Environmental Factors Affecting Brown-Spot Infection on Longleaf Pine

A. G. Kais

Plant Pathologist, Southern Forest Experiment Station, Forest Service, U.S. Department of Agriculture, Gulfport, Mississippi 39501.

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ABSTRACT

Indirect evidence suggested that light enhances infection of *Pinus palustris* by stimulating the opening of stomata through which the brown-spot fungus *Scirrhia acicola* penetrates the needles. In the absence of light, the fungus functions primarily as a wound parasite. Exposure of seedlings to high humidity both before and after inoculation shortened the incubation period and greatly increased the degree of infection. Delaying the high-humidity treatment of inoculated seedlings did not decrease infection. Although infection occurred over a wide range of temperature, a regimen of 35 C day and 27 C night was inhibitory. Maximum infection occurred under a 30 C day and a 21 C night. An efficient inoculation procedure is proposed for brown-spot studies.

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Additional key words: Lecanosticta acicola, environmental effects, artificial inoculation.

Brown-spot needle blight, caused by *Scirrhia acicola* (Dearn.) Siggers, is a major obstacle in the regeneration of longleaf pine (*Pinus palustris* Mill.) in the South. Partly as a consequence, longleaf has been supplanted by other species and now occupies about 25% of the acreage it once dominated (8). The disease has also become serious in Christmas tree plantations of Scotch pine (*P. sylvestris* L.) in the north and middle central States (9, 10).

The discovery of heritable resistance in longleaf pine (3) suggests the use of resistant trees to control the disease. Field testing, which heretofore has been relied upon exclusively, is expensive and time-consuming, requiring about 4 years per test. Identification and release of resistant progeny would be greatly facilitated by artificial inoculation techniques that would allow rapid screening tests.

Although several researchers have reported successful

inoculations (1, 5, 14, 15, 16), attempts to adapt or utilize their techniques have yielded inconsistent results (2, 4, 7, 12). The work described here had a two-fold purpose: (i) to determine the optimal conditions of light, moisture, and temperature required for significant infection in the greenhouse, and (ii) to define a simple but efficient inoculation technique for use in research.

Effects of light have not been evaluated critically, even though the pathogen apparently enters through stomata (7, 11, 13, 16, 17). The literature does not indicate the optimal humidity conditions for infection, and there are no reports on the role of temperature.

MATERIALS AND METHODS.—*Preparation of inocula and seedlings.*—*Scirrhia acicola* isolates were obtained from longleaf pine seedlings growing on the Harrison Experimental Forest, Saucier, Mississippi. These isolates, all from single spores, were maintained on malt agar slants at 25 C. The inoculum for each test was a mixture of three or more isolates. Spore suspensions were obtained by rinsing 3- to 6-week-old cultures with sterile distilled water and then screening through four thicknesses of cheesecloth. The individual suspensions from each isolate were adjusted to 10^5 to 2×10^5 spores/ml and then equal volumes were combined. About 4 ml of the spore suspension were atomized on each seedling.

Longleaf pine seeds randomly selected from a bulk collection were germinated in 5-cm diameter peat pots and then transplanted into 10-cm diameter plastic pots. Potting medium was a 4:1 (v/v) ratio of sandy loam to vermiculite. Plants were grown in the greenhouse under a 14-hour day and inoculated after the formation and maturation of fascicled needles (4-6 months). In all tests, disease was evaluated 4 months after inoculation.

Light.—Seedlings were kept in light or darkness for 7 or more days immediately following inoculation in a series of five tests; they were covered with polyethylene bags to maintain high humidity. In Tests 1 and 2, plants were wounded prior to inoculation by clipping some needles to lengths of 10 cm. In Tests 3 and 4, only plants maintained in darkness had some needles clipped. In all four tests, seedlings were kept at 25 C. Bags were removed and plants were placed in the greenhouse for observation after the 7-day humidity treatment. Final evaluations were based on the occurrence and number of lesions on inoculated plants. Infection was confirmed when acervuli formed on the lesions.

In Test 5, seedlings growing in 5-cm diameter peat pots were placed in plastic canisters, inoculated, covered with Saran wrap or black polyethylene, and held under one of four treatments: (i) 5 days of darkness, (ii) 5 days of normal daylength, (iii) 10 days of darkness, and (iv) 10 days of normal daylength. Seedlings were then transplanted to 10-cm plastic pots and maintained in the greenhouse. Each treatment was applied to four replicates of five seedlings each.

Humidity.—The effect of high humidity was evaluated in greenhouse experiments in which polyethylene bags provided high humidity. Inoculated seedlings were exposed to six different daily periods of high humidity (4, 8, 12, 16, 20, and 24 hours) for 3, 6, or 10 consecutive days in Test 6 and to various combinations of both pre- and postinoculation high-humidity periods in Tests 7 and 8. Test 9 evaluated spore viability and subsequent disease development after exposure to high humidity had been postponed for various periods up to 10 days after inoculation.

