Branch Dieback and Cone and Seed Infection Caused by *Fusarium moniliforme* var. *subglutinans* in a Loblolly Pine Seed Orchard in South Carolina

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ABSTRACT

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Incidence of shoot dieback in a loblolly pine seed orchard varied by clone. All but the most susceptible clones recovered from the dieback. *Fusarium moniliforme* var. *subglutinans* was isolated from branches, conelets, mature cones, and seeds. Pathogenicity tests on loblolly and Virginia pine seedlings indicated that the same pathotype of *F. moniliforme* var. *subglutinans* causing shoot dieback also caused deterioration of pine reproductive structures. The pattern of fungus isolation and the histological data indicated that outbreaks of shoot dieback and conelet deterioration could occur independently. A comparison of radiographs showed only slight differences in shape between infected and noninfected seeds. Fungus-damaged seeds occurred only in cones atypical in shape or having necrotic areas on the scales.

Additional key words: pitch canker.

Seed orchards of loblolly pine (*Pinus taeda* L.) and other pine species supply genetically improved seed for replanting harvested forests in the southeastern United States. During the approximate 19-mo period between production of female strobili and maturation of fully developed pine seed, there are numerous opportunities for different biotic and abiotic factors to reduce potential seed yields. Disease impacts are increasing in spite of (and sometimes because of) the specialized practices used to force seed production and to harvest the crop (15).

Branch dieback in the upper crown, the predominant symptom of pitch canker on loblolly pine, has been found in several seed orchards since major outbreaks of the disease were reported in the mid-1970s (7). The causal organism of pitch canker, *Fusarium moniliforme* Sheld. var. *subglutinans* Wollenw. & Reink., is also a component of an insect-disease complex that reduces the quality and quantity of viable seed (14). The pathogen causes strobilus mortality and deterioration of internal seed tissues (13). Preliminary observations on slash pine (*P. elliottii* Engelm. var. *elliottii*) indicate that the amount of infection in a given seedlot may vary from 0 to 98% (3). At present, the relationship between branch and shoot mortality caused by *F. m.* var. *subglutinans* and strobilus mortality and seed deterioration by the same organism is not known.

We report here on an outbreak of pitch canker in a loblolly pine seed orchard in South Carolina. The objectives of this study were: to monitor the severity of shoot dieback symptoms over time, to determine if *F. m.* var. *subglutinans* infected the pine reproductive structures, to describe histopathology of naturally infected branches and reproductive structures, and to determine whether isolates of *F. m.* var. *subglutinans* collected from branches and reproductive structures of the pine hosts were the same pathogenic type.

MATERIALS AND METHODS

Disease survey—1983. Surveys were conducted in a 21-yr-old loblolly pine seed orchard located in the Piedmont near Rock Hill,

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SC and owned by the Catawba Timber Company. An outbreak of pitch canker occurred in the autumn of 1982. In March 1983, all ramets in the orchard were surveyed for shoot dieback and rated for disease severity by using the Kelley and Williams (14) rating system. An average severity rating was calculated for each clone in the orchard. Twelve clones (K-18-106, G-1-573, H-18-3, L-1-575, D-1-513, A-1-521, C-1-515, P-1-581, B-1-561, 0-1-536, N-3-43, and M-3-6), ranging from low (<1%) to high (>50%) disease incidence, were selected for sampling branches, conelets, and cones. In December 1983, another disease survey was conducted, and the 12 clones were rated again based on new infections since March 1983.

In September 1983, 24 branches bearing 5-mo-old conelets were randomly collected from each clone (six branches from each of four ramets). The number of conelets per branch was recorded and the conelets were removed and split longitudinally. One-half of each conelet was stored in FPA (40% formaldehyde-proprionic acid-ethanol [5:5:90, v/v]) (8) for histological examination, the other half was surface sterilized in 70% ethanol, flamed for 2 sec, and cultured on a medium selective for *Fusarium* (1) to detect the presence of the pathogen. Following conelet removal, the branches were cut into 0.5- to 1.0-cm sections, one-half of each section stored in FPA and one-half cultured.

