Zoospore Inoculum Density of *Phytophthora cinnamomi* and the Infection of Lateral Root Tips of Shortleaf and Loblolly Pine

S. W. Fraedrich, F. H. Tainter, and A. E. Miller

Former graduate research assistant, professor, and assistant professor, respectively; Department of Forestry, Clemson University, Clemson, SC 29634-1003. Present address of first author: Forestry Sciences Laboratory, USDA Forest Service, P. O. Box 70, Olustee, FL 32072.

A statistical routine, EXST100, developed by Dr. P. M. Burrows of the Experimental Statistics Unit, Clemson University, was employed for parameter estimation of the logistic relationship functions and determination of the goodness of fit.

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ABSTRACT

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The relationship of spore concentration of *Phytophthora cinnamomi* to frequency of infection of shortleaf and loblolly pine roots was determined. Pine seedlings grown in sand culture were root-dip inoculated for 3 hr in spore suspensions ranging from 14 to 2,000 spores/ml. Shortleaf pine roots were more susceptible to infection by *P. cinnamomi* than were roots of loblolly pine. Comparison of fitted regression functions for the two species indicated that the regression lines were not coincident. Differences between the species were evident at spore concentrations of 80–280 spores/ml. At spore concentrations outside this range, differences in susceptibility between loblolly and shortleaf pine were undetectable, because either too high or too low a percentage of roots of both species was infected. Spore concentrations required to infect 50% of lateral roots were approximately 100 and 178 spores/ml for shortleaf and loblolly pine, respectively.

Loblolly pine (*Pinus taeda* L.) is considered to be less susceptible to the disease complex known as littleleaf than is shortleaf pine (*Pinus echinata* Mill.) (2). Edaphic and climatic factors, as well as fine root mortality caused by *Phytophthora cinnamomi* Rands, are believed to be involved in the development of littleleaf disease (2,27). Species difference to this disease may be due partly to a difference in susceptibility of roots to infection by *P. cinnamomi* (27,28). Zak and Campbell (28) indicated that shortleaf pine is more susceptible to *P. cinnamomi* than is loblolly pine. Marx (20), however, found that shortleaf and loblolly pine nonmycorrhizal roots were equally susceptible to *P. cinnamomi* and were infected at equivalent frequencies.

Inoculum density is an important consideration in assessing resistance to plant pathogens. Low inoculum densities enhanced sensitivity for resistance screening to *Cronartium quercuum* f. sp. *fusiforme* on loblolly pine and slash pine (*Pinus elliotii*) (19). Likewise, Griggs et al (10) found that optimum inoculum concentrations of *C. q. fusiforme* were essential for detecting differences in slash pine families and assessing types of resistance reactions. Hodgson (14) emphasized the importance of using a proper concentration of spores when studying resistance in potatoes (*Solanum tuberosum*) to *P. infestans*. At high inoculum concentrations, plants of all cultivars became infected, and differences in resistance were not apparent. Similarly, Eye et al (6) studied the effect of zoospore concentrations of *P. megasperma* var. *sojae* on disease incidence in various cultivars of soybean. They found that 100% of 2-day-old seedlings of a susceptible cultivar were infected at all zoospore concentrations tested; however, in resistant cultivars, disease incidence increased as inoculum concentration increased. At the highest concentration, all seedlings were infected, regardless of cultivar. Their conclusion, similar to that of Hodgson (14), was that resistance can be

overcome with sufficient inoculum. In the studies by Eye et al (6) and others (9,25), the rapidity and/or severity of disease development has been related directly to the quantity of spores used in plant inoculations.

No studies have examined the effect of spore concentration of *P. cinnamomi* on susceptibility of shortleaf and loblolly pine roots to infection. Objectives of our study were to characterize the relationship between zoospore concentration of *P. cinnamomi* and frequency of infection of shortleaf and loblolly pine lateral root tips, and evaluate relative susceptibility of the two pine species.

MATERIALS AND METHODS

Seedling production. Pots used in this study were 12 cm deep and tapered from 11 cm in diameter at the top to 9 cm at the bottom. Holes in the bottom of pots were plugged with nonabsorbent cotton wrapped in gauze to permit free drainage. Procedures for sand preparation were adopted from Hewitt (11). Sand was soaked in a 5% HCl solution for 1 wk to eliminate impurities, rinsed with tap water until the pH had risen to at least 5.5, and then dried at 105 C for 24 hr; 1,175 g of sand was placed into each pot. Pots with sand were autoclaved (248 C, 104 kPa) for approximately 90 min on 3 consecutive days to eliminate potential pathogens.