In both the humidity and the light experiments, bagged seedlings were kept in a greenhouse. Air temperatures within bags ranged from 30 to 38 C between 1000 hours and 1600 hours during July. These temperatures were approximately 6 to 10 C above ambient air. Bagging for long periods (up to 17 days) apparently caused no adverse effects on the seedlings.

Temperature.—Effects of temperature were determined in growth chambers maintained at five regimes: (i) day 24 C, night 16 C; (ii) day 30 + 2 hours 38 C, night 21 C; (iii) day 30 C, night 21 C; (iv) day 35 C, night 27 C; and (v) day 30 C, night 21 C + 2 hours 10 C. Daylengths were 16 hours. Seedlings assigned to each

TABLE 1. Occurrence of lesions on longleaf pine seedlings held under moist conditions in light[a] or darkness for 7 days after inoculation with *Scirrhia acicola*

Test and treatment[b]	Infected plants/ total tested	Total number of infected needles	Total number of lesions on—	
			Clipped ends of needles	Unwounded portions of needles
Test 1				
Light, clipped	5/5	22	3	58
Dark, clipped	8/8	59	58	1
Test 2				
Light, clipped	2/4	2	0	2
Dark, clipped	2/2	4	4	0
Test 3				
Light	4/4	12	...	20
Dark, clipped	1/3	1	1	0
Test 4				
Light	28/37	94	...	187
Dark, clipped	21/30	60	46	22

[a]2,152 lx (200 foot-candles).
[b]Clipped plants had apical 5-cm portions of needles cut off.

treatment were incubated, either bagged or unbagged, for 7 days prior to inoculation and were bagged and returned to the chambers for 10 days after inoculation.

Analysis.—Data were subjected to analysis of variance, and means were compared by Duncan's multiple range test. All differences discussed in the text were significant, $P = 0.05$.

RESULTS.—*Effect of light.*—In Tests 1 to 4, brown spot lesions developed on plants kept in light or darkness (Table 1). On plants held in darkness, lesions generally occurred near the cut ends of fascicled needles, with only a few appearing on unwounded needles. In contrast, lesions occurred anywhere on unwounded needles on plants kept in light, with only a few near the cut ends. Levels of infection were low in Test 2, but the pattern was similar to that in the other tests. In Test 5, plants were heavily infected when held under a normal daylength for 10 days (3.19 lesions/needle), moderately infected under normal daylength for 5 days (0.41 lesion/needle), and almost clean under 5 days of darkness (0.01 lesion/needle). Plants kept in the dark for 10 days were killed by root rot.

These results indicate that infection of unwounded needles is significantly increased by light. The occurrence of typical lesions near the cut ends of needles on seedlings incubated in the dark indicates that *S. acicola* functions as a wound parasite under certain conditions.

Effect of humidity.—Infection increased with postinoculation exposure to high humidity. Thus, in the 3-day exposures, seedlings became severely diseased only when the saturated atmosphere was maintained for 24 hours per day (Table 2). In the 10-day treatments, 8-hour periods of humidity sufficed.

High humidity before inoculation also increased infection (Table 3). In Test 7, plants with no preinoculation exposure had much lighter infection than the others. When plants were given 3 days of exposure before inoculation and 1 to 10 days afterwards, infection generally increased with total time exposed (Test 8). Thus, infection was increased with combined pre- and postinoculation exposure.

Postponing exposure to high humidity for periods up to 10 days after inoculation appeared to have no effect. With no delay, the mean infection was 35.0%, with a 5-day delay it was 27.4%, and with the maximum delay of 10 days it was 31.0%. These differences were not significant. Spores remained viable for at least 10 days and could germinate and cause severe infection when humidity became high.

Effect of temperature.—Maximum infection occurred on plants held in growth chambers with temperature regimes similar to field conditions; i.e., a day temperature of 30 C and a night temperature of 21 C (Table 4). Addition of a 2-hour nighttime period at 10 C did not alter results, but infection appeared to decline when a 2-hour period of 38 C was interjected into the day sequence. Less infection occurred on plants held at 24 C in the day and 16 C at night. Lack of infection on plants held at 35 C during the day and 27 C at night indicated that long periods of high temperatures are lethal to germination or germ-tube growth. Finally, exposing seedlings to high humidity both before and after inoculation resulted in much greater infection than exposure only after inoculation.

DISCUSSION.—It is generally accepted that *Scirrhia*

TABLE 2. Mean number of lesions per needle on longleaf pine seedlings subjected to different daily periods of high humidity following inoculation with *Scirrhia acicola*[a]

Duration of treatments (days)	Hours per day of high humidity					
	4	8	12	16	20	24
3	0.12 de	0.11 de	0.20 de	0.39 de	0.48 de	1.03 cde
6	0.01 e	0.30 de	1.07 cde	0.47 de	1.46 cde	2.35 cde
10	0.34 de	2.40 cd	5.18 ab	3.13 bc	5.34 a	5.13 ab

[a]Mean of six plants per treatment. Values not followed by same letters are significantly different ($P = 0.05$).

