DISEASE-SUPPRESSIVE VERMICOMPOST INDUCES A SHIFT IN GERMINATION OF *PYTHIUM APHANIDERMATUM* ZOOSPORANGIA

A Thesis

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by

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ABSTRACT

Composts are commonly used in agriculture to minimize losses from soil-borne plant pathogens such as *Pythium aphanidermatum*, the causal agent of seed and seedling rot in a wide range of host plants. Currently, there is a lack of understanding in how composts suppress pathogens and diseases, and the mechanisms by which suppression occur remain unclear. The goal of this study was to examine the developmental responses of *Pythium aphanidermatum* zoosporangia when exposed to compost to understand how suppression of Pythium seedling disease is expressed. Mature zoosporangia were exposed to liquid vermicompost extracts (VCE) and the developmental responses were monitored using time lapse photomicroscopy. Sterile and non-sterile VCEs inhibited viable zoospore production by inhibiting indirect germination and causing vesicles and zoospores to immediately lyse while at the same time stimulating direct germination and germ tube production. Additional treatments were tested to determine factors that stimulate direct germination or inhibit indirect germination. The pH (5-9 at 0.001 M) and ionic strength (0.1-0.0001 at pH 6) of potassium phosphate buffer did not alter zoosporogenesis compared to sterile water. However, decreasing the osmotic potential in glucose and sucrose from -248 to -2,712 kPa or in polyethylene glycol 8000 from -0.335 to -105 kPa caused indirect germination to decrease with a corresponding increase in direct germination. Significant infection was observed within 1 h when cucumber seeds exposed to zoosporangia germinating indirectly to produce zoospores in sterile water and directly to produce germ tubes in sucrose solution (0.5 M). Germ tube infection was suppressed after 1 h in treatments of VCE, but significant disease was observed after 2 h. Germ tube infection was greater in sucrose solutions than VCE at 1, 2, and 3 h of inoculum exposure. My data show that VCEs suppress

zoosporogenesis and stimulate direct germination; however, this did not lead to long-term disease suppression.

BIOGRAPHICAL SKETCH

Eric A. Carr was born February 11, 1983, in Front Royal, Virginia. In 2001, he began undergraduate studies at Millersville University where he majored in biology, minored in chemistry, and pursued a concentration in botany. While studying at Millersville University, Eric completed an honors thesis on plant-mycorrhizae associations. Eric received his B.S. in biology in 2005. For two years, he was employed as a research technician on dendropyroclimatological projects in the Department of Geography, Penn State University, State College, Pennsylvania. From 2007 to 2010 Eric was employed in the Department of Plant Pathology & Plant-Microbe Biology, Cornell University as a research technician on projects relating to fungal and bacterial pathogens of onion. During that time, he became a Master Composter of Tompkins County through the Cornell Cooperative Extension. Shortly after, Eric obtained a technician position in the laboratory of Dr. Eric Nelson where he assisted projects related to vermicompost-mediated disease suppression of *Pythium aphanidermatum*. In 2010, Eric began his graduate studies with Dr. Eric Nelson as his advisor. His Master's thesis examined the developmental responses of *Pythium aphanidermatum* zoosporangia when exposed to vermicompost to understand how suppression of Pythium seedling disease is expressed.

To my Mother and Father for all their guidance and encouragement.

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Introduction

Composts and other organic amendments are increasingly being used in agriculture to minimize losses from plant diseases (32). However, disease suppression with composts has been quite variable, limiting the widespread adoption of compost applications to control plant diseases in diverse cropping systems (3,33). Developing a better understanding of how composts suppress pathogens and diseases could increase the predictability of compost-induced disease suppression and thereby improve their effectiveness. Despite numerous investigations of the mechanisms of compost-induced disease suppression, our understanding of the multiple mechanisms that may lead to disease suppression remain insufficient to predict the pathosystems, soil conditions, and environmental conditions for which disease suppression may occur.

Previous approaches to understand the mechanisms of disease suppression have focused largely on the comparative bulk properties of suppressive and non-suppressive composts to identify factors involved with suppression (3). A consistent pattern that has emerged from these studies is the central role of microbial activity in compost-induced disease suppression (27,33). For Pythium seedling diseases in particular, the subset of bacteria that colonize seeds shortly after sowing may be the most critical for disease suppression (4,6) because of their direct interactions with the pathogen in the infection court, which interferes directly with pathogenesis (5). The significance of this spatial and temporal synchrony between plant-associated microbial activities and developmental responses of pathogens during infection emphasizes the importance of understanding these direct interactions to further identify how composts suppress disease.

Pythium aphanidermatum causes seed and seedling rots in over 60 different genera of plant hosts (14). Zoospores of *P. aphanidermatum* and many other zoosporic pathogens are often considered the main developmental stage responsible for plant infection (11,26) and therefore,

the most likely stage affected by disease-suppressive agents. During zoosporogenesis, zoosporangia germinate through an indirect germination mode by first giving rise to spherical vesicles into which zoosporangial cytoplasm is transported and where zoospores are subsequently differentiated and released (19). Once released, it is believed that zoospores are attracted to various sugars and amino acids present in seed and root exudates and/or electrical fields surrounding roots where they swim chemotactically towards the host surface (12,45). After reaching the host surface, zoospores attach, encyst, germinate, and then penetrate host tissues (11,19,44).

While many of the studies aimed at understanding the suppression of zoosporic oomycete pathogens have focused on the suppression of zoospore chemotaxis (23) and arrival at the host surface (17,18), any disruption of zoosporangial development, vesicle development, zoospore release or chemotaxis, attachment, encystment, and germination could equally result in the suppression of disease. However, this has not been explored previously. The factors that influence host-pathogen interactions after zoospores are released have been described (12), but the factors that influence the interactions before zoospore release are not well understood. Even the factors that influence the entire process of zoosporogenesis are poorly understood. Although *Pythium* zoosporogenesis has been observed empirically to be influenced by the transfer of cultures from nutrient-rich to nutrient-poor media or to water, changes in water relations and fluxes of cations (40,41,46), the specific mechanisms by which zoosporogenesis can be suppressed remain unknown.

The goal of this study was to examine the developmental responses of *P. aphanidermatum* zoosporangia when exposed to compost to understand how suppression of Pythium seedling disease occurs. A vermicompost substrate that has been shown to suppress

infections caused by *P. aphanidermatum* zoospore inoculum (25) was used in this study. It is currently unknown if zoosporangial development prior to zoospore release is susceptible to suppression. I predict that inhibition of any stage during zoosporogenesis (zoosporangial germination and cytoplasmic emptying, vesicle formation, zoospore differentiation, or zoospore release) would decrease the potential for zoospore inoculum to infect plants, ultimately leading to a reduction in disease. In addition, I attempt to identify additional biological and biophysical factors that influence zoosporogenesis and address how they relate to compost-induced disease suppression.

