

# Density of *Pythium myriotylum* Oospores in Soil in Relation to Infection of Rye

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## ABSTRACT

Suspensions of oospores of *Pythium myriotylum* were sonificated to leave only oospores as viable propagules. Autoclaved Arredondo fine sand was infested with oospores, and prepared inoculum density levels were checked by plating soil samples on a selective medium.

Percentages of rye infection after 5 days at 30 C in growth chambers at inoculum densities of 0, 1, 10, 25, 50, 100, 150, and 250 oospores per gram of soil were  $0, 2 \pm 2, 34 \pm 3, 56 \pm 1, 71 \pm 3, 81 \pm 5, 100,$  and  $100,$  respectively. Infection percentages after 14 days in pots in the greenhouse at the same inoculum levels were the same or slightly lower. No

alterations of original inoculum densities by the formation of zoospores from oospores were observed. Although infection occurred at 25, 30, or 35 C, death of rye seedlings did not normally result at temperatures below 30 C. Plant density (one to five plants per container) did not influence the percentage of infection in short term studies, and approximately the same percentages of infection occurred at the same inoculum densities in artificially infested, untreated field soil as in infested autoclaved soil.

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*Additional key words:* soil-borne fungus, inoculum density.

Plant infection by *Pythium* spp. is influenced by soil moisture, soil temperature, pH, cation composition, light intensity, presence of other microorganisms, and inoculum density (10). While most of these factors, especially the influence of soil moisture and soil temperature, have been studied widely, the influence of inoculum density of *Pythium* spp. on root infection has received little critical attention.

Since zoospores and sporangia survive for only short or intermediate periods of time in soil and oospores normally serve as the main mechanism of survival for longer periods, oospores are considered to be the primary units infecting roots in soil (3, 10, 22).

Factors such as oospore germination prior to host stimulation, proliferation of infectious propagules by zoospore formation from germinating oospores, or secondary spread subsequent to primary colonization of a single plant, may complicate the determination of the relationship of oospore density in soil to the incidence of root infection. It has been observed, however, that oospores of several species of *Pythium* that have been

carefully observed do not germinate in nonamended soil in the absence of host roots, and that zoospores are not formed from oospores in the absence of nutrients or flooded soil (3, 22). A critical evaluation of the relationship of oospore density to incidence of infection can thus be made if the following considerations are met: (i) only carefully quantified suspensions of oospores free of all other viable forms of the pathogen should be used for infesting non-amended soil, (ii) the *Pythium* spp. tested should require stimulation by host-produced nutrients for oospore germination, (iii) soil moisture should be maintained below saturation, (iv) host roots should be kept physically separated or should be removed at timed intervals to evaluate secondary spread, (v) disease incidence should be evaluated as the percentage of plants infected rather than by symptoms or a disease index, and (vi) samples of infested soil assayed on a selective medium for *Pythium* spp. should reveal the same or some fraction of the population originally established.

The objectives of this study were to determine the relationship of oospore density in soil to the incidence of

infection, and the effects of temperature, time of host exposure, and plant density on that relationship. Rye (*Secale cereale* L.) and *Pythium myriotylum* Drechs. were selected for the host-pathogen combination in this system because the fungus produces abundant oospores in culture and is pathogenic to many crops (13), and rye allows rapid assay because of its fast growth.

**MATERIALS AND METHODS.**—The single-oospore isolate of *P. myriotylum* (Pm-1) used in this study was obtained from a diseased peanut root and maintained on V-8 juice agar.

Oospores used in inoculum density studies were obtained from mycelial mats grown in 15 ml of Schmitthenner's liquid medium (21) in 250-ml Erlenmeyer flasks at 25 C in darkness. After 3 weeks of incubation, the cultures, which contained numerous oospores attached to the hyphae, were rinsed three times in sterile distilled water and homogenized in a glass tissue grinder. The homogenates were diluted to 50-ml volumes and subjected to 40% maximum sonification with a Biosonik III ultrasonic system for 40 seconds to leave only oospores as viable propagules in the suspension. To confirm that growth originated only from oospores after sonification, a sample of the oospore suspension was plated on a selective medium (PV) which was modified from that of Tsao and Ocana (23) and contained 5 mg pimaricin (Delvocid, Gist-Brocades, Delft, Holland), 300 mg vancomycin hydrochloride (Vancocin, Eli Lilly & Co.) and 17 g Difco cornmeal agar in 1 liter of water. The number of oospores in a suspension was determined by counting six fields for each of 12 samples in a standard haemocytometer.

