Relationships of Numbers of Spores of *Phytophthora parasitica* var. *nicotianae* to Infection and Mortality of Tobacco

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Portion of Ph.D. thesis by the senior author, University of Florida.
Florida Agricultural Experiment Stations Journal Series Paper 2277.
Accepted for publication 8 June 1980.

ABSTRACT

Kannwischer, M. E., and D. J. Mitchell. 1981. Relationships of numbers of spores of *Phytophthora parasitica* var. *nicotianae* to infection and mortality of tobacco. Phytopathology 71:69-73.

Sonicated chlamydospores of *Phytophthora parasitica* var. *nicotianae* were mixed into autoclaved Astatula sand to achieve defined inoculum densities. Individual 4-wk-old susceptible tobacco plants were exposed to infested soil. After 50 days at 25 C in a growth room, the percentages of infection were 23, 27, 50, 81, 92, 98, and 100 for plants originally exposed to 50, 75, 100, 250, 500, 1,000, and 5,000 chlamydospores per kilogram of soil, respectively. Motile zoospores were added to individual plants growing in flooded sand. After 28 days at 25 C in a growth room, the percentages of infection were 10, 22, 35, 55, 82, 93, and 100 for plants originally exposed to 5, 15, 25, 50, 100, 200, and 300 zoospores per plant, respectively. Sonicated oospores that were frozen and either left untreated or treated with cellulase or Gluculase (glucuronidase and sulfatase) enzymes were mixed into autoclaved soil at densities of 500 to 100,000 spores per kilogram of soil. The oospores were plated on various media to determine germination percentages. None of the enzyme-treated or untreated oospores germinated after 96–128 hr at 25 C and 12 hr light on various media and none of the tobacco plants were infected after exposure to infested soil for 50 days at 25 C in a growth room.

Additional key words: epidemiology, *Nicotiana tabacum, Phytophthora nicotianae* var. *nicotianae.*

Phytophthora parasitica var. *nicotianae* (Breda de Haan) Tucker (= *P. nicotianae* Breda de Haan var. *nicotianae* Waterhouse), the causal agent of black shank of tobacco (*Nicotiana tabacum* L.), produces several types of spores. Quantitative information about the roles that zoospores, chlamydospores, and oospores of *P. parasitica* var. *nicotianae* play in infection and subsequent disease development is limited (12,16).

Although it was proposed that zoospores of *P. parasitica* var. *nicotianae* were the primary inocula in the field because they were the most frequently observed type of spore (5,8), there is little evidence to substantiate this claim. Recent studies indicate that zoospores of species of *Phytophthora* and *Pythium* may not be the primary source of inoculum (4,6,16,19), but they are considered to be important in the secondary spread of disease in the field (16,19). Gooding and Lucas (7) showed that the incidence of black shank increased under laboratory conditions with an increase in zoospore inoculum concentration, but that disease indices (based on the number of plants killed and the rate of death) at a given inoculum concentration varied significantly in different experiments. Since zoospores must be close to a plant for chemotaxis and infection to occur, their inoculation technique, in which zoospores were injected into sand around plant roots, may have reduced the contact of motile zoospores with roots and thus may have been responsible for the inconsistencies in the disease development observed. Recent studies where motile zoospores were added to the standing water over flooded soil (or vermiculite), which was drained through the soil after several hours, showed that low numbers of zoospores per plant could result in higher percentages of plant infection and mortality (19,20). Conditions that favor zoospore motility (ie, careful handling to reduce encystment and soil moisture conditions that provide water filled channels through which zoospores can move) result in greater infection of plants at a given zoospore concentration than do conditions that induce zoospore encystment or reduce active movement of zoospores (6,11,15,19,20). Thus, it is important to consider the inoculation technique used when disease or inoculum is evaluated quantitatively.