Loblolly and shortleaf pine seeds were obtained from the International Forest Seed Co. (Birmingham, AL). Seeds were soaked for 2 days in distilled water, surface disinfested in 30–35% H_2O_2 for 15 min, rinsed with sterile distilled water, and stratified for 30 days at 5 C. Seeds were germinated on moist, sterile filter paper. Seven germinated seeds of either shortleaf or loblolly pine were placed in each pot. Pieces of dark polyethylene plastic (11 cm diameter), with holes cut to allow for seedling growth, were placed over the germinated seeds. This procedure effectively prevented algal growth on the sand surface. Seedlings were watered daily with 25–50 ml of distilled water for the first 2–3 days after planting to permit establishment. Thereafter, seedlings were watered daily with 100 ml of nutrient solution, modified from that of Ladiges (18). Macronutrients were supplied as follows: nitrogen, 30 mg/L as NH_4NO_3; calcium, 40 mg/L as $CaCl_2$; potassium, 25 mg/L as K_2SO_4; magnesium, 12 mg/L as $MgSO_4$; and phosphorus, 5 mg/L as NaH_2PO_4. Micronutrients were supplied as described by Hoagland and Arnon (13) with the exception of iron chelate (FeEDTA), which was formulated as described by Hewitt (11) and supplied at the rate of 2.5 mg/L. The nutrient solution was adjusted to pH 5.2 with 0.1 N NaOH or 0.1 N HCl.

Plants were grown in growth chambers at 24 ± 1 C, with 16-hr photoperiods for 6–7 wk. Light intensity in growth chambers was approximately 1,750 ±150 lx.

Zoospore production. Zoospores were produced axenically by a procedure modified from that of Byrt and Grant (1). The fungus initially was grown on V-8/oatmeal agar (75/25%, respectively) for 30 days (20). Mycelial mats were then stripped from the agar medium and placed in 250-ml flasks containing 100 ml of V-8 broth. Flasks were shaken on an orbital shaker at 160 rpm for 19 hr at 23 C. Mycelial mats then were washed five times with a mineral salts solution (3), placed in 100 ml of this salt solution, and returned to the orbital shaker for 24 hr. Zoospore liberation was induced by placing mycelial mats in 100 ml of sterile distilled water chilled to 18 C and incubating at 18 C for 2–3 hr. Zoospores were separated from mycelial fragments by filtering the zoospore suspension through four-ply sterile gauze. Zoospore concentrations were determined from three 10-ml samples of the stock solutions. Ten counts were made on each sample with the aid of a hemacytometer. Mean concentrations of zoospores in stock solutions among replicates of both experiments were 44,481 ± 5,041 spores/ml.

Inoculation, incubation, and infection assessment. Plants to be inoculated were removed from sand culture by immersing pots in water and permitting the sand to wash out of pots and away from the root systems. Intact plants were removed from the pots without exerting force, and seedlings were placed in distilled water at room temperature until they were inoculated (<30 min). Inoculations were conducted by root-dip into a series of spore concentrations of *P. cinnamomi*. Four shortleaf and four loblolly pine seedlings were inoculated together at each inoculum concentration.

A preliminary inoculum density/root infection experiment was conducted to determine a suitable range of spore concentrations for further experiments. Six zoospore concentrations were used, ranging from 2.4×10^{-1} to 2.4×10^{4} spores/ml by factors of 10. Results of this experiment indicated that spore concentrations in the range of 2.4×10^{1} to 2.4×10^{3} spores/ml were best to characterize the relationship between inoculum density and root infection for the two species. At concentrations less than 2.4×10^{1} spores/ml, no roots of either species were infected, whereas at concentrations of 2.4×10^{3} spores/ml and greater, all roots of both species were infected.

Two subsequent experiments were conducted, each with three replicates. In each replicate, inoculum concentrations were approximately 14, 80, 160, 280, 570, and 2,000 spores/ml. Roots of seedlings were maintained in their respective spore suspensions for 3-hr in the dark at 24 C. Spore suspensions were not mixed during the inoculation period.

