QUANTITATIVE STUDIES ON SPORE PRODUCTION AND HOST INFECTION BY *PYTHIUM* SPP.

BY

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Oospore production by *Pythium aphanidermatum*, *P. debaryanum*, *P. myriotylum*, and *P. polymastum* was evaluated on two natural media and on two synthetic media. Abundant oospores were produced in the natural media and in the synthetic medium that contained cholesterol. Cholesterol was required by all isolates for oospore production in the synthetic medium. The amount of cholesterol required for oospore production differed with each isolate and ranged from 1 to 1,000 ug per ml of medium. Oospore production was inhibited when the cholesterol concentration was greater than the amount required for optimum production. Optimum temperatures for oospore production ranged from 15 to 35 C and were 5 to 10 C lower than the optimum temperatures for vegetative growth. Maximum oospore production occurred after 7 to 21 days of incubation in the dark at 25 C. After 3 weeks of incubation under continuous darkness or light, there were no differences in the number of oospores produced.

Oospore germination and host infection were not affected adversely when either of the following methods were used to prepare oospore suspensions that were free of viable mycelial fragments: filtering and sonicating suspensions at 40 to 80% of maximum intensity of a Biosonic III ultra-sonic system for up to 60 sec; or treating suspensions with cellulase-hemicellulase solutions or with solutions of commercial snail enzymes. Oospore germination and host infection were, however, reduced by freezing. In vitro germination of oospores and host infection in soils were greater with oospores of *P. myriotylum* that were fed to water snails than with comparable numbers of untreated oospores; with *P. aphanidermatum*, no increases in germination of oospores or host infection were observed when oospores were fed to water snails.

When the four *Pythium* spp. were used in a host range test with cabbage, corn, rye, and tomato, *P. aphanidermatum* and *P. debaryanum* infected all plants tested but caused root weight reduction only in tomato. *Pythium myriotylum* infected and caused root weight loss in all plants tested, but *P. polymastum* infected and caused root weight loss only in cabbage.

The relationships of the density of oospores in soil to the incidence of infection were determined for *P. aphanidermatum* and *P. debaryanum* with tomato, for *P. myriotylum* with rye, and for *P. polymastum* with cabbage. Between 16 and 64 oospores per g of soil were required for 50% infection of the host plants by the four *Pythium* spp. Percentages of infection of tomato and rye by *P. aphanidermatum* and by *P. myriotylum* respectively, were affected by the temperature, time, and the watering frequency while the hosts were exposed to the inocula. Infection of rye by *P. myriotylum* also was affected by isolates that differed in the ability to infect and

by the time that the isolate had been maintained in culture previous to inoculation.

In addition, the relationships of numbers of motile or encysted zoospores (cysts) of *P. aphanidermatum*, *P. myriotylum*, and *Phytophthora palmivora* to infection of tomato were determined. Fewer numbers of motile zoospores were required to obtain the same levels of infection that occurred with cysts. The numbers of motile zoospores required per plant for 50% infection of tomato seedlings were 275 for *P. aphanidermatum*, 166 for *P. myriotylum*, and 1,505 for *P. palmivora*. The numbers of cysts required per plant for 50% infection of tomato seedlings were 23,419 for *P. aphanidermatum*, 10,988 for *P. myriotylum*, and 47,424 for *P. palmivora*. Optimum temperatures for infection of tomato by motile zoospores of *P. aphanidermatum*, *P. myriotylum*, and *P. palmivora* ranged from 20 to 30 C.

Percentages of infection of all hosts exposed to oospores or zoospores increased with increasing inoculum levels, but the ratio of infection to amount of inoculum decreased as the inoculum increased. Slopes of regression lines of $\log_{10}\log_e (1/1-y)$, where y equals the proportion of infected plants, vs $\log_{10}$ of inoculum level of oospores or motile and encysted zoospores were between 0.62 and 0.69.

PART 1

OOSPORE PRODUCTION BY *PYTHIUM APHANIDERMATUM*, *P. DEBARYANUM*, *P. MYRIOTYLUM*, AND *P. POLYMASTUM* IN LIQUID MEDIA

Introduction

Various physical and nutritional factors influence oospore production by *Pythium* spp. The specific numbers of oogonia and oospores produced by several different species of *Pythium* in natural or synthetic media under different cultural conditions rarely have been determined precisely.

Although oospores are produced readily by most *Pythium* spp. in natural media such as V-8 juice broth or hemp seed broth, synthetic media may be desired for some studies which require a minimum of organic debris or nutrients in oospore preparations, or which require reproducible defined levels of medium components. Lenny and Klemmer (36) produced mature oogonia of *P. graminicola* Subr. in a synthetic liquid medium, but they did not indicate if oospores were formed. Yang and Mitchell (67) observed the formation of mature oogonia of *P. debaryanum* Hesse, *P. irregulare* Buis., and *P. ultimum* Trow in a chemically defined medium. Adams (1) and Schmitthenner (56) produced oospores of *P. aphanidermatum* (Edson) Fitz. in a synthetic medium for germination studies. Subsequently, Schmitthenner's medium has been used for the production of oospores of several other *Pythium* spp., including *P. debaryanum*, *P. myriotylum* Drechs., and *P. ultimum* (3, 44, 54, 55).

Several basic requirements must be met for oospore production in synthetic media. For example, various carbon and nitrogen sources are required by different species. The best carbon sources for oospore production by P. debaryanum are lactose, glucose, and fructose, and the best nitrogen sources are dl-isoleucine, l-leucine and dl-asparagine (66). Pythium butleri Subram. produces oospores when amine asparagine is used, but does not when isoleucine or leucine is used (35). The carbon to nitrogen ratio may be more important for oospore production than the source or concentration of these elements (34). Oospore production by both Phytophthora and Pythium spp. is favored by a high carbon to nitrogen ratio (34, 36, 55). Since Pythium spp. are incapable of synthesizing sterols, cholesterol or some other suitable sterol must be incorporated into synthetic media for oospore production (24, 25). Calcium is essential for the production of oogonia by P. debaryanum, P. graminicola, P. irregulare, and P. ultimum (36, 67).

Optimum oospore production by Pythium spp. occurs near neutral pH; oospores are produced typically at the same hydrogen ion concentrations at which growth of these fungi occurs (1, 2). Al-Hassan & Fergus (2) reported growth and oospore production by P. artotrogus (Mont.) deBary over the pH range of 4.5 to 8.5. The optimum initial pH of the culture medium ranged from 5.0 to 5.5 and the final pH ranged from 5.9 to 6.2.

Light may be an important environmental factor that affects oospore formation by Pythium spp. High light intensities (10,760-13,988 lx) inhibit oospore production by P. ultimum (42).

The range of favorable temperatures for oospore production by pythiaceous fungi is narrower than that for growth (2, 3, 28). In general, temperatures lower than those for optimum vegetative growth

favor oospore formation, differentiation, and maturation by both homothallic and heterothallic *Pythium* spp. The time required for initiation of antheridia and oogonia depends upon the temperature of incubation. Gametangial initiation in some heterothallic *Pythium* spp. begins after 3 days of incubation at 25 C (36). Germinable oospores of *P. aphanidermatum* can be obtained after 5 days of incubation at 26 C (3).

Large quantities of oospores of *P. aphanidermatum*, *P. debaryanum*, *P. myriotylum*, and *P. polymastum* produced under defined environmental conditions were needed in our laboratory for studies on oospore germination, fungal survival in soil, and plant infection. The objective of this study was to evaluate the cultural conditions in liquid media that are most favorable for abundant production of viable oospores free of other reproductive structures.

Materials and Methods

Pythium aphanidermatum and *P. debaryanum* were isolated from tomato seedlings (*Lycopersicon esculentum* Mill.), *P. myriotylum* was isolated from a peanut pod (*Arachis hypogeae* L.), and *P. polymastum* came from a cabbage seedling (*Brassica oleracea* L.). Cultures of these fungi were maintained on V-8 juice agar.

Hemp seed decoction broth was prepared as follows: 20 g of heat sterilized hemp seeds (*Cannabis sativa* L.) were cracked by placing the seeds in a Waring blender with 40 ml of distilled water (DW) and turning the blender on and off at maximum speed ten times during a 10 sec period. The cracked seeds with the water were then autoclaved at 121 C for 30 min. After autoclaving, the mixture was filtered through four layers of cheese cloth while still hot and the filtrate was adjusted to a 40-ml volume with

DW. Twenty milliliters of this solution were mixed with 980 ml of DW and 15 ml of broth were added to each 250-ml Erlenmeyer flask.

Half strength V-8 juice broth was prepared by mixing 300 ml of V-8 juice (Campbell Soup Co., Camden, New Jersey) with 4.5 g of $CaCO_3$ and centrifuging the mixture at 5,860 X g for 5 min. One hundred milliliters of supernatant were added to 900 ml of DW.

Yang's synthetic medium was prepared as described by Yang and Mitchell (67). It was composed of 0.5 g of KH_2PO_4, 0.5 g of K_2SO_4, 0.6 g of $MgCl_2 \cdot 6\ H_2O$, 5.9 g of glucose, 1.3 g of dl-asparagine, and 1,000 ml DW. The cultures were grown in 15 ml of this medium in 250-ml flasks for 7 days at 25 C in the dark, rinsed three times with sterile distilled water (SDW) and then resuspended in 50 ml of replacement solution which contained 0.001 M Ca^{++} in the form of a chloride salt ($CaCl_2 \cdot 2\ H_2O$). The cultures were incubated for an additional 14 days.

Schmitthenner's synthetic liquid medium consisted of 0.15 g of KH_2PO_4, 0.15 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7\ H_2O$, 4.4 mg of $ZnSO_4 \cdot 7\ H_2O$, 1.0 mg of $FeSO_4 \cdot 7\ H_2O$, 0.07 mg of $MnCl_2 \cdot 4\ H_2O$, 2 mg of thiamine HCl, 10 mg ascorbic acid, 30 mg of $CaCl_2$, 2.4 g of sucrose, 0.27 g of asparagine, 30 mg of cholesterol, and 1,000 ml of DW (56). Cholesterol was added to 15 ml of medium in each 250-ml flask as 0.45 mg of cholesterol in 1 ml of dichloromethane after the addition of the medium and before autoclaving.

Cultures were initiated for each fungus by adding a 6-mm agar disc cut from the perimeter of a 24 to 48-hr-old culture to each of four 250-ml flasks containing 15 ml of a liquid medium. The agar medium used for inoculum production was the same as the respective liquid medium used in each test except for the addition of 17 g of Difco agar per liter of medium. All media were adjusted to pH 6.5 with 0.1 N NaOH or 0.1 N HCl

before autoclaving. After inoculation, the cultures routinely were maintained at 25 C for 21 days under continuous darkness unless otherwise treated.

After 3 weeks of incubation, each culture was washed on a nylon screen with SDW, resuspended in SDW, and homogenized with a glass tissue grinder. The final homogenate from each culture was adjusted to a 50-ml volume with SDW. This suspension then was subjected to 40% of maximum intensity of a Biosonic III ultrasonic system for 40 sec to disrupt mycelial fragments and to disperse the spores more evenly in water. The numbers of oogonia and oospores within oogonia in the suspension were determined immediately after sonication by counting four fields for each of ten samples from each culture on a standard hemacytometer. Aborted oospores and oospores with excessive vacuoles were not included in the oospore counts.

The ability of the Pythium spp. to grow on different media was observed on agar plates. Cultures for radial growth determination were initiated by placing a 6-mm inoculum disc obtained from the margin of a 24- to 48-hr-old culture grown on the same medium as that used in the test. Each petri dish contained 15 ml of medium. Four replicates of each isolate were grown on each test medium. After 24 or 48 hrs of incubation, four measurements of each colony diameter were taken and measurements for each isolate on a particular medium were averaged.

The effects of cholesterol on colony diameter and on oogonium and oospore production by each Pythium spp. were determined in Schmitthenner's medium with or without agar. Concentrations of cholesterol ranging from 0.1 to 1,000 ug per ml of substrate were tested.

The time required for initiation of oogonia was determined at six temperatures. Individual cultures were examined at 24-hr intervals and

the numbers of oogonia were recorded.

The effect of time on the production of oogonia and oospores was determined by harvesting cultures after 1, 2, 3, 6, or 12 weeks of incubation and counting the spores.

The effect of light was studied in a growth chamber illuminated with fluorescent lamps producing 10,760 lx at the level of the cultures. One set of cultures was illuminated continuously and one set was wrapped in aluminum foil.

The standard medium and incubation conditions (unless otherwise indicated) were as follows: Schmitthenner's liquid medium was used for oogonium and oospore production; Schmitthenner's agar was used for growth determination; cultures were maintained in the dark at 25±1 C; cultures in liquid medium were incubated for 21 days and those on agar for 24 or 48 hrs. Each experiment was repeated, and each treatment was replicated four times. The results in tables represent data from one of the two experiments since the results were similar. Duncan's multiple-range test was used to determine significant differences between treatments. Analyses of data in the form of percentages were performed with the data transformed to arcsin degrees.

Results

Generally, the natural media were the best substrates for oospore production by the *Pythium* spp. studied (Table 1). More oospores of *P. aphanidermatum* and *P. polymastum* were produced in V-8 juice broth than in the other media. The best medium for oospore production by *P. debaryanum* was hemp seed broth. Schmitthenner's medium supported good oospore production by all isolates. There were no significant differences (P=0.05) in the number of oospores produced by *P. debaryanum* and *P. myriotylum* in Schmitthenner's medium or in V-8 juice broth. No oospores of *P. myriotylum* or *P. polymastum* and very few oospores of *P. aphanidermatum* and *P. debaryanum* were produced in Yang's replacement medium.

