of nutrients for germination and growth of encysted zoospores (3). Competition for nutrients from exudates of roots or seeds may be a common interaction between bacteria and pathogens on the root and may be responsible for some of the biocontrol by introduced bacteria (5,17,28). Competition for suitable sites for zoospore accumulation and encystment also may occur on the root surface between the zoosporic pathogen and the introduced bacterium.

The purpose of this study was to determine the effects of introduced rhizosphere bacteria on the behavior of zoospores and zoospore cysts of *P. aphanidermatum* in root exudates and on the roots of cucumbers prior to penetration.

**MATERIALS AND METHODS**

Bacterial and fungal cultures. All bacterial strains used in this research were isolated from the rhizosphere of *Cucumis sativus* L. 'Corona' and were cultured and stored as previously described (18). Bacteria were identified using the Biolog GN Log microplates and the microcomputer database (Biolog, Inc., Hayward, CA). These bacteria included *Pseudomonas corrugata* (strains 13 and 35), *P. fluorescens* subgroup C (strains 15 and 16), and *P. fluorescens* subgroup E (strain 27). Zoospores of *P. aphanidermatum* were produced by growing the fungus on V8 juice agar in petri plates at 26 C for 48 h. The culture was cut into 1-cm-wide strips, one-half of the strips were transferred to an empty petri plate, and both plates were flooded with 20–25 ml of sterile distilled water. After 0.5–1 h, the water was removed and replaced with the same amount of water. Plates were incubated at 35 C under fluorescent lights for 18 h, then at 20 C for 4 h to stimulate zoospore release. The final concentration of zoospores ranged from 10,000 to 80,000/ml.

Exudate of cucumber roots. Seeds of cucumber cv. Corona were germinated on moist filter paper in a petri dish for 2 days in the dark at 26 C and for two additional days on a growth
bench (26°C and 16-h photoperiod with combined incandescent and fluorescent light). The seedlings were then transferred into sterile test tubes (16 × 125 mm) filled with a plant nutrient solution containing 0.97 g/L of Peter’s Hydro-sol fertilizer (5-11-26) (W. R. Grace, Fogelsville, PA) and 0.64 g/L of Ca(NO₃)₂. Two layers of Parafilm were wrapped around the top of the tubes to secure the seedlings. Tubes were placed on a rotary shaker (80 rpm) on the growth bench. One day after the seedlings were transferred, a bacterial suspension was added to the tubes to give a final concentration, determined by measuring optical density, of 1.0 × 10⁶ cells per milliliter of nutrient solution. The following six treatments were tested: control (no bacteria added) and bacterial strains 13, 15, 16, 27, and 35. Each treatment consisted of eight seedlings and was divided into two subtreatments. Twenty-four hours after addition of bacteria, one-half of the cucumber seedlings were transferred to tubes with fresh nutrient solution with no added bacteria (changed), after gently blotting the roots with a sterile paper towel to remove excess water. The other plants remained in the same test tubes with the bacterial suspension (no change). Forty-eight hours after the addition of bacteria, plants were removed from the tubes and the nutrient solutions were filter-sterilized through a 0.2-μm hydrophilic nylon membrane in a syringe filter unit. The filtrates containing exudates from the roots of each plant were used for the tests of attraction and germination of zoospores and will be referred to as root exudates.

Attraction of zoospores to root exudates. A sample of root exudate from each of four seedlings per treatment was tested. An autoclaved solution of 0.5% purified water agar (Difco) was added to the cucumber root exudate sample to give a final exudate concentration of 10% (v/v). The agar-exudate mixture was used to fill capillary tubes (5 μl-micropropettes; Drummond Scientific Co., Broomall, PA), which were used to test the attraction of zoospores to root exudates, as described previously by Paulitz et al. (18). One end of the filled tubes was held for 1 h in a suspension of 5 × 10⁶ zoospores per milliliter. Tubes were then placed horizontally on a glass slide in a closed container filled with vapor of formaldehyde to kill the zoospores. Beginning with the end that was immersed in the zoospore suspension, the number of zoospores in each of five 0.9-mm sections in each tube was counted with the aid of an ocular micrometer and a compound microscope. Root exudate samples were taken from each of four replicate plants in each treatment. Root exudates from plants not treated with bacteria served as a control. For two additional controls, distilled water and Peters Hydrol-sol nutrient solution were mixed with 0.05% purified water agar at 10% (v/v) and tested in the capillary tubes.

