Colonization of Cones and Seed of Loblolly Pine Following Inoculation with *Fusarium subglutinans*

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ABSTRACT

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Recovery of *Fusarium subglutinans* from seeds of loblolly pine (*Pinus taeda*) cones inoculated 6, 15, 16, or 17 mo after pollination was significantly more frequent in the 15-mo inoculation treatment (24%) than in all other treatments (0–4%). Inoculation of the cone apex or of scales at the middle of the cone produced infections of scales and seeds; inoculation of peduncles did not. Wounding of uninoculated cones increased the incidence of seed infection with *F. subglutinans*. Isolation of *F. subglutinans* from seeds of naturally colonized cones collected from four seed orchards averaged 1–34%. *F. proliferatum* was commonly isolated from seeds and scales of treated cones and from naturally colonized cones.

Additional keywords: *Fusarium moniliforme* var. *intermedium*, *Fusarium moniliforme* var. *subglutinans*, pitch canker

Seed orchards of loblolly pine (*Pinus taeda* L.) supply genetically improved seeds for forest plantations in the southeastern United States. During the approximately 19-mo period between production of female strobili and maturation of pine seed, there are numerous opportunities for pathogens to reduce potential seed yields (4,11). An additional concern is that the sowing of infected seed could result in production losses in the nursery and field.

Shoot dieback in the upper crown, the predominant symptom of pitch canker on loblolly pine, has been found in seed orchards since major outbreaks of the disease were reported in the mid-1970s (7,11,13). The causal organism of pitch canker, *Fusarium subglutinans* (Wollenweb. & Reinking) Nelson, Toussoun & Marasas (=*F. moniliforme* J. Sheld. var. *subglutinans* Wollenweb. & Reinking), requires a wound as an infection court (5,9,12). In loblolly pine seed orchards, *F. subglutinans* has been isolated not only from branches and shoots, but also from conelets, mature cones, and seeds of loblolly pine (3,14). Patterns of fungus isolation and histological data indicate that outbreaks of dieback and conelet deterioration can occur independently (3). Currently, the disease cycle on loblolly pine reproductive structures is not known.

This paper describes inoculation trials of loblolly pinecones with *F. subglutinans*. The goal was to describe colonization patterns of the pathogen. Specific objectives were to determine if inoculation of different tissue areas on cones influenced disease development, to evaluate the effects of inoculating cones at several stages of development, and to compare naturally colonized cones with artificially inoculated cones. A preliminary report (2) on a portion of this research has been published.

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MATERIALS AND METHODS

Tissue study. In July 1984 and 1985, a loblolly pine seed orchard (17 yr old in 1984) in the Piedmont, near Rock Hill, South Carolina, was chosen for study. Sixteen-month-old cones were wounded with a dissecting needle (single puncture, 3 mm deep) in one of three tissues—apical scales, central fertile scales, or peduncle. Cones were inoculated by spraying to runoff with 10^6 conidia/ml of *F. subglutinans* in sterile water. Control cones were wounded and sprayed with sterile water.

Six ramets of clone A-1-521 were randomly selected in the seed orchard for study. This clone was chosen for the study because it is a prolific cone producer (3). On each ramet, six cone-bearing branches were selected to receive one each of the following six treatment combinations: wound location (apical scales, central fertile scales, or peduncle) × inoculation (*F. subglutinans* or sterile water). One to six cones per branch were available for treatment. Thirty-six branches were treated in a given year. Eighty-one and 75 cones were treated in 1984 and 1985, respectively.

The inoculum came from a culture derived from a single spore of *F. subglutinans* recovered from a pitch canker on loblolly pine. The culture was grown on potato-dextrose agar for 7 days on a 12-hr photoperiod at 24 C. Conidia were washed from the culture plates with sterile, deionized water, and the number of conidia per milliliter was adjusted with a Coulter electronic particle counter (model ZBI; Coulter Electronics, Inc., Hialeah, FL).

Ten weeks after treatment (October 1984 and 1985), the mature cones were harvested by clipping them off the branches. Each cone was assessed for colonization by removing three to six scales adjacent to the inoculation point and culturing them on a *Fusarium*-selective medium (1) for 7 days on a 12-

hr photoperiod. Each isolate recovered from the samples was identified from a single spore grown on a sterile oat grain on 2% agar (10 days, 12-hr photoperiod, 24 C) and observed directly under a Leitz microscope (×100). Presence of mono- and polyphialides was confirmed on slides observed at higher magnification (×400). Identification was based on the taxonomic key of Nelson et al (15).