Materials and Methods

Maintenance and production of *Pythium aphanidermatum* zoosporangia. To maintain pathogen virulence and prevent bacterial contamination, surface-disinfested non-treated cucumber seeds (*Cucumis sativus* cv "Marketmore 76") were inoculated with *P. aphanidermatum* zoospores by placing 9 seeds in a dish containing mature zoosporangia grown for 3-4 days on green bean agar (GBA; described below). Ten milliliters of sterile water was added to the culture medium, which was then incubated at 27°C for 24 h. Seeds that were visibly infected (discolored and/or rotted radicle) were transferred to sterile plastic Petri dishes. Solidified KWARP medium, which consisted of 2% agar with 0.025 mg/ml kanamycin sulfate, 0.015 µg/ml rifampicin, and 0.015 µg/ml penicillin was placed on top of the seeds, incubated at 27°C for 2-3 days, and then hyphal tips were transferred to GBA. To prevent carry-over of antibiotics from KWARP to GBA, an additional subculture step was carried out (4 mm diameter culture plugs from GBA to fresh GBA plates) and incubated at 27°C for 3 days before use in experiments.

Pythium aphanidermatum (Edson) Fitzp. (Pa58) (2) was cultured on GBA, a medium modified from Nelson and Craft (30), prepared first by boiling 20 g of 1.0 cm-long frozen cut green beans in 1 liter of water for 20 min. This boiled mixture was then filtered through four layers of cheesecloth. Ten grams of agar (Difco) was then added to 500 ml of filtrate along with the following ingredients, each separately dissolved in 100 ml of sterile water: 5 ml of 0.1780 M K_2HPO4, 5 ml of 0.1176 M KH_2PO_4, 50 µl of 0.0501 M thiamine HCl, 5 ml of 0.4011 M $(NH_4)_2SO_4$, 5 ml of 0.10 M $CaCl_2$, 5 ml of 0.20 M $MgSO_4*7H_2O$, 0.5 ml of 0.0999 M asparagine, and 5 ml of 0.0999 M D-glucose. The final pH of GBA was 6.87. The GBA was then autoclaved for 30 min. Before cooling, 3 ml of sterile GBA was aliquoted to sterile 5 cm glass Petri dishes. This resulted in a thin layer of medium suitable for subsequent microscopic examination. Using a 4 mm diameter corer borer, 4 culture plugs were evenly transferred to GBA, approximately 5 mm from the edge of the dish, and incubated at 27°C for 3 days at which time zoosporangia had formed for use in subsequent experiments.

Compost preparation and use in experiments. Solid vermicompost was prepared at RT Solutions, Inc., Avon, NY using a dairy manure feedstock. Dewatered dairy manure solids were mixed with spoiled corn and hay silage, and cured thermophilic dairy manure compost at a ratio of 7:1:1, respectively. The mixture was composted thermogenically in a forced-air system for up to 2 weeks. This material was then fed every 3-4 days in 5 cm layers to a continuous flow-through vermicomposting system stocked with the earthworms *Eisenia fetida* and *Dendrobaena venata*. Finished vermicompost was removed 75 days after the initiation of thermophilic composting from the underside of the continuous flow-through system and sieved to 10 mm particle size. Vermicompost was stored at -20^0C, and then thawed at room temperature for 24 h in the dark prior to use in experiments. The percent dry weight of the vermicompost was 42%.

My studies necessitated the use of liquid vermicompost extracts (VCE) instead of the solid material because of the need to visualize rather rapid developmental changes during zoosporogenesis that could not be monitored easily in the solid material. Therefore, a series of non-sterile VCEs were prepared consisting of 1.7, 2.7, 5.3, 27.6 or 30.5 g in 100 ml Nanopure® water. The solid vermicompost was measured, added to a plastic container, and then 100 ml of Nanopure® was added to the container. The surface to volume ratio of the container was 0.686. Mixtures were gently mixed, then incubated at 27°C for 4 h. The supernatant was then filtered twice through Whatman #1 filter paper to remove fine particulates. The final pH of non-sterile VCE (30.5% w/v) was 8.4.

Sterilized VCEs (30.5% w/v) were also prepared in two ways. First, heat-sterilized VCE was prepared by autoclaving the solid vermicompost three times prior to producing extracts as described above. Second, filter-sterilized VCE was prepared by filtering the non-sterile extract through a 0.2 µm filter. All liquid extracts were prepared fresh for each zoosporangial germination assay.

Zoosporangial germination assays. Zoosporangial germination was monitored using time lapse photomicroscopy. The influence of pH on zoosporangial germination was evaluated by preparing a range of potassium phosphate buffers to create aqueous solutions at pH 5, 6, 7, 8 and 9. To evaluate the impact of ionic strength on zoosporangial germination, a series of ten-fold dilutions were prepared in sterile water from the 0.1 M KPO_4 solution (pH 6) to obtain ionic concentrations of 0.1, 0.01, 0.001 and 0.0001 M. All liquid solutions were autoclaved for 30 min.

The influence of osmotic potential on zoosporangial germination was evaluated using solutions of polyethylene glycol 8000 (PEG), sucrose, and glucose. PEG was prepared in 100 ml sterile water to obtain osmotic potentials of -0.335 (0.08 g), -4.0 (0.8 g), and -105.0 kPa (8.0 g).

Glucose and sucrose solutions were prepared in water to obtain osmotic potentials of -247.6 (0.1 M), -1,238 (0.5 M), and -2,467 (1 M) kPa for glucose, and -271.2 (0.1 M), -1,356 (0.5 M), and -2,712 (1.0 M) kPa for sucrose. The osmotic potentials for PEG were calculated using the following equation: $\psi=1.29[PEG]^2T-140[PEG]^2-4.0[PEG]$, where ψ=osmotic potential (bars), [PEG]=g PEG/g H_2O, and T=temperature (°C) (29). The osmotic potentials for glucose and sucrose were calculated using the following equation: $\psi=-CiRT$, where ψ=osmotic potential (mPa), C=solute concentration (mol/L), i=solute ionization constant, R=gas constant, and T=temperature (K) (31). All osmotic potentials were converted to kPa. Sterile water was used as a control for inducing indirect germination, viable vesicle formation, and subsequent zoospore release. All liquid solutions were autoclaved for 30 min.