Pythium aphanidermatum consistently produced the highest percentage (70%) of oogonia with oospores that appeared normal, *P. debaryanum* (43%) and *P. myriotylum* (37%) were intermediate and *P. polymastum* produced the lowest percentage of oogonia with oospores (24%). When *P. aphanidermatum*, *P. myriotylum*, or *P. polymastum* were cultured in hemp seed broth, V-8 juice broth, or Schmitthenner's medium, there were no significant differences in the percentages of oogonia with oospores. However, the percentages of oogonia with oospores of *P. debaryanum* differed with each medium used. *Pythium debaryanum* produced the highest percentage of oogonia with oospores in hemp seed broth (66%) and the lowest percentage in Yang's medium (3%). Forty percent of the oogonia of *P. debaryanum* that were produced in V-8 juice broth contained oospores and 20% of those produced in Schmitthenner's medium contained oospores.

A significant increase in oospore production by all isolates was observed when cholesterol was added to Schmitthenner's basal medium at concentrations greater than 1.0 ug per ml (Table 2). *Pythium aphanidermatum*

and P. debaryanum produced the most oospores at 200-300 and 10-1,000 ug of cholesterol per ml of medium, respectively. Maximum production of oospores by P. myriotylum and P. polymastum occurred at 100-300 and 10 ug per ml of medium, respectively.

At concentrations of cholesterol greater than that required for optimum oospore production, oospore production by most isolates was inhibited. Only P. polymastum did not produce oospores at high concentrations of cholesterol. No inhibition was observed when P. debaryanum was cultured in media containing up to 1,000 ug of cholesterol per ml of medium. The proportion of aborted oospores of P. aphanidermatum and P. polymastum increased when the concentration of cholesterol was greater than the concentration required for optimum oospore production. The percentages of oogonia of P. debaryanum and P. myriotylum with oospores that appeared normal were not significantly greater at 1,000 ug of cholesterol than at 10 ug per ml of medium (Table 2).

Vegetative growth by P. debaryanum, P. myriotylum, and P. polymastum appears to be stimulated slightlv when the medium contained concentrations of cholesterol ranging from 0.1 to 10 ug per ml of medium. No stimulation of vegetative growth of P. aphanidermatum was observed when cholesterol was added (Table 2).

For two isolates the range of temperatures for optimum oospore production was broader than the range of temperatures for optimum growth. The optimum temperatures for growth were 35 C for P. aphanidermatum and P. myriotylum, 25 C for P. debaryanum, and 30 C for P. polymastum (Table 3). Temperatures for optimum oospore production were 20-30 C for P. aphanidermatum, 15 C for P. debaryanum, 25 C for P. myriotylum, and 15-25 C for P. polymastum.

The earliest production of oogonia by all isolates in Schmitthenner's medium occurred at 25 C (Table 4). Pythium aphanidermatum produced oogonia after 2 days at 25, 30, and 35 C; P. debaryanum, P. myriotylum, and P. polymastum required an additional 2 to 3 days at 20 or 30 C. Only P. aphanidermatum and P. myriotylum produced oogonia at 35 C. All isolates produced oogonia at 15 C, but longer incubation times were required.

Pythium aphanidermatum and P. myriotylum produced maximum numbers of oospores after 7 days of incubation at 25 C. Pythium debaryanum and P. polymastum required 14 to 21 days of incubation, respectively, for maximum oospore production (Table 5). The number of oogonia per culture remained the same after the first and second week of incubation. With the exception of P. myriotylum, the number of oospores per culture and the percentage of oogonia with oospores that appeared normal remained relatively constant or declined slightly during the 12 week study. Aborted oospores were observed in all cultures after the first week of incubation. As time progressed the number of aborted oospores of P. myriotylum steadily increased.

Cultures of Pythium spp. incubated under continuous darkness produced 4 to 25% more oospores than those incubated under continuous light; however, the increased production was not significant (P=0.05). There were no differences in the number of oospores produced when the four isolates were exposed to the following light regimes: 3, 7, and 14 days of continuous light followed with 18, 14, and 7 days of darkness, respectively, or 3, 7, or 14 days of darkness followed with 18, 14, and 7 days of continuous light. When cultures of the four isolates were exposed to strong light (10,760 lx), growth was inhibited slightly.

None of the four *Pythium* spp. grew or produced oospores in Schmitthenner's medium adjusted to pH 4.0, but all isolates grew and produced abundant oospores in media with initial hydrogen ion concentrations ranging from pH 4.5 to 8.5. The final pH of the filtrates obtained from cultures that produced oospores were all near neutrality, and the number of oospores produced by each isolate in cultures with different initial hydrogen ion concentrations did not differ.

Table 1. The influence of media on colony diameter and production of oogonia and oospores by *Pythium* spp. after 21 days in the dark at 25 C.

Medium	Colony[x] diameter (mm)	Number of oogonia per culture[y]	Number of oospores per culture	% oogonia with oospores
		Pythium aphanidermatum		
Hemp seed	67 b[z]	1.2×10^6 b	0.8×10^6 c	70 a
V-8 juice	73 a	3.1 C 10^6 a	2.3×10^6 a	74 a
Schmitthenner's	61 c	2.6×10^6 a	1.7×10^6 b	65 a
Yang's	55 d	0.4×10^6 c	0.1×10^5 d	2 b
		Pythium debaryanum		
Hemp seed	61 b	1.1×10^6 a	0.7×10^6 a	66 a
V-8 juice	70 a	0.5×10^6 c	0.2×10^6 b	40 b
Schmitthenner's	61 b	0.9×10^6 b	0.2×10^6 b	20 c
Yang's	62 b	0.2×10^6 d	0.1×10^5 c	3 d
		Pythium myriotylum		
Hemp seed	72 b	1.0×10^6 b	0.3×10^6 b	32 a
V-8 juice	81 a	1.9×10^6 a	0.7×10^6 a	40 a
Schmitthenner's	61 c	2.1×10^6 a	0.8×10^6 a	39 a
Yang's	55 d	0 c	0 c	0 b
		Pythium polymastum		
Hemp seed	40 a	1.4×10^5 b	0.5×10^5 b	27 a
V-8 juice	29 b	5.6×10^5 a	1.1×10^5 a	19 a
Schmitthenner's	21 c	0.7×10^5 bc	0.3×10^5 b	27 a
Yang's	16 d	0.1×10^5 c	0 c	0 c

[x]Growth after 24 hrs (48 hrs for *P. polymastum*) of incubation on 1.7% agar incorporated into the respective liquid media (described in text).

[y]The cultures were incubated in 250-ml Erlenmeyer flasks containing 15 ml of liquid medium; all media were adjusted to pH 6.5 before autoclaving.

[z]Means within a column not followed by the same letter are significantly different (P=0.05) as determined by Duncan's multiple-range test; all percentages were converted to arcsin degrees before analyses.

Table 2. The influence of cholesterol on colony diameter and production of oogonia and oospores by *Pythium* spp. in Schmitthenner's medium in the dark at 25 C.

Cholesterol (ug/ml)	Colony[x] diameter (mm)	Number of oogonia per culture[y]	Number of oospores per culture	% oogonia with oospores
		Pythium aphanidermatum		
0.0	59 a[z]	0.4×10^5 d	0 f	0 f
0.1	59 a	0.4×10^5 d	0.2×10^5 f	56 cde
1.0	60 a	0.5×10^5 c	0.3×10^5 ef	59 bcde
10.0	59 a	2.4×10^6 a	1.5×10^6 c	62 bcd
30.0	59 a	2.6×10^6 a	1.7×10^6 bc	65 bcd
100.0	61 a	2.5×10^6 a	1.8×10^6 bc	71 abc
200.0	60 a	2.6×10^6 a	2.0×10^6 ab	76 ab
300.0	59 a	2.8×10^6 a	2.3×10^6 a	86 a
500.0	59 a	1.4×10^6 b	0.7×10^6 d	49 de
1000.0	61 a	1.4×10^6 b	0.6×10^6 de	41 e
		Pythium debaryanum		
0.0	55 b	0.9×10^5 c	0 b	0 b
0.1	55 b	7.1×10^5 b	0 b	0 b
1.0	59 ab	7.8×10^5 ab	0 b	0 b
10.0	62 a	7.9×10^5 ab	1.3×10^5 a	16 a
30.0	61 a	8.5×10^5 a	1.7×10^5 a	20 a
100.0	60 a	8.3×10^5 a	1.8×10^5 a	22 a
200.0	58 ab	8.6×10^5 a	2.0×10^5 a	23 a
300.0	58 ab	8.5×10^5 a	2.1×10^5 a	25 a
500.0	59 ab	8.5×10^5 a	1.7×10^5 a	20 a
1000.0	59 ab	8.3×10^5 a	1.7×10^5 a	20 a
		Pythium myriotylum		
0.0	49 b	1.7×10^6 c	0 e	0 c
0.1	56 a	2.0×10^6 bc	0.4×10^5 de	2 c
1.0	60 a	2.0×10^6 bc	0.2×10^6 d	16 b
10.0	59 a	2.0×10^6 bc	0.6×10^6 c	32 a
30.0	60 a	2.1×10^6 bc	0.8×10^6 bc	40 a
100.0	60 a	2.4×10^6 ab	0.9×10^6 ab	36 a
200.0	58 a	2.5×10^6 a	1.0×10^6 a	40 a
300.0	58 a	2.8×10^6 a	0.9×10^6 ab	32 a
500.0	59 a	2.5×10^6 a	0.8×10^6 bc	30 a
1000.0	60 a	2.6×10^6 a	0.8×10^6 bc	29 a

Table 2 - continued.

Cholesterol (ug/ml)	Colony[x] diameter (mm)	Number of oogonia per culture[y]	Number of oospores per culture	% oogonia with oospores
		Pythium polymastum		
0.0	25 b[z]	0 c	0 d	0 d
0.1	25 b	0 c	0 d	0 d
1.0	26 b	0.2×10^4 c	0 d	0 d
10.0	30 a	5.4×10^5 a	7.2×10^4 a	13 b
30.0	31 a	1.0×10^5 b	2.6×10^4 b	28 a
100.0	29 a	0.8×10^5 bc	0.8×10^4 c	9 c
200.0	31 a	0.7×10^5 bc	0.6×10^4 c	7 c
300.0	30 a	0.2×10^4 c	0 d	0 d
500.0	31 a	0 c	0 d	0 d
1000.0	32 a	0 c	0 d	0 d

[x]Growth after 24 hrs (P. polymastum 48 hrs) of incubation in the dark at 25 C on 1.7% agar incorporated into the respective liquid media (described in text).

[y]The cultures were incubated for 21 days in 250-ml Erlenmeyer flasks containing 15 ml of liquid medium; all media were adjusted to pH 6.5 before autoclaving.

[z]Means within a column not followed by the same letter (s) are significantly different (P=0.05) as determined by Duncan's multiple-range test; percentages were converted to arcsin degrees before analyses.

Table 3. The influence of temperature on colony diameter and production of oogonia and oospores by Pythium spp. in Schmitthenner's medium.

Temperature (C)	Colony[x] diameter (mm)	Number of oogonia per culture[y]	Number of oospores per culture	% oogonia with oospores
		Pythium aphanidermatum		
15	21 f[z]	1.2×10^6 c	5.0×10^5 b	41 b
20	37 e	2.7×10^6 a	1.7×10^6 a	63 a
25	61 c	2.6×10^6 a	1.6×10^6 a	62 a
30	70 b	2.5×10^6 a	1.6×10^6 a	67 a
35	77 a	1.7×10^6 b	5.0×10^5 b	28 c
40	52 d	0 d	0 c	0 d
		Pythium debaryanum		
15	33 d	1.1×10^6 a	6.6×10^5 a	59 a
20	51 c	7.4×10^5 b	4.7×10^5 b	63 a
25	61 a	8.5×10^5 b	1.7×10^5 c	20 b
30	56 b	4.5×10^5 c	2.4×10^4 d	5 c
35	11 e	0 d	0 d	0 c
40	6 f	0 d	0 d	0 c
		Pythium myriotylum		
15	20 f	1.6×10^6 b	1.1×10^5 d	7 d
20	31 e	2.2×10^6 a	5.3×10^5 b	24 c
25	61 c	2.1×10^6 a	8.1×10^5 a	39 a
30	75 b	1.5×10^6 bc	4.8×10^5 b	31 b
35	87 a	1.2×10^6 c	3.3×10^5 c	28 bc
40	54 d	1.2 C 10^6 c	0 d	0 d
		Pythium polymastum		
15	24 d	7.0×10^4 a	2.2×10^4 a	33 a
20	26 c	9.8×10^4 a	3.1×10^4 a	30 a
25	31 b	9.5×10^4 a	2.6×10^4 a	27 a
30	39 a	9.2×10^4 a	1.4×10^4 b	16 b
35	6 e	0 b	0 c	0 c
40	6 e	0 b	0 c	0 c

[x]Growth after 24 hrs (48 hrs for *P. polymastum*) on 1.7% agar incorporated into the respective liquid media.

[y]The cultures were incubated for 21 days in the dark in 250-ml Erlenmeyer flasks containing 15 ml of liquid medium; all media were adjusted to pH 6.5 before autoclaving.

[z]Means within a column not followed by the same letter are significantly different (P=0.05) as determined by Duncan's multiple-range test; all percentages were converted to arcsin degrees before analyses.