Chlamydospores of many species of *Phytophthora* serve as survival structures in soil or in plant debris and have been considered to be the primary inoculum in nature (4,11,13,16,19,22). It was reported previously that an inoculum density of 500 chlamydospores of *P. parasitica* var. *nicotianae* per kilogram of soil resulted in 93% mortality of susceptible tobacco plants and an apparent infection rate of 0.9/unit/day under growth-room and greenhouse conditions (12). The numbers of chlamydospores required for specific amounts of infection and mortality of tobacco (in particular, the inoculum densities required for 10, 50, and 90% infection and mortality) have not been reported.

Little is known about the role of oospores of *P. parasitica* var. *nicotianae* in the development of black shank, and there are no known reports of oospores of this fungus in nature (17). Oospores of *P. parasitica* var. *nicotianae* and other heterothallic *Phytophthora* spp. are formed in abundance only in paired cultures of isolates of opposite mating types. The oospores from crosses rarely germinate in vitro (usually 0–2% germination); thus, it is difficult to study the genetics of these fungi, determine the possible pathogenic variability of *Phytophthora* spp., or to learn the role of oospores in the various cycles of the diseases caused by these fungi. Pathogenicity studies have been conducted with oospores of homothallic *Phytophthora* spp. (2,3,14).

The objective of this study was to determine the relationships of numbers of chlamydospores, oospores, and zoospores to the percentages of infection and mortality of tobacco plants.

MATERIALS AND METHODS

The isolates of *P. parasitica* var. *nicotianae* used in chlamydospore and zoospore studies (P-230) and in oospore studies (P-230 and P-583) were originally isolated from tobacco and obtained from the collection of *Phytophthora* spp. maintained at the Department of Plant Pathology, University of California, Riverside. The cultures were maintained on V-8 juice agar and transferred monthly.

Chlamydospores were produced in liquid culture by the method of Tsao (24). Known numbers of chlamydospores, free of viable hyphal fragments and other spores, were obtained by the method

0031-949X/81/01006905/$03.00/0

described by Ramirez and Mitchell (22). The infested-soil-layer technique used by Mitchell (18) was modified to allow uninjured tobacco roots to grow into Astatula sand that had been autoclaved for 2 hr on 2 successive days, aged for 30 days, and infested with the pathogen. Sixty-five grams of soil were infested with 0, 50, 75, 100, 250, 500, 5,000, or 50,000 chlamydospores per kilogram of soil and layered over 15 g of autoclaved builder's sand in a 100-ml polypropylene beaker. The infested soil was covered with 35 g of noninfested soil, and a 1-mo-old tobacco seedling of the susceptible cultivar Hicks was transplanted into the noninfested soil. Plants were maintained in a plant-growth room at 25 C and 12 hr light (4,000 lx at the level of the plants) and were watered daily and fertilized weekly as described previously (12). Plants were observed daily for wilting and blackening of the stems. Experiments consisted of 15 or 30 plants per inoculum level, and the experiments were conducted six times.

Oospores of *P. parasitica* var. *nicotianae* were produced in V-8 juice broth by the method developed by Honour and Tsao (10). A paired culture of isolate P-230 (A_1 mating type) and P-583 (A_2 mating type) was initiated by adding 1 ml of a suspension of mycelium of each isolate to 15 ml of V-8 juice broth in a 200-ml prescription bottle and incubating the cultures for 14 days in the dark at 25 C. To obtain oospores free of viable hyphal fragments, 2-wk-old cultures were washed and suspended in a 10^{-3}M solution of 2-(*N*-morpholino)-ethanesulfonic acid (MES) in deionized water (adjusted to pH 6.2 with 1 N KOH). The suspensions were comminuted for 1 min at maximum speed in a Waring Blendor, homogenized in a tissue grinder, and sonicated for 1 min at 240 W with a Bronsonic 1510 sonicator (B. Braun Instruments, S. San Francisco, CA 94080). The resulting suspension was left undisturbed for approximately 1 hr to allow the spores to settle, and the suspended mycelial debris was discarded. The spores and remaining debris then were resuspended in a larger volume of buffer, the spores again were allowed to settle, and the debris was removed. This process was repeated four times, and the final spore suspension was subjected to low-speed centrifugation. The pellet containing the spores was placed in 5–10 ml of buffer and agitated to resuspend the spores; the suspension was then frozen at −20 C to eliminate other viable spores. The spore preparation was thawed after 18–24 hr, and 1-ml samples were removed and either diluted (1:1, v/v) with cellulase (1% solution of Cellulysin in MES [Calbiochem-Behring Corp., San Diego, CA 92112]) or diluted (1:1 or 1:2, v/v) with 2% Gluculase (a mixture containing 163,604 units glucuronidase and 14,995 units sulfatase per milliliter; Endo Laboratories Inc., Garden City, NY 11530). After 24–72 hr of incubation at 25 C in the dark, enzyme-treated spores were washed, suspended in the buffer, and concentrated four times by using low-speed centrifugation to remove the enzymes from the spore preparations. Soil was infested with 0, 500, 5,000, 50,000, or 100,000 oospores per kilogram and plants were transplanted and maintained as described for plants in the chlamydospore experiments.