In October 1983, 12 mature 19-mo-old cones were randomly collected from each clone and subjected to standard cone analysis techniques (6). All cones were dried and stored until the scales separated. The cones that failed to open after drying were drilled through the cone axis in order to extract the seed. Seeds from each cone were attached to paper and radiographed. The radiographs were used to separate the seeds into the following categories: full, empty, insect damaged, or fungus damaged. Full and fungus-damaged seeds were surfaced sterilized (3 min in 0.5% sodium hypochlorite) and the seed coats were removed aseptically. The seeds were then separated into lots for either culturing or histological examination.

Histological procedure. Fixed tissue samples of branches, conelets, and seeds were dehydrated in a graded series of *t*-butyl alcohol solutions and embedded in Paraplast Plus (Scientific Products Div., American Hospital Supply Corp., Evanston, IL) (8). The paraffin blocks were stored in a softening solution (10% glycerin-1% sodium lauryl sulfate) (2) in a refrigerator for 2 wk to 2 mo. Transverse and tangential sections 8–12 μm thick were cut serially on a rotary microtome and mounted on glass slides with Haupt's adhesive (8). Alternate slides were stained with a modified Triarch's stain (Triarch Inc., Ripon WI) without crystal violet or with Pianeze's III b stain (17). Stained sections were examined with a Leitz microscope and photographed with an attached Wild MP5 515 camera with Kodak technical pan film 2415.

Seedling inoculation. One-year-old seedlings of Virginia pine (*P. virginiana* Mill.) and loblolly pine were obtained from the Georgia Forestry Commission and transplanted into plastic flats (33 × 13 × 11 cm) that contained a mixture of fumigated soil, pine bark, and sand (2:1:1, v/v). Ten seedlings were planted in each of 93 flats for each species. Flats were kept in a greenhouse for 3 mo prior to treatment.

Thirty isolates of *F. m.* var. *subglutinans* were recovered from pitch canker infections on branches in the seed orchard, from 5-mo-old conelets, from internal seed tissues, and from mature cone scales. Cultures derived from a single spore for each isolate were grown on noncommerical PDA 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 to 10^6 as measured with a Coulter electronic particle counter (model ZBI; Coulter Electronics, Inc., Hialeah, FL).

Seedlings were inoculated 2.5 cm above the node, on the epicotyl formed after seedlings were moved to the greenhouse. Inoculation was accomplished by hypodermic needle puncture of the epidermis through a 1-μl droplet of inoculum. Seedlings in control treatments were wounded by a needle through a 1-μl droplet of sterile deionized water. All seedlings in each flat received a single treatment (one isolate). The thirty isolates plus a control constituted the study treatments.

After inoculation, the seedlings were placed in a mist chamber at 20 C for 24 hr and then moved to a bench in a lath house outdoors where the flats were randomized. There were three replications (flats) per treatment per species.

Eight weeks after inoculation, the number of seedlings killed to the node and the number of live seedlings with a lesion at the inoculation point were recorded. Three stems with lesions were randomly collected from each flat and cultured to confirm colonization by the pathogen.

The data for the surveys, field collections, and the greenhouse experiment were each submitted to analysis of variance for a completely random design. Differences among means were determined by Duncan's multiple range test or Kramer's extension of Duncan's multiple range test. The relationship between the two surveys was explored through regression analysis and correlation coefficient (11,16).

RESULTS

Disease survey. From the survey in March, 12 clones were selected for additional study because they represented a wide range

TABLE 1. Average disease severity ratings of shoot mortality caused by *Fusarium moniliforme* var. *subglutinans* on clones of loblolly pine in a seed orchard[a]

Clone	Ramets (no.)	March rating[b]	December rating[c]
M-3-6	8	4.5 a	4.0 a
N-3-43	26	3.6 b	1.9 b
0-1-576	15	3.2 b	1.3 c
B-1-561	26	3.0 b	2.0 b
P-1-581	16	2.3 c	1.1 c
C-1-515	24	2.3 c	2.3 b
A-1-521	34	1.8 cd	1.1 c
D-1-513	24	1.6 cde	1.2 c
L-1-575	25	1.0 e	1.1 c
H-18-3	14	1.0 e	1.0 c
G-1-573	19	1.0 e	1.1 c
K-18-106	17	1.0 e	1.0 c

[a] Five-point scale after Kelley and Williams (10): 0 = no disease, 1 = <1% shoots exhibiting dieback, 2 = 1–10%; 3 = 11–20%; 4 = 21–50%; and 5 = >50%. Within columns, means not followed by the same letter are significantly different ($P = 0.01$) according to Kramer's (11) extension of Duncan's multiple range test to group means with unequal numbers of replications (ramets).