Table 4. Time required for initiation of oogonium production by four *Pythium* spp. at six temperatures.

Pythium spp.	Temperature (C)[x]					
	15	20	25	30	35	40
	Days after inoculation					
P. aphanidermatum	8	3	2	2	2	--[y]
P. debaryanum	7	5	3	4	--	--
P. myriotylum	7	5	2	3	3	--
P. polymastum	6	4	3	4	--	--

[x]The cultures were maintained in Schmitthenner's liquid medium in the dark.

[y]No oogonia were produced during 21 days of incubation.

Table 5. The effect of time of incubation at 25 C on oogonium and oospore production by *Pythium* spp. in Schmitthenner's liquid medium.

Days after inoculation	Number of oogonia per culture[y]	Number of oospores per culture	% oogonia with oospores
	Pythium aphanidermatum		
7	2.5×10^6 a[z]	1.9×10^6 a	76 a
14	2.4×10^6 a	1.7×10^6 ab	70 ab
21	2.6×10^6 a	1.7×10^6 ab	65 ab
42	2.5×10^6 a	1.6×10^6 b	63 b
84	2.5×10^6 a	1.5×10^6 b	62 b
	Pythium debaryanum		
7	7.2×10^5 b	1.0×10^5 b	14 b
14	8.4×10^5 a	2.0×10^5 a	24 a
21	8.5×10^5 a	1.7×10^5 a	20 a
42	8.8×10^5 a	1.7×10^5 a	19 ab
84	8.9×10^5 a	1.7×10^5 a	19 ab
	Pythium myriotylum		
7	2.3×10^6 a	1.0×10^6 a	44 a
14	2.2×10^6 a	9.3×10^5 ab	42 a
21	2.1×10^6 a	8.1×10^5 b	39 a
42	2.2×10^6 a	3.5×10^5 c	16 b
84	2.2×10^6 a	2.9×10^5 c	13 b
	Pythium polymastum		
7	5.2×10^4 b	1.3×10^4 b	25 a
14	5.4×10^4 b	1.6×10^4 b	28 a
21	9.5×10^4 a	2.6×10^4 a	27 a
42	9.9×10^4 a	2.2×10^4 ab	22 a
84	1.0×10^5 a	1.8×10^4 ab	18 a

[y]The cultures were incubated in 250-ml Erlenmeyer flasks containing 15 ml of liquid medium, the medium was adjusted to pH 6.5 before autoclaving.

[z]Means within a column not followed by the same letter (s) are significantly different (P=0.05) as determined by Duncan's multiple-range test; percentages were converted to arcsin degrees before analyses.

Discussion

The natural media used most often for culturing pythiaceous fungi are: cornmeal, hempseed, lima bean, oatmeal, pea, potato-carrot, soybean or V-8 juice (2, 22, 27, 28, 42, 50, 52). Although these media provide the nutritional requirements for growth and sporulation of pythiaceous fungi they are defined poorly and are subject to change with locality, source of ingredients, manufacturing, and consumer trends. Agar contains calcium, nitrogen and other contaminants. Unless purified, it is unreliable for use in studies with fungi that have exacting nutritional requirements. Thus, chemically-defined liquid media are preferred over natural liquid or solid media for nutritional or physiological studies. Synthetic liquid media provide a homogenous environment for fungi, can be duplicated, and can be separated more easily from the organism (27).

Optimum oospore production by the species of *Pythium* tested in this study varied with the media used. The natural media were better than the synthetic media for oospore production by most species. The replacement medium of Yang and Mitchell (67) supported production of very few oospores but the synthetic medium developed by Schmitthenner (56) sustained abundant production by all species.

The percentage of oogonia with oospores changed when the cholesterol concentration in Schmitthenner's medium was altered. No oospores were produced when the cholesterol was omitted, but most isolates produced oogonia. Ayers and Lumsden (3) obtained mature oospores of *P. aphanidermatum* and *P. ultimum* in this medium when it lacked cholesterol; however, their production was much greater when they added the sterol.

The results obtained by Ayers and Lumsden (3) on oospore production in V-8 juice broth with *P. aphanidermatum* were similar to ours: 71% of

the oogonia in their study and 74% in this study contained mature oospores. Similar numbers of oospores of _P. myriotylum_ were formed in the two studies; however, only 15% of the oogonia in their study compared to 40% of the oogonia in our experiments produced mature oospores.

The differences that exist between the two studies are understandable since the isolates (20, 40) and the methods used were different. All cultures used by Ayers and Lumsden (3) were grown in 25 ml of media in petri dishes for only 14 days, while the fungi in this study were grown in 15 ml of media in 250-ml Erlenmeyer flasks for 21 days. In some of their tests, cultures were comminuted in a Tekmar Tissuemizer before oospores were counted in a hemacytometer; in other tests oospores were counted directly from mats. All cultures used in our tests were comminuted with a glass tissue grinder and sonicated before counts were made. This procedure eliminated the variability due to non-uniform distribution of oospores on mats encountered by Ayers and Lumsden (3), but the use of a tissue grinder for comminuting fungal mats probably destroyed some oospores (55). Loss of oospores due to grinding appears to be uniform since counts among replicates of a treatment were not singnificantly different.

Of the physical environmental factors studied, temperature had the greatest influence on oospore production and growth. For two isolates the ranges of temperatures for optimum oospore production were broader than for growth. Temperature ranges for optimum growth were higher than temperatures required for optimum oospore production.

The use of oospores obtained from cultures older than 2 weeks for studies on oospore germination in vitro or on inoculum density in soils reduces the possibility of introducing immature oospores or unfertilized oogonia, which may lead to quantitatively inaccurate results. Oogonium

germination or growth from antheridia not yet moribund has been reported to occur in vitro with several pythiaceous fungi (3, 9, 52). Furthermore, oospores older than 2 weeks have been reported to germinate at a higher rate than those obtained from younger cultures (1, 3).

Pythiaceous fungi have the ability to alter the hydrogen ion concentration of the medium in which they are cultured (2, 31, 32, 39). All Pythium spp. grown in Schmitthenner's medium neutralized the hydrogen ion concentration. Since the phosphate buffer systems used were not adequate to maintain the initial hydrogen ion concentration, better buffer systems (12) should be evaluated in the future.

The presence or absence of light greatly influences oospore production by Phytophthora spp. (10, 24, 27), but it does not appear to have a significant effect on oospore production by the Pythium spp. tested in this study.

Various physical and nutritional factors influence oospore production by Pythium spp. Schmitthenner's liquid medium is a good medium for oospore production by all of the Pythium spp. used in this study, but for optimum oospore production the cholesterol level and the incubation temperature should be adjusted to suit the individual isolate.

PART 2

AN EVALUATION OF METHODS FOR OBTAINING MYCELIUM-FREE OOSPORES OF *PYTHIUM APHANIDERMATUM* AND *P. MYRIOTYLUM*

Introduction

Techniques for obtaining oospores free of mycelial fragments and other viable propagules are useful for studies on oospore germination, population dynamics, and the relationship of inoculum densities of various species of *Phytophthora* and *Pythium* to infection and disease incidence.

Some of the methods that have been used by other workers to separate oospores of various oomycetes from mycelial fragments are centrifuging an oospore suspension in a sucrose density gradient after freezing the culture to kill the mycelium (48); forcing cultures through a 50-u sieve and separating oospores from mycelial fragments on a column of water (14); coupling sonication and isopycnic centrifugation of an oospore suspension (66); grinding mats in a tissue grinder and sonicating the suspension to disrupt mycelial fragments (44, 54); grinding mats and treating the suspension with commercial snail enzymes or combinations of cellulase and hemicellulase (58, 60); and feeding the mycelial mats to snails such as species of *Helisoma*, *Helix*, *Planorbarius*, and *Planorbis* (7, 19, 57, 60).

The objectives of this study were to evaluate several methods for producing oospore inocula free of viable mycelial fragments and to evaluate the effect of the inocula on root infection.

Materials and Methods

The isolate of *Pythium aphanidermatum* (Edson) Fitz. used in this study was isolated from a tomato seedling (*Lycopersicon esculentum* Mill.) and the isolate of *P. myriotylum* Drechs. was isolated from a peanut pod (*Arachis hypogeae* L.). Single oospore isolates of these fungi that had been isolated 11 to 13 months before they wereused in this study were maintained on V-8 juice agar (200 ml of clarified V-8 juice, 800 ml of H_2O, 17 g of Difco agar) in the dark at 20 C.

Oospores were produced in 250-ml Erlenmeyer flasks containing 15 ml of Schmitthenner's liquid medium (56). Mycelial mats free of oospores were produced either in Difco nutrient broth or in Schmitthenner's liquid medium without cholesterol. All cultures were incubated for three weeks in the dark at 25 C.

Before use, all of the mycelial mats were washed three times in sterile deionized water and homogenized with a glass tissue grinder. Sterile deionized water was added to the homogenate to provide a final concentration of 10,000 oospores per ml of suspension. The suspension was divided then into six 50-ml aliquots and each aliquot was subjected to one of the treatments listed in Table 6.

At the termination of each treatment, the number of oospores remaining in each suspension was determined by counting six fields for each of 10 samples in a standard hemacytometer. The final concentration was adjusted to 1,000 oospores per ml and 10 plates containing a selective medium (PV) modified from that of Tsao and Ocana (62) were each inoculated with 1 ml of the suspension. The medium contained 5 mg of pimaricin (Delvocid, Gist-Brocades, Delft, Holland), 300 mg of vancomycin hydrochloride (Vancomycin, Eli Lilly & Co.), and 17 g of

of Difco cornmeal agar in 1 liter of sterile deionized water. After 24 and 36 hrs of incubation at 30 C in the dark, the plates were examined microscopically for growth from mycelial fragments or for oospore germination.

Autoclaved Arredondo fine sand (pH 6.5, measurement obtained from a 1:2 suspension of soil in 0.01 M $CaCl_2$) was infested with oospores to give a final density of 25 oospores per g of soil for each test using P. aphanidermatum inocula and 50 for those using P. myriotylum inocula. Before the inocula were introduced into the soil, the water content of the soil was adjusted to 5% (w/w).

The system developed by Mitchell (44) was used to expose noninjured roots of tomato ('Bonny Best') and rye (Secale cereale L. 'Wesser') seedlings to treated inocula of P. aphanidermatum and P. myriotylum. respectively. Tomato seedlings were maintained for 7 days and rye seedlings for 5 days in growth chambers at 30 C under 12 hrs of daylight (10,760 lx at the level of the plants). Fifty plants were used in each treatment.

At the termination of each experiment, seedlings were harvested by gently washing the soil away from the roots with a steady stream of tap water. After the shoots were cut off, the roots were submerged in 70% ethyl alcohol for 5 sec and rinsed three times in sterile deionized water. The roots were blotted dry on sterile paper towels and plated on the PV medium. The plates were incubated in the dark at 30 C and examined for growth of Pythium after 24 and 36 hrs.

All experiments were repeated at least twice. Those dealing with root infection were repeated three times. The data presented on the following tables are means of the experiments.

Results

Sonication of oospore suspensions at 20% of maximum intensity for periods in excess of 100 sec, or at 40, 60, or 80% of maximum intensity for 20 sec or longer, resulted in suspensions that contained only oospores as viable propagules (Table 7). At the lower sonication intensities or at shorter time intervals, some hyphal fragments gave rise to new growth. All growth, at the higher intensities or at longer sonication time intervals, was traced to germinated oospores. Pythium myriotylum oospores were slightly less resistant to high sonication intensities than were those of P. aphanidermatum. Oospores of P. aphanidermatum, but not those of P. myriotylum, germinated after being subjected to intensities as high as 80% of maximum for up to 100 sec.

No oospores or hyphal fragments of P. myriotylum produced new growth when the suspension of the culture was frozen, but some growth was observed from hyphal fragments and oospores of P. aphanidermatum (Tables 8, 9). No infection of rye seedlings occurred with inoculum of P. myriotylum that had been frozen (Table 9). Only 7% of the tomato seedlings were infected after exposure to soil infested at 25 oospores per g of soil with inoculum of P. aphanidermatum that had been frozen, but 70-80% of the seedlings were infected after growth in soil infested at the same inoculum density with oospores that had been exposed to the other treatments (Table 8).

Although hyphal fragments from fresh mats produced new growth on PV, seedlings exposed to soil infested with only hyphal fragments remained healthy and were not infected (Tables 8, 9).

When oospores of P. myriotylum were recovered from pond snails and used to infest soil, a significant increase in host infection occurred

(Table 9). Oospores of _P. myriotylum_ that were treated with the enzymes or fed to the pond snails germinated better in vitro than those that were treated by the other methods (Table 9). No increase in oospore germination or host infection was observed with _P. aphanidermatum_ inoculum that was treated with enzymes or fed to pond snails (Table 8).

Table 6. Treatments used for the preparation of oospore inocula.

Treatment	Description
Untreated suspension	The suspension was used immediatelv after grinding in a glass tissue grinder.
Filtered suspension	The suspension was filtered twice through 4 layers of cheese cloth to remove mycelial fragments.
Frozen suspension	The suspension was frozen at -5 C for 48 hrs to kill mycelial fragments.
Sonication	The suspension was subjected to 40% maximum intensity of a Biosonic III ultrasonic system for 40 sec at 20-30 C.
Cellulase & hemicellulase	The suspension was centrifuged (6,000 g for 2 min) and resuspended in 20 ml of a 2.0% buffered solution (0.1 M phosphate buffer at pH 6.0) of cellulase[a] and hemicellulase[b]. After 48 hrs of incubation at 30 C in the dark, the oospores were washed three times in sterile distilled water.
Snail enzymes	The suspension was centrifuged (6,000 g for 2 min) and resuspended in 20 ml of a 5.0% buffered solution of snail intestinal fluid (0.1 M acetate buffer at pH 5.0) obtained from *Helix pomatia*[c]. After 48 hrs of incubation at 30 C in the dark, the oospores were washed three times in sterile distilled water.
Snail ingested	Intact mats containing oospores were fed to small pondsnails that had been starved overnight. After 48 hrs, the fecal pellets were collected and the oospores were washed four times in sterile distilled water that contained 200 ppm Vancomycin hvdrochloride.