After scales were sampled, each cone was placed in a labeled paper bag, and approximately 10 bags each were placed in wire-bound, plastic milk crates. The crates were dried in a shed at 26–32 C for an average of 7 days to force scales to open. Cones were stored at 20 C (<40% RH) until processed. The cones that failed to open after drying were drilled through the axis in order to extract the seeds. The number of seeds per cone extracted from scales that closed because of physiological damage was recorded.

Seeds from each cone were attached to paper and radiographed. The radiographs were used to separate the seeds into sound or abnormal categories (4). Abnormal seeds exhibited one of the following symptoms: shrunken gametophyte, missing embryo, insect injury, or empty.

All seeds were surface-sterilized according to the following schedule: 0.75% sodium hypochlorite, 15 min; sterile, deionized water rinse; sterile, deionized water, 5 min; 2% hydrogen peroxide, 5 min; sterile, deionized water rinse; and sterile, deionized water, 5 min. The seeds were then nicked at the micropylar end with a sterile razor blade and cultured, and the cultures were identified as described previously.

Cone age at inoculation. The study was conducted in the same seed orchard used for the tissue study. In September 1984 and June, July, and August 1985, cones 6, 15, 16, and 17 mo old, respectively, were wounded with a dissecting needle (single puncture, 3 mm deep) in a mid-cone scale. Cones were then inoculated by spraying to runoff with 10^6 conidia/ml of *F. subglutinans* in sterile water. Control treatments were wounded and sprayed with sterile water.

Six ramets of clone A-1-521 were randomly selected for study. On each ramet, eight cone-bearing branches were selected to receive one each of the following eight treatment combinations: cone age after pollination (6, 15, 16, or 17 mo) × inoculation (*F. subglutinans* or sterile water). One to six cones per branch were available for treatment. A total of 79 cones on 48 branches were treated. At the beginning of the study, one branch per ramet was also designated as a nonwounded control (six branches; 15 cones).

In October 1985, when the cones matured, all were harvested by clipping them off the branches. Four to six scales adjacent to the inoculation point on each cone were sampled and tested for colonization by *F. subglutinans* as described for the tissue study. Cones were then dried; seeds were extracted, radiographed, and cultured, and the cultures were identified as previously described.

Naturally colonized cones. In October 1985, mature cones exhibiting symptoms of fungal infection were collected from four loblolly pine seed orchards, three in South Carolina and one in Alabama, for a general comparison with the artificially inoculated cones. Subsamples of six cones from each of the South Carolina orchards and 36 from the Alabama orchard were processed as described for the earlier studies.

Analysis of data. Effects of inoculation were subjected to analysis of variance (ANOVA). Percentages were transformed to arcsines for the ANOVA to correct for heterogeneity. In the tissue study, treatment means were separated by Duncan's multiple range test. In the cone-age inoculation study, data were subjected to Abbott's adjustment (8) to compensate for the amount of infection present in the nonwounded cone samples. Effects of wounding alone were tested in chi-square analyses. Because the cone-age inoculations evaluated susceptibility of various distinct developmental stages of the cones (determined by cone size and relative succulence), treatments were considered qualitative, and Duncan's multiple range test was used to separate treatment means (16).

RESULTS

Tissue study. *F. subglutinans* and a closely related species, *F. proliferatum* (Matsushima) Nirenberg (=*F. moniliforme* var. *intermedium* Niesh & Leggett), were isolated from scales adjacent to wounds in the apex and mid-cone but not from the peduncle or the scales adjacent to it (Table 1).

Approximately three to 10 scales per cone adjacent to apical and mid-cone wounds in both control and inoculated cones were necrotic and remained closed after cones were dried (Fig. 1A). Hyphae were frequently observed on cones inoculated with *F. subglutinans* (Fig. 1B).

Data for 1984 and 1985 were pooled for ANOVA. Neither treatment nor tissue type had a significant main effect on the quantity of seeds extracted from closed vs. open scales nor on the number of seeds rated as abnormal on the radiographs.