The method of handling zoospores differed slightly between the first and second experiments. In the first experiment, attempts were made to maintain zoospore motility when spores were diluted to specific concentrations; however, these attempts were unsuccessful. Upon separation of zoospores and mycelial mats, zoospores were stirred gently and three 10-ml samples were removed. After spore counts were made, stock solutions were mixed gently by slowly stirring and pouring the spore solution from one flask to another, and dilutions of spores were made from the stock solutions. Immediately after placing seedlings in the inoculum, microscopic observations of spores were made from the concentrations of 570 and 2,000 spores/ml. Most zoospores had encysted at this time, although an occasional motile zoospore was observed. Observations of spores at 1 hr after inoculation of seedlings indicated that only a very low percentage (~15%) of spores had germinated. In the second experiment, zoospores in stock solutions were encysted by shaking flasks for approximately 1 min. Microscopic examination of spores from stock solutions indicated that this procedure induced encystment of all zoospores. Concentrations of spores were estimated in three 10-ml samples withdrawn from the stock solution, and dilutions were made to specific concentrations for inoculation. Germ tubes were initiated with this procedure in approximately 75% of cysts within 40 min after shaking.

After inoculation, plants were incubated under hydroponic conditions in holding chambers for 48 hr. Holding chambers were Plexiglas cylinders, with walls 7 mm thick, a height of 5 cm, and a diameter of 16 cm. Tops of chambers had 12 22-mm-diameter holes through which seedlings were placed. Styrofoam plugs, 3 cm in diameter and slit lengthwise to the middle, served as supports for seedlings. Chambers were wrapped with aluminum foil to maintain roots in constant darkness. Chambers had a 1-cm-diameter hole in the side wall into which a 1-cc hypodermic syringe was inserted. Ambient air was pumped through the syringe into the hydroponic solution in a stream of fine bubbles to maintain oxygen at a high concentration. Each chamber contained a stir bar and was placed on top of a stirrer set at slow speed to maintain a constant circulation of nutrients and oxygen across root surfaces. Nutrient solution within the holding chambers, and light and temperature conditions for incubations, were as described previously for seedling production. Shortleaf and loblolly pine seedlings, which had been inoculated with the same spore concentrations, were incubated together in the same chambers.

At the end of the incubation period, root tips, 1–1.5 cm long, were severed, surface-disinfested in 0.25% sodium hypochlorite for 30 sec (8), and plated on PCH agar, a medium selective for isolation of *Phytophthora* spp. (24). Root tips were evaluated 48 hr after plating and scored for the presence or absence of *P. cinnamomi*. Root tips that were evaluated for infection were

from first-order roots according to the classification scheme established in the morphometric root analysis system developed by Fitter (7). These roots also would be classified as lateral roots (17), and only root tips of lateral roots longer than 3 cm were assessed for infection. Roots of the four seedlings for each species and inoculum concentration were pooled within each replicate. There was an average of 9.5 root tips for each shortleaf pine seedling and 11.3 for each loblolly pine seedling.

Data analysis. Weighted least squares, as described by Cox (4) for a linear logistic model, was used to characterize the relationship between spore concentration (concomitant variable x) and frequency of root infection (response variable y). Linear functions were of the form $y = \alpha + \beta x$, where y is the logit of the proportion of infected roots, α is the intercept, β is the slope, and x is the natural logarithm ($\log_e$) of the spore concentration. Calculated spore concentrations varied slightly among replicates, and each replicate within an experiment was treated as an approximate repeat (5). Mean spore concentrations with standard errors were 13.7 ± 0.3, 77.8 ± 3.7, 160.2 ± 1.25, 282.0 ± 1.9, and 568.5 ± 2.6 spores/ml. Values at a spore concentration of 2,000 spores/ml were dropped from the analysis, because most roots of both species were infected at this concentration. Tests for lack of fit were conducted as described by Neter et al (23).

Regression functions were compared for loblolly and shortleaf pine within each experiment. Linear, straight-line functions were reparameterized to the form $y = \Gamma + \beta(x - \bar{x})$, where Γ is the logit of the proportion of infected roots at the mean of the $\log_e$ spore concentration, and $\bar{x}$ is the mean of the log spore concentration. Fitted regression functions for shortleaf and loblolly pine were compared by evaluating for coincidence of lines and commonality of each parameter estimate (Γ,β) as described by Neter et al (23).