[a]"Onuzuka R-10", Calbiochem, P. O. Box 12087, San Diego 92112.

[b]"Rhozyme Hp-150", Rohm & Haas Co., Philadelphia 19105.

[c]"Glusulase", Endo Laboratory, 1000 Stewart Street, Garden City, N. J.

Table 7. The effects of time and intensity of sonication on growth from hyphal fragments and oospore germination of *Pythium aphanidermatum* and *P. myriotylum*.

Time (s)	Hyphal growth and oospore germination after sonication															
	20%[v]				40%				60%				80%			
	Hyphal[w] growth		Oospore[x] germination		Hyphal growth		Oospore germination		Hyphal growth		Oospore germination		Hyphal growth		Oospore germination	
	P.a.[y]	P.m.[z]	P.a.	P.m.	P.a.	P.m.	P.a.	P.m.	P.a.	P.m.	P.a.	P.m.	P.a.	P.m.	P.a.	P.m.
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+
40	+	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+
60	+	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+
80	+	-	+	+	-	-	+	+	-	-	+	+	-	-	+	-
100	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	-
120	-	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-

[v]Percentage of maximum intensity of a Biosonic III ultrasonic system.

[w]Growth from hyphal fragments recorded after 24hrs of incubation in the dark at 30 C (growth= +; no growth= -).

[x]Oospore germination recorded after 24 hrs of incubation in the dark at 30 C (germination= +; no germination= -).

[y]*Pythium aphanidermatum*.

[z]*Pythium myriotylum*.

Table 8. The effect of various treatments on hyphal growth, oospore germination and infection of tomato seedlings by *Pythium aphanidermatum*.

Treatment	Hyphal growth[w]	Oospore germination[x] (%)	Frequency of infection[y] (%)
Nontreated mycelial fragments	+		0 b
Frozen suspension	+	4 b[z]	7 b
Nontreated suspension	+	82 a	77 a
Filtered suspension	+	86 a	73 a
Sonicated suspension	-	84 a	80 a
Cellulase & hemicellulase		86 a	73 a
Snail enzymes		94 a	80 a
Snail ingested		91 a	70 a

[w]Growth from hyphal fragments recorded after 24 hrs of incubation in the dark at 30 C (growth= +; no growth= -).

[x]Oospore germination recorded after 24 to 36 hrs of incubation in the dark at 30 C.

[y]Percent infection of tomato seedlings ((number of infected seedlings/ 50 plants) X 100) after 7 days of incubation in soil infested with 25 oospores per g of soil at 30 C in a growth chamber with a 12-hr day length.

[z]Within each column, entries without a common letter are significantly different (P= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 9. The effect of various treatments on hyphal growth, oospore germination and infection of rye seedlings by *Pythium myriotylum*.

Treatment	Hyphal growth[w]	Oospore germination[x] (%)	Frequency of infection[y] (%)
Nontreated mycelial fragments	+		0 c
Frozen suspension	-	0 b[z]	0 c
Nontreated suspension	+	4 b	40 b
Filtered suspension	+	6 b	43 b
Sonicated suspension	-	4 b	47 b
Cellulase & hemicellulase		14 ab	43 b
Snail enzymes		12 ab	40 b
Snail ingested		21 a	67 a

[w]Growth from hyphal fragments recorded after 24 hrs of incubation in the dark at 30 C (growth= +; no growth= -).

[x]Oospore germination recorded after 24 to 36 hrs of incubation in the dark at 30 C.

[y]Percent infection of rye seedlings ((number of infected seedlings/ 50 plants) X 100) after 5 days of incubation in soil infested with 50 oospores per g of soil at 30 C in a growth chamber with a 12-hr day length.

[z]Within each column, entries without a common letter are significantly different (P= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Discussion

It is important to have oospore suspensions free of viable hyphal fragments for in vitro studies on oospore germination so that slow germinating oospores are not overgrown, and for inoculum density studies to ensure that propagule counts are as accurate as possible. The presence of mycelial fragments from cultures 21-dav-old or older in non-amended soil, however, does not appear to influence the infection rates at defined oospore densities in soil. Laviolette and Athow (33) also noted that mycelia obtained from older cultures of *Pvthium* spp. are not infective.

In our experiments *P. aphanidermatum* oospores were not affected by any of the treatments and germinated readily in vitro. In contrast to *P. aphanidermatum* oospores, *P. myriotylum* oospores germinated poorly and were not affected greatly by any of the treatments. This difficulty in germinating *P. myriotylum* oospores in vitro has been reported previously (3, 44).

Although the percentages of infection of rye seedlings exposed to *P. myriotylum* oospores from most treatments used in this study at 50 oospores per g of soil were low compared to the 71±3% infection observed by Mitchell (44, 45) at the same oospore density of this fungus, the latter value was close to the 67% infection obtained with oospores ingested by pond snails. Oospores of two isolates of *P. myriotylum* (Part 3 of this dissertation) gradually lost the ability to infect rye roots after varying times (3 to 18 months) in culture or in soil in pots under greenhouse conditions, but no changes were observed in the oospores after inoculum was produced as described above. Thus, the higher infection of rye seedlings with oospores that had been passed through

pond snails may be due, in part, to a screening effect produced by the snails wherein damage or aborted oospores that are not discerned easily visually from healthy oospores are destroyed and those retrieved from fecal pellets are mostly germinable oospores. Counts of total oospores recovered from fecal pellets were about 40% less than those from mats that only had been homogenized. No increase in infectivity was observed when oospores of an isolate of P. aphanidermatum that had not been observed to lose its ability to infect tomato seedlings were ingested by snails.

This study shows that sonication provides a rapid reliable method for destruction of mycelial fragments for work requiring suspensions of oospores of P. aphanidermatum and P. myriotylum. Sonication has been used to prepare inocula of P. debaryanum Hesse, P. irregulare Buisman, and P. polymastum Drechs. which contained only oospores as viable propagule (44, 45, 54). This method has also been used to obtain chlamydospore suspensions free of hyphal fragments of several Phytophthora spp. (45, 47, 49, 51).

PART 3

EFFECT OF SOME ENVIRONMENTAL AND CULTURAL FACTORS ON INFECTION OF SEVERAL HOSTS BY *PYTHIUM* SPP.

Introduction

Under environmentally favorable conditions for host infection in growth chambers, populations or *Pythium* spp. of 15 to 50 oospores per g of soil were required for 50% infection of several hosts (43, 44, 53). Disease under natural conditions, however, may not occur in soils that contain abnormally high populations of *Pythium* spp. propagules if the environmental conditions are not favorable (23). Although information is available on variability in host susceptibility to *Pythium* spp. (23, 40), on intraspecific variability in pathogenicity of the fungi (23, 40), on the number of oospores required for infection of plants in soils (44, 45), and on certain environmental conditions that contribute to infection of host plants during exposure to oospores (23, 37, 44, 55), little or no information is available on the influence of environmental conditions during oospore production on subsequent infection of host roots.

The objectives of this study were: (i) to evaluate the effect of four *Pythium* spp. at two inoculum levels on infection and disease development in four plant species; (ii) to determine quantitatively the relationship between numbers of oospores of *P. aphanidermatum*, *P. debaryanum*, *P. myriotylum*, and *P. polymastum* to infection

of tomato, rye, and cabbage; and (iii) to evaluate the influence of oospore age, different isolates of a species, media used for oospore production, temperature used for oospore production, and of certain environmental factors during exposure of oospores to host plants on infection.

Materials and Methods

Pythium aphanidermatum (Edson) Fitz. and P. debaryanum Hesse were isolated from tomato (Lycopersicon esculentum Mill.), P. myriotvlum Drechs. was isolated from a peanut pod (Arachis hypogeae L.) and P. polymastum Drechs. was isolated from a cabbage seedling (Brassica oleracea L.). Hyphal-tipped cultures of these fungi were maintained at 20 C on V-8 juice agar and transferred at monthly intervals.

The media and methods used for production and preparation of oospores for soil infestation are described in Parts 1 and 2 of this dissertation.

Arredondo fine sand (pH 6.5, measurement obtained from a 1:2 suspension of soil in 0.01 M $CaCl_2$) that had been sieved through a 20 mesh sieve and autoclaved at 15 psi and 120 C twice for 4 hrs at 24-hrs intervals was used throughout this study. Before inocula was introduced, the water content of the soil was adjusted to 5% (w/w) by adding sterile deionized water. The inocula were suspended in various volumes of sterile deionized water and added to moist soils; the final water content of the soil was 10%.

Cabbage ('Early Jersey Wakefield'), corn (Zea mays L. 'Fla 200 N'), rye (Secale cereale L. 'Wesser') or tomato ('Bonny Best') seeds that were surface-disinfested with 1% sodium hypochloride for 30 sec and

rinsed three times with sterile deionized water were placed on a layer of sterile paper towels, moistened, wrapped in aluminum foil and incubated at 30 C for 24 hrs.

The following methods were used for greenhouse experiments where cabbage, corn, rye, and tomato were exposed to oospores of P. aphanidermatum, P. debaryanum, P. myriotylum, or P. polymastum. Fifty grams of soil infested with 100 or 1,000 oospores per g of soil were layered over 200 g of autoclaved soil packed in the bottom of a 10-cm (diameter) clay pot. A 200 g layer of autoclaved soil was distributed evenly and packed over the infested soil, and five germinated seeds were placed on the surface of the layer. The seeds were covered with 50 g of autoclaved soil. Ten pots were planted at each inoculum level for each treatment. The pots were randomized, placed in saucers, and maintained in the greenhouse for 3 weeks at temperatures which fluctuated between 28 and 37 C. Every 48 hrs the saucers were filled with tap water and after 15 min the excess water was removed.

For experiments on the relationship of inoculum density to infection of cabbage, rye and tomato or on recovery of oospores from soil, the infested-soil layer technique developed by Mitchell (44) was used. Fifteen grams of infested soil were layered over 100 g of autoclaved coarse-builder's sand packed in the bottom of 100-ml polypropylene beakers that each had 3 small holes at the base for water movement. Five germinated seeds were placed on a 15 g layer of autoclaved soil over the infested soil. The seeds were covered with 5 g of vermiculite and the beakers were placed in a nylon pan. Ten beakers were used for each treatment; an additional two beakers were used to follow soil populations of Pythium spp. These beakers contained 30 g of infested soil

layered over the builder's sand and no autoclaved soil was used to separate the seeds from the infested soil. No seeds were planted in one of the two beakers used for soil population studies. The pans containing 12 beakers each were maintained in growth chambers with 12 hrs of light (10,760 lx) at 30 C except for those containing cabbage seedlings which were held at 25 C. The plants were kept in the growth chambers for 7 days except for rye seedlings which were maintained for 5 days. The plants were watered every 48 hrs by filling the pans with tap water. After 15 min, the water was drained and the pans were returned to the growth chambers. Unless otherwise indicated, these were the standard conditions for all tests.

At the termination of each experiment, plants were harvested by gently washing the soil and sand away from the root systems with a stream of tap water. Root systems that were visibly free of adhering soil particles were surface disinfested by dipping the roots for 5 sec in 70% ethanol, rinsing three times in sterile deionized water, and blotting on paper towels to remove excess water. Two root systems were plated on each plate that contained a selective medium (PV) that was modified from that of Tsao and Ocana (67). This medium contained 17 g of Difco corn meal agar, 5 mg of Pimaricin (Delvocid, Delft, Holland) and 300 mg of vancomycin hydrochloride (Vancocin, Eli Lilly & Co.) in a liter of sterile deionized water. The inoculated plates were incubated in the dark at 30 C and examined for growth of *Pythium* spp. after 24 and 36 hrs.

Populations of *Pythium* spp. in soil were evaluated by a soil dilution method using dilutions of 1:10 and 1:20. Samples taken from soils with or without plants were mixed with 200 ml of 0.3% water agar that contained 300 mg vancomycin hydrochloride and 3.68 g of $CaCl_2 \cdot 2\ H_2O$

in a liter of deionized water. For each dilution a 1-ml aliquot was spread onto each of 10 PV plates with a bent glass rod, and the plates were incubated in the dark. After 36 hrs at 30 C (unless otherwise indicated), the soil-agar mixture was washed from the surface of the plates under a slow stream of tap water and the colonies were counted.

To evaluate the influence of the hydrogen ion concentration of the dilution medium on recovery of propagules of *Pythium* spp. from soil, the 0.3% water agar dilution was adjusted to hydrogen ion concentrations ranging from pH 2.5 to 11.5 with 0.1 N HCl or 0.1 N NaOH.

All data presented in this study are means of at least two experiments. The data presented for inoculum density studies at various levels were repeated from three to eight times.

