Inoculum Source and Density Influence Assessment of Fusiform Rust Resistance in Slash Pine

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ABSTRACT

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Open-pollinated seedlings from resistant, moderately resistant, and susceptible slash pines (*Pinus elliottii* var. *elliottii* Engelm.) were inoculated with basidiospores of *Cronartium quercuum* f. sp. *fusiforme* from two geographic sources at three densities. Variation among pine families, inoculum densities, and sources was assessed in terms of presence or absence of initial symptoms (purple spots on stems and needles), seedlings galled 6 and 9 mo after inoculation, gall lengths, and seedling recovery. Recovery was indicated either by lack of gall formation or small galls that ceased to grow. More seedlings of the resistant and moderately resistant parents than of the susceptible parent developed initial symptoms without subsequently forming galls ("early recoveries"). Frequency of early recovery varied with inoculum density. Fewer seedlings from the resistant parent were galled 9 mo after inoculation, but the distinction between families diminished with increasing density. Most seedlings with small galls (<2.5 cm long) were free of external symptoms and active rust mycelium 33 mo after inoculation ("late recoveries"). Family effects on seedlings with small galls were significant. Family differences were increased when late recoveries were deducted from those galled at 9 mo. Results confirm that uniform, appropriate inoculum densities are prerequisite to accurate resistance tests and indicate that tests with varied sources and densities will help discern resistance types, degrees, and stabilities.

Resistance to fusiform rust (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*) is an important trait in selection and breeding of slash (*Pinus elliottii* var. *elliottii* Engelm.) and loblolly (*P. taeda* L.) pines. Development of artificial inoculation techniques facilitated large-scale resistance screening of progenies from selected trees (21). The early tests permitted rapid identification of resistant and susceptible materials (2,6), but their limited flexibility with regard to inoculum density and source generally has restricted their sensitivity and prevented detection of different resistance reactions.

Several methods for providing control over inoculum source and density have been developed (5,14,17,24). Laird and Phelps (15), using the concentrated basidiospore spray (CBS) method, varied inoculum density and found that lower densities enhanced sensitivity of screening slash pine seedlings for resistance. The families segregated into three distinct categories: resistant, intermediate, and susceptible. As a result, inoculum densities were lowered in tests using the CBS system. Matthews et al (16), also using the CBS system and one inoculum source, found an additive relationship between percentage of seedlings galled and inoculum density (probit-disease vs. $\log_{10}$ dose) for each of four open-pollinated slash pine families. Several investigators (8,22,23,26) have shown that resistance ratings for families vary when slash pine seedlings are artificially inoculated with *C. quercuum* f. sp. *fusiforme* from several sources. Few attempts have been made, however, to evaluate the impact of varying *both* inoculum density and source on the accuracy of resistance tests or their utility as aids in discerning differences in resistance reactions, degrees, or stabilities.

This study evaluates the utility of varying inoculum source and density for detection of family differences in fusiform rust reactions in slash pine.

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MATERIALS AND METHODS

Open-pollinated progeny of three slash pines from southern Mississippi were inoculated (using the forced-air techniques of Snow and Kais [24]) with two sources of basidiospores at three densities. One slash pine parent (8-7) was considered highly resistant (1), but its offspring varied greatly in their response to diverse inocula (4,8,22). A second parent (9-2) was considered moderately resistant (12,22,24). Varied responses of its offspring to varying inoculum densities and sources have been noted (3,22,23) but to a much lesser extent than for 8-7. The third parent (18-62) was highly and uniformly susceptible throughout the research cited above.

One inoculum source (LM) was derived from five single-gall aeciospore collections made near Laurel, MS. The second (MG) was produced from five single-gall collections made near Macon, GA. Aeciospores from individual galls were processed and stored separately (20). In an effort to apply more uniform numbers of basidiospores per gall than would have resulted from simply mixing aeciospores, two water oak (*Quercus nigra* L.) seedlings were inoculated with each aeciospore collection. Three leaves bearing uniform numbers of telia were removed from the two oak seedlings representing each LM gall and distributed at random in the leaf chamber of the forced-air inoculation apparatus (24). Basidiospores shed from these leaves thus constituted the LM inoculum source. The same procedure was employed to obtain and apply mixtures of MG basidiospores.