To examine the impacts of these aqueous treatments as well as the impacts of sterile and non-sterile VCE treatments on zoosporangial germination, 10 ml of solution (described above) was applied to a glass Petri dish containing zoosporangia formed after 3 days on GBA and incubated at 25°C for 4-7 h. Subsequent germination was captured using a 10x water-immersion lens on a Zeiss compound microscope outfitted with an Olympus digital DP71 camera and images were processed using Olympus DP2-BSW software. The observation location, which was selected typically from the margins of a culture plate, was chosen to maximize the quantity of zoosporangia (> 20 zoosporangia) within a 1.0 mm^2 viewing area. A single time lapse video was recorded each time a treatment was applied to zoosporangia (hereafter referred to as a flooding event). Based on preliminary observations, greater than 95% of zoosporangia germinated indirectly in sterile water 4 h after flooding; therefore, a 4-hour time lapse duration was set for osmotic potential treatments and all VCE treatments. To observe any prolonged effects on zoosporangial germination, a 7-h time lapse was used for ionic strength and pH experiments. The

interval for image capture was 10 s for indirect germination and vesicle formation in sterile water and 5 min for all other germination observations.

At the end of each flooding event, zoosporangia were scored either as (i) viable and indirectly germinating (vesicles formed with subsequent germ tube production and/or swimming zoospores), (ii) non-viable and indirectly germinating (vesicles or zoospores lysed and/or no activity observed from vesicles), (iii) directly germinating (germ tubes present), or (iiii) non-germinated (no zoosporangial germination or other activity observed). For most analyses, both viable and non-viable indirectly germinating zoosporangia were combined as one category designated as indirect germination. The percentage of zoosporangia displaying each germination mode was calculated for each flooding event. For example, the percent of direct germination = (# zoosporangia germinating directly/total # of observable surface zoosporangia on GBA)*100. The mean for each percent germination mode per treatment across replicates was determined. At least three biological replicates (= three replicate flooding events) were used for each treatment except for the ionic concentration experiment where only two replicates were used. The growth rate of germ tubes from directly germinating zoosporangia was determined using time lapse photomicroscopy. Ten replicate zoosporangia from each of 3 flooding events were selected for germ tube growth rate measurements, which were taken during the first 30 min of germ tube growth.

Bioassay design. Non-treated cucumber seeds (*Cucumis sativus* cv "Marketmore 76) were used in all bioassays. Prior to use in experiments, seeds were sorted to remove damaged seeds, individually screened to a weight range of 0.02-0.03 g/seed, surface disinfested with 0.5% sodium hypochlorite for 2.5 min, rinsed with sterile water, and air-dried before use. Quartz sand was initially dry-sieved to a 0.5-1.0 mm diameter particle size range, wet-sieved to remove fine

particulates, and then autoclaved for 30 min three times before use. Zoosporangia were grown on GBA for 3 days as described above and the surface zoosporangia were quantified and ranked for abundance on a scale of 0-5, with each increasing rank category corresponding to an approximately 10x increase in the number of surface zoosporangia. Such quantification facilitated the selection of three replicate culture plates with relatively equal numbers of zoosporangia per plate. Six cucumber seeds were added to each culture plate containing zoosporangia. Zoosporangia were forced to germinate indirectly in 3 ml of sterile water to produce swimming zoospore inoculum, and directly in 3 ml of either sterile sucrose solution (0.5 M, -1,356 kPa) or 30.5% (w/v) VCE to produce germ tube inoculum. For making comparisons between inoculum types as they relate to disease suppression, 30.5% (w/v) VCE was used in bioassays because it was the only VCE treatment to completely inhibit viable indirect germination and zoospore production. Non-inoculated treatments served as controls. Flooded plates were incubated at 27°C for 1, 2, 3 and 4 h. Three replicate plates were used per incubation time per treatment.

At hourly intervals, 3 ml of sterile sand was placed in each well of a 12-well plate, an individual seed was placed in each well, an additional 3 ml of sand was added over the seed, and then each well received 1.25 ml of sterile water. The culture plate lids were sealed with Parafilm®, placed in clear plastic bins (23.5Wx10.5Hx31.0L cm, 5 plates per bin), and then incubated at 27°C for 3 days with 14 h light. Tissue culture plate lids were removed after 3 days and 250 ml of distilled water was added to the bottom of the plastic bins to create a moisture chamber. The bin lids were then re-attached and sealed with Parafilm®. Bins were then incubated at 27°C for an additional 4 days. After incubation, seed/seedling survival was recorded as a binary variable to account for varied disease symptoms and strengthen statistical analyses.

The following seed/seedling conditions received a value of 0: a) non-germinated seeds, b) radicle present but shoot absent, c) seed/seedling completely rotten, or d) seedling still standing but with symptoms on both the cotyledons and the shoot. The following seedling conditions received a value of 1: a) symptoms present only on the cotyledons, b) symptoms present only on the shoot, or c) no observable symptoms. All bioassay experiments were repeated three times.

Statistical analysis. Differences between the different modes of zoosporangial germination within a treatment and differences in germ tube growth rates were determined using ANOVA for which means were separated using Tukey-Kramer HSD test (JMP Pro 9.0). The dominant mode of germination (indirect, direct, or non-germinated) for each flooding event was calculated from the original percent germination data. For example, if the dominant mode of germination during a single flooding event was indirect, than the total number of zoosporangia germinating indirectly was greater than the sum of germination modes direct and non-germinated. The Fisher's exact test (Chi Squared analysis, JMP Pro 9.0) was used to determine if a treatment significantly altered the dominant mode of germination compared to sterile water. A binary logistical regression model (SAS, least squares means) was used to determine significant differences in bioassay survival data.

Results

Zoosporogenesis in sterile water. Maximum zoosporangial growth was obtained after 3 days incubation on GBA at 27°C (data not shown). However, despite attempts to normalize the numbers of zoosporangia per plate, the total numbers of zoosporangia varied significantly from plate to plate. For example, from 208 culture plates, the quantity of zoosporangia ranged from 2-99 per plate, with a mean of 41 zoosporangia. Despite this variation, zoosporangial germination

in sterile water (25°C) was dominated by indirect germination ($P < 0.0001$) and differed significantly from all other modes of germination ($P < 0.05$) (Fig. 1).