Zoospore germination in vitro. A zoospore suspension produced on V8 juice agar was passed through one layer of Kimwipes tissue (Kimberly-Clark, Mississauga, Ont.) and filtered through a nylon membrane filter (0.45 μm). Zoospores on the filter were then washed out with sterile distilled water, and the concentration of the zoospore suspension was adjusted to 10⁶ zoospores per milliliter. A 0.45-ml aliquot of filtered root exudate from each plant was mixed with the zoospore suspension (0.05 ml) in a 1.5-ml microcentrifuge tube, which was shaken on a vortex shaker for 30 s to allow zoospores to encyst. Thirty 0-μl drops of the suspension were placed in individual rings on a ceramic ring slide. Slides were incubated in a moist chamber for 2 h before a drop of 95% ethanol was added to the ring to kill the cysts. Germination of cysts was determined by using a compound microscope to count 100 cysts in each drop.

Zoospore behavior on the root surface. Three-day-old cucumber seedlings were transferred to the plant nutrient solution in glass tubes (16 × 125 mm). The bottom opening of each tube was blocked with a rubber serum-bottle stopper, and the top was sealed with Parafilm. Seedlings were treated with a bacterial suspension of strain 13, 15, 16, 27, or 35, to give a final concentration of 1.0 × 10⁶ cells per milliliter of nutrient solution. A treatment without added bacteria served as a control. Each treatment contained six replicate plants. Tubes were placed on a rotary shaker (80 rpm) on a controlled-temperature growth bench at 26°C under a 16-h light/dark cycle with combined incandescent and fluorescent light. One day after the seedlings were transferred and treated with bacteria, a syringe and needle were used to inject a zoospore suspension into each tube through the rubber stopper. Because zoospores are negatively geotactic, they were injected into the tube bottom. This method also put the zoospores in proximity to the root tip without agitating or mixing the solutions, which would cause zoospore encystment. The final concentration of zoospores in the tubes was 1,000/ml. Two hours after addition of zoospores, the plants were removed and the main roots were cut and fixed in a solution of formaldehyde-acetic acid-ethanol (FAA). The fixed roots were stained for 5 min with 0.5% malachite green, washed with distilled water, and then counterstained for 20 min with 0.001% acridine orange in a boric acid-borax buffer (pH 8.6). Stained roots were observed under UV light with an epifluorescence microscope (200X magnification). A black and white video camera (JVC TK-S300) and a black and white monitor (JVC TM 920) were used to observe the roots under the microscope, and the images were recorded with a Sony SVO 140 video recorder. The video camera recorded a 0.3-mm-diameter field of view, and 20 mm of the primary root of each seedling, starting from the root tip, was recorded section by section. The number of zoospore cysts in each field of view was rated using the following encystment classes: 0 = no cysts, 1 = 1–2 cysts, 2 = 3–5 cysts, 3 = 6–10 cysts, 4 = 11–20 cysts, 5 = 21–30 cysts, 6 = 31–40 cysts, 7 = 41–50 cysts, and 8 = >50 cysts per each 0.3 mm of root length. Germination of cysts was rated using the following classes: 1 = no germination, 2 = <25% cysts germinated, 3 = 25–50% cysts germinated, 4 = 51–75% cysts germinated, and 5 = >75% cysts germinated per 0.3-mm root section. Percent frequency of occurrence of each rating class on each primary root was calculated using 100 × n/NZ, i = 1, 2, ... 8 for number of zoospore cysts and 100 × n/NZ, i = 1, 2, ... 5 for germination of zoospore cysts, where n is the number of sections in ith class, i is the number of the class, and NZ is the number of the total sections on the 20-mm-long root (68 sections).