Individual pine seedlings were grown in peat pots of 5 cm diameter for inoculation at 5–6 wk of age. On each of 3 days (replicates), a set of 24 seedlings from each family was inoculated with basidiospores at each of three concentrations from each of the two sources. Thus 72 seedlings from each family received each of the six possible combinations of density and source. Inoculation sequences were determined randomly each day, with source chosen first, density second, and family last. Basidiospore densities averaged 5, 15, and 25 spores (ranging, respectively, from 3 to 7, 12 to 18, and 22 to 28) per square millimeter of inoculated stem surface.

Spores were deposited by the forced-air apparatus at the juncture of the stem and base of the terminal tuft of juvenile needles on each seedling. Spore numbers were monitored after every eighth seedling and adjusted when necessary to ensure close adherence to prescribed densities (24). After inoculation, seedlings were transplanted to plastic pots of 10 cm diameter containing a 1:3 mixture of vermiculite and sandy loam. Potted seedlings were grown in a greenhouse for observation and eventual transplanting to the field. Individual seedlings were scored for the presence or absence of

initial symptoms (purple spots) on stems, needles, or both, 1 and 2 mo after inoculation, and galls at 2, 3, 6, and 9 mo. Gall length was measured to the nearest millimeter at 9 mo.

Previous work showed that resistance is expressed not only by the absence of galls but also by small galls that eventually cease to grow. In a sense, the seedlings "recover" (3,13,22). To evaluate this "late-recovery" phenomenon, all seedlings without galls and all seedlings with galls 2.5 cm or less in length at the end of the 9-mo observation period were transplanted to the field on the Harrison Experimental Forest in south Mississippi. Several seedlings with vigorous galls from each treatment combination using LM inoculum were included as checks. All transplanted seedlings were scored for the presence of growing galls after 2 yr in the field, and seedlings that had recovered or remained free of infection were noted as well. In addition, at least 10% of the seedlings given each combination of density and source were dissected and examined histologically (13) to confirm field scores. Each seedling was then assigned to one of four classes.

Class 1. Seedlings that remained free of initial symptoms (purple spots) or galls throughout the experiment.

Class 2. Early recoveries—seedlings that had initial symptoms (purple spots) on stems and/or needles but never developed galls. Such seedlings were considered susceptible to penetration of the fungus but resistant to its further development.

Class 3. Late recoveries—seedlings that had galls less than 2.5 cm long at 9 mo, and the galls ceased to grow thereafter. The subsamples examined histologically contained only dead hyphae, and affected tissues were overgrown by normal tissues free of the fungus.

Class 4. Seedlings with galls that were longer than 2.5 cm by 9 mo and continued to enlarge throughout the experiment.

For each set of 24 seedlings, the number falling into each of the four classes was recorded. Seedlings that died during the experiment were not considered in any calculations. To test for differences among inoculum sources, densities, and families, proportional data for each class were subjected to analysis of variance for a split-split-plot design with three replicates. Inoculum sources were the main plots, densities the subplots, and families the subsubplots. Differences among average responses to individual factors were checked for significance using Fisher's least significant difference (LSD). Relationships of interest among several variables were explored through correlation and regression analyses. All tests were made at the 0.05 level of probability.

RESULTS

Over all families, 2.5% of all seedlings were assigned to class 1, 14.7% to class 2, 19.4% to class 3, and 63.4% to class 4.

Class 1. Only 32 of 1,296 seedlings remained free of rust symptoms. Variation among families and sources was not significant, but significant effects of density were observed. Frequency varied inversely with density and 26 of the 32 seedlings were observed at the lowest density. This dependence on density and absence of family or source effects indicated that such seedlings were escapes and not resistant. Consequently, for further analyses, the 32 seedlings were deducted from the denominator before the percentages in classes 2–4 were computed.