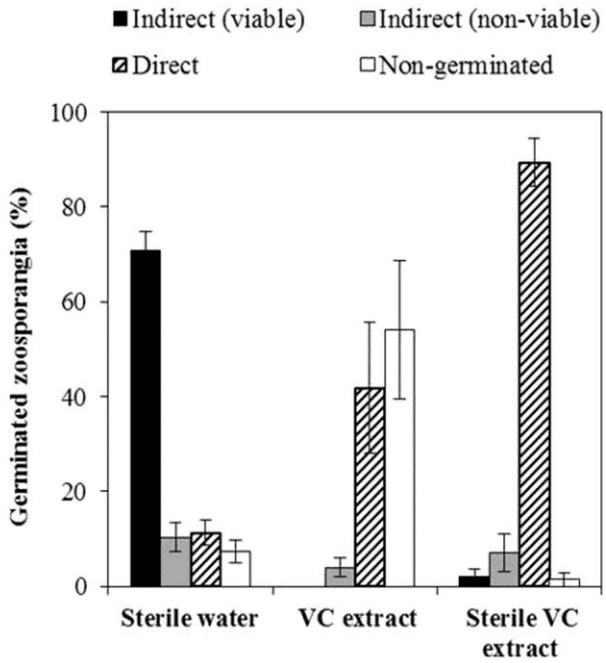
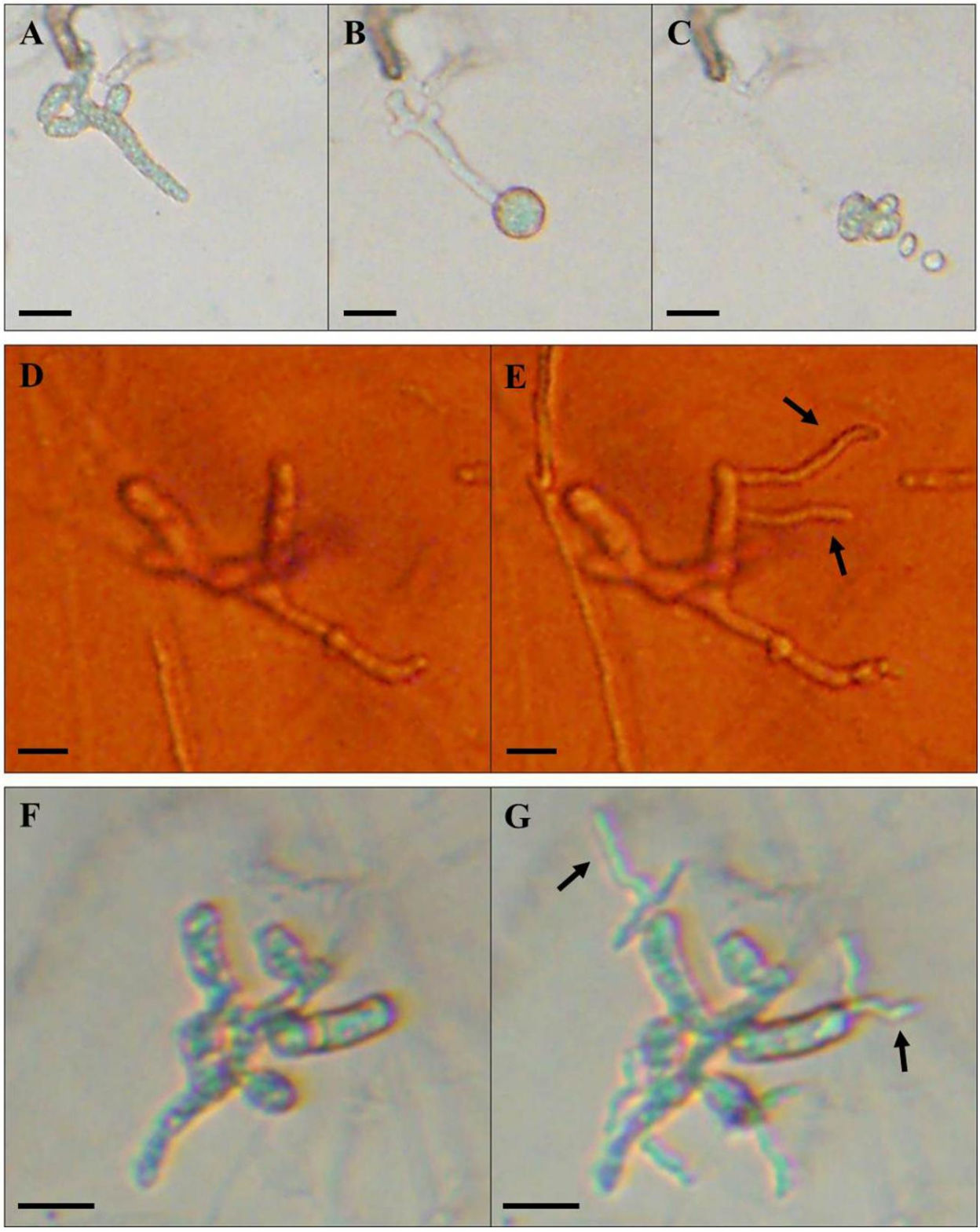


Figure 1. Effects of vermicompost (VC) extracts on *Pythium aphanidermatum* zoosporangial germination. Ten milliliters of 30.5% (w/v) sterile or non-sterile VC extracts were applied to zoosporangia and the mode of germination was recorded 4 h later using time lapse photomicroscopy. Viable indirectly germinating zoosporangia produced vesicles with subsequent germ tubes and/or swimming zoospores. Non-viable indirectly germinating zoosporangia produced vesicles and/or zoospores that lysed in solution. Directly germinating zoosporangia produced germ tubes. Sterile water served as a control. n=10 for water, 8 for VC extract, and 5 for sterile VC extract treatments. Bars represent standard errors.

Indirect germination in sterile water was characterized by the formation of vesicles in which zoospores differentiated and were subsequently released (Illustration 1A-C). Only low levels of directly germinating or non-germinated zoosporangia were observed. Vesicle formation occurred 78.0 min (± 6.8 SE) after flooding with sterile water followed by zoospore release 20.8 min (± 0.4 SE) later. During this period, zoosporangial cytoplasm was translocated into the vesicle and then immediately differentiated into approximately 5-20 motile zoospores, depending on the initial size of the zoosporangium. Within seconds after differentiation, the vesicle membrane burst and swimming zoospores were released. At this time, vesicles were no longer visible and

Illustration 1. Germination of *Pythium aphanidermatum* zoosporangia. **(A-C)** Indirect germination of a zoosporangium flooded with sterile water, scale bars = 25 µm. A vesicle was produced followed by zoospore release. Elapsed time was approximately 20 min. Direct germination and germ tube production was initiated in treatments of **(D-E)** 30.5% (w/v) non-sterile vermicompost extract, scale bars = 25 µm and **(F-G)** sterile 0.5 M sucrose solution, scale bars = 30 µm. Arrows point to zoosporangial germ tubes.


A
B
C
D
E
F
G

zoosporangial walls were only faintly visible. Figure 2 shows the average vesicle production of 5 flooding events with sterile where vesicle production continued intermittently but at a relative constant rate up to 7 h, by which time nearly all zoosporangia had germinated indirectly.