Arredondo fine sand with a pH of 6.5 (measurement obtained from a 1:2 suspension of soil in 0.01 M CaCl<sub>2</sub>) was infested with *P. myriotylum* to establish various inoculum densities by thoroughly mixing a known number of oospores into a specific weight of soil which had been autoclaved for 1 hour on two successive days and adjusted to a final water content of 5% (w/w).

A system was developed to expose noninjured roots of rye plants to various densities of oospores in soil maintained at the same moisture level in many different plant containers. Fifteen grams of infested soil were layered over 100 g of autoclaved, coarse, builder's sand packed in the bottom of a 100-ml polypropylene beaker

that had three small holes at the base for water movement. Rye seeds were surface-sterilized for 3 minutes in 0.525% (w/w) sodium hypochlorite, rinsed in sterile distilled water, and incubated at 30 C on moist filter paper for 24 hours. Five germinated seeds were placed on a 15-g band of sterile soil that was distributed evenly and packed over the infested soil. The seeds were covered with approximately 5 g of vermiculite and the 10 beakers for each treatment group were placed in a nylon pan (30 × 18 × 16 cm).

Oospore germination in soil was followed by sampling additional beakers prepared as described above, except that infested soil was used for the entire soil complement (30 g) in each beaker. Half of the beakers were seeded with rye so that the influence of the host on oospore germination in soil could be evaluated.

Every 48 hours 500 ml of tap water was added to each pan, and after 10 minutes excess water was removed from the pan and the cups were allowed to drain. The cups were maintained in growth chambers at 30 C under 12 hours of daylight [10,760 lux (1,000 ft-c) at the level of the plants] for 5 days.

The incidence of infection at various inoculum densities was also evaluated in the greenhouse by exposing five rye seedlings in each of ten clay pots (10-cm diameter) to each inoculum level. Germinated seeds were placed on 100 g of autoclaved soil layered over 200 g of oospore infested soil in the bottom of each pot. The seeds were covered with 100 g of autoclaved soil and the pots were watered when the surface of the soil dried (approximately every 48 hours). The pots were maintained at greenhouse temperatures fluctuating between 26 and 37 C for 14 days.

At the termination of each experiment, roots of harvested seedlings were washed in running tap water, weighed, dipped in 70% ethyl alcohol for 15 seconds, rinsed three times in sterile distilled water, dried on paper towels, and plated on PV. The plates were observed for the growth of *P. myriotylum* from the roots after 36 hours of incubation in the dark at 30 C.

The population of *P. myriotylum* in infested soil was determined by preparing a soil dilution of 1:20 (w/v) in 0.3% water agar containing 300 mg/liter vancomycin and 3.68 g/liter CaCl<sub>2</sub>·2H<sub>2</sub>O. The pH of the soil suspension was adjusted to 5.5, and a 1-ml aliquot was spread onto

TABLE 1. The effects of time and intensity of sonification on growth from hyphal fragments and oospore germination of *Pythium myriotylum*

Time (sec)	Growth and oospore germination after sonification					
	20% <sup>a</sup>		40%		60%	
	Hyphal <sup>b</sup> growth	Oospore <sup>c</sup> germination	Hyphal growth	Oospore germination	Hyphal growth	Oospore germination
0	+	+	+	+	+	+
20	+	+	+	+	—	+
40	+	+	—	+	—	+
60	+	+	—	+	—	+
80	+	+	—	+	—	+
100	—	+	—	+	—	—

<sup>a</sup>Percentage of maximum sonification with Biosonik III ultrasonic system.

<sup>b</sup>Growth from hyphal fragments recorded after 24 hours of incubation at 30 C.

<sup>c</sup>Oospore germination recorded after 24 hours of incubation at 30 C.

each of 10 PV plates per sample with a sterile glass rod. After incubation in the dark at 30 C for 36 hours, the soil-agar suspension was washed from the surface of the plates under a slow stream of tap water, and the colonies of *P. myriotylum* were counted.

The influence of the presence of host roots on oospore germination was further investigated by placing envelopes of No. 20 Nitex monofilament nylon screen cloth (16) containing sonicated oospores between the two 15-g soil layers in the beakers. The envelopes were removed periodically from beakers with or without plants and examined microscopically for oospore germination.

All experiments were repeated three times except those on the relationship of inoculum density to root infection in the beakers which were repeated eight times. The data presented in this paper are means of the experiments.