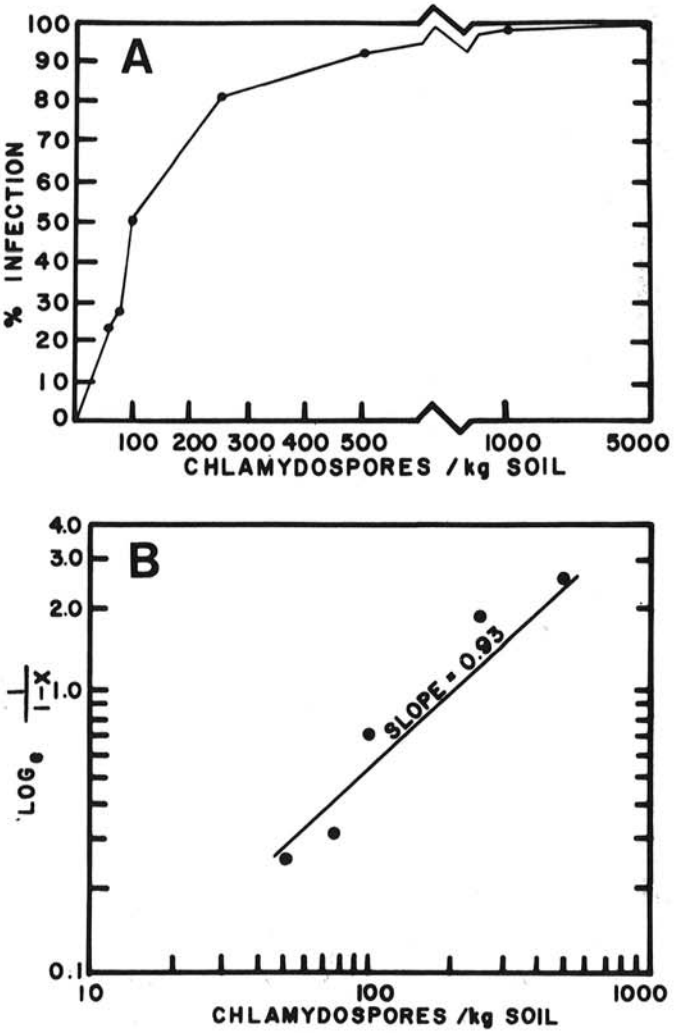

Fig. 1. The relationship of density of chlamydospores of *Phytophthora parasitica* var. *nicotianae* in soil to percentage infection of tobacco plants 50 days after planting: **A,** percentage infection (arithmetic), and **B,** percentage infection adjusted for multiple infection (logarithmic) and inoculum density (logarithmic).