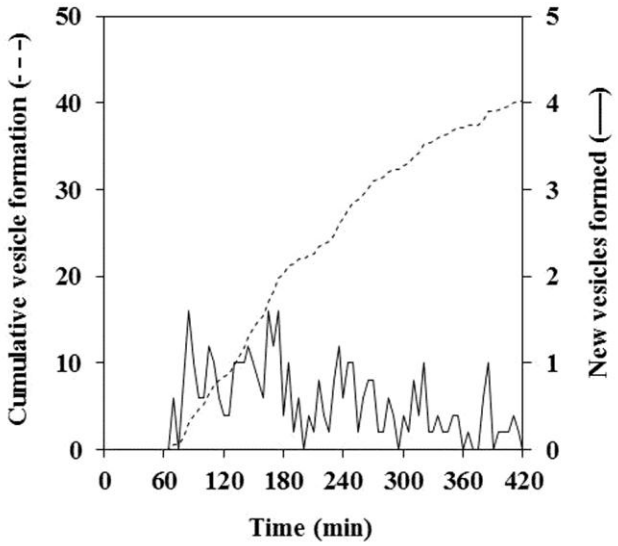


Figure 2. Mean vesicle formation by *Pythium aphanidermatum* after flooding with sterile water. Data were averaged from five flooding events with a mean of 36 zoosporangia observed per plate.

Zoosporangial germination assays using vermicompost extracts (VCE). Non-sterile VCEs significantly ($P < 0.0001$) altered the mode of zoosporangial germination compared to that observed in sterile water. At low VCE concentrations (1.7-5.3%, w/v), germination was unchanged and nearly all zoosporangia germinated indirectly by forming vesicles and zoospores. However, at a concentration of 27.6% (w/v), VCEs induced an average of 39% (± 14.6 SE) indirect germination (3% viable indirect and 36% non-viable indirect germination), whereas an average of 31% (± 7.0 SE) germinated directly by the formation of a germ tube. The remaining zoosporangia were non-germinated. Direct germination of zoosporangia was characterized by the production of multiple germ tubes (often three per zoosporangium) (Illustration 1D-E). At a VCE concentration of 30.5% (w/v), zoosporangia remained viable but indirect germination was

completely inhibited while the level of direct germination increased to an average of 42% (± 13.8 SE). The level of non-germinated zoosporangia in 30.5% (w/v) VCE was 54% (± 14.6 SE) (Fig. 1).

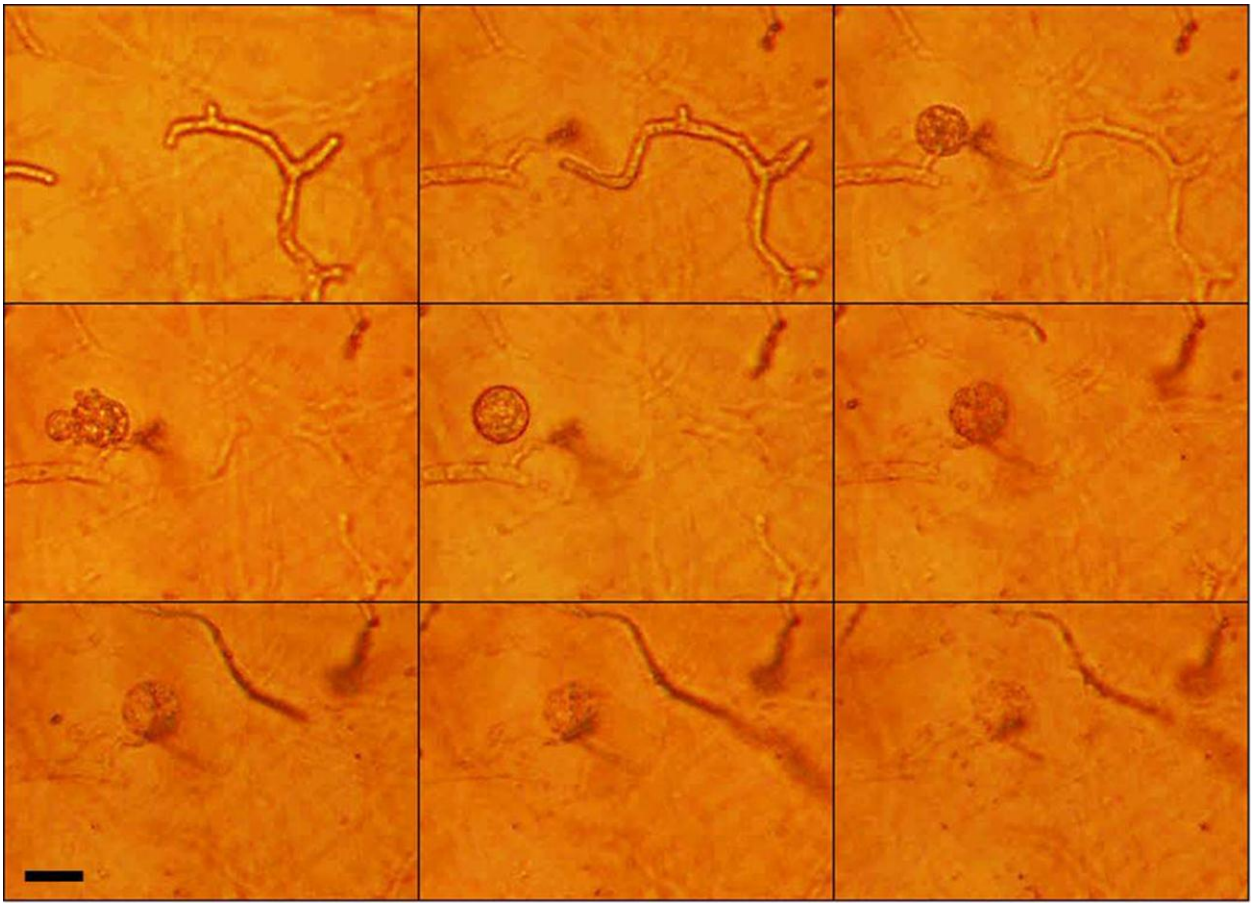


Illustration 2. Non-viable indirectly germinating zoosporangia of *Pythium aphanidermatum* in non-sterile vermicompost extract (30.5% w/v). The series of images, viewed left to right, top to bottom, represent the temporal progression of zoosporangial germination and vesicular lysis. Approximate elapsed time was 35 min. Scale bar = 25 µm.