Results

The four plant species tested in this study differed greatly in susceptibility to P. aphanidermatum, P. debaryanum, P. myriotylum, and P. polymastum (Table 10). Although cabbage, corn, rye or tomato seedlings were infected when grown in soils infested with P. aphanidermatum or P. debaryanum at 100 oospores per g of soil, root weight loss and mortality occurred only in tomato. All plants grown in soils infested with oospores of P. myriotylum were infected and developed disease symptoms. Although no dead corn seedlings were observed in this study, root weight loss occurred when they were grown in soil infested with 1,000 oospores per g of soil of P. myriotylum. Pythium polymastum infected and caused root weight loss only in cabbage.

Percentages of root infection of tomato, rye, and cabbage grown in soils infested with 10 to 150 oospores per g of soil of P. aphanidermatum or P. debaryanum, P. myriotylum, and P. polymastum, respectively, increased with increasing inoculum levels (Fig. 1). However, the proportion of infection to inoculum density decreased with increasing inoculum densities (Fig. 1, and Table 11).

Points of proportion of infection (y) transformed to $\log_{10} \log_e 1/1\text{-}y$ plotted against $\log_{10}$ of the inoculum density lie in a straight line between 10 and 150 oospores per g of soil for all four host-pathogen combinations (Fig. 2). The slopes of the data calculated by linear regression analyses were between 0.66 and 0.68 (Fig. 2). Interpolated inoculum levels required for 50% infection of the host plants (ID_{50}) were 16, 64, 22, and 51 for P. aphanidermatum, P. debaryanum, P. myriotylum, respectively.

The isolate of P. polymastum used in this study rapidly lost the ability to infect cabbage seedlings. When the experiment on the relationship of the number of oospores of P. polymastum per g of soil to infection of cabbage seedlings was repeated, 23 times more oospores were required to infect 50% of the seedlings than were required previously. At that time, the isolate of P. polymastum had been in culture for 4 months. The experiment was repeated a third time but no infection of cabbage occurred. The data presented for P. polymastum on cabbage in Table 11 is from the first experiment.

The effects of maintaining P. aphanidermatum and P. myriotylum in culture for 1, 2, 4, and 8 months on infection of their respective hosts were evaluated (Table 12). During the study, no noticeable decrease in the infection of tomato by P. aphanidermatum at 10 or 100 oospores per g of soil was observed, but a considerable decrease in the percentages of infection of rye by P. myriotylum at 25 and 100 oospores per g of soil occurred. After 6 months in culture, approximately three times more oospores of P. myriotylum were required to produce the same percentages of infection that were obtained when the isolate was freshly isolated.

When a 2-year-old culture of P. myriotylum (Pm-1)that had been observed to lose its ability to infect rye was compared to six cultures that were freshly isolated from peanut pods, considerable variations in their ability to infect rye were observed (Table 13). Only Pm-15 behaved similarly to the original isolate Pm-1 before it lost the ability to infect rye. Isolates Pm-10 and Pm-12 did not differ from Pm-1, after it had lost the ability to infect rye, in their ability to infect rye. Isolates Pm-11, Pm-13, and Pm-14 were relatively non-infective.

In an experiment to determine the influence of time of exposure of host roots to inoculum on incidence of infection, maximum infection ot tomato by P. aphanidermatum occurred after 7 days, and maximum infection of rye occurred after 4 days (Table 14). No increases in infection of tomato or rye seedlings were observed when the seedlings were exposed to infested soil for longer times.

Maximum infection of tomato by P. aphanidermatum or rye by P. myriotylum occurred when the plants were incubated at 35 C (Table 15). Lower infection rates were observed when the plants were incubated at lower temperatures.

When weight of roots obtained from rye seedlings that were grown in soils infested with P. myriotylum at 25 or 150 oospores per g of soil and maintained at temperatures ranging from 20 to 35 C were compared, the largest loss in root weight was from seedlings that were grown in soil infested with 150 oospores per g of soil and maintained at 35 C. The optimum temperature for root growth was at 30 C.

Tomato and rye seedlings that were exposed to infested soils that were watered every 24 hrs had higher levels of infection than those that were watered every 48 or 72 hrs (Table 16). The moisture content of the soil 30 min after watering was 23.6% (w/w). After 24, 48, 72, and 96 hrs of incubation at 30 C in the growth chamber, the moisture contents were 21.1, 20.1, 17.2, and 16.4%, respectively.

The percentages of tomato seedlings infected when grown in soils infested with oospores of P. aphanidermatum that were produced in hemp seed broth, V-8 juice broth, or Schmitthenner's liquid medium did not differ. But, when rye seedlings were grown in soils that were infested with oospores of P. myriotylum that were produced in hemp seed broth or

V-8 juice broth, slightly greater infection occurred than in soils infested with oospores that were produced in Schmitthenner's medium (Table 17).

Slightly higher infection rates occurred when tomato or rye seedlings were grown in soils infested with oospores of P. aphanidermatum or P. myriotylum, respectively, produced at 20 to 25 C than when they were grown in soils infested with oospores produced at 30 or 35 C (Table 18).

The rate of recovery of P. aphanidermatum from soils infested with oospores produced in hemp seed decoction broth was lower than from soils infested with oospores produced in V-8 juice broth or Schmitthenner's medium (Table 17). No differences in recovery rates of P. myriotylum from soils infested with oospores produced in hemp seed decoction broth, V-8 juice broth, or Schmitthenner's liquid medium were observed.

When soils infested with oospores of P. aphanidermatum, or P. myriotylum were incubated at 30 C in a growth chamber with a 12-hr day length and plated at 24-hr intervals, maximum recovery of P. aphanidermatum from infested soils occurred after 5 days of incubation and maximum recovery of P. myriotylum occurred after 4 days (Table 14).

A higher population of P. aphanidermatum occurred in infested soils that were maintained at 30 C than at 20, 25, or 35 C (Table 15). However, the highest populations of P. myriotylum occurred in soils that were maintained at 35 C.

Lower rates of recovery of P. aphanidermatum or P, myriotylum were observed from soils that contained plants than from soils that did not contain plants (Table 19). But, maximum recovery of propagules from soils that contained plants occurred earlier than from soils that were without plants.

Optimum recovery rates were observed when the pH of the medium used for making soil dilutions ranged from 3.5 to 10.5. At pH 2.5 or 10.5 reductions in the rates of recovery occurred (Table 20).

Incubation temperature and the length of time that oospores in soil suspensions were maintained on the selective medium affected the percentage of propagules recovered from soil. Germinated oospores of P. aphanidermatum and P. myriotylum were observed on PV medium 6 hrs after plating (Table 21). The percentages of germinated oospores increased with time of incubation at 30 C in the dark. Maximum germination percentages occurred after 30-36 hrs of incubation, after which no additional germinated oospores were observed. Better recovery of P. aphanidermatum occurred when the selective medium was maintained at 30 C than at 20, 25, or 35 C (Table 22). Maximum recovery of P. myriotylum occurred at 30 C but the rate of recovery at that temperature was not significantly different to recovery rates at 25 or 35 C (Table 22).

Table 10. Infection and disease of several hosts by four *Pythium* spp.

	Inoculum density					
	100 oospores per g soil			1000 oospores per g soil		
	Infection[x] (%)	Root weight loss (%)	Mortality (%)	Infection (%)	Root weight loss (%)	Mortality (%)
Cabbage						
Pythium aphanidermatum	85 a[y]	0 c	0 c	100 a	0 c	0 b
Pythium debaryanum	62 b	0 c	0 c	100 a	0 c	0 b
Pythium myriotylum	66 b	33 a	30 a	90 a	65 a	33 a
Pythium polymastum	96 a	18 b	17 b	100 a	23 b	28 a
Corn						
Pythium aphanidermatum	86 a	0 b	0 a	100 a	0 b	0 a
Pythium debaryanum	68 b	0 b	0 a	100 a	0 b	0 a
Pythium myriotylum	80 a	14 a	0 a	100 a	40 a	0 a
Pythium polymastum	0 c	0 b	0 a	0 b	0 b	0 a
Rye						
Pythium aphanidermatum	100 a	0 a	0 a	100 a	0 b	0 b
Pythium debaryanum	50 c	0 a	0 a	100 a	0 b	0 b
Pythium myriotylum	78 b	10 a	8 a	100 a	56 a	14 a
Pythium polymastum	0 d	0 a	0 a	0 b	0 b	0 b
Tomato						
Pythium aphanidermatum	96 a	65 b	88 a	100 a	91 a	92 a
Pythium debaryanum	63 b	58 b	84 a	100 a	89 a	92 a
Pythium myriotylum	85 a	90 a	22 b	100 a	99 a	34 b
Pythium polymastum	0 c	0 c	0 c	0 b	0 b	0 c

[x]Percentages are based on 50 plants per treatment.

[y]Values in a column followed by the same letter are not significantly different ($\underline{P} = 0.05$) as determined by Duncan's multiple-range test; percentages were converted to arcsin degrees before analyses.

Table 11. The effect of inoculum density on percentage root infection.

Pythium spp.	Plant	Infection: Inoculum density (oospores/g soil) 10 (%)	25 (%)	50 (%)	100 (%)	150 (%)
Pythium aphanidermatum	Tomato	46±3	65±3	75±4	84±6	96±6
Pythium debaryanum	Tomato	17±4	33±2	43±2	64±3	69±5
Pythium myriotylum	Rye	34±3	56±1	71±3	80±5	100
Pythium polymastum[x]	Cabbage	24	30	50	85	---[y]

[x]Data from one experiment.

[y]No data.

Table 12. The effect of culture age of *Pythium aphanidermatum* on infection of tomato seedlings and of *P. myriotylum* on infection of rye seedlings.

	Infection[x]			
Age of culture[y] (months)	*Pythium aphanidermatum* oospores/g of soil		*Pythium myriotylum* oospores/g of soil	
	10 (%)	100 (%)	25 (%)	100 (%)
1	44 a[z]	90 a	38 a	86 a
2	41 a	84 a	29 a	74 a
4	49 a	87 a	6 b	44 b
8	38 a	89 a	0 b	53 b

[x]Tomato and rye seedlings were harvested after 7 and 5 days, respectively, of incubation in a growth chamber at 30 C with a 12-hr day length.

[y]Hyphal tipped isolates of *P. aphanidermatum* and *P. myriotylum* were maintained on V-8 juice agar at 20 C and transferred at monthly intervals to fresh agar slants.

[z]Within each column, entries without a common letter are significantly different (*P*= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 13. The effect of different isolates of *Pythium myriotylum* on infection of rye seedlings.

Isolate[y]	Infection[x] (oospores/g soil) 25 (%)	150 (%)
Pm-1	2 b[z]	16 b
Pm-10	0 b	19 b
Pm-11	0 b	3 c
Pm-12	2 b	23 b
Pm-13	0 b	0 c
Pm-15	54 a	98 a

[x]Seedlings were harvested after 5 days of incubation in a growth chamber at 30 C with a 12-hr day length.

[y]All isolates were obtained from different peanut pods collected from a field in Levy County, Florida, and were maintained on V-8 juice agar at 20 C.

[z]Within each column, entries without a common letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 14. The effect of time of exposure of tomato seedlings to soil infested with *Pythium aphanidermatum* and of rye seedlings to soil infested with *P. myriotylum* on percentages of infection.

	Frequency of isolation[w]					
	Pythium aphanidermatum			*Pythium myriotylum*		
	Tomato		Soil	Rye		Soil
Time[x] (days)	10 opg[y] (%)	100 opg (%)	100 opg (%)	25 opg (%)	100 opg (%)	100 opg (%)
0	0 b[z]	0 d	84 a	0 c	0 d	4 c
1	2 b	4 d	17 b	7 c	20 c	4 c
2	8 b	14 cd	14 b	9 c	36 c	8 c
3	14 b	25 c	16 b	25 b	69 b	28 b
4	39 a	67 b	20 b	43 a	82 a	41 a
5	39 a	77 ab	86 a	56 a	80 a	44 a
6	44 a	74 ab	81 a	54 a	78 ab	57 a
7	46 a	84 a	89 a	49 a	82 a	39 ab
14	41 a	84 a	83 a	45 a	79 a	42 a
21	45 a	81 a	89 a	56 a	93 a	42 a

[w]Seedlings were incubated in beakers in growth chambers at 30 C with a 12-hr day length and watered every 48 hrs.

[x]After 1 to 21 days, 50 seedlings were harvested and the root systems were plated on a selective medium (PV); soil samples were assayed for populations after 0 to 21 days, only soils that did not contain plants were sampled.

[y]opg= oospores per g of soil.

[z]Within each column, entries without a common letter are significantly different (*P*= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 15. The effect of temperature on percent infection of tomato by *Pythium aphanidermatum* and of rye by *P. myriotylum*.

Temperature[v] (C)	Frequency of isolation					
	Pythium aphanidermatum[w]			*Pythium myriotylum*[x]		
	Tomato		Soil	Rye		Soil
	10 opg[y] (%)	100 opg (%)	100 opg (%)	25 opg (%)	100 opg (%)	100 opg (%)
20	26 b[z]	49 c	62 b	0 c	17 c	16 c
25	39 ab	69 b	74 ab	29 b	47 b	21 c
30	46 a	84 ab	89 a	56 a	80 a	46 b
35	44 a	91 a	52 b	61 a	84 a	62 a

[v]Seedlings were incubated in beakers in growth chambers with a 12-hr day length at temperatures ranging from 20 to 35 C.

[w]Tomato seedlings and infested soil without plants were maintained for 7 days.

[x]Rye seedlings and infested soil without plants were maintained for 5 days.

[y]Oospores per g of soil.

[z]Within each column, entries without a common letter are significantly different (*P*= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 16. The effect of watering frequency on percent infection of tomato by *Pythium aphanidermatum* or of rye by *P. myriotylum*.

