Rapid Germination of Sporangia of *Pythium* Species in Response to Volatiles from Germinating Seeds

Eric B. Nelson

Assistant professor, Department of Plant Pathology, University of Arkansas, Fayetteville 72701. Present address: Department of Plant Pathology, Cornell University, Ithaca, NY 14853. I wish to thank Dr. J. B. Murphy for performing the gas chromatographic analysis of cotton seed volatiles. Cucumber seeds were supplied by Petoseed Co., Inc., Saticoy, CA 93004. Pea seeds were supplied by Asgrow Seed Co., Kalamazoo, MI 49001. Accepted for publication 9 February 1987 (submitted for electronic processing).

ABSTRACT


Sporangia of *Pythium ultimum*, *P. sylvaticum*, and *P. irregularare* germinated in response to volatile seed exudates. Germination was most rapid at 25 C with germ tubes evident 2 hr after exposure. Maximum germination occurred within 6-8 hr. Germination was delayed at 15 C but suppressed at 35 C even though sporangia were 100% germinable. Volatiles evolved during seed germination were separated by gas chromatography, identified, and quantified. Acetaldehyde and ethanol were the only components detected after 2 hr of imbibition, whereas ethane, acetone, and methanol were also detected after 6 hr of imbibition. Ethane production was detected only from seeds germinated at 15 C. Levels of all other volatiles increased as imbibition temperature increased. Acetaldehyde was not stimulatory to sporangium germination at concentrations of 1-6,200 nmol/ml. Ethanol was stimulatory only at concentrations of 1-7 nmol/ml, whereas higher concentrations appeared to be inhibitory.

*Pythium* species are principal pathogens involved in seed rots and preemergence damping-off of a wide variety of plant species (10). Both oospores and sporangia serve as major survival structures during intersubstrate periods (29,31,32), and among those species with globose sporangia, sporangia may persist to the same extent or longer than oospores (29).

Sporangia in soil are exogenously dormant and will germinate rapidly in response to introduced nutrient stimuli or upon alleviation of fungistasis (1,32). In soil at 24 C, sporangia of *P. ultimum* can germinate as early as 1.5 hr after addition of bean seed exudates with maximum germination occurring after 3 hr and host penetration occurring within 24 hr (32). Similarly, Nelson et al (20) observed colonization of cucumber seeds by *Pythium* species as early as 4 hr after planting in soil with maximum seed colonization occurring 8 hr after planting.

This rapid response has typically been attributed to stimulation of germination and growth by soluble exudates (i.e., amino acids and sugars) from germinating seeds (5,12,16,17,23,25). However, volatile exudate components have largely been overlooked in the initiation of host-pathogen interactions despite reports of stimulation of fungal spore germination and growth by volatile compounds (3,4,9,21).

The purpose of the present study was to determine if volatiles from germinating seeds were stimulatory to sporangium germination among several *Pythium* species in an attempt to explain the rapid response of *Pythium* to germinating seeds.
MATERIALS AND METHODS

Preparation of sporangia. The following Pythium species were used: *Pythium ultimum* Trow, isolate P4 (20); *P. irregulare* Buik., isolate 180; and *P. sylvaticum* Campbell and Hendrix, isolate 179. *P. irregulare* and *P. sylvaticum* were recovered from hypocotyls of damaged-off cotton seedlings, and *P. ultimum* was recovered from diseased snap beans (20). Cultures were maintained on grass extract agar (GEA), which was prepared by boiling 10 g of freshly cut feces in 1 L of distilled water for 5 min. The mixture was filtered through two layers of cheesecloth and then through Whatman No. 1 filter paper. The filtrate was brought to 1 L and amended with 20 g of agar. The pH was adjusted to 6.4, and cultures were incubated at 25 C.

Sporangia were raised on 5-mm-diameter noble agar (2%, w/v) disks on which 48-h-old GEA cultures of the appropriate *Pythium* species had been placed. Disks were incubated at 25 C for 4 days. They were then leached in sterile distilled water for 30 min (six changes of water), removed aseptically, and incubated an additional 3 or 4 days when abundant sporangia had formed. Sporangia were then assayed for germination within 24 hr.