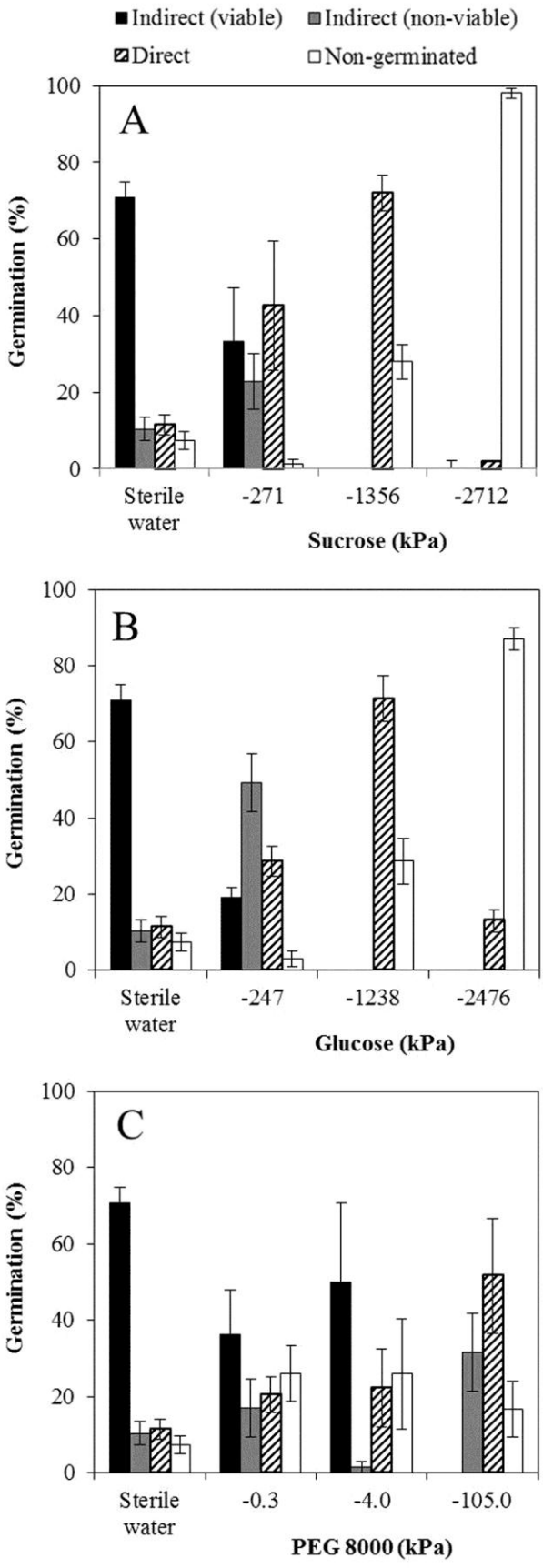
In the presence of heat- or filter-sterilized VCEs (30.5%; w/v), germination mode was significantly ($P = 0.0003$) altered compared to that in sterile water. There was no significant difference ($P > 0.05$) in germination response between heat- and filter-sterilized VCEs (data not shown). As a result, their percent germination data were combined as one category designated as sterile VCE. In the presence of sterilized VCEs, 89% (± 5.0 SE) of the zoosporangia germinated

directly by way of germ tubes (Fig. 1). The lysis of vesicles and/or zoospores (i.e. non-viable indirect germination) was observed in the presence of both sterile and non-sterile VCEs (30.5% w/v) (Illustration 2). Furthermore, in the presence of sterile VCE, the time required for germination (23.2 min) was significantly reduced relative to that in sterile water (78.0 min). Germ tube growth rates did not differ significantly ($P > 0.05$) between sterile and non-sterile VCEs. The average germ tube growth rate from all directly-germinating zoosporangia exposed to sterile and non-sterile VCEs was 116.9 µm/h (± 13.2 SE).

Zoosporangial germination assays to test the factors influencing direct germination. The germination mode and the time required for germination were unaffected by pH across the range of 5 to 9 ($P = 0.7059$) or by the molar concentration of a potassium phosphate buffer solution (from 0.001 to 0.1 M) ($P = 0.5455$) compared to that in sterile water. Although viable indirect germination was nearly inhibited in 0.1 M potassium phosphate buffer (pH 6), non-viable indirect germination persisted and the overall trend in zoosporangial germination was not significantly ($P > 0.05$) different from that in sterile water (data not shown). However, osmotic potentials generated with either sucrose ($P < 0.0001$), glucose ($P < 0.0001$), or PEG ($P = 0.0046$) significantly altered germination mode but not the time required for germination relative to that in sterile water (Fig. 3). As osmotic potential decreased, the level of viable indirect germination decreased with a corresponding increase in direct germination (Illustration 1F-G); however, indirect germination was observed in all osmotic potentials generated in PEG but not all those generated in sucrose and glucose. The average germ tube growth rate in sucrose solution (0.5 M, -1,356 kPa) and glucose solution (0.5 M, -1,238 kPa) were 119.3 µm/h (± 7.6 SE) and 122.3 (± 18.4 SE), respectively, and did not differ significantly ($P > 0.05$) from growth rates observed in

VCEs. At an osmotic potential of -2,712 kPa in sucrose (1.0 M), nearly all of the zoosporangia remained non-germinated.

Figure 3. *Pythium aphanidermatum* zoosporangial germination at different osmotic potentials. Ten milliliters of **(A)** sucrose solution, **(B)** glucose solution, or **(C)** polyethylene glycol 8000 were applied to zoosporangia and the germination mode was recorded 4 h later using time lapse photomicroscopy. Viable indirectly germinating zoosporangia produced vesicles with subsequent germ tubes or swimming zoospores. Non-viable indirectly germinating zoosporangia produced vesicles and/or zoospores that lysed in solution. Directly germinating zoosporangia produced germ tubes. Sterile water served as a control. n=10 for water, 5 for sucrose treatments, 3 for glucose treatments, and 3 for PEG treatments. Bars represent standard errors.