Watering[y] frequency (hrs)	Infection			
	Pythium aphanidermatum		*Pythium myriotylum*	
	oospores/g of soil			
	10 (%)	100 (%)	25 (%)	100 (%)
24	49 a[z]	100 a	73 a	94 a
48	44 a	74 b	54 b	78 b
72	41 a	79 b	54 b	72 b

[y]After 24, 48, or 72 hrs, the pans containing tomato or rye seedlings were taken out of the growth chamber and were filled with tap water; after 15 min, the water was drained and the pans were returned to the growth chamber at 30 C with a 12-hr day length for 6 days.

[z]Within each column, entries without a common letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 17. The effect of the media used for oospore production on percent infection of tomato by *Pythium aphanidermatum* or of rye by *P. myriotylum*.

Medium[v]	Frequency of isolation					
	Pythium aphanidermatum[w]			*Pythium myriotylum*[x]		
	Tomato		Soil	Rye		Soil
	10 opg[y] (%)	100 opg (%)	100 opg (%)	25 opg (%)	100 opg (%)	100 opg (%)
Hemp seed	41 a[z]	78 a	56 b	47 a	94 a	51 a
V-8 juice	43 a	84 a	86 a	41 a	88 a	55 a
Schmitthenner's	46 a	84 a	89 a	35 a	80 a	46 a

[v]Cultures of *P. aphanidermatum* and *P. myriotylum* were incubated in the dark at 25 C for 21 days.

[w]Tomato seedlings and infested soil without plants were maintained at 30 C for 7 days in a growth chamber with a 12-hr day length.

[x]Rye seedlings and infested soil without plants were maintained at 30 C for 5 days in a growth chamber with a 12-hr day length.

[y]Oospores per g of soil.

[z]Within each column, entries without a common letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 18. The effect of the temperature at which cultures were maintained for oospore production on percent infection of tomato by *Pythium aphanidermatum* or of rye by *P. myriotylum*.

Temperature[v] (C)	Frequency of isolation					
	Pythium aphanidermatum[w]			*Pythium myriotylum*[x]		
	Tomato		Soil	Rye		Soil
	10 opg[y] (%)	100 opg (%)	100 opg (%)	25 opg (%)	100 opg (%)	100 opg (%)
20	52 a[z]	89 a	84 a	41 a	92 a	34 b
25	46 a	84 a	89 a	34 ab	80 ab	46 a
30	37 ab	66 b	89 a	28 ab	74 b	38 ab
35	28 b	41 c	81 a	24 b	65 b	17 c

[v]Oospores of *P. aphanidermatum* and *P. myriotylum* were produced in Schmitthenner's liquid medium; the medium was incubated in the dark at temperatures ranging from 20 to 35 C for 21 days.

[w]Tomato seedlings and infested soil without plants were maintained at 30 C for 7 days in a growth chamber with a 12-hr day length.

[x]Rye seedlings and infested soil without plants were maintained at 30 C for 5 days in a growth chamber with a 12-hr day length.

[y]Oospores per g of soil.

[z]Within each column, entries without a common letter are significantly different (*P*= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 19. The influence of the presence of host plants on recovery of *Pythium aphanidermatum* or *P. myriotylum* from artificially infested soils.

Plant[x]	Percent recovery	
	Pythium aphanidermatum[y] (%)	*Pythium myriotylum* (%)
+	39 b[z]	17 b
-	86 a	46 a

[x]Populations of *P. aphanidermatum* or *P. myriotylum* were assayed from soils that contained plants and from soils that were without plants (Plants= +, no plants= -); initial inoculum was 100 oospores per g of soil.

[y]Soil infested with *P. aphanidermatum* or *P. myriotylum* was maintained at 30 C in a growth chamber with a 12-hr day length for 7 or 5 days, respectively.

[z]Within each column, entries without a common letter are significantly different (*p*= 0.05) as determined by "T" test; analyses were performed with data transformed to arcsin degrees.

Table 20. The effect of the hydrogen ion concentration of the suspension used for making soil dilutions on recovery of *Pythium aphanidermatum* or *P. myriotylum* from artificially infested soils.

	Percent recovery[y]	
	Pythium aphanidermatum	*Pythium myriotylum*
pH	(%)	(%)
2.5	55 c[z]	15 b
3.5	96 a	36 a
4.5	100 a	42 a
5.5	100 a	47 a
6.5	89 ab	46 a
7.5	86 ab	46 a
8.5	83 ab	42 a
9.5	80 b	34 a
10.5	77 b	32 a
11.5	58 c	19 b

[y]Oospores of *Pythium aphanidermatum* and of *P. myriotylum* were incorporated in Arredondo fine sand (pH 6.5) and maintained in a growth chamber at 30 C for 7 days; no plants were used in this test; soil samples containing 100 oospores per g of soil were plated on a selective medium (PV) using water agar solution with a pH ranging from 2.5 to 11.5.

[z]Within each column, entries without a common letter are significantly different (*P*= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 21. The effect of time on the selective medium on recovery of oospores of *Pythium aphanidermatum* or *P. myriotylum* from artificially infested soils.

Time[x] (hrs)	Percent recovery[y] *Pythium aphanidermatum* (%)	*Pythium myriotylum* (%)
6	3 c[z]	1 b
12	12 c	4 b
18	52 b	10 b
24	77 a	31 a
30	89 a	43 a
36	89 a	46 a

[x]Sixty petri dishes that each contained 15 ml of a selective medium (PV) were inoculated with a suspension of soil infested with 100 oospores of *Pythium aphanidermatum* or *P. myriotylum* per g of soil and incubated in the dark at 30 C; after 6, 12, 18, 24, 30, and 36 hrs of incubation, 10 plates were removed from the incubator and examined under magnification (200 X) for germinated oospores.

[y]Prior to plating the soil on a selective medium, the cups that contained the infested soil were maintained at 30 C in a growth chamber with a 12-hr day length for 7 days for *P. aphanidermatum* and for 5 days for *P. myriotylum*; only soil from cups that did not contain plants was sampled.

[z]Within each column, entries without a common letter are significantly different (*P*= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

Table 22. The effect of temperature during the incubation of fungi on the selective medium on percent recovery of *Pythium aphanidermatum* or *P. myriotylum* from artificially infested soils.

Temperature[x]	Percent recovery[y]	
	Pythium aphanidermatum	*Pythium myriotylum*
(C)	(%)	(%)
20	62 b[z]	24 b
25	74 b	42 a
30	89 a	46 a
35	44 c	39 ab

[x]Inoculated petri dishes that contained a selective medium were incubated at temperatures ranging from 20 to 35 C in the dark for 36 hrs.

[y]Soil infested with *P. aphanidermatum* or *P. myriotylum* without plants was maintained at 30 C for 7 or 5 days, respectively, prior to plating.

[z]Within each column, entries without a common letter are significantly different (*P*= 0.05) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

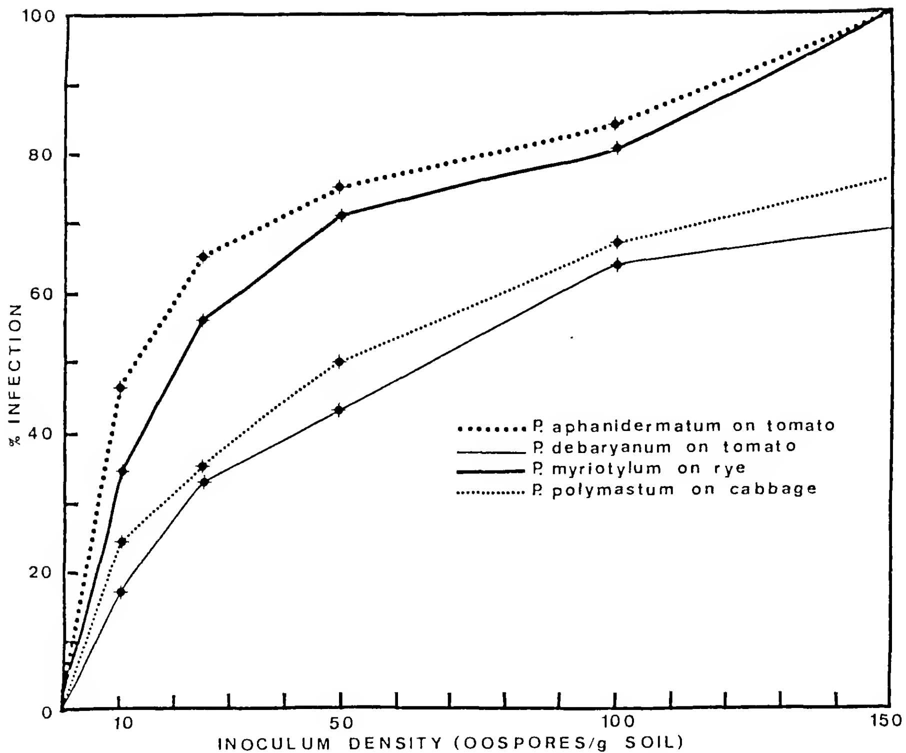

Fig. 1. The relationship of incidence of infection (arithmetic) of several hosts to densities of oospores (arithmetic) of several *Pythium* spp.

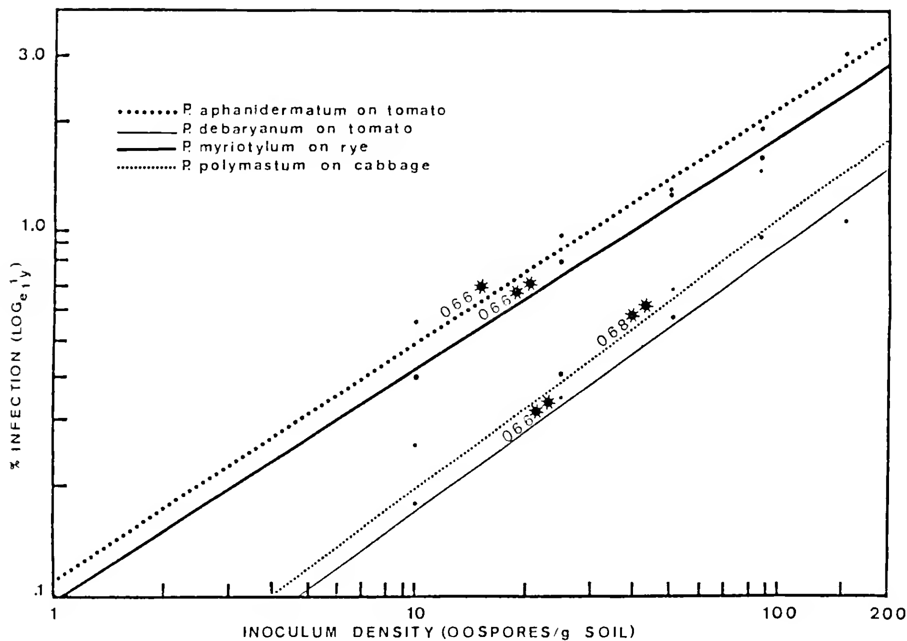

Fig. 2. The relationships of percentages of infection adjusted for multiple infections (logarithmic) to densities of oospores of several Pythium spp. (logarithmic); the linear correlation coefficient was significant at P= 0.05 (*) or P= 0.01 (**).

Discussion

In this study reductions in the rates of infection occurred when factors that affect the susceptibility of the host, the pathogenicity of the pathogen or the favorability of the environment were adversely altered. The curvilinear arithmetic and the log-log plots drawn from data obtained from experiments with the four host-pathogen systems studied were similar to the results obtained by other workers for host-pathogen systems involving non-motile inoculum and motile infection courts (4, 44, 45). The slope values of the log-log transformations were between 0.66 and 0.68. Baker (4) predicted that in systems where pathogen response to the host operates in the rhizoplane the slope of the log-log transformation of the inoculum density-disease curve should be near 0.67.

The pathogen response to the host in the four host-pathogen system studied may operate only in the rhizoplane as postulated by Baker (4), but, as pointed out by Mitchell (45), a rhizosphere effect modified by various factors that adversely affect host infection could result in similar slopes. In an ideal system each propagule that is encountered by a growing root will germinate and infect that root and a slope of 1.0 will be attained (63). Since 100% efficiency is rare in nature, a slope of less than 1.0 is expected.

When the interpolated ID_{50}'s of the four host-pathogen systems were compared with those obtained by Mitchell (45), our values were in the same range. Mitchell (45) reported ID_{50}'s ranging from 15 to 50 oospores per g of soil with *Pythium* spp.

Variability in pathogenicity of the different *Pythium* spp. to to different plant species was observed when the four plant species were

inoculated at two inoculum levels with oospores produced by the four *Pythium* spp. Variability among species of *Pythium* is not unusual even though many have wide host ranges (23). McCarter and Littrell (40) observed basic differences in pathogenicity of *P. aphanidermatum* and *P. myriotylum* to different crops and found large intraspecific variability in the pathogenicity of these fungi.

During the course of this study, a gradual loss in the ability of *P. myriotylum* and *P. polymastum* to infect rye and cabbage, respectively, was observed. Although loss of pathogenicity among pythiaceous fungi in culture is of common occurence, it rarely has been documented (55, 64). No loss in the ability of *P. aphanidermatum* or *P. debarvanum* to infect tomato occurred during this study. However, Mitchell (personal communication) observed loss of the ability to infect host plants in several isolates of *P. aphanidermatum* that were kept in culture for a long time. When Laviolette and Athow (53) inoculated soybean seedlings with pieces of mycelium produced by several isolates of *P. ultimum* that had been maintained in cultures for several years, they did not observe any loss of pathogenicity.