[b] First survey in March 1983; all current infections (wilted shoots with red needles) were counted.

[c] Second survey in December 1983; new infections since March were counted (needles on old infections had turned grey). Disease severity ratings were correlated with the March ratings ($r = 0.80$, $P = 0.01$).

TABLE 2. Recovery of *Fusarium moniliforme* var. *subglutinans* from branches and 5-mo-old conelets in 12 clones of loblolly pine grown in a seed orchard[a]

Clone	Branches infected[a] (avg. %)	Conelets/branch (no.)	Conelets (no.)	Conelets infected (avg. %)
B-1-561	66.7 a	0	0	0
M-3-6	65.8 a	0.21	5	100
O-1-536	58.3 ab	0	0	0
P-1-581	45.8 abc	0	0	0
N-3-43	33.3 bcd	1.12	19	31.5
G-1-575	25.0 cde	1.75	28	0
C-1-515	16.7 de	0.96	22	45.4
D-1-513	12.5 de	0.71	16	6.3
A-1-521	4.2 e	1.92	36	5.5
H-18-3	0 e	1.04	22	0
K-18-106	0 e	1.21	24	0
L-1-575	0 e	1.07	22	4.5

[a] Based on 24 branches (replications) per clone. Means not followed by the same letter are significantly different ($P = 0.05$) according to Duncan's multiple range test.

in disease severity (Table 1). The second survey in December 1983 indicated disease incidence declined for seven of the 12 clones, but the severity ratings for new infections among the clones matched those made in March ($r = 0.80$, $P = 0.01$).

F. m. var. *subglutinans* was recovered from branches and conelets collected in September 1983. Recovery from branches was highest in clones P-1-581, O-1-536, B-1-561, and M-3-6 (Table 2). Except for clone M-3-6, however, branches sampled from these clones had no conelets. The conelets in clone M-3-6 were 100% necrotic, but infected conelets from other clones (K-18-106, G-1-573, H-18-3, L-1-575, D-1-513, A-1-521, C-1-515, N-3-43) did not exhibit disease symptoms. Recovery of *F. m.* var. *subglutinans* from conelets in the other clones was not correlated with recovery from the branches. Clone C-1-515, for example, averaged 16.7% branches infected and 45.5% conelets infected with *F. m.* var. *subglutinans*. Conversely, 25% of the branches sampled from clone G-1-575 were colonized by the pitch canker fungus, but *Fusarium* was not recovered from the conelets.

Cone harvest in the orchard in 1983 was 458 L (13 bu) compared with an average 7,040 L/yr (200 bu/yr) for the previous 5 yr (Catawba Timber Company records). Clone A-1-521 (34 ramets) produced a total of 422.8 L (12 bu), and clones B-1-561, C-1-515, H-18-3, M-3-6, and N-3-43 (total 98 ramets) produced 35.2 L (1 bu). Cones collected from clones A-1-521, B-1-561, C-1-515, H-18-3, M-3-6, and N-3-43 were separated into healthy and diseased categories, based on symptoms and recovery of *F. m.* var. *subglutinans* from the scales. Diseased cones from clone M-3-6 were stunted compared with noninfected cones. Symptoms on diseased cones from the other clones ranged from a terminal necrosis to resinous lesions on the cones, to misshapen cones with purple discoloration.

Radiographs, and subsequent seed culture, indicated that fungus-infected seeds were found in cones that were misshapen, discolored, or with resinous lesions. Infected seeds on the radiographs had slightly faded embryos and shrunken gametophytes compared with noninfected seeds (Fig. 1A and B).

