Relationship of Density of Chlamydospores and Zoospores of Phytophthora palmivora in Soil to Infection of Papaya

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ABSTRACT

Autoclaved or nontreated soil was infested separately with suspensions of cultures of *Phytophthora palmivora* that had been sonicated so that chlamydospores were the only viable propagules which remained. After 8 days of incubation in moist paper towels, and an additional 7 days of exposure to infested soil in plastic beakers at 30 C in growth chambers, 50% of the 15-day-old seedlings were infected at 0.5 chlamydospores/g of soil, and 95% were infected at 10 chlamydospores/g of soil. Under the same conditions, the same results were obtained with nontreated, infested soil as with autoclaved, infested soil. Fifty and 95% of plants in pots in the greenhouse were infected at 0.1 and 10 chlamydospores/g of soil, respectively, after 45 days of growth in autoclaved soil and an additional 30 days exposure to infested soil. Although percentages of infection were greater with increasing age of papaya seedlings after 30 days of exposure to infested soil, the percentages of mortality decreased with increasing age of the seedlings. Synergism between chlamydospores of *P. palmivora* was suggested when inoculum density-disease incidence transformations were analyzed. Percentages of infection of 45-day-old seedlings exposed for an additional 30 days to zoospore-infested sand in the greenhouse increased with increasing densities of zoospores, and 75% of the seedlings were infected at 10^5 zoospores per container. Mortality was first observed at 10^4 zoospores per plant and increased to 40% at 10^5 zoospores per plant. Populations of *P. palmivora* after 1 week in infested soil without plants were 70-100 percent of the original chlamydospore densities. Populations in infested soil with papaya seedlings were four to five times greater after 1 week than the original chlamydospore density.

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Additional key words: inoculum density, soil-borne diseases, epidemiology.

Phytophthora palmivora Butler causes damping-off of seedlings and fruit-, trunk-, and root-rot of papaya (*Carica papaya* L.) (9, 23). Trujillo and Hine (23) showed that disease development on papaya as measured by amount of root rot, plant height, and mortality rate, was more severe with increasing inoculum densities of *P. parasitica*. The relationship between known numbers of propagules and disease incidence was not, however, established quantitatively.

Low populations of various species of *Phytophthora* in soil can result in significant disease development (3, 4, 7, 13, 20). Hendrix and Kuhlman (7) found populations of 1-30 propagules of *P. cinnamomi* per g in nursery soil where 50% of a stand of *Abies fraseri* had been killed by the fungus. Burley 21, a cultivar of tobacco susceptible to *P. parasitica* var *nicotianae,* was killed after 2 weeks in pots of naturally infested soil containing an initial population of 34 propagules per g of soil (3), or 3-4 weeks after transplants were placed in a field which contained less than 10 propagules per g of soil (4).

In inoculum-density studies with zoospores of several *Phytophthora* spp., approximately 10^6 motile zoospores per plant were required to kill more than 90% of inoculated seedlings (5, 10). Little information is available, however, on the relationship of known concentrations of oospores or chlamydospores in soil to specific levels of disease incidence under carefully controlled environmental conditions. Ko and Chan (12) recently observed 49% mortality of papaya seedlings which had been inoculated by the addition of a chlamydospore-mycelial fragment suspension containing 2.4×10^4 chlamydospores per plant container.

The objectives of this study were to determine the relationships of the densities of chlamydospores and zoospores of *P. palmivora* in soil to the incidence of disease and mortality of papaya seedlings.

MATERIALS AND METHODS.—The culture of *P. palmivora* (P-455) used in this study was obtained from the collection of *Phytophthora* species maintained at the Department of Plant Pathology, University of California, Riverside. It was maintained on V-8 juice agar and transferred monthly.