Seed preparation. Seeds of cotton (*Gossypium hirsutum* L. 'Acala SJ-2') were used in all experiments. Other plants used were soybean (*Glycine max* L. Merr. 'Lee 74'), pea (*Pisum sativum* L. 'Venus'), cucumber (*Cucumis sativus* L. 'Poinsett 76'), and okra (*Abelmoschus esculentus* L. Moench 'Jefferson'). Seeds were not visible in a field of 1 cm above the germinating seeds. Sufficient sterile distilled water was added to each flask so that all was taken up during an 8-hr incubation period (1.5, 1.4, and 1.0 ml per flask for 35, 25, and 15 C, respectively). Flasks were sealed with Teflon tape and rubber septum stoppers and incubated in the dark at 15, 25, and 35 C. Controls consisted of flasks containing sporangia but no seeds.

Sporangium germination assay. All glassware used in germination assays was washed with a chromic acid solution before use. Agar disks containing sporangia were placed on sterile 12×65-mm glass slides (three disks per slide), which were placed aseptically at an angle into 50-ml Erlenmeyer flasks containing 20 surface-sterilized imbibed seeds. With this configuration, disks with sporangia were approximately 1-3 cm above the germinating seeds. Sufficient sterile distilled water was added to each flask so that all was taken up during an 8-hr incubation period (1.5, 1.4, and 1.0 ml per flask for 35, 25, and 15 C, respectively). Flasks were sealed with Teflon tape and rubber septum stoppers and incubated in the dark at 15, 25, and 35 C. Controls consisted of flasks containing sporangia but no seeds.

Sporangial germination was determined by removing sporangial disks from flasks at 1- or 2-hr intervals and staining them with 0.03% acid fuchsin in 85% lactic acid. Disks were examined microscopically (100X) and the number of germinated and ungerminated sporangia recorded. Sporangia were considered germinated if a developing germ tube was visible. Ten random microscope fields containing sporangia (25-50 per field) were counted from disks in each of three replicate flasks for a total of 2,000-2,500 sporangia counted for each determination. In each experiment, germinability of sporangia was determined by adding 50 μl of 10% yeast extract solution to each dish in a duplicate set of flasks (three replications) and determining percent germination after 3 hr of incubation at 25 C.

To test the influence of temperature on sporangium germination, 50 μl of 10% yeast extract solution, preincubated at the appropriate test temperature, was added to each dish. Disks were incubated at temperatures from 5 to 40 C for 3 hr. Sporangium germination was determined at hourly intervals.

To test the influence of various concentrations of acetate and ethanol on sporangium germination, disks with sporangia were placed in empty 50-ml flasks of known total volume and sealed as described above. Reagent grade ethanol or acetate as well as syringes were cooled to -20 C before use. Fifty microliters of ethanol or acetate was injected into a test tube (14.4 ml total volume) and allowed to volatilize. Serial dilutions were prepared with the aid of a gas-tight syringe. Flasks were incubated at 25 C. Percent sporangium germination was determined after 6 hr and approximate concentrations of ethanol or acetaldehyde calculated based on the original flask volume. All sporangium germination assays were repeated at least once.

Analysis of cotton seed volatiles. Seeds were selected and surface sterilized as described above, placed in 50-ml flasks, sealed, and incubated at 15, 25, or 35 C. After 2 or 6 hr of incubation, 1-ml aliquots of volatiles were removed from the headspace using a gas-tight syringe and injected into a Hewlett Packard 5880A gas chromatograph equipped with a flame ionization detector and a 183-× 0.32-cm column packed with Poropak R (Alltech Associates, Deerfield, IL). The column was maintained at 60 C for 1.75 min, increased to 95 C for 2 min, and then increased from 95 to 115 C at a rate of 5 C per minute. Nitrogen at 35 ml/min was used as the carrier gas. Peaks were recorded and integrated on an SP 4100 integrator (Spectra-Physics, San Jose, CA). Peaks were compared with known standards and amounts of individual volatiles determined from standard curves, adjusted for actual headspace volume, and expressed as nanomoles or nanoliters per milliliter. Data are means of three replicate determinations. Collections were repeated twice.