TABLE 3. Infection of longleaf pine seedlings exposed to continuous high humidity before and after inoculation with *Scirrhia acicola*

Test 7[x]			Test 8[y]		
Days of exposure to high humidity		Infection[z] percentage	Days of exposure to high humidity		Infection[z] percentage
Before	After		Before	After	
7	10	18.9 a	3	10	38.6 a
5	10	13.2 b	3	9	37.6 ab
3	10	9.1 bcd	3	8	30.6 abc
1	10	9.1 bcd	3	7	32.2 abc
0	10	6.7 cde	3	6	31.0 abc
7	8	10.8 bc	3	5	27.0 bc
5	8	8.9 bcd	3	4	31.4 abc
3	8	10.7 bc	3	3	28.0 abc
1	8	6.8 cde	3	2	23.6 cd
0	8	3.1 ef	3	1	14.6 d
7	6	12.1 bc			
5	6	4.6 def			
3	6	6.8 cde			
1	6	3.0 ef			
0	6	0.8 f			

[x]Mean of nine plants per treatment.
[y]Mean of five plants per treatment.
[z]Mean visual estimate of infected tissue as a percent of needle tissue inoculated. Values not followed by same letters are significantly different ($P = 0.05$).

TABLE 4. Effect of temperature and high humidity on infection percentage[z] of longleaf pine seedlings by *Scirrhia acicola*

Day/night temperatures (C)	Exposed to high humidity 7 days before and 10 days after inoculation	Exposed to high humidity only for 10 days after inoculation
24/16	21.4 ab	8.3 bc
30 + 2 hr @ 38/21	21.5 ab	4.2 bc
30/21	34.5 a	5.2 bc
35/27	0.0 c	0.0 c
30/21 + 2 hr @ 10	34.4 a	4.8 bc

[z]Mean visual estimate of infected tissue as a percent of needle tissue inoculated. Values are means of two replications (five plants each replication). Those not followed by the same letter are significantly different ($P = 0.05$).

acicola penetrates needles through the stomata. Killebrew (7) found germ tubes with appressorium-like structures positioned above stomata. Wolf and Barbour (17) postulated stomatal penetration after observing that mycelium is initially localized in substomatal chambers, while Snow (16) and Setliff and Patton (13) showed hyphae entering stomatal cavities.

The present data suggest that the fungus normally enters needles through the stomata. In light, and with high humidity and favorable temperatures, infection occurred and characteristic symptoms developed. Periodic microscopic examination of inoculated needle tissue confirmed the presence and buildup of hyphae in substomatal chambers. Accordingly, it is thought that light promotes infection by stimulating the opening of stomata, which in turn favors penetration by the germ tube of the pathogen.

In darkness, needle penetration and infection via stomata significantly decreased, probably because stomata were closed. Infection was generally confined to the cut ends of needles, but microscopic examination did not indicate how penetration occurred.

Exposure of seedlings to high humidity both before and after inoculation was necessary for frequent penetration and subsequent lesion development. The occurrence of substantial infection only after inoculated plants were exposed to high humidity for at least 3 days (Table 2) is probably related to two characteristics of conidial behavior of *S. acicola*. Conidia require 14-52 hours for germ tubes to appear (7, 15, 17), and germ tubes may meander over the needle surface prior to stomatal entrance (7, 11).

The high rate of infection due to the exposure of seedlings to high humidity both prior to and after inoculation can probably be attributed to: (i) changes in host metabolism which would facilitate initial penetration by the fungus; and (ii) optimal environmental conditions favoring spore germination and development within the host.

Since field infection of longleaf pine is not precluded by lack of light or inoculum (6), moisture and temperature are probably the most important factors. Temperature and humidity conditions in the ranges explored in these studies frequently coincide at times when susceptible host tissue is present in the forest. That is probably why natural infection develops annually, and why the disease is found throughout the range of longleaf pine.

Inoculation technique.—As a result of these experiments, the following technique is proposed for inoculating pines with *Scirrhia acicola* under greenhouse conditions. Four-month-old pine seedlings are enclosed in moist plastic bags for 3 days, inoculated by atomizing spore suspensions from 3-week-old cultures to the point of run-off, and then bagged for 10 days. Plants are grown and maintained under 14-hour daylength until final disease readings are taken 4 months after inoculation.

With this technique, high levels of infection are obtained at any time of year. Longleaf, shortleaf (*Pinus echinata* Mill.), slash (*P. elliottii* var. *elliottii* Engelm.), loblolly (*P. taeda* L.), sand [*P. clausa* (Chapm.) Vasey], jack (*P. banksiana* Lamb.), red (*P. resinosa* Ait), and Scotch pines have been infected. Total time required for an experiment is approximately 8-10 months. This procedure is currently being used to study pathogenic variability and host-pathogen relationships.

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