Chlamydospores were produced with the submerged culture method described by Menyonga and Tsao (16). After 4 weeks of incubation, the cultures were washed on a 20-μm nylon screen with sterile distilled water and comminuted twice for 30 seconds in a micro-blender with 25 ml of sterile distilled deionized water per culture. The resulting suspension was homogenized in a glass tissue grinder, passed 10 times through a single layer of cheesecloth to remove mycelial fragments, and subjected to 60% maximum sonication with a Biosonic III (Bronwill Scientific Co.) ultrasonic system for 60 seconds to disrupt mycelial fragments. The number of chlamydospores in the suspension was determined immediately after sonication by counting six fields for each of 12 samples in a standard hemacytometer. Five 1-ml samples were spread over a pimaricin-vancomycin selective medium (24), and the plates were observed after 12 hours at 25 C in the dark to assure that chlamydospores were the only viable propagules in the suspension.

Cultures for zoospore production were initiated by inoculating petri plates containing 15 ml of V-8 juice broth (20% Campbell's V-8 juice plus 4.5 g/liter $CaCO_3$ and cleared by centrifugation at 1,275 *g*) with three 7-mm plugs from the margin of a three-day-old culture of *P. palmivora* on V-8 juice agar (V-8 juice broth + 17 g/liter Difco agar). After incubation at 30 C in the dark for 72 hours, the medium was drained from the plates and the mycelia were washed three times with 25-ml aliquots of sterile distilled deionized water and incubated in a growth chamber under 12 hours of light [10,760 lx (1,000 ft-c) at the level of the cultures] at 25 C for 48 hours. After two additional washings, the mycelia were suspended in 25 ml of sterile distilled deionized water and placed at 9 C for 30 minutes. The cultures were returned to 25 C for 1 hour and ten 1-ml samples of the zoospore suspension were removed for counting. The samples were agitated in test tubes for 60 seconds on a vortex mixer to induce motile zoospores to encyst (22). Concentrations of zoospores were determined by counting five fields of four samples from each of the ten tubes on a standard hemacytometer.

In one set of experiments, the infested-soil layer technique used by Mitchell (17) was employed to allow noninjured papaya roots to grow into soil containing various densities of chlamydospores. Arredondo fine sand with a pH of 6.5 (measurement obtained from a 1:2 suspension of soil in 0.01 M $CaCl_2$) was sieved through a 0.97 mm (20-mesh) sieve, and, unless otherwise indicated, was autoclaved twice for 1 hour on two successive days. Soil was infested with *P. palmivora* to establish various inoculum densities by thoroughly mixing a known number of chlamydospores into specific amounts of soil with a final water content of 5% (w/w). Fifteen grams of the infested soil were layered over 100 g of autoclaved, coarse, builder's sand packed in the bottom of a 100-ml polypropylene beaker that had three small holes at the base for water movement. Two seedlings of the papaya cultivar Solo were placed on a 15-g band of sterile soil packed over the infested soil. Seedlings with 10-20 mm radicals were selected after seeds had been soaked in tap water for 16 hours, treated with 0.525% sodium hypochlorite for 30 minutes, rinsed in sterile distilled water, wrapped in moist paper towels, and incubated at 30 C for 7 days. The seeds were covered with approximately 5 g of vermiculite, and ten beakers for each treatment were placed in a nylon watering pan.

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

Every 48 hours, 500 ml of tap water was added to each pan, and after 10 minutes the excess water was removed from the pan and the beakers were allowed to drain. The beakers were maintained in growth chambers at 30 C with 12 hours of light (10,760 lx) for 7 days.

The incidence of disease in relation to chlamydospore inoculum level was also evaluated in the greenhouse using seedlings of different ages. Germinated seeds were placed in pans of vermiculite in the greenhouse, and watered every 48 hours. After 15 days, two seedlings were placed in 100 g of autoclaved soil layered over 200 g of chlamydospore-infested soil in the bottom of each of 20 clay pots (10-cm diameter) for each inoculum level. The remaining 15-day-old seedlings were removed from the

vermiculite and planted in 10-ml polypropylene beakers filled with autoclaved soil. After 30 days of growth in the beakers, the 45-day-old seedlings were removed and the root system of each seedling together with the soil from the beaker was surrounded with 75 g of autoclaved soil layered over 200 g of chlamydospore-infested soil in the bottom of each pot. Twenty pots were prepared for each inoculum level. The plants were watered every 48 hours, and maintained under greenhouse temperatures which fluctuated between 26 and 37 C.