Specific combinations of treatment and tissue type significantly affected ($P = 0.05$) the percentage of seeds infected with *F. subglutinans*. The fungus was recovered more often from inoculated than from control cones, and inoculation at cone apex and mid-cone resulted in significantly more infected seeds than inoculation at the peduncle. *F. proliferatum* was also recovered from internal seed tissues. Percentage of recovery of *F. subglutinans* and *F. proliferatum* from seeds was generally higher in 1984 than 1985.

Cone age at inoculation. *F. subglutinans* was isolated from scales inoculated 15 and 17 mo after pollination. The highest recovery occurred in the 17-mo control and inoculated treatments (32 and 53%, respectively). *F. proliferatum* was isolated from scales wounded 15, 16, and 17 mo after pollination. Table 2 summarizes the recovery of the two *Fusarium* species by treatment and age.

Scales on cones inoculated 15, 16, and

Table 1. Isolation of *Fusarium subglutinans* (Fs) and *F. proliferatum* (Fp) from scales and seeds of loblolly pine cones wounded in apical scales, mid-cone scales, or peduncle and inoculated with *F. subglutinans* 16 mo after pollination and harvested 10 wk later

Date	Treatment-position[b]	No. of cones	Scales[a]			Seeds		
			No.	%Fs	%Fp	No.[c]	%Fs	%Fp
1984								
	C-Apex	17	66	24	35	1,305	3	6
	I-Apex	13	60	78	15	730	11	1
	C-Mid	13	72	6	31	1,191	0	5
	I-Mid	13	81	61	22	1,527	12	2
	C-Ped	14	70	0	0	1,355	3	0
	I-Ped	11	55	0	0	829	2	1
1985								
	C-Apex	13	78	3	6	1,154	4	0
	I-Apex	13	75	47	9	1,149	4	1
	C-Mid	10	54	0	0	986	2	0
	I-Mid	13	69	0	18	1,110	2	3
	C-Ped	16	75	0	0	1,523	0	0
	I-Ped	10	53	0	0	1,310	1	1

[a] Three to six scales were sampled per wound site per cone.
[b] Cones were wounded by a 3-mm-deep puncture with a dissecting needle at the site indicated. C = control, I = inoculated; Apex = apical scales; Mid = mid-cone scales; and Ped = peduncle.
[c] All seeds (open and closed scales) were sampled.

Table 2. Isolation of *Fusarium subglutinans* (Fs) and *F. proliferatum* (Fp) from scales and seeds of loblolly pine cones wounded in the mid-cone scales inoculated with *F. subglutinans* 6, 15, 16, and 17 mo after pollination and harvested 10 wk after the last inoculation date

Cone age (mo.)	Treatment[x]	No. of cones	Scales[w]			Seeds		
			No.	%Fs	%Fp	No.[y]	%Fs[z]	%Fp
6	C	7	35	0	0	550	0 b	0
	I	8	40	0	0	480	0 b	0
15	C	11	55	0	27	969	2 b	1
	I	10	49	12	0	662	24 a	3
16	C	10	54	0	0	986	2 b	1
	I	13	69	0	18	1,110	1 b	2
17	C	9	57	32	2	948	3 b	1
	I	11	66	53	1	882	4 b	0

[w] Four to six scales were sampled per wound site per cone.
[x] Cones were wounded by a 3-mm-deep puncture with a dissecting needle in a mid-cone scale. C = control, I = inoculated.
[y] All seeds (open and closed scales) were sampled.
[z] There was a significant treatment by date interaction (ANOVA, $P = 0.05$). The means for cones inoculated 15 mo after pollination differed significantly from all other treatment-date combinations (Duncan's multiple range test, $P = 0.05$).