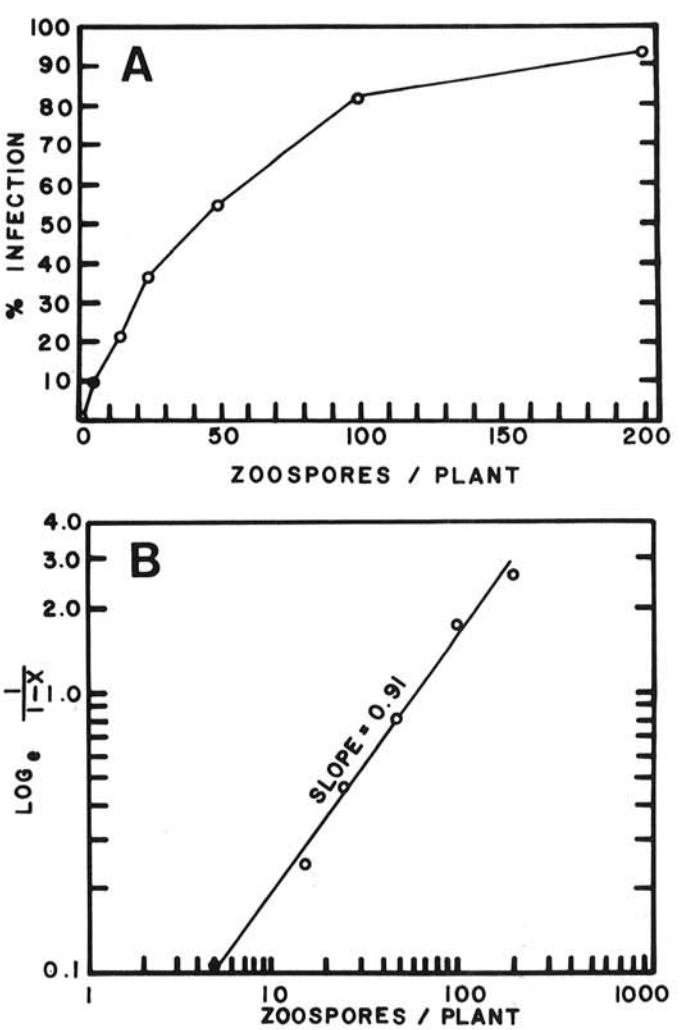

Fig. 2. The relationship of numbers of zoospores of *Phytophthora parasitica* var. *nicotianae* per tobacco plant to percentage infection 28 days after inoculation: **A,** percentage infection (arithmetic), and **B,** percentage infection adjusted for multiple infections (logarithmic) and zoospore concentration (logarithmic).

Zoospores were produced in cultures initiated by placing six 2-mm-diameter plugs from the margin of a 2-day-old culture of *P. parasitica* var. *nicotianae* on V-8 juice agar in petri plates containing 15 ml of V-8 juice broth. Cultures were incubated at 25 C and 12 hr light (4,000 lx at the level of the cultures). The medium was drained from the plates after 48 hr and each mycelial mat was washed three times with 25 ml of an autoclaved solution of 10^{-4}M MES (pH 6.2) and flooded with 10 ml of the buffer. After incubation for an additional 48 hr to induce sporangium formation, the cultures were rinsed once and flooded with 25 ml of the buffer and chilled in a refrigerator at 3 C for 15 min. Zoospores were released 10–30 min after the cultures had been returned to room temperature. The zoospore suspension from the plates was diluted 1:1 with the buffer, and 3-ml samples were removed and agitated on a vortex mixer to induce spore encystment (23). The encysted spores were counted by using the hemacytometer and micro-drop methods described by Mitchell et al (20). The zoospore suspension then was diluted with the buffer to give 50 zoospores per milliliter (one zoospore per 20-μl drop), and the motility of zoospores was checked by observing spores in six 20-μl drops. The procedures for inoculation of plants with zoospores were designed to simulate flooded conditions in the field where zoospores move in the surface water to stems or are carried to the roots as infested water drains through the soil. Individual 1-mo-old Hicks plants were transplanted into 60 g (30-ml volume) of washed, autoclaved builder's sand in 50-ml polypropylene beakers with small holes in the bottom to allow water movement. Beakers were placed in nylon trays (15 per tray) and were maintained in a growth room at 25 C and 12 hr light (4,000 lx at the level of the plants) for 1–2 wk prior to inoculation. Plants were watered daily and fertilized once a week with modified Hoagland's solution (9). Immediately before inoculation, the trays were filled with tap water to a level that provided 1 cm of standing water above the surface of the sand in the beakers. Five, 15, 25, 50, 100, 200, or 300 zoospores were added to each of 15 beakers per tray by pipetting 0.1, 0.3, 0.5, 1.0, 2.0, 4.0, or 6.0 ml, respectively, of a zoospore suspension that contained 50 zoospores per milliliter into the surface water of each beaker. Experiments consisted of two trays of each inoculum level. After 3 hr the water was drained from the trays which allowed the zoospore-infested water to move down through the sand in the beakers. Plants were maintained in the plant-growth room for 14–28 days.