For studies with zoospore inoculum in the greenhouse, seedlings were grown for 15 days in vermiculite, and then transplanted to 100-ml polypropylene beakers filled with autoclaved, coarse, builder's sand. The plants were watered daily and fertilized weekly with 10 ml of Hoagland's solution (8). After 30 additional days of growth, 10 ml of sterile distilled deionized water containing various concentrations of zoospores were added to each beaker with a 10-ml syringe.

All plants in the chlamydospore and zoospore studies in the greenhouse were observed daily for mortality. Collapsed plants were harvested when observed, and the living plants were harvested 30 days after inoculation.

After the plants were removed from beakers or pots, the roots were washed, weighed, dipped in 70% ethyl alcohol, rinsed in sterile distilled water, blotted dry on paper towels, and plated on the selective medium. The

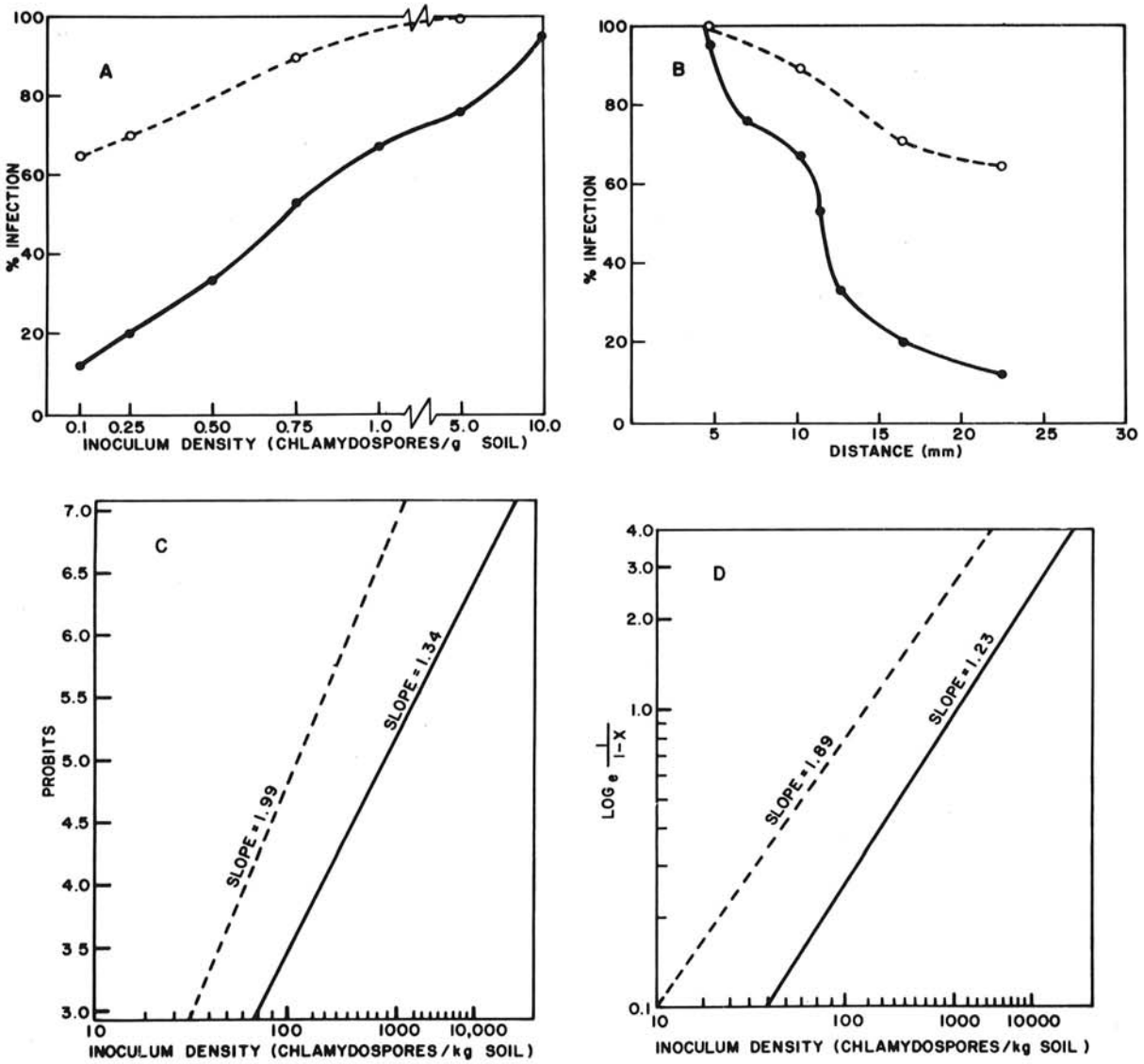

Fig. 1-(A to D). The relationship of density of chlamydospores of *Phytophthora palmivora* to disease incidence in 15-day-old papaya seedlings (—) and 75-day-old papaya seedlings (----). **A)**Percentage infection (arithmetic) and inoculum density (arithmetic). **B)** Percentage infection (arithmetic) and inoculum density (distance between oospores). **C)** Infection (Probits) and inoculum density (logarithmic). **D)** Infection (logarithmic) and inoculum density (logarithmic).

TABLE 1. The influence of host age and inoculum density on the percent of infection, percent of weight loss in roots, and percent of mortality[a]

Inoculum density (chlamydospores/g soil)	Infection (%)		Weight loss (%)[c]		Mortality (%)	
	45[b] Days	75 Days	45 Days	75 Days	45 Days	75 Days
0	0	0	0	0	0	0
1	75	96	0	29	0	5
10	89	100	19	64	10	0
25	100	100	20	74	15	0
50	100	100	30	78	35	0
100	100	...	58	...	55	...
250	100	100	54	76	65	5