Loss of pathogenicity by an isolate sustained while it is maintained in culture sometimes may be regained by inoculating susceptible plants and reisolating from affected plants (64). When this procedure was tried with *P. myriotylum*, it did not recover the ability to infect rye. Loss of pathogenicity by *P. myriotylum* and by *P. polymastum* may be due to a deficiency of a substance that is required in minute quantities; thus, when this substance is exhausted, oospores that are produced subsequently may be unable to germinate and infect host plants. Progressive changes in pathogenicity may result through genetic recombination in the same

way that an avirulent strain of Phytophthora infestans can change to a virulent strain (64). The loss of the ability of P. myriotylum to infect rye may be attributed to a loss in the ability of this fungi to produce germinable oospores. Perhaps if rye seedlings were inoculated by inserting a piece of mycelium into an incision in the hypocotyl of the plant, as was done by Laviolette and Athow (33) with soybean, no loss in the ability of P. myriotylum to infect rye would have been detected.

Hendrix and Campbell (23) regarded soil temperature and moisture as the two most important factors that influence infection. Maximum infection of tomato by P. aphanidermatum and of rye by P. mvriotylum occurred when the seedlings were incubated at 35 C. These results are in agreement with those reported by Littrell and McCarter (38) for infection of rye and tomato by P. aphanidermatum and by P. myriotylum, respectively, and with those reported by Mitchell (44) for infection of rye by P. myriotylum. Although disease caused by Pythium spp. usually is low in soils maintained near water saturation (23), disease caused by Pythium spp. increases as the percent moisture holding capacity (MHC) of the soil is increased (5, 53). Bateman (5) observed that the development of poinsettia root rot increased when the percent MHC of a soil that held 56% moisture expressed on a dry weight basis was increased to 70% MHC. When the percent MHC of a soil was increased from 15 to 100%, damping-off of red pine seedlings increased linearly (52). In this study, maximum infection of tomato by P. aphanidermatum and or rye by P. myriotylum occurred when the soil was maintained at 89-100 MHC (soil watered every 24 hrs). When the soil was maintained at 85-89 MHC or at 73-85% MHC (soil watered every 48 or every 72 hrs, respectively) the percentages of infection were lower.

Temperatures and media that favored oospore production resulted in oospores that were more infective. Although there were no great differences in the rates of infection of tomato or rye when grown in soils infested with oospores that were produced in the various media, slightly higher rates of infection occurred when the seedlings were grown in soils infested with oospores that were produced at 20-25 C than at 30-35 C. The media used for producing inocula have been reported to be important when the inocula consist of blended fungal mats. Mildenhall et al (43) obtained marked differences in disease incidence and severity of carrot with identical clones of any of three *Pythium* spp. that were cultured in two different natural media.

The reductions in inoculum densities that occurred in soils which contained plants may have been due to microbial lysis of oospores that had been stimulated to germinate by root exudates. Stanghellini and Burr (59) observed complete lysis of hyphae and/or germ tubes that originated from oospores of *P. aphanidermatum* 96 hrs after amending artificially infested soils with either bean seed exudate or nutrient solutions. Reductions in inoculum densities of *P. myriotylum* in field soils was observed also by Frank (15) following cyclic wetting and drying of naturally infested field soils. Similarly, Burr (11) found a significant decrease in the populations of oospores of *P. aphanidermatum* in naturally infested soils after cyclic wetting and drying, or after amending soils with asparagine.

In this study, we have shown that many cultural and environmental factors can alter the rates of infection by *Pythium* spp. Since these factors can alter the results of an experiment, they should be considered when establishing tests with defined inoculum levels to evaluate certain chemicals for disease control or when evaluating host resistance or tolerance to these fungi.

PART 4

RELATIONSHIPS OF THE NUMBERS OF MOTILE AND ENCYSTED ZOOSPORES OF *PYTHIUM APHANIDERMATUM*, *P. MYRIOTYLUM*, AND *PHYTOPHTHORA PALMIVORA* TO INFECTION OF TOMATO

Introduction

In recent studies where care was taken to prevent or reduce the rate of encystment of zoospores during inoculation procedures, a lower number of zoospores was required for infection of plants by pythiaceous fungi than was expected from previous studies (45,46). Less than 300 zoospores per plant were required for 50% infection of cotton, tomato, or watercress by *Pythium ostracodes* Drechs., *P. aphanidermatum* (Edson) Fitz., or *Phytophthora cryptogea* Pethyb. and Laff., respectively (45). Formerly, a minimum of 1.25×10^4 zoospores per plant were required for 50% infection of papaya (50). The large difference between the studies may be due to the method used for inoculation. In the former study, zoospores were introduced to the plants while they were under flooded conditions as opposed to injection of the inoculum directly into nonflooded soil.

Most studies involving specific numbers of zoospores as inocula have had results reported as percentages of disease incidence or mortality of host plants rather than percentages of infection (6, 16, 17, 29, 30, 31). However, when percentages of infection were compared with percentages of mortality, at least ten times more zoospores were required to kill seedlings than were required for infection (45, 46, 49).

Fewer motile zoospores than encysted zoospores are required to cause specific levels of infection or disease incidence. For example, 98% of the papaya seedlings inoculated with 6 X 10^4 motile zoospores of *P. palmivora* Butler were killed, but only 11% of the seedlings were killed when exposed to a similar number of encysted spores (29).

Among the many factors that can cause zoospores to encyst during inoculation procedures are changes in hydrogen concentration, ion or nutrient concentration, water current, and temperature (8, 16, 17, 21, 23, 41, 46, 61). Temperature may be one of the most important factors that causes immobilization of zoospores. For example, no zoospores of *P. aphanidermatum* or *P. myriotylum* Drechs. were motile after 1 hr of incubation at 37 C while 70% of the zoospores were motile after 18 hrs of incubation at 19 C (40). Immobilization of *P. palmivora* occurred after 0.5 hr at 33 C, 2.5 hrs at 28 C, or 6 hrs at 8 or 12 C (8, 29).

The objectives of this study were to determine the relationships of the numbers of motile and encysted zoospores of *P. aphanidermatum*, *P. myriotylum*, and *P. palmivora* to percentages of infection of tomato (*Lycopersicon esculentum* Mill.) seedlings, and to evaluate the effects of temperature on root infection and encystment of zoospores.

Materials and Methods

The isolates of *Pythium* spp. used in this study were obtained from diseased plants found in Florida. *Pythium aphanidermatum* was isolated from pigeon pea (*Cajanus cajan* (L.) Mill.) and *P. myriotylum* was isolated from a peanut pod (*Arachis hypogeae* L.). The strain of *Phytophthora palmivora* (P-455) examined as a comparison to *Pythium* spp. was isolated from papaya (*Carica papaya* L.), and was obtained through the courtesy of G. A. Zentmeyer (University of California). All fungal isolates were maintained on V-8 juice agar in the dark at 20 C; the cultures were transferred monthly to fresh agar slants.

Zoospores of *P. aphanidermatum* and *P. myriotylum* were produced by a method described by McCarter and Littrell (41). Mycelium-bearing V-8 juice agar discs (15 mm in diameter) were cut from the periphery of 24-hr-old colonies (grown at 30 C in the dark) with a sterilized cork borer, and four discs were placed aseptically in an inverted position in a sterile petri dish containing 10 ml of sterile deionized water. The dishes were incubated for 15 hrs at 30 C in the dark, removed from the incubator, the water was replaced with fresh sterile deionized water, and the cultures were incubated for an additional 3 hrs at room temperature.

Zoospores of *P. palmivora* were produced from cultures prepared by a method used by Ramirez and Mitchell (49). Each culture was initiated by adding three 15 mm discs cut from the margin of a three-day-old culture of *P. palmivora* on V-8 juice agar to a 250-ml Erlenmeyer flask containing 15 ml of V-8 juice broth. After incubation at 30 C in the dark for 48 hrs, the medium was drained from the flask and the mycelia were washed three times with 50-ml aliquots of sterile deionized water and incubated for an additional 48 hrs in a growth chamber at 25 C

under continuous illumination (10,760 lx at the level of the cultures). The water in the flasks was removed and the mycelia were resuspended in 15 ml of fresh sterile deionized water, chilled at 9 C for 30 min and returned to the growth chamber for 1 hr.

Four 1.0-milliliter samples were removed from suspensions containing motile zoospores for counting. The samples were placed in test tubes and immersed for one min in a water bath at 46 C to induce rapid encystment. The concentrations were estimated by counting five fields of four samples from each of the four tubes on a standard hemacytometer

For comparisons of motile and encysted spores, zoospores were induced to encyst by a method used by Tokunaga and Bartnicki-Garcia (61). Samples of each fungus were agitated in test tubes for 60 sec on a Vortex mixer set at maximum speed.

Immediately after the counts were made, dilutions were prepared with sterile deionized water at ambient temperature and inoculation were performed.

Tomato seeds were soaked in tap-water for 30 min, surface-disinfested by soaking the seeds for 3 min in 0.5% (w/w) sodium hypochlorite. rinsed in sterile deionized water, and incubated at 30 C on moist paper towels for 2 days. One 2-day-old seedling was planted in 75 g of autoclaved coarse builder's sand in a 50-ml polypropylene beaker that had three small holes at the base for water movement. After planting, 4 g of vermiculite were layered around the base of the seedling to prevent rapid dying, and the beakers were placed in a nylon pan (30 X 18 X 16 cm). The pan was placed in a growth chamber at 30 C with a 12-hr-day-light (10,760 lx at the level of the plants) and maintained for 5 days. All plants were watered daily by filling the pan with sterile deionized water,

maintaining the flooded condition for 10 min, and draining the pan. Fifteen beakers were placed into each pan and one pan was used for each teatment.

Before inoculation, the pans were removed for the growth chambers, the layer of vermiculite was removed and the cups were flooded by filling the pan with sterile deionized water. One milliter of a known concentration of zoospores was pipetted onto the surface of the standing water (16.6 cm^2 X 0.5 cm deep) approximately 2 cm away from the stem of the tomato seedlings. The zoospores were allowed to disperse in the sterile deionized water for 5 min, the water was drained off and the pans were returned to the growth chambers. Plants that were inoculated with zoospores of *P. aphanidermatum* or *P. myriotylum* were maintained in the growth chamber for 2 days while those inoculated with zoospores of *P. palmivora* were maintained for 3 days. In tests conducted to determine the effects of temperature on infection, the pans were maintained in growth chambers at 15, 20, 25, 30, 35, and 40 C.

Tomato seedlings were harvested by washing the sand from the roots under running tap water. Each stem and root system was surface-disinfested by dipping in 70% ethyl alcohol for 5 sec, rinsing in sterile deionized water, and blotting on paper towels to remove excess water. The root systems were plated then on a selective medium (PV) which was modified from that of Tsao and Ocana (62) and contained 5 mg pimaricin (Delvocid, Gist-Brocades, Delf, Holland), 300 mg vancomycin hydrochloride (Vancocin, Eli Lilly & Co.) and 17 g of Difco cornmeal agar in 1 liter of deionized water. Plates were observed for growth of *P. aphanidermatum* or *P. myriotylum* after 1 to 2 days of incubation at 30 C, or for growth of *P. palmivora* after 3 to 4 days of incubation at 30 C.

The effects of time and temperature on encystment of zoospores were evaluated after 1, 3, 6, 12, 18, 24, and 48 hrs of incubation in the dark at 10, 15, 20, 25, 30, 35, and 40 C. Samples (2.5 ml) of a zoospore suspension that contained 1 X 10^5 zoospores per ml were placed into each of four stender dishes (37 X 25 mm) for each treatment and incubated at the appropriate temperature and time intervals; the percentage of motile zoospores was estimated under magnification (200 X). The stender dishes were returned to the incubator for additional incubation time and the samples were estimated again for zoospore motility.

All experiments were repeated twice except those on the relationship of the number of zoospores per plant to root infection which were repeated at least three times. The data presented in this paper are means of the experiments.

Results

Higher percentages of root infection were obtained with motile than encysted zoospores (Fig. 3). From 32 to 78 times more encysted zoospores than motile zoospores were required to produce the same level of root infection. The number of zoospores required to infect 50% of the plants with motile and encysted zoospores of P. aphanidermatum, P. myriotylum, or P. palmivora were 275 and 23,419, 166 and 10,988, or 1,505 and 47,424, respectively. Percentages of seedling infection increased with increasing numbers of zoospores per plant (Fig. 3-A, 3-B). Percentages of infection of tomato seedlings inoculated with 50, 100, 250, 500, 1,000, and 10,000 motile zoospores of P. aphanidermatum were 18±6, 30±8, 51±9, 64±9, 78±9, 98±3, and 98±3%; percentages of infection with P. myriotylum at the same inoculum levels were 26±8, 42±15, 59±11, 73±5, 92±5, 98±3, and 100%; and the percentages of infection with P. palmivora were 0, 13, 20±14, 31±11, 42±13, 76±8, and 89±8%. Tomato seedlings inoculated with 1, 5, 10, 50, 100, and 500 (X 10^3) encysted zoospores per plant had percentages of infection of 2±3, 20±6, 34±5, 66±7, 87±5, and 98±3% with zoospores of P. aphanidermatum; and 14±5, 33±5, 48±7, 87±5, 93±5, and 100% with zoospores of P. myriotylum. Percentages of infection of tomato seedlings inoculated with 10, 50, 100, and 500 (X 10^3) encysted zoospores of P. palmivora were 20±6, 53±6, 73±6, and 95±3.