The percentages of germination of chlamydospores, oospores, or zoospores from inocula or infested soil were determined by plating samples on a selective medium (PARP) which contained the following ingredients: 10 mg pimaricin (Delvocid, 50% a.i., Gist-Brocades N.V., Delft, Holland), 250 mg ampicillin (Polycillin-N, 81% a.i., Bristol-Meyers Co., Syracuse, NY 13201), 10 mg rifampicin (Rifamycin SV, 100% a.i., Sigma Chemical Co., St. Louis, MO 63178), 100 mg pentachloronitrobenzene (Terraclor, 75% a.i., Olin Mathieson Chemical Corp., Little Rock, AR 72203), and 17 g Difco cornmeal agar (Difco Laboratories, Detroit, MI 48201) in 1.0 L of deionized water. The plates were incubated at 25 C in the dark for 24–48 hr and observed for colony development. Oospore suspensions also were plated on cornmeal agar, Difco Noble agar, Difco Bacto-agar, V-8 juice agar, and the $P_{10}VP$ medium of Tsao and Ocaña (25), and incubated at 25 C with 12 hr of light (4,000 lx at the level of the plates) for 96–148 hr.

Plants in all experiments were observed daily and considered dead when they had wilted permanently and had blackened stems. The roots and stems of surviving plants that had been inoculated with chlamydospores, oospores, or zoospores were washed in running water, dipped in 70% ethyl alcohol for 15 sec, rinsed twice in deionized water, and blotted dry on paper towels. Plants were plated on the selective medium (PARP), and incubated for 48 hr at 25 C in the dark; the plates then were observed for the growth of *P. parasitica* var. *nicotianae* from the stems and roots of infected plants.

RESULTS

The percentages of infection and mortality of tobacco increased when the numbers of chlamydospores of *P. parasitica* var. *nicotianae* (Fig. 1A) to which the plants were exposed were increased from 50 to 5,000 chlamydospores per kilogram of soil. After 50 days, 23 and 100% of the plants were infected at 50 and 5,000 chlamydospores per kilogram of soil, respectively. The infection data were transformed to $\log_e 1/(1-x)$, in which x equals the proportion of the plants infected, to adjust for multiple infections (26). When $\log_{10}(\log_e 1/(1-x))$ was plotted against $\log_{10}$ of the number of chlamydospores per kilogram of soil (Fig. 1B), points of the log-log transformation plot lay in a straight line between 50 and 1,000 chlamydospores per kilogram of soil. The slope of the line determined by linear regression analysis was 0.93 ($P=0.001$). The inoculum density required for 50% infection of the plants was interpolated to be 132 chlamydospores per kilogram of soil. Data presented are from experiments conducted in autoclaved Astatula sand, but similar levels of infection were obtained at the corresponding inoculum levels in raw or methyl bromide-treated Astatula sand.

Recovery of the fungus from chlamydospore-infested soil increased from 30–40% of the artificially established densities when the soil was plated immediately following infestation to 70–90% when the soil was plated 1 day after infestation.