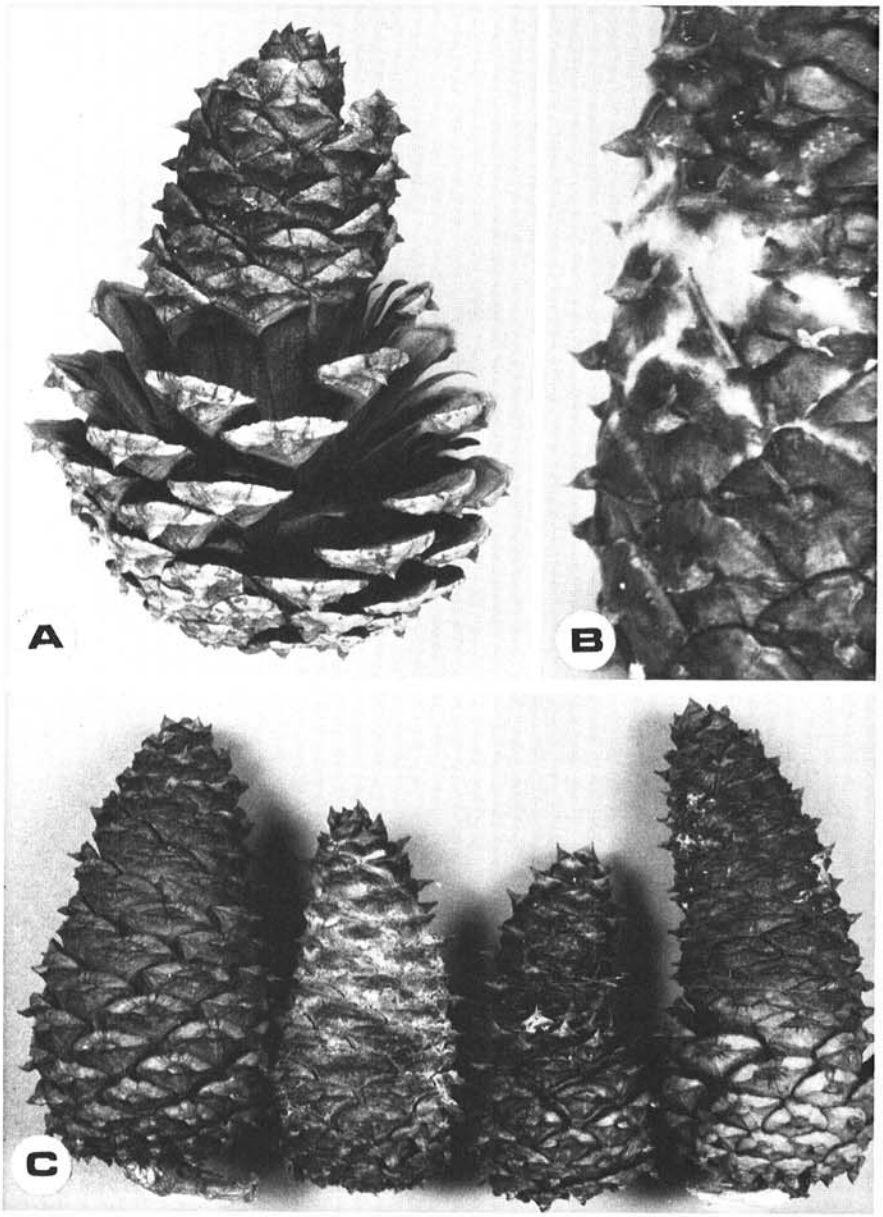

Fig. 1. Symptoms of infection by *Fusarium subglutinans*: **(A)** scales adjacent to the inoculation point permanently closed; **(B)** hyphae on inoculted and infected scales; and **(C)** closed scales, resinosis, discoloration, and hyphal blooms on naturally infected cones.

17 mo after pollination developed symptoms identical to those described for the tissue study. Cones inoculated 6 mo after pollination showed no symptoms of disease.

Cone age significantly affected numbers of seeds rated abnormal and seeds in closed scales (ANOVA; $P = 0.05$ and $P = 0.01$, respectively). Treatment at 15 mo (June 1985) differed significantly from the other dates in both average percentage of seed extracted from closed scales (33 vs. 2–8) and in average percentage of abnormal seeds (33 vs. 6–9).

There was a significant treatment × cone age interaction with seed infection by *F. subglutinans* (ANOVA, $P = 0.05$). Average infection of seeds (21%) from cones inoculated 15 mo after pollination (June 1985) was significantly greater than all other treatment-age combinations (0–4%; Duncan's multiple range test, $P = 0.05$). *F. proliferatum* also was isolated at a low rate in several of the seed lots.

Comparison of wounded, noninoculated cones with nonwounded cones indicated that wounds affected both the quantity of seeds rated abnormal and the quantity infected by *F. subglutinans*. Two general statements about wounded (3,453 seeds) vs. nonwounded (1,168 seeds) cones are supported by chi-square analyses ($P < 0.01$). First, abnormal seeds occurred more frequently than expected under the hypothesis of independence in wounded cones than they did in nonwounded cones. Second, infection of internal seed tissues by *F. subglutinans* occurred more frequently than expected under the hypothesis of independence in wounded cones than it did in nonwounded cones.