Fig. 1. Effect of strains of *Pseudomonas* spp. and changing of nutrient solution on the attraction of zoospores of *Pythium aphelendermatum* to cucumber root exudates in capillary tubes. Seedlings were treated for 24 h with bacterial strain 13, 15, 16, 27, or 35 (10⁶ cells per milliliter) or no bacteria (Cont) and transferred to fresh nutrient solution for another 24 h (changed). Another set of seedlings with the same treatments were left in the nutrient solution for 24 h (no change). Root exudates from each seedling were filter-sterilized, mixed with 0.05% purified water agar (10%, v/v), and drawn into 5-μl capillary tubes. One end of the tube was placed in a zoospore suspension, and the number of encysted spores was counted in each section of the tube after 1 h. Tubes filled with water or nutrient solution (Hydro) with the same concentration in water agar as the root exudates served as controls. Values are means of four replicates. Bars with the same shading and the same lowercase letters are not significantly different from the nonbacteral control according to Duncan’s multiple range test (*P* = 0.05).
Data analyses. Data were analyzed with SAS, using the general linear models procedure, including analysis of variance, Duncan's multiple range test, and LSD test ($P = 0.05$). Percentage data for the zoospore germination experiment in root exudates were arcsine-square root transformed before analysis. All experiments in this report were performed twice, and $F$ tests showed no significant differences between the variances of the experiments. The data presented in the figures were from the first trial. The Kruskal-Wallis test, and Dunn's multiple comparison procedure were used to analyze differences in class frequencies among bacterial treatments, since these data did not satisfy the assumptions of analysis of variance.

RESULTS

Attraction of zoospores to root exudates. Both bacterial treatment and transfer of seedlings into fresh nutrient solution after 24 h had a significant effect on the attraction of zoospores to root exudates, and there was a significant interaction between these two variables. In the plants not transferred to fresh nutrient solution (no change), treatment with strain 13, 15, 27, or 35 significantly reduced the average number of zoospores per section of capillary tube, compared with seedlings not treated with bacteria (Cont, Fig. 1). The tubes containing water or nutrient solution (Hydro) also attracted fewer zoospores than did tubes containing root exudates from plants not treated with bacteria and not transferred to fresh nutrient solution. However, there was no significant effect of bacterial treatments on root exudates from seedlings transferred to fresh nutrient solution. Bacterial treatments also influenced the distance that zoospores swam in capillary tubes, and there was a significant interaction between these two factors (Fig. 2). Treatment with strain 13, 15, 27, or 35 significantly reduced the number of zoospores found in the distal sections of the capillary tube, farthest from the zoospore suspension, when compared with the nonbacterial treatment (Cont). However, bacterial treatment had no effect on the number of zoospores in the proximal sections of the capillary tube. Water or nutrient solution (Hydro) attracted fewer zoospores than root exudates from nontreated seedlings in all sections of the capillary tube.

Zoospore germination in vitro. Germination of zoospores cysts of *P. aphanidermatum* in root exudates from plants treated or not treated with bacteria was determined in vitro. Percent germination was significantly higher in root exudates from the no-bacteria control seedlings than in roots treated with bacteria, with one exception, strain 16 in the changed solution (Fig. 3). This reduction of cyst germination in exudates from bacteria-treated roots was seen in treatments in which the nutrient solution was changed or not changed, and there was no significant interaction between the changing treatments and strains. Cyst germination in distilled water was significantly less than in exudates from roots not treated with bacteria, but germination in nutrient solution (Hydro) was not significantly different from the no-bacteria control.

Zoospore behavior on the root surface. Zoospores were not evenly distributed on the root surface. The ratio of the variance to the mean number of zoospores per section on roots not treated with bacteria was 33, indicating a highly aggregated, nonrandom distribution. More zoospores were found on the zone of elongation than on the root tip or zone of differentiation. In addition, clusters of zoospores were observed around the lateral root initials or small lateral roots. Treatment with bacteria for 24 h did not change the overall morphology or length of the root. On roots of the no-bacteria control, 69% of the sections did not contain zoospores. On roots treated with bacterial strains, a significantly greater number of sections were devoid of zoospore cysts (82-87%, data not shown). Most sections had fewer than five zoospores (Fig. 4). The percent frequency of sections with 21-30 zoospores per section was significantly lower on roots treated with strain 13 or 27 than on roots of the no-bacteria control. All bacteria treatments, except strains 16 and 35, had significantly fewer sections with >50 zoospores per section than the no-bacteria control. Treatments with strain 13 had fewer sections with >20 zoospores per section than the no-bacteria control.