Histology of infected tissues. Natural infections on branches looked similar to those previously described by Barrows-Broaddus and Dwinell (5) on loblolly pine seedlings artificially inoculated with the pitch canker fungus. In resinous lesions on live branches, parenchyma cells in the cortex, rays, and pith were attacked by the pathogen. Xylem tracheids were filled with resin. Peridermlike tissue in the cortex and reaction parenchyma in the xylem formed

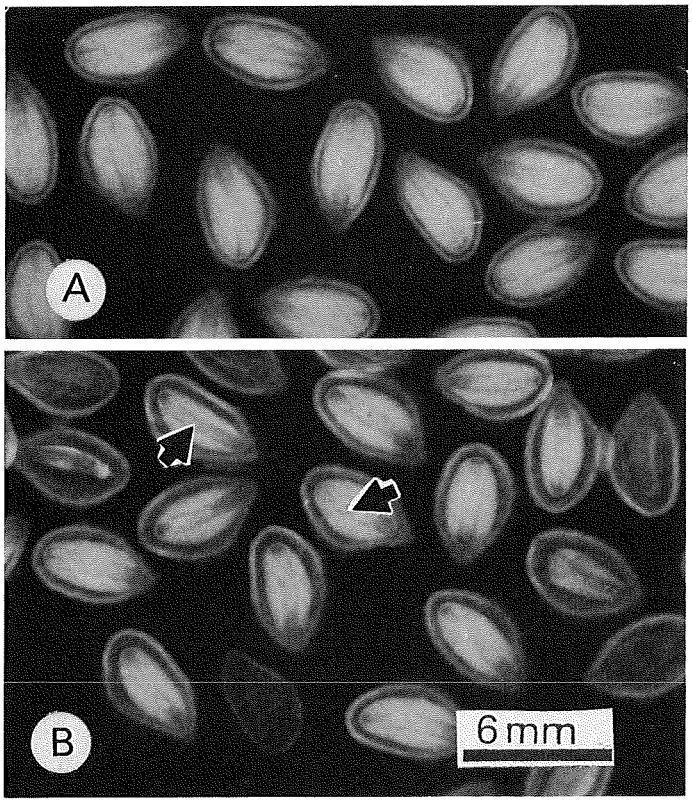

Fig. 1. Radiographs of seeds of loblolly pine infected with *Fusarium moniliforme* var. *subglutinans*. **A,** Radiograph of noninfected seeds with gametophyte and embryos intact. **B,** Radiograph of infected seeds. Gametophyte tissue (right arrow) is shrunken from the seed coat and the embryo (left arrow) appears slightly faded.