[a]Percentages based on 10 plants.
[b]Age of papaya seedlings at harvest time after 30 days of exposure to infested soil.
[c]Root weight as mean percent of control plants for the surviving plants.

plates were observed for growth of *P. palmivora* from the roots after 48-96 hours of incubation in the dark at 30 C.

The populations of *P. palmivora* in infested soil in beakers were determined by spreading 1-ml aliquots of 1:1 or 1:10 (w/v) dilutions of soil in 0.3% water agar containing 300 mg/liter vancomycin over each of 10 plates of the selective medium per sample. After 48 hours of incubation in the dark at 30 C, the soil-agar suspension was washed from the surface of the plates under a slow stream of tap water and the fungal colonies were counted. Isolations from representative colonies were transferred to corn meal agar and paired with opposite mating types of *P. palmivora* to obtain oospores for the identification of the colonies (25).

The influence of the host on chlamydospore germination was observed by placing sonicated chlamydospores contained in envelopes of #20 Nitex monofilament nylon screen cloth (18) between 15-g layers of soil in beakers with or without plants. The envelopes were removed periodically and examined microscopically for chlamydospore germination.

All experiments were repeated twice with the exception of those on the relationship of inoculum density to root infection in the beakers which were repeated three times.

RESULTS.—Higher percentages of root disease incidence occurred at all inoculum levels with 75-day-old plants grown in pots in the greenhouse than with 15-day-old seedlings grown in polypropylene beakers in growth chambers (Fig. 1-A). Percentages of papaya infection in the greenhouse at all inoculum levels were also lower with 45-day-old seedlings than with 75-day-old seedlings (Table 1).