Class 2. Numbers of seedlings with initial symptoms but no galls (early recoveries) varied significantly among densities and families. Source effects were not significant even though more than twice as many seedlings were recovered early for the LM source as for MG—probably an outcome of the experimental design providing too few degrees of freedom for the contrast. Frequencies of class 2 seedlings varied inversely with density, regardless of source or family (Fig. 1, top). Most of the variation resulted from the occurrence of more early recoveries at 5 rather than at 15 or 25 spores per square millimeter. Family differences were dramatic, with 8-7 and 9-2 always having a higher proportion of early recoveries than 18-62, the susceptible parent (Fig. 1, top). The source × family interaction was also significant—apparently a result of 1) early recovery occurring much more frequently after exposure to LM inoculum, 2) 8-7 having more early recovery than 9-2 for LM inoculum, and 3) 9-2 having more early recovery than 8-7 for MG inoculum.

Class 3. For seedlings that developed galls but showed arrested gall growth (length <2.5 cm, late recovery), there were significant differences among families but not among densities or inoculum sources (Fig. 1, bottom). The significant family × inoculum interaction apparently was caused by a scale effect rather than large family rank changes, because more late recoveries occurred with the MG inoculum group than with the LM inoculum group for all three families. Although families 9-2 and 8-7 had roughly similar proportions of early recoveries (class 2), family 8-7 had more than twice as many late recoveries as family 9-2.

Class 4. Proportions of seedlings that never recovered varied significantly only among families and densities (Fig. 2, top). Family differences were large regardless