Naturally colonized cones. *F. subglutinans* was isolated from 7 to 61% of scales on cones exhibiting symptoms of fungal infection, and *F. proliferatum* was isolated from 12 to 88%. Both species were also isolated from internal seed tissues (seeds from open and closed scales); 1–34% yielded *F. subglutinans* and 0–16% yielded *F. proliferatum* (Table 3).

Symptoms observed included scales closed because of physiological damage (frequently the entire cone), resinosis, discolored scales, and hyphal blooms on the scale surfaces (Fig. 1C). No symptoms of insect damage, particularly by *Dioryctria* sp., were observed on these cones.

DISCUSSION

Among cones inoculated at various ages, *F. subglutinans* was recovered at the highest rate from internal seed tissues of cones inoculated at 15 mo after pollination (June 1985). Cones in both control and inoculated treatments also had the highest number of abnormal seeds and closed scales. At this stage of

Table 3. Isolation of *Fusarium subglutinans* (Fs) and *F. proliferatum* (Fp) from scales and seeds of loblolly pine cones with symptoms of infection collected from seed orchards in South Carolina and Alabama

Location	No. of cones	Scales[a]			Seeds[b]		
		No.	%Fs	%Fp	No.	%Fs	%Fp
Alabama	36	180	50	33	541	25	7
South Carolina-1	12	60	17	88	497	18	16
South Carolina-2	12	60	61	22	427	34	0
South Carolina-3	12	60	7	12	1,197	1	0

[a]Five scales per cone were sampled.
[b]All seeds (open and closed scales) were sampled.

development, the cones had begun a rapid growth phase to attain their full size. Entire cones were succulent and the seed coats were not yet hardened. Inoculations before and after this date probably resulted in significantly fewer infected seeds because the cone tissue was either dormant (before 15 mo) or had begun to harden (after 15 mo). Maturation of cones varies considerably among clones of loblolly pine. The clone used in this study matures early (late September to early October), and the time of maximum susceptibility to infection by *F. subglutinans* may have been affected by this variable.

In the tissue study, inoculation of scales resulted in infection of scales and, to a lesser degree, of seeds, regardless of location. Inoculation at the peduncle had no effect on scales or seeds. Although conelets can become infected from diseased branches through connective tissues such as a peduncle (3), in this study seed and scales did not become infected after peduncle inoculation. Peduncle tissues were hardened, and this may have influenced tissue susceptibility. Percentages of seeds infected were low (<13%) both years because the inoculation dates used in this study (16 mo after pollination, July 1984 and 1985) were probably not optimal.

Puncture wounds of scales on developing cones adversely affected seed quality. More seeds became naturally infected with *F. subglutinans* in wounded than in nonwounded cones. This result indicates that the pathogen requires a wound for entry into the cone as it does on the vegetative portions of the tree (6).

Numbers of closed scales and seeds rated abnormal in the radiographs were not useful parameters for determining the impact of inoculation on seed quality. Combining all types of seed damage from the radiographs into one category introduced too much variation in the data. Radiography can reveal infected embryos (10), but empty seeds may be caused by several factors in addition to infection. In both studies, empty seeds comprised the majority rated abnormal on the radiographs.

Except for the cone inoculations at 15 mo, percentage isolation of *F. subglutinans* from scales and seeds of naturally infected cones collected from the four seed orchards generally exceeded the recovery rates from inoculated cones. The moderate resistance of clone A-1-521 to shoot dieback caused by *F. subglutinans* (3) may have contributed to the low proportions of seed infection reported here. Another explanation may relate to differences in the frequency (or type) of wounding between the natural and experimental cones. The number of wounds on the naturally colonized cones is unknown and could have easily exceeded the single wound used in the study. Timing and techniques of inoculation also need further refining to more accurately determine the disease potential of *F. subglutinans*.

F. proliferatum was isolated from scales and seeds in wounded and inoculated cones in both studies and also from the naturally infected cones. *F. proliferatum* and *F. subglutinans* are closely allied species in section Liseola (15) and apparently occupy similar ecological niches in pine seed orchards. The results of this study, however, do not prove that *F. proliferatum* is actually pathogenic. Separate inoculation trials are required to determine what type of symptoms, if any, it causes. The data do indicate that *F. proliferatum* is probably a resident on pine tissues and that in the process of wounding, it gained entry. This species may also play a role in the succession of microflora on wounds.

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