Collection and analysis of soluble cotton seed exudates. A sterile leukophor apparatus similar to that described by Weir and Lacy (27) was used to collect soluble exudates from germinating cotton seeds. All components of the system were autoclaved before exudate collection. Chromatography columns (15×2.5 cm) were filled with 20 ml of glass beads (<1 mm diameter). Distilled water served as the eluent and was pumped through the column at the rate of 5 ml/hr with the aid of a peristaltic pump. Water flow was initiated and the entire system allowed to equilibrate for 30 min before introducing seeds. Two undamaged seeds were surface sterilized as described above and placed aseptically 1 cm deep in the sterile glass bead matrix. The column was covered with aluminum foil to exclude light. Five-milliliter fractions were collected in presterilized tubes at hourly intervals for a 48-hr period. Leachings were then frozen and freeze-dried. Exudates were dissolved in 1 ml of sterile distilled water and analyzed for carbohydrates using the anthrone reagent (19) as modified by Loewus (15) and using glucose as the standard. Levels of carbohydrates released were expressed as micrograms of glucose equivalents per seed. Collections were repeated two additional times.

RESULTS

Effect of seed volatiles on germination of sporangia. Sporangia of *P. ultimum* germinated in response to volatiles from all plant species evaluated (Table 1). The first sign of germination (i.e., appearance of germ tubes) was apparent 2-4 hr after exposure to volatiles. The most rapid rate of germination was observed in response to volatiles from large-seeded plants. For example, 8 hr after exposure to volatiles from cotton, soybean, and pea, 88.7, 92.4, and 72.7% of sporangia had germinated, respectively. In contrast, volatiles from okra and cucumber stimulated only 28.4 and 19.4% germination, respectively, after an 8-hr exposure. Response to volatiles was not nearly as rapid as the response of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4 hr</th>
<th>8 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.0 f</td>
<td>8.4 c</td>
</tr>
<tr>
<td>No treatment</td>
<td>100.0 a</td>
<td>100.0 a</td>
</tr>
<tr>
<td>10% yeast extract</td>
<td>34.6 c</td>
<td>92.4 b</td>
</tr>
<tr>
<td>Soybean</td>
<td>33.4 b</td>
<td>72.7 c</td>
</tr>
<tr>
<td>Pea</td>
<td>21.0 cd</td>
<td>88.7 d</td>
</tr>
<tr>
<td>Cotton</td>
<td>14.1 de</td>
<td>28.4 e</td>
</tr>
<tr>
<td>Okra</td>
<td>8.8 e</td>
<td>19.4 de</td>
</tr>
</tbody>
</table>

TABLE 1. Germination of sporangia of *Pythium ultimum* in response to seed volatiles from different plant species

Volatiles from 20 seeds germinating in a volume of 57.5 ml. Means in each column followed by the same letter are not significantly different (P = 0.05) according to Duncan's multiple range test.
sporangia to yeast extract. Four hours after exposure, 100% of the sporangia exposed to yeast extract had germinated, whereas exposure to volatiles resulted in germination percentages ranging from 8.8 to 53.4%.

Effect of temperature on the response of sporangia to cotton seed volatiles. Sporangia of all three Pythium species germinated when exposed to cotton seed volatiles (Table 2). However, each species responded differently to temperature. For example, 8 hr after exposure to cotton seed volatiles, maximum germination of sporangia of P. ultimum occurred at 25 C, whereas maximum germination of sporangia of P. sylvaticum occurred at 35 C. It was frequently observed that the smallest sporangia and hyphal swellings of all three species were the first to germinate; germination was generally evident 1 to 2 hr after exposure. Larger sporangia germinated later. Germination of sporangia of P. ultimum was evident 2, 4, and 6 hr after exposure to cotton seed volatiles at 25, 15, and 35 C, respectively (Fig. 1). Germination of sporangia of P. ultimum and P. irregularis was significantly reduced at 35 C.

Effect of temperature on germination of sporangia of P. ultimum. Sporangium germination was evaluated at temperatures ranging from 5 to 40 C (Table 3) to determine whether the response of sporangia of P. ultimum was due to the inability of sporangia to germinate at the desired test temperatures. As incubation temperatures increased, the rate of sporangium germination increased with the most rapid rate of germination occurring at 35 C. Germination was 100% after 2 hr at 25 and 35 C, whereas 4 hr was required to 100% germination at 15 C (data not shown). At 5 C, 49% of the sporangia had germinated by 24 hr. No germination was observed at 40 C after 24 hr of incubation.