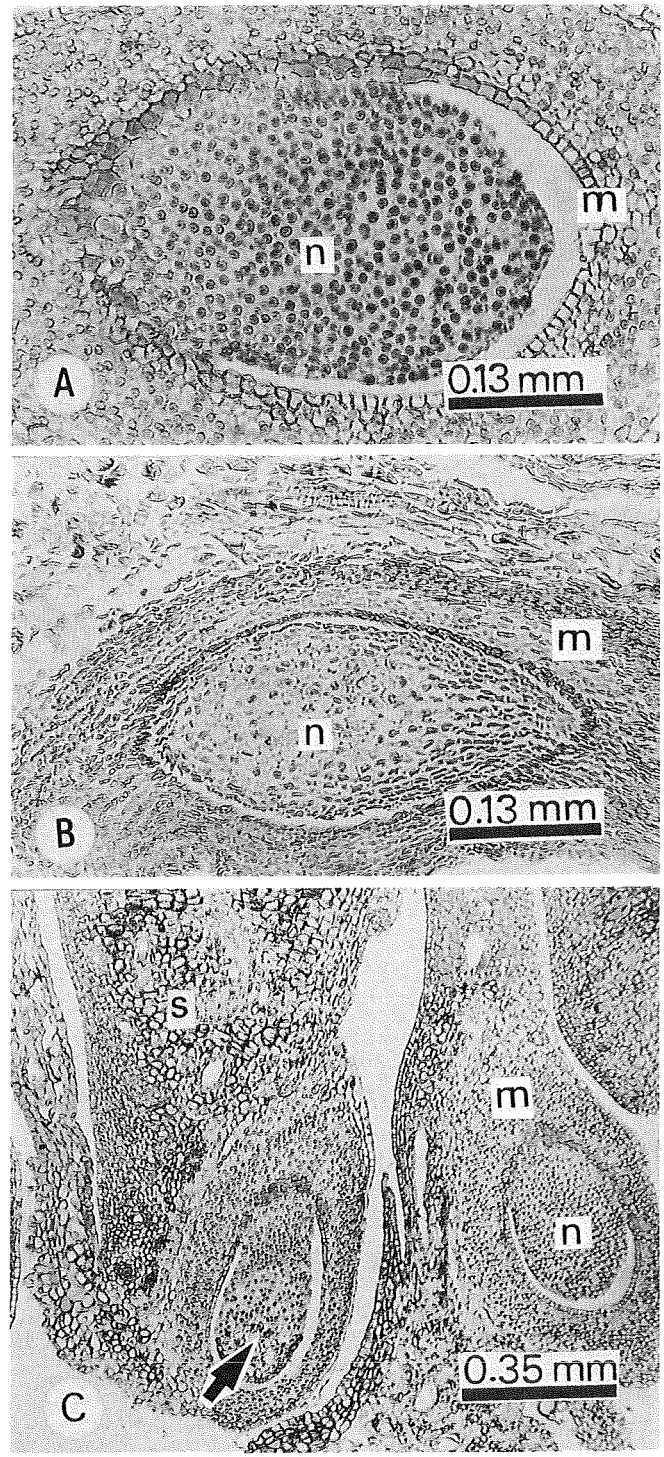

Fig. 2. Tangential sections of loblolly pine conelets infected with *Fusarium moniliforme* var. *subglutinans* 5 mo after pollination . **A,** Nucellus (n) and megasporangium (m) of a healthy ovule. **B,** Nucellus and megasporangium are shrunken in an ovule from a necrotic conelet of clone M-3-6. **C,** Scale (s) of an asymptomatic conelet where the ovule is necrotic in the nucellus adjacent to the micropyle (arrow).

by the host in response to infection were also attacked by the pathogen. The formation of callus-like cells at the margins of the lesion appeared to restrict spread of the pathogen above and below the visible lesion. In some instances, necrotic tissue (containing hyphae) within the lesion was sloughed off.

Conelets attached to infected branches also became infected when the parenchyma connecting the conelet to the branch was colonized by the pathogen. The ovules and surrounding tissue in necrotic conelets (clone M-3-6) were shrunken compared with noninfected conelets (Fig. 2A and B). In asymptomatic conelets colonized by *F. m.* var. *subglutinans*, the nucellus adjacent to the micropyle was necrotic in approximately one to three ovules per conelet (Fig. 2C).

The tissues of mature seeds colonized by *F. m.* var. *subglutinans* lacked cellular organization compared with tissues in noninfected seeds (Fig. 3A and B). Hyphae were observed in the embryo cavity (Fig. 3C) and between the gametophyte and the integument. Both embryo and gametophyte tissues were filled with hyphae (Fig 3D).

Seedling inoculation. There were significant differences among isolates in mortality of inoculated shoots of Virginia pine seedlings (range 14.8 to 84.3%, $P = 0.05$). The differences were by individual isolates, not by groups from particular sources (e.g., branches versus conelets versus seeds). For loblolly pine seedlings, there were no significant differences in mortality of inoculated shoots among the isolates (range 0 to 13.3%).

Lesions formed on all inoculated seedlings for both species. Lesions observed on live shoots at the end of the experiment ranged from no visible difference from a control wound (no wound enlargement, resin production, or discoloration), to typical pitch cankers (enlarged wound, copious resin flow, and purple discoloration of contiguous tissues). All lesions sampled from inoculated seedlings produced cultures of *F. m.* var. *subglutinans* following isolation. No shoots died in control treatments, and no isolates of *F. m.* var. *subglutinans* were recovered from the wounds.