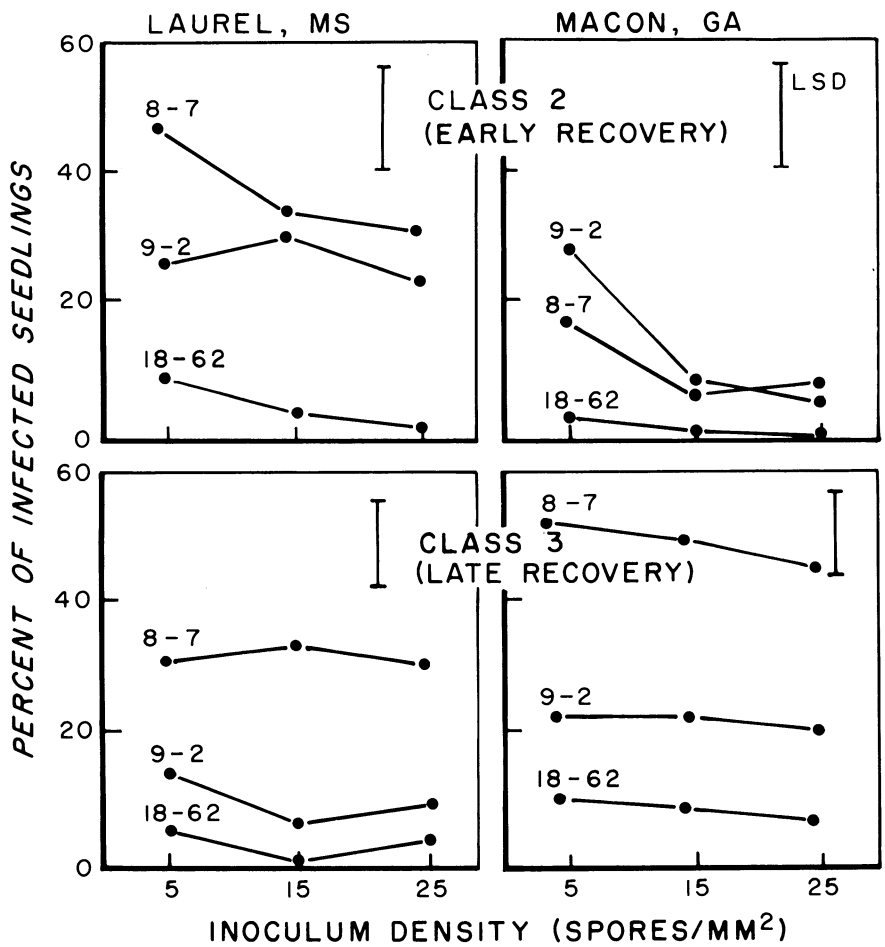

Fig. 1. Differential response of seedlings from three open-pollinated slash pine families to inoculation with two sources of *Cronartium quercuum* f. sp. *fusiforme* at three densities: class 2 (initial symptoms [purple spots] only); class 3 (galls <2.5 cm long). For least significant difference (LSD), $P = 0.05$.

of density or source, and the families were differentiated clearly from one another: 8-7 = resistant, 9-2 = moderately resistant, and 18-62 = susceptible. Fewer seedlings were galled at the lowest density than at the others, and essentially no difference occurred between the two higher densities.

Several variations were noted in the generalized pattern of frequencies in class 4. For example, density effects were nonexistent for the susceptible family—90% or more infection occurred regardless of density. Thus, very few spores of either source were required for successful infection of offspring from 18-62. The pattern for the moderately resistant parent, on the other hand, tended to differ with source. The curve was distinctly flat for LM inoculum but followed the general pattern for MG inoculum. Thus, relatively few LM spores were required to cause the highest level of infection on 9-2 offspring, whereas several times more MG spores were required to produce the same level. A yet different pattern occurred for 8-7 offspring; infection tended to increase across all LM densities, whereas it plateaued at the intermediate density of MG inoculum.