RESULTS

Regression functions and coefficients of determination are presented for shortleaf and loblolly pine in Figure 1A for experiment one and in Figure 2A for experiment two. Tests for lack of fit of the linear model were nonsignificant for both species in each experiment. Statistical comparisons of regression functions indicated that regression lines for shortleaf and loblolly pine were not coincident ($P < 0.01$). Parameter estimates of the slopes of the regression functions for the two species were not different ($P > 0.05$). However, estimates of the parameter Γ differed between species ($P < 0.01$), indicating that infection levels of shortleaf and loblolly pine roots were different at the mean of the $\log_e$ spore concentration. Back-transformed parameter estimates of Γ in experiment two indicated that 58% of shortleaf and 35% of loblolly pine roots would be expected to be infected at the mean $\log_e$ spore concentration. Similar estimates for Γ were obtained in experiment one. The ID_{50} values for loblolly and shortleaf pine in experiment one were 221 and 114 spores/ml, respectively. In experiment two, ID_{50} values were lower for both species (i.e, 100 spores/ml for shortleaf pine and 178 spores/ml for loblolly pine).

The mean percentage of infected roots with standard errors for each spore concentration are presented in Figures 1B and 2B for experiments one and two, respectively. Discernible differences in relative susceptibility between shortleaf and loblolly pine were exhibited best at spore concentrations in the range of 80–280 spores/ml. At lower or higher spore concentrations, ability to detect differences in relative susceptibility between the two species decreased.

The most obvious visual symptom of infected roots was a grayish or grayish-brown discoloration. The severity and rate of development of discoloration usually depended on spore concentration, with symptom development progressing rapidly in lateral roots at high inoculum concentrations. In the preliminary experiment, distinct symptoms of infection were noted within 24 hr after inoculation in both species exposed to 2.4×10^4 spores/ml. At 36 hr, most lateral roots of both species inoculated with 2.4×10^3 spores/ml showed symptoms of disease. At lower concentrations, few or no symptoms of disease were observed at the end of 48 hr, even though the fungus was recovered from the roots at 24 and 240 spores/ml. Similar observations were noted among replicates of the two subsequent experiments. At a concentration of approximately 2×10^3 spores/ml, and occasionally at 570 spores/ml, symptom development proceeded rapidly and symptoms were observed within 36–48 hr. At lower inoculum densities, discernible infections usually were not noticeable at 48 hr, even though relatively high percentages of roots may have been infected.