In sections with a small number of widely spaced zoospores, percent germination was very low. In contrast, percent germination was higher in the sections where zoospores were clustered together. In general, no significant differences in the frequency of germination classes were found between the bacteria treatments and the no-bacteria control, except for strains 13 (higher frequency of sections with no germination) and 27 (lower frequency of sections with 51-75% germination) (Fig. 5).
DISCUSSION

More zoospores entered the capillary tubes filled with root exudate from plants not treated with bacteria than those filled with distilled water or Hydro-sol nutrient solution. This suggests that zoospores of *P. aphanidermatum* exhibit chemotaxis to cucumber root exudates in the hydroponic system. Cucumber root exudates and nutrient solution also stimulated the germination of zoospore cysts (Fig. 3). Similar results were reported when pea root exudates were tested (3). When the nutrient solution was not changed, zoospore attraction to exudates from roots treated with some bacterial strains was weaker than to exudates from the no-bacteria control. However, this effect was less pronounced in treatments where the nutrient solution was changed, suggesting that the high population density of bacteria in the nutrient solution of the nontransferred plants (10^6 cells per milliliter) may be more effective than the bacteria that adhered to the rhizoplane in the treatments where the solution was changed. Also, cyst germination was lower in root exudates from the bacterial treatments than in those from the no-bacteria control. The weaker attraction and lower germination in root exudates tested in vitro implies that bacteria may reduce the number of zoospores that accumulate and encyst on the root surface and reduce the germination of encysted zoospores. The reduction in attraction and encystment of zoospores was also seen in situ on cucumber roots (Fig. 4). However, the effect of bacteria on zoospore germination on the rhizoplane was less dramatic, since only strains 13 and 27 significantly reduced germination. On the root surface, bacteria may have a stronger influence on attraction and encystment than on germination. Our observations also confirmed the findings of other researchers (10) that encysted zoospores of *P. aphanidermatum* are distributed in a nonrandom or aggregated pattern on the rhizoplane. Bacteria also have a nonrandom or aggregated distribution on the root (13,16). This aggregation may reflect the sites of maximum exudation on the rhizoplane, to which the zoospores are preferentially attracted and where bacterial colonies can grow. Another hypothesis is that zoospores autoaggregate because of a signal released from an initial encysting zoospore (26).

The reduction in zoospore attraction and germination of encysted zoospores in root exudates from bacteria-treated plants may be due to competition between zoospores and bacteria for carbon and nitrogen. Bacteria, especially fluorescent pseudomonads, are nutritionally versatile and grow rapidly in the rhizosphere (27,28). The *Pseudomonas* strains used in this study were initially isolated from the rhizosphere of cucumbers (18). Large populations of bacteria established on roots become a partial sink for carbon substrates, thus reducing the amount of substances that attract, trigger, or stimulate fungal spore germination or provide energy for penetration (3,4). In our experimental system, the roots were the sole source of carbon substrates. The bacteria may also alter the levels of cations such as calcium that can influence zoospore encystment and germination (2,8). A high level of zoospore germination was observed in nutrient solution with or without exudates from nonbacterial treated plants (Hydro and Cont, Fig. 3), and germination was reduced by the addition of bacteria. Besides competing for root exudates, the bacteria may have reduced the level of cations responsible for high germination in the nutrient solution. Also, the zoospores and bacteria on the cucumber root may have competed for space. Bacteria were estimated to cover only 4-10% of the root surface in natural soil (22). However, certain areas on the root, such as cell junctions and points of emergence of lateral roots, are favored for bacterial colonization (25). Clusters of bacteria on these areas were observed on cucumber roots in our study. These areas are also the most vulnerable to attack by *Pythium* zoospores (7). We observed zoospore clusters in these areas, as did Wester et al (29). The bacteria that colonized the root surface may have occupied some of the sites suitable for the accumulation and encystment of zoospores. Bacteria in the nutrient solution and on the root surface also may have consumed substances secreted by plant roots, resulting in less attraction of the zoospores to the root and fewer

