Wilt of Loblolly Pine Inoculated with Blue-Stain Fungi of the Genus Ceratocystis

H. G. Basham

Graduate Student, Department of Plant Pathology, North Carolina State University, Raleigh. Present address: Department of Plant Pathology, Cornell University, Ithaca, New York 14850.

Journal Series Paper Number 2728, North Carolina State University Agricultural Experiment Station, Raleigh.

Prepared as an M.S. Thesis at North Carolina State University under the direction of E. B. Cowling. Financial support in part provided by a fellowship from the National Defense Education Act.

Grateful acknowledgement is made to R. W. Davidson for several isolates of *Ceratocystis*.

Accepted for publication 6 October 1969.

ABSTRACT

Isolates of *Ceratocystis ips, C. minor, C. montia,* and *C. pilifera* caused a deeply penetrating blue stain of the sapwood of loblolly pine (*Pinus taeda*) and killed the trees. *C. olivacea, C. multiannulata,* and *C. coerulescens* caused little blue stain and no mortality. This is the first evidence that blue-stain fungi are pathogenic to loblolly pine and that *C. pilifera* is pathogenic to any host.

Seedlings were more susceptible than saplings in terms of per cent of trees killed and average time to death. Vigorous saplings (rated by crown class and stem diam) were more resistant than weaker saplings.

Blue-stain fungi cause wilt by a blockage of the ascent of sap. Dye conduction in the stem of the host occurred only in tissues not colonized by the fungi. Moisture content of the sapwood of infected trees was reduced in and above the infected portion of the stem. A high percentage of the pits was aspirated in and above the region colonized by the fungi. Symptoms of seedlings killed by withholding water were indistinguishable from those of inoculated seedlings. Color reactions indicative of phenolic compounds were observed in the infected region of the stems of living inoculated or wounded trees, but not in trees killed by the fungi. Phytopathology 60:750-754.

Blue-stain fungi associated with bark beetles cause significant economic damage to many gymnosperms. The bark beetles are vectors of the fungi, distributing the spores throughout their galleries in the inner bark of the trees. The fungi colonize the cambium and grow inward to the sapwood-heartwood transition zone (17). Trees so attacked die within 1 year, and in some cases in a few weeks. Girdling of the cambium by the bark beetles does not explain this rapid death (13). Shortleaf pine (*Pinus echinata* Mill.), slash pine (*P. caribea* Morlet), and Virginia pine (*P. virginiana* Mill.) were killed after inoculation with *C. ips* (Rumb.) C. Moreau, and *C. minor* (Hedgc.) Hunt (2, 3, 14, 15); ponderosa pine (*P. ponderosa* Laws.) was killed by *C. ips, C. minor, C. montia* (Rumb.) Hunt, and possibly by *C. schrenciana* (Hedgc.) C. Moreau (11). Inoculated trees died only after a complete cross section of the functional xylem was infected. Dye solutions were not conducted through infected areas of the stem; there was a marked drying of infected areas (2, 3, 12, 14). The means by which the ascent of sap is blocked in infected trees has not been determined.

The present study was undertaken to determine the susceptibility of loblolly pine (*P. taeda* L.) seedlings and saplings to seven species of blue-stain fungi, and to investigate the mechanisms of pathogenicity and resistance involved in this disease.

MATERIALS AND METHODS.—*Pathogenicity of isolates of Ceratocystis.*—Twelve isolates of *Ceratocystis* were selected: (i) three species causing penetrating sapstain of living trees and reported as pathogens of pine (12); two isolates of *C. ips,* one isolate of *C. montia,* and three isolates of *C. minor*; (ii) two species causing penetrating sapstain in logs and lumber but not reported as pathogens of pine (8); two isolates of *C. pilifera* (Fries) C. Moreau and two isolates of *C. coerulescens* (Munch) Bakshi; and (iii) two species causing nonpenetrating sapstain of logs and lumber (8); single isolates of *C. olivacea* (Mathieson) Hunt and *C. multiannulata* (Hedgc. & Davids.) Hunt. Cultures were maintained on 2% malt-extract agar and transferred every 6 months.

The loblolly pines were of two age groups: (i) 13-year-old saplings growing in a 3.5-hectare (7-acre) plot near Rougemont, North Carolina; and (ii) 2-year-old seedling growing in pots in a greenhouse in Raleigh, North Carolina.