Carbohydrate exudation from cotton seeds. Carbohydrates could not be detected in leachates from germinating cotton seeds until after 9 hr of germination. Maximum levels of carbohydrates were released 15 hr after placing seeds in the glass bead matrix. For example, by 12 hr, 19.8 μg of glucose equivalents per seed was detected, whereas at 15 hr, 135.1 μg of glucose equivalents per seed was detected. By 18 hr, levels of carbohydrates had declined to 25.2 μg of glucose equivalents per seed. Levels of carbohydrates continued to decline thereafter. After 42 hr of germination, carbohydrates could no longer be detected.

Composition of cotton seed volatiles. Volatiles collected from the headspace of germinating cotton seeds are listed in part in Table 4. Peak retention times were 1.22, 6.03, 7.43, 14.52, and 16.53, which corresponded to ethanol, acetone, methanol, and ethylene, respectively. After 2 hr of germination, only acetdehyde and ethanol could be detected. However, after 6 hr of germination, ethylene, methanol, and acetone were also detected. Only a trace of methanol could be detected at 35 C after 6 hr. Acetdehyde and ethanol were the most abundant volatiles detected at all incubation temperatures. As temperatures increased, the levels of individual volatiles increased. Ethylene, on the other hand, was produced in lower levels as temperatures increased.

Germination in response to ethanol and acetdehyde. The two major components detected in cotton seed volatiles (acetdehyde and ethanol) were tested for their ability to stimulate germination of sporangia of P. ultimum in the absence of other volatile components (Table 5). Over the concentration range tested, no germination of sporangia occurred after 6 hr when exposed to acetdehyde. However, sporangia exposed to ethanol were stimulated to germinate, but as concentrations of ethanol decreased, sporangium germination increased. For example, only 7% of the sporangia exposed to 70 nmol/ml of ethanol had germinated after 6 hr, whereas 22% exposed to 1 nmol/ml had germinated after 6 hr. Concentrations greater than 6,200 nmol/ml were not stimulatory to sporangium germination.

![Fig. 1. Germination of sporangia of Pythium ultimum in response to volatiles from cotton seeds germinating at three different temperatures.](image-url)

* - * = 15 C, △ = 25 C, ■ = 35 C. LSD (P = 0.05) at 8 hr 13.9%.

1110 PHYTOPATHOLOGY
DISCUSSION

The ability of *Pythium* species to respond rapidly to germinating seeds is well documented (7,14,18,20,22,25,29,31,33,34). This response involves a series of events that are initiated by osmopore or sporangium germination, followed by growth to the seed, colonization of seed coats, and finally infection of the embryo (7). Sporangium germination in soil can occur as early as 1–3 hr after seeds are planted (39–32), and embryo infection can occur within 24 hr (32). The rapid activation from a dormant sporangium to a rapidly developing thallus has typically been attributed to stimulation by sugars and amino acids present in seed exudates (1,26,32), yet only rarely have studies addressed carbohydrate or amino acid exudation during initial stages of imbibition and their effects on *Pythium* species. For example, Short and Lacy (27) detected carbohydrates in exudates from germinating pea seeds as soon as 1 hr after imbibition began with maximum levels detected between 3 and 4 hr of imbibition. Similarly Schlub and Schmittchen (26) detected significant levels of carbohydrates and amino acids in leachates from soybean seeds after 3 hr of imbibition. However, the relationship between early exudation of carbohydrates or amino acids and their effects on *Pythium* species can only be inferred in these studies.

In this study, sporangium germination in response to volatile exudates from seeds was evident by the emergence of a germ tube after as little as 2 hr of seed imbibition at 25 C. However, even though sporangia germinate on cotton seed coats as early as 2 hr after planting, no carbohydrates can be detected in exudates from cotton seeds germinating in a glass bead matrix until after 9 hr of imbibition at 25 C. Despite this, 100% of cotton seeds removed from *Pythium*-infested soil are colonized by *Pythium* after 8–10 hr (E. B. Nelson, unpublished). This suggests that sporangium germination and growth to the seed can occur in the apparent absence of carbohydrates and that either undetected soluble compounds at very low concentrations or volatile exudates from cotton seeds are responsible for activating sporangium germination in soil. Because germ tube emergence signals the end of the germination process, many biochemical events have occurred after sporangium activation and before germ tube emergence (36,37). For example, physiological changes within ascospores of *Neurospora tetrasperma* Shear & Dodge are evident within the first 20 min after activation (37). Therefore, sporangium activation may have occurred immediately upon exposure to seed volatiles.