Slopes determined by linear regression anaylsis of $\log_{10}\log_e$ (1/1-y), where y equals the proportion of infected plants, vs $\log_{10}$ of the number of motile or encysted zoospores of P. aphanidermatum, P. myriotylum, or P. palmivora per plant were 0.68 and 0.69, 0.67 and 0.64, or 0.62 and 0.67, respectively (Fig. 3-C, 3-D).

Optimum temperatures for root infection by zoospores of *P. aphanidermatum*, *P. myriotylum*, and *P. palmivora* were 25, 30, and 20-25 C, respectively (Table 23). No recovery of *P. aphanidermatum*, *P. myriotylum* or *P. palmivora* was observed from inoculated tomato seedlings that were maintained at temperatures below 20 or above 35 C after inoculation.

In a test in which rye (*Secale cereale* L.) seedlings were used instead of tomato seedlings, the slope of the regression line and the number of motile zoospores of *P. myriotylum* required to infect 50% of the seedlings approximated the results obtained for tomato. The percentages of infection of 7 dav-old rye seedlings inoculated with 31, 62, 125, 250, or 500 motile zoospores of *P. myriotylum* per plant were 20, 20, 33, 67, or 88%, respectively. The slope of the regression line for the log-log transformation was 0.69 and the number of zoospores per plant required to infect 50% of the seedlings was interpolated to be 209.

When the root systems of plants that were inoculated by adding the inoculum to seedlings that were flooded and left for 5 min before draining were plated on PV, the fungi were recovered from random locations on the root systems. Increasing the time of flooding after inoculation resulted in a slightly lower infection rate.

Zoospores produced by *P. aphanidermatum*, *P. myriotylum*, or *P. palmivora* retained motility longer at 20 C than at other temperatures (Fig. 4-A, 4-B, 4-C). Twenty percent of the zoospores produced by *P. aphanidermatum* and 2% of those produced by *P. palmivora* were motile after 48 hrs of incubation at 20 C. At temperatures above or below 20 C, the rate of encystment increased with rise or fall in temperature. Very

few slow moving zoospores were observed after 1 hr of incubation at 40 or 10 C. At 47 C, motility was terminated within 40 sec (Fig. 4-D). Eighty-eight to 97% of the cysts germinated after 48 hrs of incubation at temperatures ranging from 20 to 35 C. No germinated cysts were observed at 10 or 40 C after 48 hrs of incubation in the dark. At 15 C, few germinated cysts of *P. aphanidermatum* and of *P. palmivora* were observed after 48 hrs of incubation; but no germinated cysts of *P. myriotylum* were observed.

Table 23. The effect of temperature on infection of tomato seedlings by zoospores of *Pythium aphanidermatum*, *P. myriotylum*, or *Phytophthora palmivora*.

Fungus[x]	Zoospores per plant	Temperature (C) 20	25	30	35
		Infection[y]			
		(%)	(%)	(%)	(%)
Pythium aphanidermatum	100	25 a[z]	34 a	29 a	29 a
Pythium myriotylum	100	38 ab	42 ab	50 a	36 b
Phytophthora palmivora	400	27 a	27 a	22 a	7 b

[x]Seedlings were inoculated with motile zoospores of *Pythium* spp. and of *Phytophthora palmivora* while they were flooded; seedlings that were inoculated with zoospores of *Pythium* spp. were maintained for 48 hrs in the growth chambers and those inoculated with *P. palmivora* were maintained for 72 hrs.

[y]Mean of two experiments, each with 15 plants per treatment.

[z]Within each row, entries without a common letter are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test; analyses were performed with data transformed to arcsin degrees.

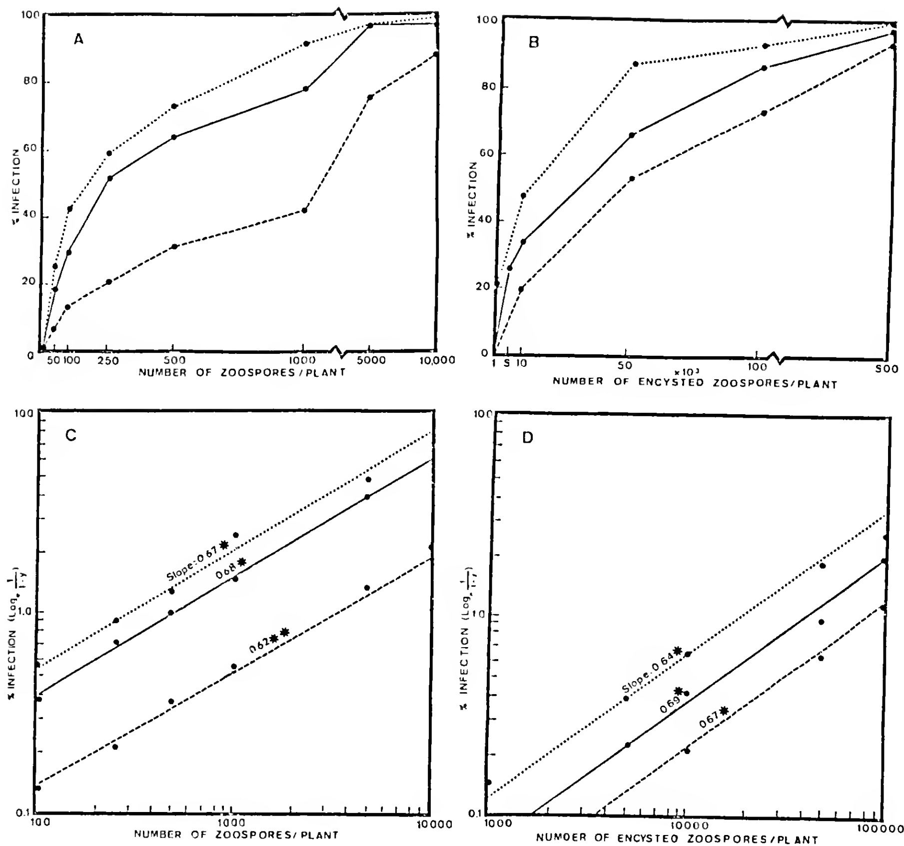

Fig. 3-(A to D). The relationship of the number of zoospores of _Pythium aphanidermatum_ (____), _P. myriotylum_ (····), and _Phytophthora palmivora_ (----) to infection of tomato seedlings. A) Percentage infection (arithmetic) and number of motile zoospores per plant (arithmetic). B) Percentage infection (arithmetic) and number of encysted zoospores per plant (arithmetic). C) Percentage infection (logarithmic) and number of motile zoospores (logarithmic). D) Percentage infection (logarithmic) and number of encysted zoospores (logarithmic). C & D) The linear coefficient was significant at _P_= 0.05 (*) or _P_= 0.01 (**).

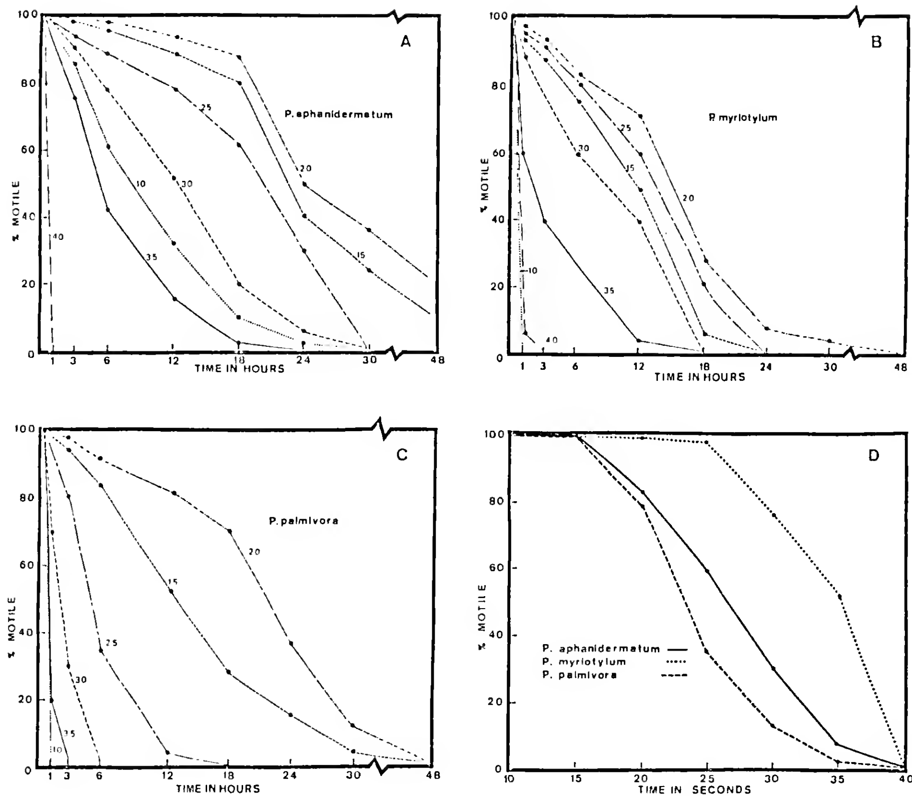

Fig. 4-(A to C) The effect of temperature on motility of zoospores of (A) Pythium aphanidermatum, (B) P. myriotylum, (C) Phytophthora palmivora. (D) The time in seconds for encystment of zoospores of P. aphanidermatum, P. myriotylum, and of P. palmivora at 47 C.

Discussion

Fewer motile zoospores generally are required to produce the same levels of infection obtained with encysted zoospores. From 32 to 85 times more encysted zoospores than motile zoospores were required to infect 50% of the tomato seedlings in this study. Kliejunas and Ko (29) reported that ten times more encysted than motile zoospores of *P. palmivora* were required to kill the same percentage of papaya seedlings, but they inoculated 25 plants per container instead of individual plants.

The percentages of infected seedlings increased as the numbers of zoospores per plant were increased but the ratio of infection to number of zoospores decreased as the number of zoospores increased. The arithmetic and the log-log plots obtained from our data were similar to those reported by other workers for host-pathogen systems involving motile inocula (4, 45, 46). The slopes of data in the log-log transformations were between 0.62 and 0.69. These values support the predictions of Baker (4) since they are near the expected value of 0.67.

The results of this study support previous work with zoospores of several *Pythium* and *Phytophthora* spp. which indicates that fewer than 300 zoospores per plant are required for 50% infection of various hosts (45, 46). There were no indications of quantitative differences in infection of different hosts by a *Pythium* sp. or in infection of the same host by two different *Pythium* spp. The number of zoospores of *P. myriotylum* required for 50% infection of rye was similar to the number of zoospores required for 50% infection of tomato seedlings. Similar numbers of zoospores of *P. aphanidermatum* and *P. myriotylum* were required for comparable levels of infection of tomato.

The relatively great number of zoospores required by an isolate of *P. palmivora* that had been maintained in culture for several years for 50% infection of tomato seedlings may be due to a loss of pathogenicity or a loss of the ability to infect host plants since approximately five times more zoospores were required for 50% infection of this plant than were required with zoospores of other *Phytophthora* or *Pythium* spp. on various hosts (45, 46). Less than 300 zoospores per plant were required for 50% infection of watercress by *Phytophthora cryptogea* (45), milkweed vine by *P. citrophthora* (R. E. Sm. and E. H. Sm.) Leonian (Mitchell and Ridings, unpublished) or tobacco by *P. parasitica* var *nicotiana* (Dastur) var. *nicotianae* (Breda de Haan.) Tucker (Kannwischer and Mitchell, unpublished).

Low or high temperatures were unfavorable for root infection of tomato by *P. aphanidermatum*, *P. myriotylum*, or *P. palmivora*. The reduction in the percentages of infection of tomato by these fungi may be related to a rapid encystment of zoospores at low or high temperatures. At 20 C, zoospores of *P. aphanidermatum*, *P. myriotylum*, or *P. palmivora* were motile for a longer time in vitro than at other temperatures used in the tests. McCarter and Littrell (41) reported that at 19 C, zoospores of *P. aphanidermatum* and *P. myriotylum* were motile longer than at other temperatures. They also noted that the time of motility decreased with fluctuations in temperatures. Zoospores of *Pythium iwayamai* S. Ito remained motile for 16 days in unfrozen water at 0 C (37). In a 1% dextrose solution, zoospores of *P. parasitica* were motile for more than 7 hrs at 20 C (18). Bimpong and Clerk (8) reported that zoospores of *P. palmivora* were motile for 84 hrs in distilled water at 17 C.

In soils, zoospores encyst very rapidly (26). The period of motility of zoospores of P. palmivora, however, has been reported to be as long as 4.5 hrs at 25 C (29). Although the movement of zoospores through soils may not be as important as the passive transport of zoospores in soil solutions, the distance that they actively move toward plant roots, even if it is only 25-35 mm, can be important since it creates the effect of increasing the inoculum level (13, 26, 29, 65).

If zoospores are to be used in studies dealing with host infection, care must be taken to insure that they are motile during and after inoculation since loss of motility can create the effect of a 10 to 90 fold decrease in inoculum (26, 29). Forcing zoospore suspensions through a cannula or a hypodermic needle into soils could cause the zoospores to encyst rapidly since they can be induced to encyst by any rapid change in their environment (8, 16, 18, 26, 29, 41, 46, 61). To avoid the problem of excessive encystment during inoculation and to simulate conditions for the dispersal of zoospores in nature, zoospores can be added to seedlings flooded with water of similar qualities to the water used for preparing zoospore inoculum.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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