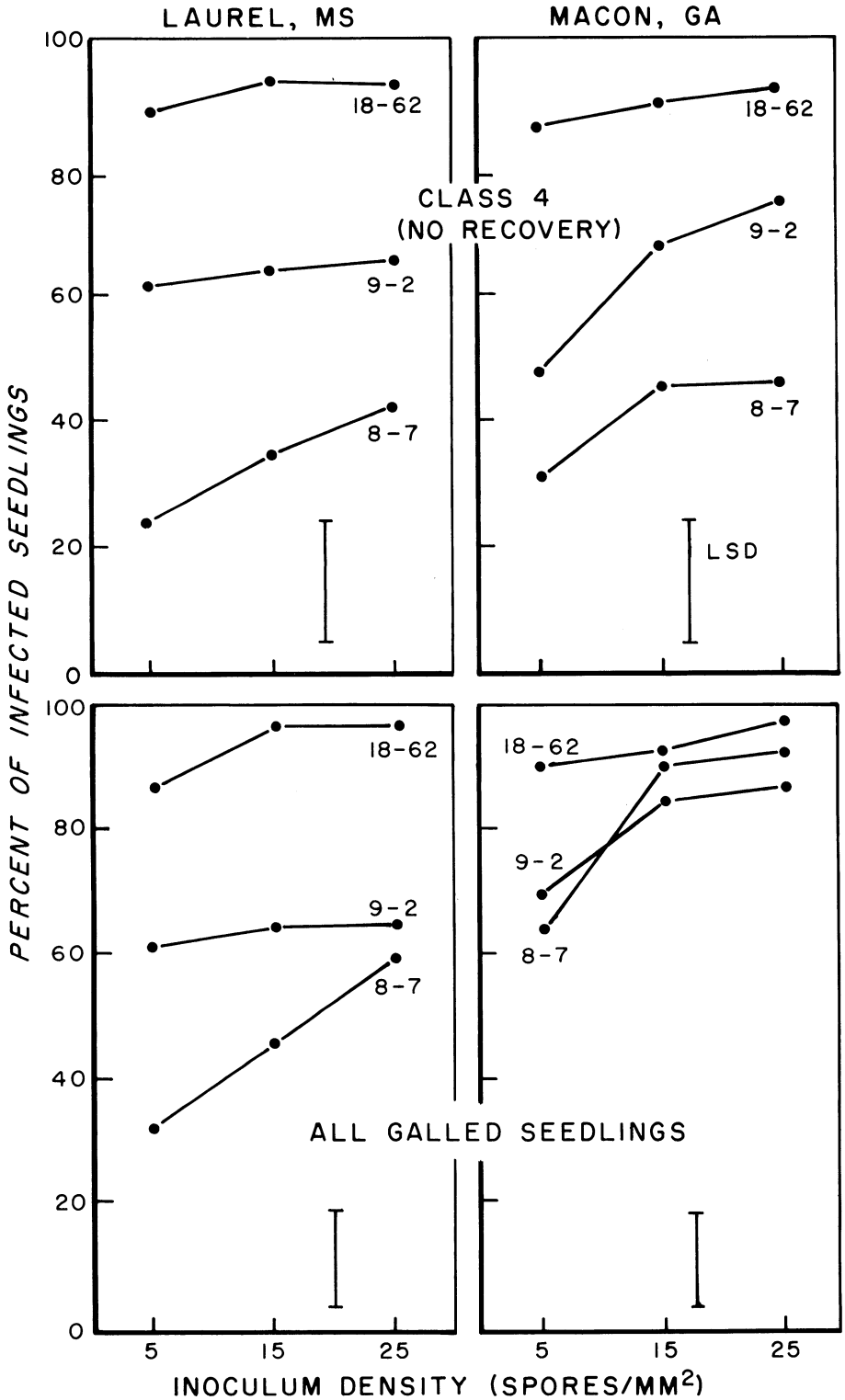

Fig. 2. Differential response of seedlings from three open-pollinated slash pine families to inoculation with two sources of *Cronartium quercuum* f. sp. *fusiforme* at three densities. **(Top)** Class 4 (galls >2.5 cm long). **(Bottom)** All seedlings galled at 9 mo, regardless of size of galls. For least significant difference (LSD), $P = 0.05$.

DISCUSSION

The fact that 2.5% of the seedlings in this experiment remained free of rust symptoms and most likely were escapes has several implications for resistance screening. The inoculation apparatus used here delivers precisely controlled numbers of spores to individual seedlings in a uniform environment. Where less precision and uniformity are possible, densities may fluctuate and escapes could be greater in number and more variable among families or inocula than reported here. Not accounting for escapes would be misleading in tests involving low densities; 81% of our escapes occurred at the lowest density. Such seedlings, however, could be ignored at higher densities without sacrificing accuracy in terms of ranking families or isolating rustfree seedlings for breeding purposes.

Class 2 seedlings had initial symptoms but did not develop galls. Initial symptoms were evidence that the pathogen invaded host tissues, at least temporarily (11). Penetration and initial colonization were successful, but further development did not occur. Family variation in early-recovery frequencies provides further evidence that such seedlings are resistant and not merely escapes. The greater early recovery occurring at the lowest inoculum density seems more than a mere density function because the probability of obtaining spore genotypes capable of overcoming resistance can be expected to rise with inoculum density in this host/pathogen system. Substantial numbers of seedlings from all three families had resistance that arrested the disease cycle in its early phases. Such resistance may be different from and stronger than that in class 3 seedlings where disease development proceeded further. In early screening trials, seedlings with early recovery were assumed to be the same as other rustfree material and are lumped with it (22,23).

The late-recovery seedlings of class 3 are of particular interest because they could be considered susceptible in routine screening trials. Scoring too early or otherwise ignoring their numbers could inflate conventional measures of family susceptibility and thereby lessen predictability of field performance. Not accounting for late recovery in seedlings may be one reason many families deemed acceptable on the basis of field performance often show unexpectedly high infection after artificial inoculation. Late-recovery seedlings were far more

numerous with MG than with LM inoculum—a result opposite to that for early-recovery seedlings (class 2). When considered within families, the total number of resistant seedlings (class 2 + 3) was about equal for each inoculum source (Table 1). Numbers per class, however, varied dramatically within each source. Thus, the observed differences resulted only from shifts of seedlings between classes, depending on inoculum source. Such differences seem to be related to the virulence of the fungus. The MG inoculum may be more virulent in that its infections proceeded further. A larger number of LM infections were stopped before galls formed, regardless of family. One would expect the pattern, not just the numbers, to differ if the inocula had different types of virulence. That 8-7 had more resistant seedlings (class 2 + 3) as well as more class 3 seedlings than 9-2 also has some interesting implications. The former parent clearly yields more resistant offspring in total, but a higher proportion of them allowed disease to proceed further—an indication that such seedlings are less resistant.