The distance (D) between chlamydospores in the infested soil was calculated with the equation

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

where (V_s) equals the volume of infested soil and (N) the number of chlamydospores per volume of soil (14). Disease incidence decreased as the distance between propagules increased (Fig. 1-B). The interpolated ID_{50} value (inoculum density required to produce 50% infection) was 11.5 mm for 15-day-old seedlings in beakers, but was greater than 25 mm for 75-day-old plants in pots.

Slopes determined by linear regression analysis were 1.23 and 1.89 for 15 and 75-day-old plants, respectively, in log-probit transformations (Fig. 1-C), and were 1.34 and 1.99 for log-log transformations (Fig. 1-D). The ID_{50} values for the log-probit and log-log transformations for 15 and 75-day-old plants were 0.81 and 0.11, and 0.50 and 0.09 chlamydospores/g of soil, respectively. Whereas the percent of seedling disease incidence and weight reduction in roots was greater with older seedlings, the percentage of mortality was greater with the 45-day-old seedlings than with 75-day-old seedlings. Few of the 75-day-old plants were killed even at 250 chlamydospores/g of soil (Table 1).

Percent infection and percent mortality of seedlings increased with increasing zoospore densities of 10^3 to 10^5 zoospores per plant (Fig. 2). The number of zoospores required per plant to produce 50% infection was approximately 1.25×10^4.

When nontreated air-dried soil was infested at the same chlamydospore densities as autoclaved soil, no significant differences in percentages of infection at specific inoculum densities were observed in plants held in beakers for 7 days.

Populations of *P. palmivora* in infested soil in polypropylene beakers containing plants were four to five

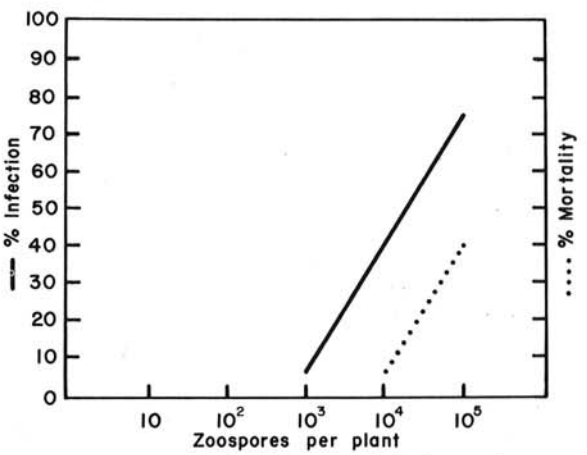

Fig. 2. The relationship of percent infection and percent mortality of 75-day-old seedlings to density of zoospores of *Phytophthora palmivora* in soil.

times greater after 1 week of incubation than the original chlamydospore density. In beakers that did not contain plants, the populations after 1 week were 70 to 100 percent of the original chlamydospore density.

Microscopic observation of the nylon screen envelopes showed that germination of chlamydospores took place in soil with or without plants. When papaya roots were present, however, empty sporangia but not zoospores were observed. Neither sporangia nor zoospores were observed in envelopes from beakers without plants.

DISCUSSION.—The percentages of infection and root weight losses were higher at all inoculum densities with older plants, but the percentages of plant mortality were greater with younger plants. Ko (11) also observed that mortality of papaya seedlings from root rot caused by *P. palmivora* decreased with plant age, and that seedlings were not killed after they reached 3 months of age. Resistance of various older host plants to *Pythium* spp. has been reported frequently (15).

Severe *Phytophthora* root rots of various hosts may occur in soils containing very low levels of the fungus (3, 4, 7, 13, 20). The results of this study indicate that chlamydospore densities of *P. palmivora* of 0.5 to 0.8 chlamydospores/g of soil with 15-day-old seedlings in beakers and 0.09 to 0.11 chlamydospores/g of soil with 75-day-old plants are required for 50% infection. All seedlings were diseased in beakers or pots when exposed to 10-25 chlamydospores/g of soil. Mitchell and Ridings (*unpublished*) also found an ID_{50} of approximately 0.5 to 1.0 chlamydospores per g of soil with *Phytophthora citrophthora* Smith and Smith and young seedlings of milk-weed vine (*Morrenia adorata* Lind.). Although populations of *Phytophthora* species may increase greatly around diseased host roots (3), soil populations of most species of *Phytophthora* are rarely greater than 5 propagules per g of soil prior to planting susceptible hosts (3, 6, 7, 13).