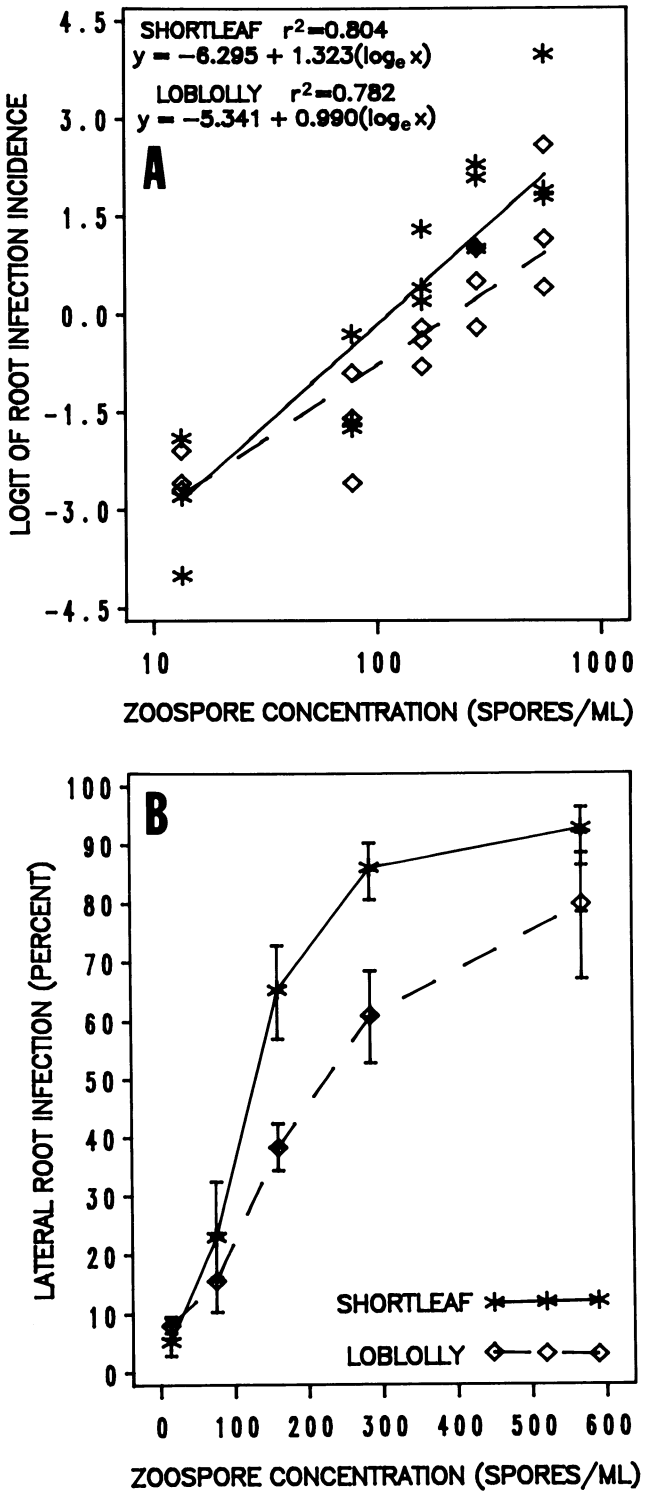

Fig. 1. The relationship between infection of lateral roots of shortleaf and loblolly pine and inoculum density of nonmotile zoospores of *Phytophthora cinnamomi* in experiment one. **A,** Logit of the proportion of infected roots, and **B,** percentage of infected roots and inoculum density.

DISCUSSION

Both loblolly and shortleaf pine are susceptible to infection by *P. cinnamomi* (20,28). However, results of our study, and that of Zak and Campbell (28), indicate that there are differences in relative susceptibility to infection. Our results indicate that spore inoculum density is critical for the determination of the difference in relative susceptibility for the two species. This difference was best exhibited at inoculum densities in the range of 80–280 spores/ml for the system employed in these experiments. The observations of Marx (20) concerning the lack of difference in susceptibility between loblolly and shortleaf pine can be related to inoculum concentration. Zoospore concentrations used in his studies ranged from 10,000–100,000 spores/ml, and 100% infection of roots of both species was obtained at those concentrations. Although the use of such high spore concentrations was appropriate for detecting differences in susceptibility of mycorrhizal versus nonmycorrhizal short roots, spore concentrations in this range apparently were inadequate for detecting differences in susceptibility of nonmycorrhizal roots of loblolly and shortleaf pine. In the study of Zak and Campbell (28), zoospore concentration was not determined; however, the quantity of inoculum introduced into their system was suitable to detect differences between the species.