Indirect (viable)
Indirect (non-viable)
Direct
Non-germinated
A
Germination (%)
100
80
60
40
20
0
Sterile water
-271
-1356
-2712
Sucrose (kPa)
B
Germination (%)
Sterile water
-247
-1238
-2476
Glucose (kPa)
C
Germination (%)
Sterile water
-0.3
-4.0
-105.0
PEG 8000 (kPa)

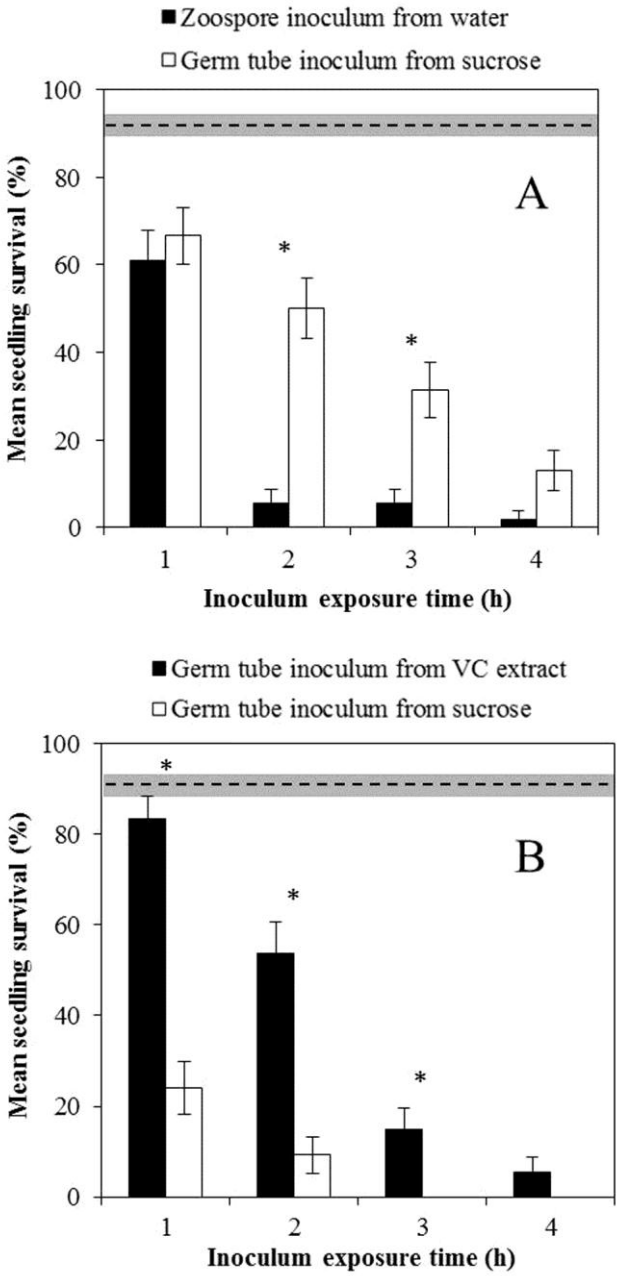
Bioassay results. Seed and seedling survival decreased significantly ($P < 0.0001$) over a 7-day period when seeds were exposed to either zoospores produced in sterile water or germ tubes produced in sucrose solution (0.5 M, -1,356 kPa) (Fig. 4A). Significantly greater seedling survival was observed with germ tube inoculum as compared to zoospore inoculum when exposed to seeds for 2 and 3 h (Fig. 4A).

Disease was suppressed during 1 h of germ tube inoculum exposure in treatments of VCE compared to non-inoculated controls (Fig. 4B). However, seedling survival decreased significantly in VCE after 2 h of inoculum exposure and continued to decrease overtime. Significantly ($P < 0.05$) greater seedling survival was observed in the presence of VCE than in the presence of sucrose solution (0.5 M, -1,356 kPa) when exposed to germ tube inoculum for 1, 2, or 3 h (Fig. 4B). After 4 h of inoculum exposure, seedling survival did not differ between sucrose and VCE treatments.

Discussion

Attempts to understand the mechanisms by which oomycete-incited diseases are biologically suppressed have centered largely on observations of zoospore behavior and either the direct lysis of zoospores by biological agents (9,15,16,18,24,47) or the inability of zoospores to reach host surfaces and infect plants (15,23,38). However, other stages of the pathogen life cycle may be equally vulnerable to microbial interference and suppression, reducing the numbers or infectivity of zoospores arriving at the host surface. Although the timing and sequence of events that I observed in *P. aphanidermatum* zoosporogenesis in the absence of vermicompost were consistent with observations of other *Pythium* species (21,28,46), the normal course of zoosporogenesis in the presence of vermicompost was severely disrupted, leading to the abortion

Figure 4. Effects of *Pythium aphanidermatum* zoosporangial germination mode on cucumber seedling survival. **(A)** Mean seedling survival after exposure to zoospore and germ tube inoculum (n=3). Zoospore formation was induced in sterile water while germ tubes were induced in sterile 0.5 M sucrose. **(B)** The suppression of germ tube inoculum of *P. aphanidermatum* by vermicompost extracts (30.5% w/v) was compared (n=3). Inoculum produced in sterile 0.5 M sucrose served as a control. For both **(A)** and **(B)**, cucumber seeds were incubated with each type of inoculum for 1, 2, 3, and 4 h at 27°C. At each hour, seeds were transplanted to sterile sand and grown for 7 days. Non-inoculated treatments served as controls. There was not a significant difference between non-inoculated treatments ($P < 0.05$). The horizontal dotted line represents the mean survival for seedlings in all non-inoculated treatments. The gray horizontal bar represents the standard error (± 1.3) of the control treatments. Vertical bars represent standard error. * indicates significant ($P < 0.5$) difference between inoculated treatments within each hour according to binomial logistic regression, least squares means test.


Zoospore inoculum from water
Germ tube inoculum from sucrose
Mean seedling survival (%)
100
80
60
40
20
0
A
*
*
1
2
3
4
Inoculum exposure time (h)
Germ tube inoculum from VC extract
Germ tube inoculum from sucrose
Mean seedling survival (%)
100
80
60
40
20
0
B
*
*
*
1
2
3
4
Inoculum exposure time (h)

and/or lysis of vesicles and complete suppression of zoospore release. The low arrival rate of zoospores on host surfaces and the ensuing low levels of disease has been explained by the presence of bacterially-produced zoosporicidal toxins (8,10,25,34,43) or antibiotics (9,15,38,47); however, such an interpretation assumes that zoospores are indeed produced during pre-infection responses to plants.

Perhaps the most interesting and puzzling result from this work was the vermicompost-induced change in the mode of zoosporangial germination. Although the indirect germination of zoosporangia was suppressed in vermicompost extracts (VCE), zoosporangia continued to germinate directly by forming germ tubes, which did not appear to be negatively affected in the presence of VCE. Observing that the mode of germination was shifted in both sterile and non-sterile VCEs suggests that the shift was induced either by non-microbial processes or from heat-stable microbial products.

Surprisingly little is known about bimodal germination in *P. aphanidermatum*. Although *P. aphanidermatum* oospores have been observed to germinate biomodally, giving rise to zoosporangia in the absence of glucose but germinating directly to produce germ tubes in the presence of glucose (35,36), this has not been described for *P. aphanidermatum* zoosporangia and, to our knowledge, direct germination has only been mentioned once in the literature (42). Stanghellini and Burr (42) observed the formation of germ tubes from *P. aphanidermatum* zoosporangia germinating adjacent to bean and sugarbeet seeds and seedlings in soil as well as in the presence of bean seed exudates, where under the conditions, concentrations of glucose and other carbon compounds would be elevated relative to soil in the absence of plants or to water where indirect germination is almost exclusively observed.