Trees in both age groups were inoculated as described by Mathre (11) as modified below. The bark was removed from a band completely encircling the tree, but strips of cambium within the band were left intact so the tree was not completely girdled. This band was 5-8 cm wide in seedlings, and 35-40 cm wide in saplings. Inoculum was grown on 2% malt-extract broth. Each seedling and sapling was inoculated with 20 and 200 ml of inoculum, respectively, added to sterile pine sawdust held in place against the debarked area of the stem with polyethylene film.

Ten saplings and eight seedlings were inoculated with each of the 12 isolates of *Ceratocystis* spp. Checks consisted of 10 saplings and eight seedlings that received no treatment and the same number of control trees inoculated with sterile malt-extract broth. Four additional untreated seedlings were not watered, so that symptoms of water deficiency could be compared with symptoms of disease.

The stem diam and crown class (19) of each sapling and the height of each seedling were recorded at the time of inoculation. Trees were harvested when most of the needles in the crown were drooping and browned

or at the termination of the experiments (300 days after inoculation with the saplings, 100 days with the seedlings). Relative pathogenicity of the isolates and species used was determined by two indexes, percentage of trees killed and average number of days to death. The relationship of these indexes to crown class and stem diam of saplings and height of seedlings was tested by regression analyses and chi-square tests for independence of means.

Mechanisms of pathogenesis and resistance.—Each dead sapling was split longitudinally, the distribution of fungal discoloration recorded, and the radial and longitudinal extension of the fungus determined by isolation. Only longitudinal distribution of the fungi was determined in the seedlings.

The pattern of dye conduction in the stems of seedlings was determined as described by Mathre (12). After 100 days, two seedlings inoculated with each isolate were immersed in water, cut just above the root collar, and placed in a solution of acid fuchsin, where they remained at least 12 hr. The pattern of dye conduction was compared with untreated, control, and unwatered seedlings treated similarly.

The moisture content of the stem above, within, and below the inoculation band was determined in saplings and seedlings. Stem segments were weighed and then dried to constant weight at 100 C. Weight loss was expressed as a percentage of dry wt.

The response of tissues to infection was studied histochemically. One seedling and one sapling inoculated with each fungal isolate as well as untreated and control trees were examined for dead parenchyma cells, aspirated pits, and the presence of starch, lipids, and phenols. Tissue from above, within, and below the inoculation band was cut into sections 28 μ thick at −20 C in a cryostat (International Equipment Co., Model CTI). Living cells in the sapwood were identified by incubating sections for 30 min at 37 C in a solution containing 0.75 mg nitro-blue tetrazolium (NBT), 1.05 ml of 0.067 M phosphate buffer at pH 7, 21.3 mg reduced nicotinamide adenine dinucleotide ($NADH_2$), 15 mg nicotinamide adenine dinucleotide (NAD), and 1.95 ml distilled water (10). In the presence of active cellular NAD diaphorase, NBT is reduced to a blue diformazan. To differentiate between enzymatic and nonenzymatic reduction of NBT, corresponding sections were incubated in the staining medium without the substrate ($NADH_2$), and steam-killed sections were incubated in the complete staining medium. Starch, lipids, and phenols were detected by placing sections in iodine-potassium iodide (IKI) (9), Sudan III (18), and diazotized tolidine (18), respectively. The percentage of pits aspirated was determined in sections mounted in lactophenol with cotton-blue. Sections stained with IKI were mounted in IKI and examined immediately. All other sections were rinsed in distilled water; killed and fixed in a solution containing 10 ml of 40% formaldehyde, 0.85 g NaCl, and distilled water to give 100 ml; and mounted in glycerin jelly (10).

RESULTS.—*Pathogenicity of isolates of Ceratocystis.*—All isolates of *C. ips, C. montia,* and *C. pilifera,* and two isolates of *C. minor* caused death in inoculated trees (Table 1). *C. minor* isolate C grew much more slowly, and developed a less dense mycelial mat than the other isolates; it was not pathogenic. No death due to blue-stain fungi occurred in control trees or those inoculated with *C. multiannulata, C. coerulescens,* and *C. olivacea.* The response of the seedlings to the various isolates was similar to that of the saplings, but there were no significant differences among pathogenic isolates in percentage of trees killed or average time to death.