The percentages of infected tobacco plants in sand increased as the inoculum was increased from five to 200 zoospores per plant (Fig. 2A). When data were transformed to $\log_e 1/(1-x)$ to compensate for multiple infections and plotted as $\log_{10}(\log_e 1/(1-x))$ against $\log_{10}$ of the number of zoospores per plant, points obtained at five to 200 zoospores per plant lay in a straight line (Fig. 2B). By linear regression analysis the slope of the line was determined to be 0.91 ($P=0.001$) and the inoculum level required for 50% infection was interpolated to be approximately 42 zoospores per plant. Greater than 70% of the zoospores were motile at the time of inoculation of the plants, and 70–90% of the zoospores germinated when samples of the inoculum were plated on the selective medium.

With few exceptions, all of the infected plants died within 14 or 50 days after the initiation of the zoospore or chlamydospore experiments, respectively. None of the plants in the oospore experiments were infected, as determined by plating plants on the selective medium, within 50 days after they were transplanted into beakers containing infested soil. None of the enzyme-treated or nontreated oospores of *P. parasitica* var. *nicotianae* germinated on cornmeal agar, Difco Bacto-agar, Difco Noble agar, V-8 juice agar, PARP medium, or $P_{10}VP$ medium.

DISCUSSION

Lower inoculum densities of *P. parasitica* var. *nicotianae* were required for 50% infection of tobacco plants in this study than have been required for the same level of infection of other hosts by various species of *Phytophthora* (19). Approximately 132 chlamydospores per kilogram of soil were required for 50% infection and mortality of young, susceptible tobacco plants grown for 50 days in individual containers under growth-room conditions. Mitchell (19) reported that 50% infection of milkweed vine by *P. citrophthora* and of papaya by *P. palmivora* required 600 and 900 chlamydospores per kilogram of soil, respectively. In his experiments plants were exposed to a smaller amount of infested soil (15 g per container) than was used in the tobacco experiments, and the length of exposure of the plants to the soil was 1–2 wk compared to 7 wk with tobacco. However, the total number of spores to which each plant was exposed that resulted in 50% infection was similar in the three systems. Only nine, nine, or 14 chlamydospores of *P. parasitica* var. *nicotianae, P. citrophthora,* or *P. palmivora* per plant were required for 50% infection of tobacco, milkweed vine, and papaya, respectively.

Gooding and Lucas (7) reported that the percentages of mortality of tobacco plants increased with an increase in zoospore inoculum. They found that 10^4 zoospores per plant were required for approximately 50% mortality of susceptible tobacco plants and that 10^6 zoospores were required for 100% mortality of plants within 2 wk after zoospores were injected into soil around the plants. Recently, however, low numbers of zoospores of *Phytophthora* spp. have been shown to be able to infect and cause

death of host seedlings when applied directly onto the plant surface in water (17,21). Pratt et al (21) found that 30% of the cotyledons of susceptible alfalfa seedlings became infected after inoculation with one zoospore of *P. megasperma* per cotyledon. McIntyre and Taylor (17) sprayed a zoospore suspension of *P. parasitica* var. *nicotianae* on whole tobacco seedlings that resulted in approximately eight viable zoospores on or near each plant. This inoculum level resulted in sufficient disease to allow distinction between susceptible and resistant varieties based on the development of disease within 5 days after inoculation. The experiments in this study were designed to provide conditions favorable for zoospore movement and presumably were similar to conditions in the field where zoospores are important in secondary spread of disease (ie, where zoospores move in saturated soil or in surface water). Only 5, 42, and 158 zoospores of *P. parasitica* var. *nicotianae* per plant were required for 10, 50, and 90% infection of susceptible tobacco plants, respectively. Mitchell (19) reported that 250–280 zoospores of *P. cryptogea, Pythium aphanidermatum,* and *Pythium ostracodes* were required for 50% infection of watercress, tomato, and cotton, respectively, under similar experimental conditions. Fewer zoospores of *P. parasitica* var. *nicotianae* may have been required for the infection of tobacco, as compared to infection in the above host-pathogen combinations, because the young tobacco plants were succulent and had lower leaves as well as stem tissue exposed to zoospore-infested water, whereas only stems were exposed in the other combinations.