Much of our understanding of bimodal germination in oomycetes comes from studies of *Phytophthora* species (7,22,37), where not only cooler temperatures induce indirect germination and higher temperatures induce direct germination, but plant extracts and exudates, carbohydrates, amino acids, inorganic salts, and low osmotic potentials also induce direct germination (7,22). Studies with *Pythium insidiosum* have shown that increasing concentrations of various cations reduce the formation and release of zoospores (39-41), although it is not known whether these patterns hold true for *P. aphanidermatum*. However, the previous studies did not determine whether direct zoosporangial germination was alternatively induced while indirect germination was suppressed. Although Shipton (39) observed decreasing zoospore release from *P. insidiosum* zoosporangia with increasing potassium ion concentration up to 0.2 M, my results with *P. aphanidermatum* demonstrated that vesicle production (and hence germination mode) were unaffected by potassium ion concentrations up to 0.1 M, indicating that other factors impact the shift from indirect to direct germination mode.

In current study, I was also able to demonstrate that low osmotic potentials could induce direct germination of *P. aphanidermatum* zoosporangia. Again, the fact that I observed this in the presence of sterile as well as non-sterile VCEs suggest that osmotic potentials and not other microbial interactions, could potentially explain the shift from indirect germination to direct germination in the presence of VCEs. Water relations are clearly one of the more important factors affecting zoosporogenesis in a wide range of *Pythium* and *Phytophthora* species (13), so it is not surprising that *P. aphanidermatum* germination mode would be influenced by osmotic potential, especially given that the vermicompost used in our study has a relatively high electrical conductivity (Worm Power, unpublished data). Although I did not estimate the osmotic

potentials of our VCEs, they are likely to be high given the relatively high levels of dissolved solids (Worm Power, unpublished data).

While our empirical understanding of the factors influencing direct germination in *Phytophthora* is relatively clear, the significance of direct germination to disease and to disease suppression is currently unknown. Hardy and Sivasithamparam (20) demonstrated that sporangia of *Phytophthora drechsleri* germinated directly in the presence of microbes that were also shown to suppress disease. However, the capacity of germ tubes to infect plants was not established and the direct suppression of germ tubes by these disease-suppressive microbes was not tested. Therefore, I felt it important to ascertain whether germ tubes of *P. aphanidermatum* could infect germinating cucumber seeds and whether I could observe any suppression of germ tube growth in the presence of VCEs. The ability to induce direct germination in the absence of vermicompost (i.e. under low osmotic potentials using sucrose) and also in the presence of vermicompost provided me with an ideal comparative system to test this hypothesis. If I had found that germ tubes could not infect seeds in VCE, this would not have provided a clear explanation of disease suppression because it would have been unclear whether the lack of infection was the inability of germ tubes to initiate infection or due to compost-induced suppression. However, contrary to my prediction, I found that germ tubes were capable of infecting seeds placed adjacent to directly germinating zoosporangia. Although greater seedling survival was observed when seeds were exposed to directly germinating zoosporangia for 1-3 h in VCE than in the absence of VCE, eventually nearly 100% seedling mortality was observed, suggesting that any initial levels of disease suppression were masked after an extended exposure to *P. aphanidermatum* germ tubes.

This result is puzzling, given that the vermicompost used in this study is highly suppressive to *P. aphanidermatum* seed and seedling disease when zoospores are used as inoculum (25). However, the apparent lack of suppression of directly germinating zoosporangia while being highly suppressive to zoosporogenesis remains somewhat of a paradox. How can a compost suppress the disease and the developmental stage of the pathogen causing the disease, but, at the same time, induce an alternate developmental stage that can still initiate infection that could not be suppressed? I believe much of this paradox may be due to artifacts of my assay system and I propose three possible explanations to help resolve this paradox. First, my assay system was not conducive for a seed-colonizing bacterial consortium, shown previously to play an important role in compost-induced disease suppression (6) and important in suppressing zoospore infections by *P. aphanidermatum* in vermicompost substrates (25). The seed-associated microbial responses require a minimum of 8 h to develop (6). In my experimental design, zoosporangia, cucumber seeds, and VCEs were incubated simultaneously, which provided little time (~1 h) for a suppressive microbial community to colonize the seed surface. Pre-exposing seeds to VCEs may have provided a more accurate experimental setup by allowing a microbial community to develop that was capable of suppressing germ tube infections. Second, the spatial distribution of directly germinating zoosporangia in the spermosphere may play a significant role in disease suppression. For example, *P. aphanidermatum* zoospores are capable of traveling at a speed of 180-210 µm/s (1), which is much faster than the growth rate of germ tubes that extend at a mean rate of 117 µm/h. The observation that *P. aphanidermatum* zoosporangia germinate indirectly at some distance from a host whereas they germinate directly when adjacent to the host (42) suggests that zoospores of *Pythium* species are the likely infective developmental stage when the distance to the host exceed the radial extent of the spermosphere and when the

influences of compost osmotic potentials are weaker. Seeds may avoid germ tube colonization in the presence of vermicompost when the distance between seed and pathogen is relatively large, preventing germ tubes from reaching the seed in advance of a suppressive seed-colonizing community developing. It is therefore possible that the suppressive effects of VCEs were obscured in my assay system because cucumber seeds were placed in such close proximity to germinating zoosporangia. Lastly, the overwhelming exposure of germ tube inoculum may have eliminated any spatial factors that could have influenced disease suppression.

In summary, although specific to one type of vermicompost and zoosporic pathogen, my results have revealed that an extract prepared from vermicompost suppress key stages of zoosporogenesis leading to the lysis of vesicles and preventing zoospore formation. Also accompanying vesicle lysis was a shift in the germination mode from indirect germination by way of zoospores to direct germination by way of germ tubes. The factors responsible for the vesicle lysis are unknown. However, low osmotic potentials likely generated by the VCE could induce the shift toward direct germination. Although I demonstrated that germ tubes are capable of initiating seed infection, the shift away from zoospores to germ tubes could explain disease suppression in vermicompost if the spatial distribution of zoosporangia is considered. Since indirect germination results in a greater number of infective units capable of reaching hosts over a larger spatial area than would occur with germ tubes, lower levels of disease would occur from directly germinating zoosporangia. My work represents one of the few reports of direct germination in *P. aphanidermatum* zoosporangia and further work is needed to determine the significance of *P. aphanidermatum* direct germination to seed and seedling diseases and the vulnerability of germ tubes to disease suppression.

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