**RESULTS.**—Sonification of oospore suspensions at 20, 40, or 60% of maximum intensity for periods in excess of 100, 40 or 20 seconds, respectively, resulted in suspensions that contained only oospores as viable propagules (Table 1). Whereas some hyphal fragments at lower sonification intensities or time intervals gave rise to

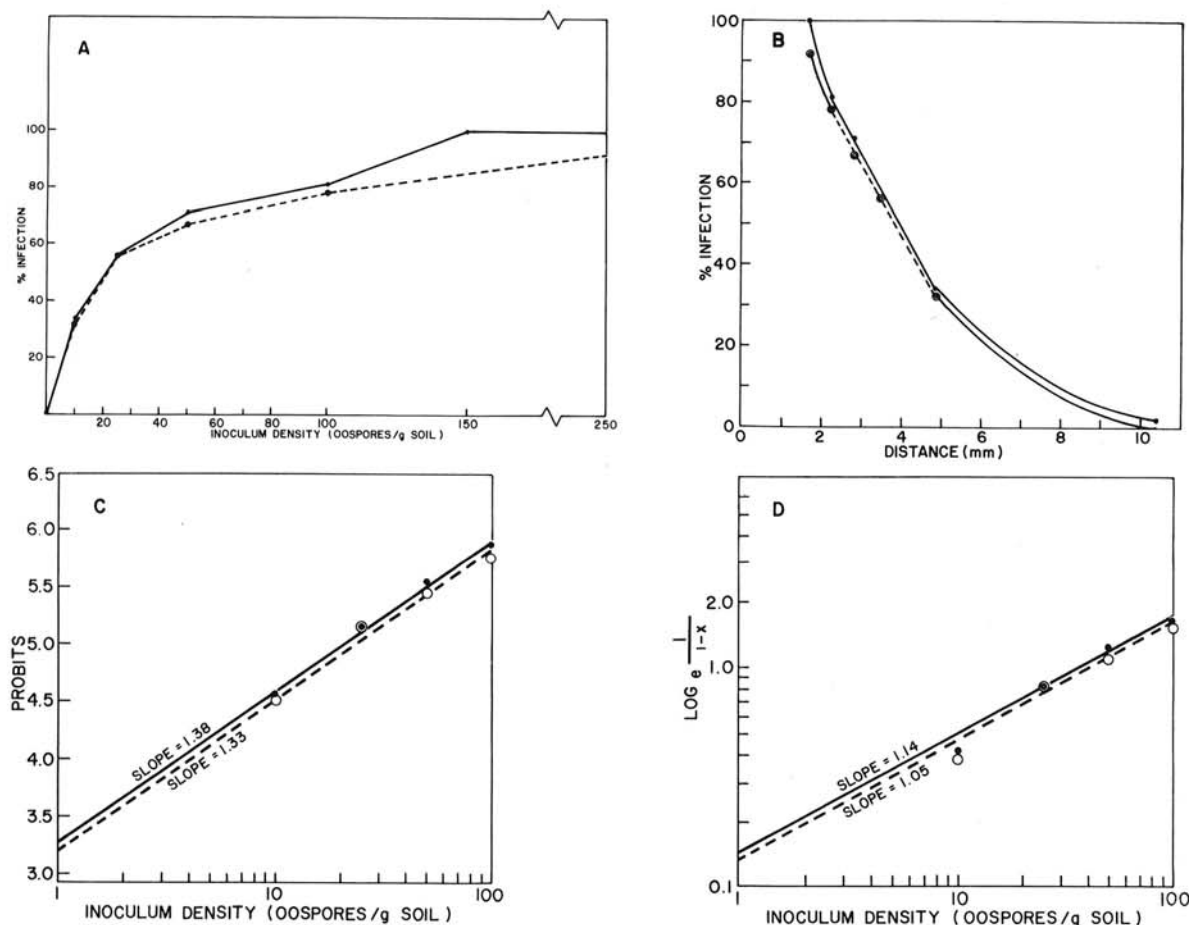
new growth, hyphal fragments treated at higher intensities or longer times were empty and nonviable. The origin of all colonies from culture suspensions treated at the higher rates and incubated for 12-24 hours on PV could be traced to germinated oospores. Oospores were not observed to germinate after exposure to a sonification intensity of 60% for 100 seconds.

Slightly higher percentages of plant infection occurred at the higher inoculum densities in beakers after 5 days than in pots after 14 days in the greenhouse (Fig. 1-A). Percentages of rye infection in beakers at inoculum densities of 0, 1, 10, 25, 50, 100, 150, and 250 oospores per gram of soil (opg) were 0, 2 ± 2, 34 ± 3, 56 ± 1, 71 ± 3, 81 ± 5, 100, and 100, respectively, and the percentages of infection in pots at the same inoculum densities were 0, 0, 32 ± 2, 56 ± 1, 67 ± 2, 78 ± 1, 87 ± 2, and 92 ± 1, respectively (Fig. 1-A).