The slopes of the log-log transformations of the relationships of both zoospores (per plant unit) and chlamydospores (per kilogram of soil) to the proportion of infected plants approached 1.0, which indicates direct proportionality. Other studies in which chlamydospores were employed as inoculum resulted in similar slopes in log-log graphs, but those in which zoospore inoculum was used resulted in log-log slopes close to 0.67 (19,20). If slopes of less than 1.0 result (when inoculum is computed on a per-plant basis) from competition for susceptible sites at higher inoculum levels or from other factors that reduce survival or effectiveness of the various spore forms in infection (1,19,20,27), the differences in slopes obtained with zoospores and various hosts under similar conditions (motile zoospores in surface water) may be explained by the availability of susceptible host tissue for infection or a reduced effectiveness of zoospores.

The role of oospores of heterothallic species of *Phytophthora* in the development of disease is unknown. In this study none of the oospores of *P. parasitica* var. *nicotianae* were observed to germinate in vitro or to infect tobacco roots in soil. Procedures similar to those employed in this study were used to obtain oospores of homothallic species of *Phytophthora* that resulted in plant infection (2,3,14). Banihashemi and Mitchell (2) obtained approximately 45 and 100% infection of safflower seedlings planted in soil infested with 10^5 or 5×10^7 oospores of *P. cactorum* per kilogram of soil, respectively. Similar densities of oospores of *P. megasperma* var. *sojae* were required for high disease incidence in soybean seedlings (14). Baumer and Erwin (3), however, found that an inoculum density of about 20 Gluculase-treated oospores of *P. megasperma* per kilogram of soil resulted in 50% death of susceptible seedlings of alfalfa within 30 days. Although the results with oospores of *P. parasitica* var. *nicotianae* indicate that no infection occurred even when the plants were exposed to up to 5×10^5 oospores per kilogram of soil for up to 75 days, additional studies still are needed to elucidate the possible role of oospores of heterothallic species of *Phytophthora* in disease development. Some of the important factors that should be examined include the age and source (various cultures or host tissue) of oospores, susceptibility of various host tissues to infection, and, most importantly, the physical, biological, and chemical factors that affect the formation, survival, and germination of oospores in the soil.

It is preferable to use raw soil in studies dealing with the epidemiology of soilborne diseases to simulate most closely the biological interactions that can occur in nature. In this study, however, it was necessary to use treated soil to eliminate other fungi pathogenic to tobacco. The autoclaved soil was held for 30 days prior to use to allow time for recolonization by airborne microorganisms. Although some seedlings damped-off in raw soil after infection with pathogens other than *P. parasitica* var. *nicotianae,* similar levels of infection and mortality of tobacco plants occurred at corresponding inoculum densities of *P. parasitica* var. *nicotianae.*

The techniques used in this study, along with the host-pathogen system of tobacco and *P. parasitica* var. *nicotianae,* supported systems with either chlamydospores or zoospores that had approximately the same percentages of both infection and mortality at specific inoculum levels. Mitchell (18) observed that the same percentages of rye plants infected by *Pythium myriotylum* subsequently died under favorable conditions; however, most studies on the relationship of inoculum densities of species of *Phytophthora* and *Pythium* to disease incidence either have not quantified percentages of infection with individual plants, or have employed host-pathogen combinations that require much higher inoculum levels for mortality than for infection (19). The tobacco-*P. parasitica* var. *nicotianae* system saves time and expense in epidemiological studies because individual plants do not have to be plated on selective media to differentiate critically between infection and death. It is also a good model to use for differentiating time or other factors required for infection as compared to death.

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