![Fig. 4](image_url)

**Fig. 4.** Effect of strains of *Pseudomonas* spp. on the attraction and encystment of zoospores of *Pythium aphanidermatum* on the rhizoplane of cucumber. Three-day-old cucumber seedlings were treated with bacterial strain 13, 15, 16, 27, or 35 (10^6 cells per milliliter) or no bacteria (Cont). Zoospores were added 1 day after bacterial treatment. Two hours after the addition of zoospores, the main root was removed, fixed, stained, and observed under an epifluorescent microscope, and recorded on videocassette. The number of zoospores were counted in each 0.3-mm-diameter field along the first 20 mm of the primary root of each seedling, starting from the root tip. Each section was placed into one of the following encystment classes: 0 = no cysts, 1 = 1 or 2 cysts, 2 = 3–5 cysts, 3 = 6–10 cysts, 4 = 11–20 cysts, 5 = 21–30 cysts, 6 = 31–40 cysts, 7 = 41–50 cysts, and 8 = >50 cysts per each 0.3 mm of root length. The frequency of each encystment class in each treatment was calculated and expressed as a percentage. Values are means of six replicate plants. Bars in each encystment class with a star symbol are significantly different from the nonbacterial control according to the Kruskal-Wallis test and Dunn’s multiple comparison procedure ($P < 0.05$).

![Fig. 5](image_url)

**Fig. 5.** Effect of strains of *Pseudomonas* spp. on zoospore germination of *Pythium aphanidermatum* on the rhizoplane of cucumber. Three-day-old cucumber seedlings were treated with bacterial strain 13, 15, 16, 27, or 35 (10^6 cells per milliliter) or no bacteria (Cont). Zoospores were added 1 day after bacterial treatment. Two hours after the addition of zoospores, the main root was removed, fixed, stained, and observed under an epifluorescent microscope, and recorded on videocassette. The percentage of cysts germinated in each 0.3-mm-diameter field was determined along the first 20 mm of the primary root of each seedling, starting from the root tip. Each section was placed into one of the following germination classes: 0 = no germination, 1 = <25%, 2 = 25–50%, 3 = 50–75%, 4 = 75–90%, 5 = >90%. Values are means of six replicate plants. Bars in each germination class with a star symbol are significantly different from the nonbacterial control according to the Kruskal-Wallis test and Dunn’s multiple comparison procedure ($P < 0.05$).
nutrients for the germination of the encysted zoospores. A smaller number of zoospores on the root surface with lower percent germination of encysted zoospores may result in less infection of plant roots treated with bacteria. Some of these same strains reduced disease severity and increased yields of greenhouse cucumbers grown in a rock wool system (19), giving further support to this hypothesis.

The possibility of antibiotic production by bacteria cannot be eliminated in these experiments. All the strains tested inhibited zoospore motility in vitro, and some inhibited zoospore germination (18). The changing of the nutrient solution (Figs. 2 and 3) may have diluted the potential antibiotic, thus explaining the reduction in inhibitory activity.

Another possibility is that the bacteria changed the morphology or physiology of the roots, thus affecting zoospore behavior. We did not see any effect of bacteria on root length or morphology, although it is doubtful that these changes would be expressed in only 24 h. Evidence for induced resistance also was found for two of these strains applied to cucumbers grown in rock wool, but these experiments were conducted over a longer period of time with more mature plants (31). Some of the strains, when applied without *P. aphanidermatum*, also increased root growth in 1-wk-old seedlings, although no consistent evidence of growth promotion has been observed in more mature plants. Many of the documented plant-growth-promoting rhizobacteria are *Pseudomonas* spp. (11). Competition for nutrients may be more important for reducing initial infection of the roots by zoospores, while induced resistance and growth promotion may function to slow the spread of the fungus to the crown of mature plants and help the plants compensate for the loss of roots to the pathogen.

The results presented here give further information on possible mechanisms of biological control of *P. aphanidermatum* in hydroponic systems but may not be applicable to soil systems. In addition, further in situ testing on older plants is required to see if these results can be extrapolated to plants growing under more normal conditions.

**LITERATURE CITED**