Seedlings were more susceptible than saplings to all pathogenic isolates in terms of percentage of trees killed and average number of days to death, except

TABLE 1. Pathogenicity of isolates of *Ceratocystis* spp. to seedlings and saplings of loblolly pine

Species	Isolate	Saplings		Seedlings	
		% Trees killed[a]	Avg days to death	% Trees killed[a]	Avg days to death
Controls		0		0	
C. ips	A	50	64	100	17
	B	60	101	100	20
C. minor	A	50	95	100	18
	B	60	65	12	50
	C	0		0	
C. montia	A	40	64	50	21
C. pilifera	A	40	80	100	15
	B	50	53	75	22
C. multiannulata	A	10[b]	180	0	
C. coerulescens	A	10[b]	180	0	
	B	0		0	
C. olivacea	A	0		0	
All pathogenic isolates taken together[c]		50	74	77	23

[a] Per cent of 10 saplings; per cent of eight seedlings.
[b] No stain was evident in these saplings.
[c] Includes all isolates of *C. ips, C. montia,* and *C. pilifera*; and *C. minor* isolates A and B.

for those inoculated with *C. minor* isolate B (Table 1). On reisolation from inoculated seedlings, *C. minor* isolate B grew more slowly and showed atypical cultural characteristics.

Saplings that were suppressed or of small stem diam were more susceptible to blue-stain disease than more vigorous saplings. There was no relationship between susceptibility and height in seedlings (Table 2).

Mechanisms of pathogenesis and resistance.—Similar symptoms developed in saplings and seedlings. Initially, the terminal drooped and needles changed from green to light green to light brown to red brown. Concomitant with these changes was a drooping and twisting of the needles. These symptoms also appeared in unwatered seedlings.

Evidence for blockage of ascent of sap in infected sapwood was obtained in the dye conduction studies. Dye solution moved from root collar to terminal in about 2 hr in untreated seedlings, control seedlings, and live infected seedlings, but was blocked in dead seedlings. Dye was conducted throughout the entire xylem in untreated and control seedlings and in untreated seedlings with the crowns cut off immediately before treatment. In living infected seedlings, conduction of dye was absent in portions of the xylem where blue stain was evident, but was conducted past these areas through unstained sapwood and then spread radially throughout the sapwood above the stained portion of the stem. In seedlings that died from infection, the dye solution was increasingly restricted from the outer portion toward the center of the stem as it moved upward, until complete blockage of dye conduction occurred about 2 cm above the lower portion of the inoculation band. Dye was not conducted through seedlings killed by withholding water.

Cytochemical changes were similar in infected saplings and seedlings. Color reactions indicated the presence of starch and lipids in untreated trees, in control trees, and above and below the infected sapwood in trees that died. These reactions were not evident, or were of limited intensity in infected tissue. The color reaction indicating phenols was very weak in infected sapwood of dead inoculated trees, and was confined to ray parenchyma and epithelial cells. In control trees, sapwood beneath the inoculation band gave a stronger reaction for phenols in parenchyma cells, epithelial cells, and tracheids of the peripheral xylem. A similar reaction occurred in infected but living trees, but the reaction extended somewhat deeper into the sapwood than in the control trees. Areas with a strong positive reaction for phenols were resin-soaked. No infected cells reacted positively to the vital stain, NBT. In trees killed by the fungi, living cells were sometimes found below the infected area of the stem, but were not found in the dry sapwood above the infected sapwood.

Wound tissue developed over the surface of the inoculation band in control seedlings and seedlings that did not die from infection. This tissue had the appearance and cytochemical reactions of normal secondary xylem except for a more intense reaction for phenols. Wound tissue was present in limited amounts in control saplings and living inoculated saplings, but did not form in infected seedlings and saplings that died.

Xylem tracheids in seedlings were examined for aspiration of bordered pit pairs. Untreated seedlings contained approximately 5% pit aspiration. Control seedlings contained 5, 16, and 43% aspirated pits below, within, and above the band, respectively; and seedling that died after inoculation contained 65, 94, and 98% aspirated pits below, within, and above the band, respectively. Pits and tracheids in trees that died after infection generally appeared to be free of material that could block the ascent of sap in the stem. Hyphae filled the ray parenchyma and caused extensive decomposition of parenchyma cells, but were only occasionally present in the tracheids.

TABLE 2. Influence of host vigor (measured as crown class and stem diameter) on the susceptibility of loblolly pine inoculated with pathogenic isolates of *Ceratocystis* spp.

Vigor class	No. trees in class	% Trees killed	Avg days to death
Crown class			
Suppressed	28	64	88
Not suppressed	43	38	111
Stem diam			
2.5-6.5 cm	34	76	83
6.5 + cm	36	22	152

DISCUSSION.—*Pathogenicity of isolates of Ceratocystis.*—This is the first experimental evidence that blue-stain fungi are pathogenic to loblolly pine, and that *C. pilifera* is pathogenic to any host. *C. pilifera* apparently is not associated with a vector; this may account for its prior lack of recognition as a pathogen. Griffin (5) has recently placed *C. schrenciana* in synonomy with *C. pilifera; C. schrenciana* may be pathogenic to ponderosa pine (11).