The disease process was arrested earliest in class 2 seedlings, regardless of family or source. Such seedlings may have more or stronger genes for resistance. Class 3 seedlings, on the other hand, seem to have fewer genes or perhaps genes with lesser effects. Seedlings in the two classes could have differing potentials and should be counted by class and kept separate for breeding purposes. The technique of varying inoculum source and density in resistance trials seems advisable because tree breeders would gain a better understanding of both the strength and nature of resistance as well as of the reliability and meaning of their trials.

Class 4 results (Fig. 2, top) differ from those that would be obtained by including all trees galled at 9 mo, as is often done in screening trials, without adjustment for late recovery, lack of initial symptoms, or death (Fig. 2, bottom). Class 4 figures show family differences that are distinct for all inocula and densities. Using only percentage of galled seedlings at 9 mo without adjustment would have inflated estimates of infection and misrepresented a family's performance. In addition, the adjusted results resemble performance of those families in field trials (7). We interpret the class 4 curves for family 18-62 to mean that 18-62 has very few resistance genes; a small number of spores was sufficient to cause numerous infections. In contrast, the curves for family 8-7 increased in slope as density increased. As density increases, so does the probability of finding a spore genotype capable of infecting 8-7 seedlings. Curves for family 9-2 were influenced by inoculum source. These findings indicate that susceptible sites on a pine seedling vary in both quantity and quality and that these variations determine the shape of the disease/inoculum density curve (25). It appears that 18-62 has many susceptible sites and that they are easily infected. Seedlings of 9-2 and 8-7 may not only have fewer susceptible sites but also have sites that are not easily infected and vary more in susceptibility (both between and within families).

Table 1. Resistance response of seedlings from three open-pollinated slash pine families to inoculation with two sources of *Cronartium quercuum* f. sp. *fusiforme*

	Number of seedlings by family and inoculum source[a]									Total seedlings		
	18-62			9-2			8-7					
Seedling class	LM	MG	Both	LM	MG	Both	LM	MG	Both	LM	MG	Both
Class 2 (early recovery)	4	1	5	30	12	42	42	13	55	76	26	102
Class 3 (late recovery)	7	15	22	22	45	67	63	99	162	92	159	251
Class 2 + class 3	11	16	27	52	57	109	105	112	217	168	185	353

[a] LM = Laurel, MS, and MG = Macon, GA.

Such findings emphasize that greater attention should be given to scoring procedures in screening tests. Making periodic observations and adjustments for small galls seems to be a simple and quick way to improve accuracy and understanding. Knowledge of the proportion of seedlings with galls longer than 2 cm at 6 mo appears useful in that results expressed in these terms bear a strong correlation ($r = 0.98$) to those provided by class 4 calculations. Similar findings were obtained in a series of experiments involving representative slash pine materials, diverse inocula, and a range of inoculum densities (3,22). At the U.S. Forest Service Resistance Screening Center, the recording of small galls (<2.5 cm) and seedlings with initial symptoms but without galls are currently incorporated in scoring procedures (26).

The approach described in this study is not new. Hodgson (9) used a similar approach to delineate different forms of resistance to *Phytophthora infestans* (Mont.) de Bary in commercial potato cultivars. Also, sequential observations like those reported here were used to classify resistance reactions in *Pinus monticola* Doug. and *P. armandii* Franch. to *C. ribicola* J. C. Fisch. ex Rabenh. (10,18,19). The use of various inoculum sources and densities can provide valuable insight into resistance and lead to refinements in family classification with regard to estimates of field performance, stability of resistance, and isolation of resistant breeding material.

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