Significant levels of both ethanol and acetaldehyde were detected in cotton seed volatiles within 2 hr of imbibition at 25 C. Additional compounds were also detected by 6 hr, but acetaldehyde and ethanol remained the most abundant compounds. These results are consistent with previous reports on the composition of volatile seed exudates (6,35,38). Vancura and Stotzky (38) detected ethanol as the major component of cotton (Deltapine 45A) seed volatiles. Similarly, Gorecki et al. (6) detected ethanol and acetaldehyde as major volatile components of pea seed exudates. Both were detected as soon as imbibition was initiated and reached a maximum between 12 and 48 hr of imbibition at 25 C.

In this study, ethanol but not acetaldehyde was stimulatory to sporangium germination of *P. ultimum*. Ethanol has been reported as an activator of spore germination in other fungi (36,37). Concentrations of ethanol between 0 and 7 nmol/ml were most stimulatory to sporangium germination. However, the level of germination was considerably less than germination in response to seed volatiles. Perhaps a greater stimulation would have been observed had concentrations lower than 1 nmol/ml been evaluated.

The lack of stimulation by acetaldehyde is in contrast to Owens et al. (22), who found acetaldehyde to be the most active component of alfalfa distillates stimulating soil respiration and increases in soil fungal populations. Similarly, Schenck and Stotzky (24) concluded that aliphatic aldehydes were the primary volatile components of seed exudates of several plant species that were stimulatory to bacterial and fungal growth. Also, Harman et al. (8)

<table>
<thead>
<tr>
<th>Approximate concentration (nmol/ml)</th>
<th>Germinated sporangia (%) at 6 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaldehyde</td>
<td>Ethanol</td>
</tr>
<tr>
<td>6,200</td>
<td>7.9 b</td>
</tr>
<tr>
<td>650</td>
<td>5.8 a</td>
</tr>
<tr>
<td>70</td>
<td>6.9 b</td>
</tr>
<tr>
<td>7</td>
<td>17.8 a</td>
</tr>
<tr>
<td>1</td>
<td>21.7 a</td>
</tr>
<tr>
<td>0</td>
<td>0.0 b</td>
</tr>
</tbody>
</table>

Means in each column followed by the same letter are not significantly different (P = 0.05) according to Duncan’s multiple range test.

suggested that various fatty acids and their lipid peroxidation products, including acetaldehyde, were the primary active components in volatile exudates from aged seeds that stimulate germination of conidia of * Fusarium solani* (Mart.) Sacc. (emend. Snyd. & H. F. sp. *pisi* and *Alternaria alternata* (Fr.) Keissler. In their system, some compounds were active at concentrations of 4 ml per petri dish. Perhaps concentrations of acetaldehyde tested in the present study were high enough to be inhibitory to sporangium germination.

Sporangium germination was consistently lower when exposed to volatiles at 35 C than to those evolved at 15 or 25 C despite the fact that sporangia were 100% germinable at 35 C. Although amounts of ethanol evolved from cotton seeds after 6 hr of imbibition at 35 C were slightly greater than 1 nmol/ml, these concentrations may not have supported complete germination, whereas lower concentrations, such as those evolved at 25 and 15 C, may have been more favorable for germination. This apparent inhibitory effect of volatiles evolved at 35 C may be a factor responsible for reduced severity of *Pythium* seed rot at elevated temperatures (2,11).

The important role that volatiles can play in spermosphere ecology has already been demonstrated (21). Volatiles can act as stimulants of fungal spore germination (3,4,9), sclerotial germination (21), fungal sporulation (18), and fungal and bacterial growth (24) and act as attractants for developing germ tubes and hyphae (13,21). On the other hand, they can also be inhibitory to spore germination and growth (4,17). This study has demonstrated the ability of volatile exudates, particularly ethanol, from germinating seeds to stimulate sporangium germination in *Pythium* species. Stimulation by cotton volatiles occurred within 2 hr of imbibition even in the apparent absence of soluble exudates. This indicates the importance of volatile exudates in rapidly initiating host-pathogen interactions in soil and suggests that volatiles, particularly ethanol, may be as important as carbohydrate exudates, if not more so, in establishing rapid infection of seeds and seedlings by *Pythium* species.

LITERATURE CITED


Vol. 77, No. 7, 1987 1111