Pathogenicity of these fungi in loblolly pine apparently is related to their capacity to penetrate living sapwood. Pathogenic isolates penetrated living sapwood readily, whereas nonpathogenic isolates did not. *C. olivacea* and *C. multiannulata* caused only localized blue stain in pine lumber or living trees (8). *C. coerulescens* causes severe blue stain of hardwood lumber (21) and sapstreak disease of maple (7), but has been isolated only occasionally from pine. All the pathogenic isolates in this work except *C. pilifera* have been previously reported to cause penetrating sapstain of pine (11).

The greater susceptibility of seedlings to pathogenic isolates may be explained by the shorter distance necessary for complete fungal penetration of the sapwood, or the greater ratio of wounded surface area to total stem volume of seedlings. Mathre (12) suggested that small ponderosa pine were more susceptible to blue-stain fungi because they contained less resin to cover wounds.

Mechanisms of pathogenesis and resistance.—The following points of evidence support the conclusion from earlier investigations that the blue-stain disease

of pine is a wilt resulting from blockage of ascent of sap in xylem (12, 14): (i) Symptoms in seedlings killed by withholding water were indistinguishable from those in infected trees that died; (ii) The moisture content of infected sapwood was well below that of uninfected sapwood; after trees died, the moisture content of their stem tissues was near the fiber saturation point within and above the region of fungal invasion, but was much higher below the point of fungal invasion; (iii) Dye was not conducted through infected portions of the sapwood and death did not occur until a complete cross section of the sapwood was invaded; and (iv) bordered pits in the region of fungal invasion were aspirated; this would block the flow of sap through the tracheids.

Although blockage of water conduction causes wilt, the means by which this blockage is induced is not known. Various types of substances have been proposed to occlude the pit membrane pores: (i) decomposition products from parenchyma cells invaded by the fungi (14); (ii) extracellular polysaccharides produced by *C. ips* and *C. minor* (12); and (iii) resin globules observed in tracheids of shortleaf pine infected with *C. minor* (2).

No obstructing materials were observed with the light microscope in tracheids or bordered pits of the trees used by Mathre (12) or in the present investigation. Failure to see occluding substances on pit membranes with a light microscope does not exclude this theory of blockage; small amounts of material that may not be visible in the light microscope could plug submicroscopic openings in the membranes between vascular elements (4). Electron micrographs of pit membranes in latewood of longleaf pine (*P. palustris* Mill.) show them to be so heavily incrusted that movement of liquid probably is restricted (20). Thus, there is still a possibility that wilt is due at least in part to occlusion of pit membranes in the vascular system.

Mathre (12) has suggested that blockage of tracheids may occur as follows: decomposition of wood rays invaded by the fungi allows evaporation of moisture from the rays, which in turn permits air to enter the tracheids. Air in the tracheids breaks the water columns, which are under tension, and columns above these breaks are withdrawn by the pull of transpiration.

It is difficult to envision how evaporation of moisture from the rays could have occurred in the trees in the present investigation, since the inoculum remained moist enough for liquid water to be present in the bottom of the inoculum poultice on most trees for the duration of the experiment. It is not difficult to envision, however, that air could be drawn into the tracheids by the pull of the transpiration stream, whether or not drying of the rays occurred. Once air entered a tracheid, pit aspiration would occur almost immediately. Since it is physically impossible for an air-water interface to be drawn through the membrane of a bordered pit by forces of the order of magnitude of those produced by transpiration, the force of transpiration on the water column above such an interface would cause aspiration of the bordered pit and thus block the flow of water through the tracheid (6). Sufficient air could enter the sapwood to seal all functional tracheids in a cross section of the stem of a tree infected by blue-stain fungi, since every tracheid in southern pine trees is adjacent to 10-30 wood rays (14). In this study, nearly all rays in trees killed by pathogenic isolates were invaded extensively by these fungi. Thus, rejection of Mathre's specific mechanism of blockage does not detract from his basic suggestion that the entry of air would interrupt the flow of water in the stem (12).