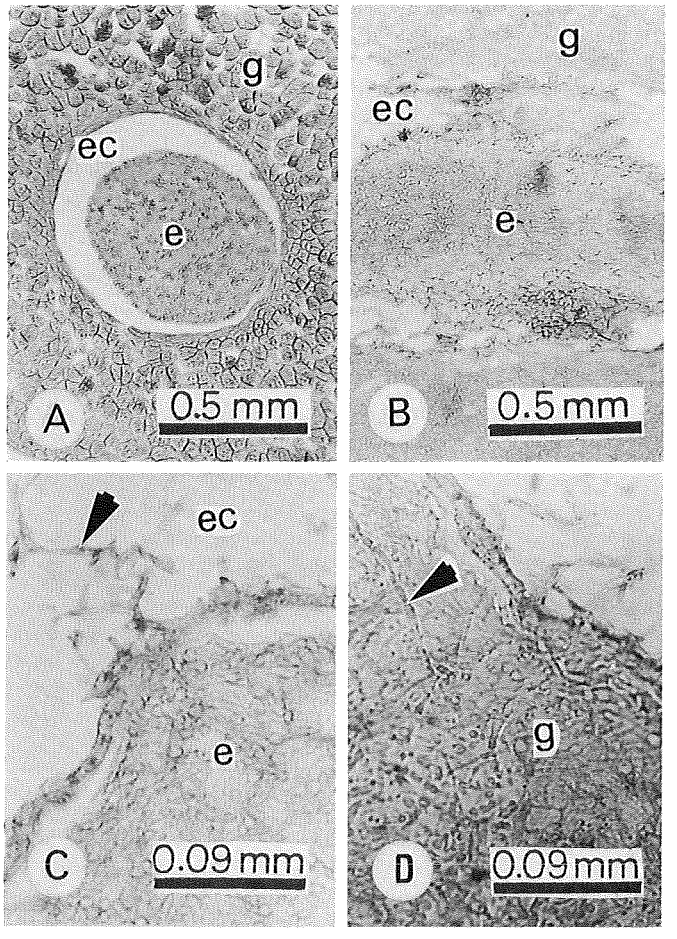

Fig. 3. Sections of seeds from mature loblolly pine cones infected with *Fusarium moniliforme* var. *subglutinans*. **A,** Embryo (e), embryo cavity (ec), and gametophyte (g) of a noninfected seed. **B,** Embryo and gametophyte of infected seed lack cellular organization. **C,** Hyphae (arrow) in the embryo cavity between the embryo and the gametophyte. **D,** Gametophyte tissue filled with hyphae (arrow). A is a transverse section; B, C, and D are tangential sections.

DISCUSSION

Incidence of *F. m.* var. *subglutinans* in branches, conelets, and cones varied by clone in the loblolly pine seed orchard. Ranking of the clones for susceptibility to branch dieback was correlated between two surveys covering a 9-mo period. The second survey indicated that disease incidence was declining. This disease pattern in loblolly pine seed orchards has been observed by others (7,10,12).

Isolates of *F. m.* var. *subglutinans* that cause deterioration of reproductive structures on loblolly pine belong to the same pathotype that causes pitch canker. The variation in virulence observed among the isolates tested was by individual isolates, not the source of the isolate, confirming earlier research (4). Therefore, an outbreak of branch dieback is a potential inoculum source for disease of reproductive structures. Conelets on infected branches may also become infected via connective tissues.

The percentage recovery of *F. m.* var. *subglutinans* from conelets was not correlated with recovery from their branches. There were instances of a higher percentage of the branches infected with *F. m.* var. *subglutinans* than the attached conelets, and vice versa. This indicates that outbreaks of shoot dieback and conelet deterioration can occur independently.

Radiographs of seeds colonized by *F. m.* var. *subglutinans* showed only slight differences in shape between infected and noninfected seeds. We found, however, that isolations from these seeds confirmed classification of seeds into infected and noninfected catagories based on the radiographs. Miller and Bramlett (14) found that slash pine seed lots with 50% of the seed classified as normal, based on radiography or cutting tests, were infected. In our cone collections, infected seeds were found only in cones with an abnormal shape or necrotic areas on the scales. After cone harvest, if abnormal cones are not culled before seed extraction, it may be difficult to later separate infected seeds from noninfected seeds. Gravity tables offer some degree of success in removing infected seeds from seed lots, but adjustments guided by radiographs must be made for each seed lot (9). Identification and culling of fungus-damaged cones during harvesting operations could increase the percentage of seed germination from improved loblolly pine seed orchards.

Results of this study demonstrated that *F. m.* var. *subglutinans* causes not only branch dieback, but also is involved in a diverse symptomatology in loblolly pine seed orchards. The pathogen infects specific vegetative and reproductive structures at different stages of maturity and produces a variety of symptoms. Because *F. m.* var. *subglutinans* infects internal seed tissues, it poses a potential threat to the quality of seeds from southern pine seed orchards. At this time, control measures are not available to prevent seed destruction by the pathogen. Determination of a complete disease cycle awaits further investigation.

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