The results of this study suggest synergism with *P. palmivora* chlamydospores and papaya seedlings because slopes derived from linear regression analysis in the log-probit transformations for plants in beakers or pots were close to the predicted value of 2.0 (19) for synergism, and were greater in the log-log transformations than the predicted value of 1.0 for this type of host-pathogen relationship (1). Benson and Baker (2) proposed that synergism between *Rhizoctonia solani* propagules resulted from rapid proliferation of hyphae stimulated by the exudates in the spermosphere after initial contact of hyphae with radish seeds. Soil assays, and the production of sporangia in the nylon envelopes in our studies, indicated that chlamydospores germinated indirectly when host roots were present and that synergism probably resulted from increased inoculum densities from zoospore production and subsequent accumulation on host roots. The inoculum density of *P. parasitica* var. *nicotianae* also increased in the presence of host roots in naturally or artificially infested soil (3, 4). Consequently root or seed exudates appear to play an important stimulatory role in synergism with *R. solani,* and *Phytophthora* spp., and other soil-borne plant pathogenic fungi.

Root exudates also stimulated the germination of oospores of *Pythium* spp. but zoospores were not produced unless soil was maintained in a flooded condition (17, 21). Mitchell (17) observed a rhizosphere effect without synergistic action for *Pythium myriotylum* infection of rye roots with slopes in the log-log analyses near 1.0. Approximately 20 oospores per g of soil were required to infect 50% of the rye plants. Thus, the chlamydospore density of *P. palmivora* required for specific levels of infection of papaya plants is much lower than the oospore density of *P. myriotylum* required for the same percent infection of rye seedlings. Additional research is needed to clarify the response of different types of spores (chlamydospores, oospores, sporangia, and zoospores) of *Phytophthora* spp. and *Pythium* spp. to exudates, and the subsequent factors in root disease.

Greater numbers of zoospores than of chlamydospores of *P. palmivora* were required for infection and death of papaya seedlings. Only 5% of the plants were infected at 10^3 zoospores per plant and approximately 1.25×10^4 zoospores per plant were required to infect 50% of the plants. More than 10^5 zoospores per plant were required to kill 50% of the seedlings. Ko and Chan (12) reported 44% mortality of papaya seedlings after inoculation with 8.3×10^4 motile zoospores of *P. palmivora* per container. They also observed that greater numbers of zoospores than of chlamydospores were required to cause death of papaya seedlings. Kliejunas and Ko (10) found that few papaya plants were killed at low levels of nonmotile zoospores (6×10^3 zoospores/container), but that 53% of the seedlings were killed at 33 C with 6×10^5 nonmotile zoospores per container. Whereas the work of Kliejunas and Ko (10) and Ko and Chan (12) differed from our work in the number of seedlings per container, plant age, and various environmental factors, the results of those studies, as well as the results of Gooding and Lucas (5) with *P. parasitica* var. *nicotianae* on tobacco, indicate that at least 10^3 zoospores per container are required for minimum mortality of hosts from root rot by *Phytophthora* spp.

Although studies on epidemiology of soil-borne diseases may best be made with natural soil (1), the interference in the recovery of *Phytophthora* spp. from soil due to overgrowth of the colonies by the faster-growing *Pythium* spp. in natural soil necessitated the use of autoclaved soil in the bulk of the experiments. When results from experiments in natural and autoclaved soil were compared, no significant differences in infection of papaya by *P. palmivora* at common inoculum densities were observed.

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