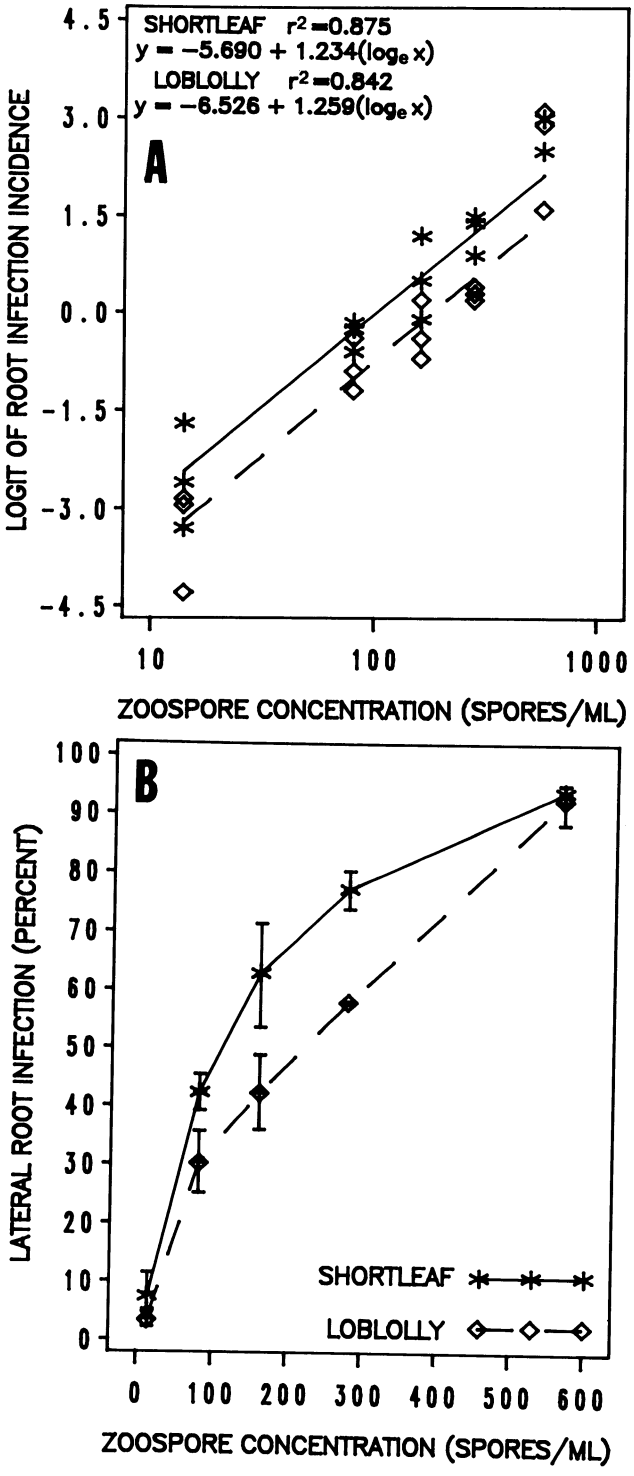

Fig. 2. The relationship between infection of lateral roots of shortleaf and loblolly pine and inoculum density of nonmotile zoospores of *Phytophthora cinnamomi* in experiment two. **A,** Logit of the proportion of infected roots, and **B,** percentage of infected roots and inoculum density.

Symptom development proceeded very rapidly at high inoculum concentrations in both species in these experiments. Rapid development of symptoms in succulent host tissues due to infection by *P. cinnamomi* is common for many diseases caused by pythiaceous fungi. Eye et al (6) reported that hypocotyls of a susceptible soybean cultivar were decayed severely within 3 days after exposure to spore concentrations of *P. m. sojae* in the range of 40–2,000 spores/ml. Ho and Zentmyer (12) reported the development of brown lesions on avocado roots within 24 hr after exposure to zoospore suspensions of *P. cinnamomi.*

The present study was conducted with nonmotile zoospore cysts; and, therefore, differences in susceptibility of the two species to infection were not due to attraction of zoospores to root surfaces. Positive attraction of cyst germ tubes toward roots has been observed (29), and exudates may have been responsible for stimulation and/or orientation of germ tubes toward roots. In theory, the types and quantities of exudates produced by roots partially may explain differences observed in susceptibility to infection for the two pine species.

Another possibility for the observed differences for shortleaf and loblolly pine may relate to the concept of susceptible sites as proposed by Vanderplank (26), who defined a susceptible site as a place on the plant where a pathogen can enter a host. The site may be a wound, natural opening, or an intact surface (26). Vanderplank further states that susceptible sites vary in both quantity and quality, and that these variations determine the shape of the disease/inoculum curves. Likewise, the relative number and quality of sites suitable for successful penetration and establishment of *P. cinnamomi* may have rendered roots of shortleaf pine more susceptible than those of loblolly pine. As inoculum concentration increased, the probability of a germinating cyst contacting a site suitable for infection also increased. If there were a greater number of conducive sites for shortleaf pine compared with loblolly pine, the probability of shortleaf pine root infection would remain consistently greater until inoculum concentrations increased to a point that ensured infection of all loblolly pine roots.

Many factors can induce zoospores to encyst, including variation in water currents, collisions with other zoospores and solid surfaces, changes in hydrogen ion concentration or temperature, and fluctuations in nutrients (22). Because spore liberation and dilutions were carried out in distilled water at 18 C, rheotaxis, associated with making dilutions, was probably of ultimate importance in zoospore encystment observed among replicates of the first experiments. Some researchers have provided estimates of the percentages of motile spores present in solutions at the time of inoculation when estimating inoculum concentrations (21,22). Encystment of all spores by agitation of the spore stock solution for 1 min provided an effective means of thoroughly mixing spores for subsequent dilutions as well as standardization of procedures among experiments. Percent germination of spores in this system was high and proceeded within 20–40 min after encystment (8). Other studies have indicated that greater numbers of zoospore cysts are required to achieve comparable levels of infection caused by motile zoospores (15,16). Lack of zoospore motility was of no consequence in obtaining root infection and in discerning differences in susceptibility to infection among hosts within the system presented here. Refinements in technique were the most

probable reason for the higher coefficients of determination for shortleaf and loblolly pine, and a lower mean square error in the second experiment as compared with the first. The use of encysted zoospores standardized conditions among experiments by providing a more homogeneous distribution of spores within stock solutions and within inoculation beakers.

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