The distance (D) between spores in soil at specific inoculum densities was estimated from the equation:

$$D = 1.1225 \sqrt[3]{\frac{Vs}{N}}$$

where (Vs) represents the volume of infested soil and (N)



**Fig. 1-(A to D).** The relationship of disease incidence in rye seedlings to density of oospores of *Pythium myriotylum* in 15 g of soil in beakers (—) or 200 g of soil in pots (----). **A)** Percentage infection (arithmetic) and inoculum density (arithmetic). **B)** Percentage infection (arithmetic) and inoculum density (distance between oospores). **C)** Infection (probits) and inoculum density (logarithmic). **D)** Infection (logarithmic) and inoculum density (logarithmic).

the number of oospores per volume of soil (14). Percentage of infection when plotted against the calculated distance between oospores (D) increased linearly between 4.84 mm (10 opg) and 2.24 mm (100 opg) (Fig. 1-B). Distances between oospores of approximately 3.85 mm in pots with 200 g of infested soil, and 3.95 mm in beakers with 15 g of infested soil were required for 50% infection of seedlings.

Points of the log-probit (5) and log-log transformations (2) lie in straight lines between 10 and 100 opg (Fig. 1-C, D). Slopes determined by linear regression analysis were 1.38 and 1.33 for beakers and pots, respectively, in the log-probit transformation (Fig. 1-C), and were close to 1.0 for the log-log transformations (Fig. 1-D). The inoculum densities required to produce 50% disease incidence ( $ID_{50}$ ) in beakers or pots were interpolated to be 20.1 and 22.5 opg in the log-probit transformation and 16.6 and 18.6 opg in the log-log transformation, respectively.

Secondary spread of *P. myriotylum* subsequent to primary infection was not observed in the beakers during short incubation periods. When 50 seedlings were harvested after incubation at 30 C in beakers containing 25 opg at 24-hour intervals, 56% infection was attained after 3-4 days, and the percentage of infection was never greater than 61% during a 14-day experiment. In addition, the percentages of dead plants after 12 days were close to the percentages of infected plants after 4 or 12 days at 30 C (Table 2), and the fungus was not isolated from living plants adjacent to infected or dying plants (Table 2). Finally, the density of plants in a beaker did not influence greatly the infection rate. Plants were infected randomly within the beakers, and the infection levels at 25 opg with 1, 2, 3, 4, or 5 seedlings per beaker were 55, 53, 57, 48, and 57%, respectively.

Maximum infection at 25 and 100 opg occurred at 30-35 C (Table 3). The lowest percentages of infection were observed at 40 C; less than 35% of the rye seedlings emerged.

Approximately the same levels of infection occurred at defined oospore levels in artificially infested, air-dried, untreated field soil and in artificially infested autoclaved soil. Percentages of infection at 25 and 100 opg were 50 and 83%, respectively, in untreated soil; and 53 and 88% at the same densities in autoclaved soil. *Pythium myriotylum* was distinguished from other *Pythium* spp. isolated from rye roots grown in natural soil by observing fungal morphology after growth in grass blade cultures (24).

Oospores germinated after 24 hours in the nylon-mesh envelopes placed next to growing rye roots in soil, but did not germinate in envelopes in soil in the absence of roots. Zoospore formation was not observed by microscopic examination of the envelopes or soil smears in any of the experiments.

When soil from the beakers was routinely plated on the selective medium, 87-100% of the original inoculum densities were recovered from soils maintained at 30 or 35 C for 5 days or longer.

DISCUSSION.—Sonification appears to provide a rapid, reliable method to obtain resting spores of Oomycetes free of other viable propagules. Bhalla and Mitchell (4) employed passage through live water snails as

TABLE 2. The effect of time of incubation<sup>a</sup> on percent infection and percent mortality of rye seedlings at three oospore densities of *Pythium myriotylum*

Incubation (Days)	Inoculum Density (Oospores/g soil)	Infection (%)	Mortality (%)
5	1	4	0
5	25	57	0
5	100	85	0
12	1	2	6
12	25	61	49
12	100	89	86

<sup>a</sup>Seedlings incubated at 30 C in beakers in growth chambers with a 12-hour day length.

TABLE 3. The effect of temperature<sup>a</sup> on percent infection of rye seedlings at two oospore densities of *Pythium myriotylum*

Temperature (C)	Infection (%) at inoculum densities of (Oospores/g soil)	
	25	100
20	5	31
25	27	56
30	51	79
35	54	84
40 <sup>b</sup>	0	34

<sup>a</sup>Seedlings incubated for 5 days in beakers in growth chambers with a 12-hour day length.