Variation in susceptibility of saplings to pathogenic isolates of *Ceratocystis* was noted in this study and has been reported in earlier investigations. This variation has been attributed primarily to differences in oleoresin exudation pressure (OEP) (12) and to resin accumulation in tissues invaded by blue-stain fungi (16). No measurements of OEP were made in the present study, but a relationship was observed between apparent vigor of the saplings (as measured by crown class or stem diam) and their susceptibility to pathogenic isolates of *Ceratocystis* (Table 2). Since OEP was not related to crown class in ponderosa pine (22) or to stem diam of slash pine (1), a second factor influencing host resistance and related to vigor of the host may have been present in the saplings used in this study. The zone of phenols and resins which appeared in the region of fungal invasion in resistant trees may account for this. When resistant lodgepole pines (*P. contorta* Dough.) were attacked by *C. montia,* primary resin flow covered the infection court. If this initial barrier was breached, colonization of the sapwood was restricted by a barrier of secondary resin which appeared in the xylem (16). A similar zone containing toxic phenols and resins is produced in vigorous loblolly pine inoculated with *Fomes annosus* (Fr.) Karst, but is not produced in suppressed trees (18). The formation of a resin barrier in sapwood during the development of blue-stain disease appears to be a vital function that could vary with vigor of the host.

LITERATURE CITED

1. Bordeau, P. F., & C. S. Schopmeyer. 1958. Oleoresin exudation pressure in slash pine: its measurement, heritability, and relation to oleoresin yield, p. 313-319. *In* K. V. Thimann [ed.] The physiology of forest trees. Ronald Press, N. Y.
2. Bramble, W. C., & E. C. Holst. 1940. Fungi associated with *Dendroctonus frontalis* in killing shortleaf pines and their effect on conduction. Phytopathology 30:881-899.
3. Caird, R. W. 1935. Physiology of pines infected with bark beetles. Bot. Gaz. 96:709-733.
4. Dimond, A. E. 1967. Physiology of wilt disease. p. 100-118. *In* C. J. Mirocha & I. Uritani [ed.] The dynamic role of molecular constituents in plant parasite interaction. Bruce Publishing Co., St. Paul, Minnesota.
5. Griffin, H. D. 1968. The genus *Ceratocystis* in Ontario. Can. J. Bot. 46:689-718.
6. Hart, C. A., & R. J. Thomas. 1967. Mechanism of bordered pit aspiration as caused by capillarity. Forest Products J. 17:61-68.
7. Hepting, G. H. 1944. Sapstreak, a new killing disease of sugar maple. Phytopathology 34:1069-1076.
8. Hunt, John. 1956. Taxonomy of the genus *Ceratocystis*. Lloydia 19:1-58.

9. JENSEN, W. A. 1962. Botan. Histochem. W. H. Freeman & Co., San Francisco. 408 p.
10. KOENIGS, J. W. 1966. Intercellular localization of individual dehydrogenases in frozen plant tissue by means of specific substrates and coenzymes. Stain Technol. 41:1-7.
11. MATHRE, D. E. 1964. Survey of *Ceratocystis* spp. associated with bark beetles in California. Contrib. Boyce Thompson Inst. 22:353-362.
12. MATHRE, D. E. 1964. Pathogenicity of *Ceratocystis ips* and *Ceratocystis minor* to *Pinus ponderosa*. Contrib. Boyce Thompson Inst. 22:363-388.
13. MILLER, J. M., & F. P. KEEN. 1960. Biology and control of the western pine beetle: a summary of the first fifty years of research. USDA Misc. Publ. No. 800. 381 p.
14. NELSON, R. M. 1934. Effect of bluestain fungi on southern pine attacked by bark beetles. Phytopathol. Z. 7:327-353.
15. NELSON, R. M., & J. A. BEAL. 1929. Experiments with bluestain fungi in southern pines. Phytopathology 19:1101-1106.
16. REID, R. W., H. S. WHITNEY, & J. A. WATSON. 1967. Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. Can. J. Bot. 45:1115-1126.
17. RUMBOLD, CAROLINE T. 1931. Two blue-stain fungi associated with bark beetle infestation of pines. J. Agr. Res. 43:847-873.
18. SHAIN, L. 1967. Resistance of sapwood in stems of loblolly pine to infection by *Fomes annosus*. Phytopathology 57:1034-1045.
19. SMITH, D. M. 1962. The Practice of Silviculture. John Wiley & Sons, N. Y. 578 p.
20. THOMAS, R. J. 1967. Structure of the pit membranes in longleaf pine: an electron microscope study. Amer. Wood Preserv. Assoc. Proc. 63:20-28.
21. VERRALL, A. F. 1941. Dissemination of fungi that stain logs and lumber. J. Agr. Res. 63:549-558.
22. VITE, J. P. 1961. The influence of water supply on oleoresin exudation pressure and resistance to bark beetle attack in *Pinus ponderosa*. Contrib. Boyce Thompson Inst. 21:37-66.