<sup>b</sup>Roots from only 34-38% of the seedlings incubated at 40 C penetrated the soil, and plant growth was greatly reduced.

well as sonification to obtain mycelium-free oospores, but the results reported here and those of Ramirez (18) indicate that suspensions containing only viable oospores of *P. myriotylum* or chlamydospores of *Phytophthora palmivora*, respectively, may be obtained by mechanical fragmentation of mycelium and sonification without additional treatment.

The infested-soil layer technique used in this study provides one approach to relating carefully prepared and monitored resting spore densities of *Pythium* spp. in autoclaved or untreated soil to specific levels of root infection in various hosts under defined environmental conditions. Large numbers of seedlings can be employed in limited space; small amounts of inoculum and infested soil are required; soil moisture can be maintained easily at the same level in many assay containers; and the test can be conducted rapidly. The results in beakers were similar to those obtained in the greenhouse under poorly controlled conditions in pots which required more infested soil, more space, and more time.

After analyzing the data of Richardson and Munnecke (19), Baker (1) postulated a rhizosphere effect without synergistic action for *P. irregulare* damping-off of peas because slopes in the log-log analysis appeared to be near 1.0. A rhizosphere effect for *P. myriotylum* infection of rye is inferred in this study by slopes with log-log transformations of 1.14 and 1.05 for plants grown in beakers or pots, respectively (Fig. 1-D). Synergistic action with *P. myriotylum* is not reflected in the log-probit transformations (Fig. 1-C), in which slopes were less than the predicted value of 2 (17), and is not apparent in the log-log transformations (Fig. 1-D), in which slopes were close to the predicted value of 1.0 (2).

Oospores of *P. myriotylum* apparently germinate under the conditions of this study in the rhizosphere and penetrate the host directly without significant increases of inoculum density through zoospore production. Barton (3) observed that oospores of *P. mamillatum* germinated in soil only in the presence of host root exudates through the formation of germ tubes. Stanghellini and Burr (22) reported that oospores of *P. aphanidermatum* likewise germinated and formed germ tubes, but not zoospores, in saturated soil containing nutrients, exudates, host seeds, or seedlings. Oospore germination did not occur in nonamended soil, and zoospores were only formed in surface water over flooded soils. Oospores of *P. myriotylum* in soil smears or nylon-mesh envelopes were observed in this study to germinate directly in the vicinity of host roots, and the fungus was never recovered on soil plates at populations higher than the original inoculum densities. Stability of inoculum density is also indicated by the lack of a synergistic effect as described above. Finally, maximum infection occurred after 3-4 days, and did not increase during 12- to 14-day experiments in beakers or during 14-day experiments in pots. Infected rye plants died after 12 days at optimum temperatures of 30-35 C (Table 2), but the fungus was not isolated from living plants contained in the same beaker as the dead plants. This effect was also observed by Gay (8), who noted that snap beans, which escaped infection in infested soil in containers in which other seedlings died, usually did not become infected with *P. myriotylum* or show symptoms when held until senescence.

Little is known about the inoculum densities of soil-borne species of *Pythium* required to obtain specific levels of disease in host plants under defined environmental conditions. While several studies indicate that populations of *Pythium* spp. of 100-1,000 propagules per g of soil are common in the field and may be required for high levels of disease (11, 12, 20) populations of many phytopathogenic species of *Pythium*, including *P. myriotylum*, are typically found to be less than 100 propagules per g of soil (6, 9, 15, 20). Under the conditions of this study, the  $ID_{50}$  for *P. myriotylum* on rye was approximately 20 opg; a similar figure was observed with untreated soil. Inoculum levels in excess of 150 opg were required for 100% infection of seedlings, but more than 30% of the plants infected at 10 opg (Fig. 1-A).

The severity of diseases caused by *Pythium* spp. depends on many environmental factors as well as on inoculum density (10, 20). Gay (8) observed that soil moisture had less effect than soil temperature on damping-off of snap beans by *P. myriotylum*; maximum damping-off occurred at 30-35 C. The highest levels of infection with 25 or 100 opg in our temperature experiments also occurred at 30-35 C (Table 3). Seedling blights of rye and other small grains caused by *P. aphanidermatum* and *P. myriotylum* are known to be more severe in the southern United States when high temperatures are reached during early fall plantings (7, 13).

After the relationships of inoculum densities of primary infection units such as oospores or chlamydospores of *Pythium* spp. and *Phytophthora* spp. to disease incidence have been determined, it will be possible to evaluate quantitatively the influence of such factors as fungicide dosage, biological control agents, and

disease tolerance on the development of diseases caused by these organisms.

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