Pathogenicity of Blue-Stain Fungi Associated with *Dendroctonus terebrans*

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ABSTRACT

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Three fungi (*Leptographium terebrantis, L. procerum*, and *Ceratocystis ips*) were consistently isolated from adult *Dendroctonus terebrans* and inner bark tissues around larval galleries in *Pinus thunbergiana* and *P. sylvestris*. Of the three fungi tested, only *L. terebrantis* was pathogenic to *P. thunbergiana* and *P. sylvestris* seedlings. The fungus caused inner bark necrosis and interruption of water conduction, as measured by xylem pressure potential and diffusive resistance, before foliar symptoms and blue stain developed in the xylem. Seedlings inoculated with *C. ips* and *L. procerum* developed necrosis of the inner bark tissues around the inoculation sites, but no effect on diffusive resistance or xylem pressure potential was detected.

Many species of bark beetles (Coleoptera:Scolytidae) maintain associations with specific blue-stain fungi (5,12,13). Most of these fungi belong to the genus *Ceratocystis* or to closely related hyphomyceteous genera (5,10, 12,13). Certain blue-stain fungi may contribute to the death of trees infested with bark beetles. Pathogenicity of some species to specific conifer hosts in the absence of the insect vector has been demonstrated (6,9,14,16,19).

Dendroctonus terebrans Oliv. has been associated with dying conifers in southeastern Massachusetts. This bark beetle attacks *Pinus thunbergiana* Franco and *P. sylvestris* L. in both forest and landscape environments (8) and vectors at least one blue-stain fungus, *Leptographium terebrantis* Barras & Perry (1). The purpose of this study was to identify the fungal associates of *D. terebrans* and determine the pathogenicity of these fungi to *P. thunbergiana* and *P. sylvestris*. Effects of these fungi on water relations of inoculated trees were also investigated.

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MATERIALS AND METHODS

Field isolations. Forty adult *D. terebrans* were collected from three naturally infested *P. sylvestris* in Falmouth, MA. The insects were removed with sterile forceps from their overwintering galleries in large buttress roots and maintained at 9 C in sterile polyethylene bags until isolations were performed (2–3 days). The insects were

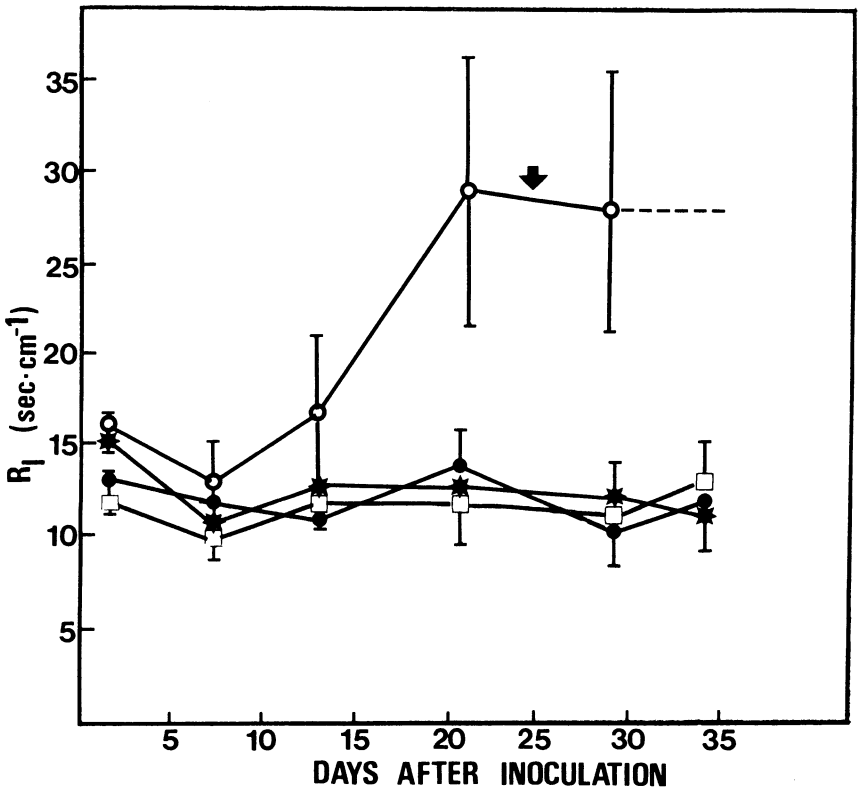

Fig. 1. Mean diffusive resistances (with standard errors) of 1-yr-old foliage of *Pinus thunbergiana* seedlings inoculated with sterile malt agar (closed circle), *Leptographium terebrantis* (open circle), *L. procerum* (closed star), and *Ceratocystis ips* (open square). Arrow indicates average date of symptom onset. Each point is the mean of eight seedlings.

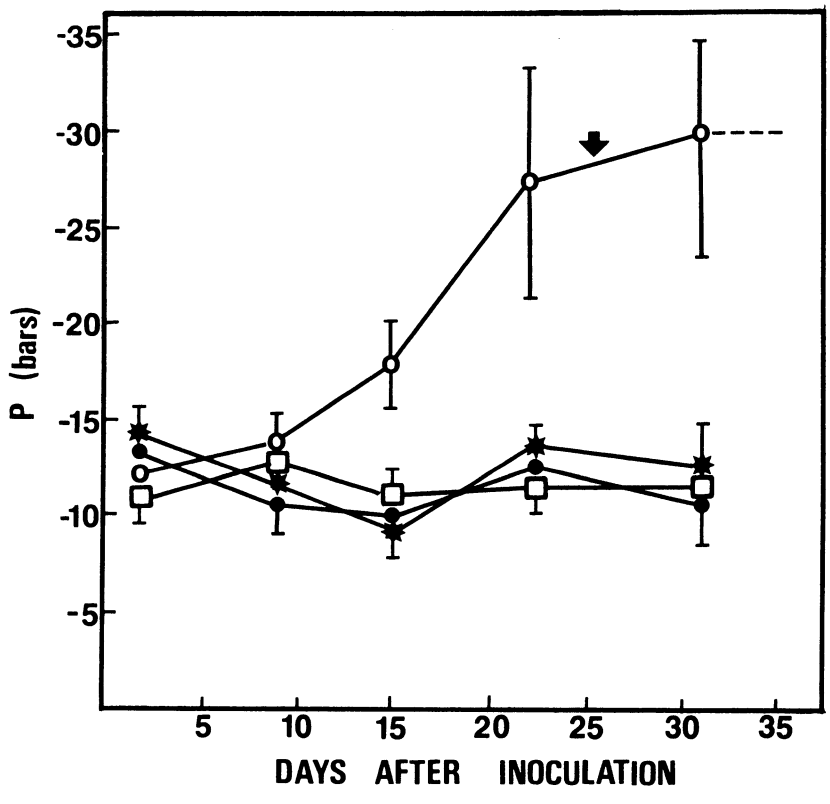

Fig. 2. Mean xylem pressure potentials (with standard errors) of 1-yr-old foliage of *Pinus thunbergiana* seedlings inoculated with sterile malt agar (closed circle), *Leptographium terebrantis* (open circle), *L. procerum* (closed star), and *Ceratocystis ips* (open square). Arrow indicates average date of symptom onset. Each point is the mean of eight seedlings.

rinsed in 10 ml of sterile distilled water, and 0.5-ml aliquots of rinse water were transferred to 2% malt-extract agar (MEA), 2% MEA acidified with 80% lactic acid to pH 4.5, and a selective medium for *Ceratocystis* sp. containing cyclohexamide and streptomycin (7).

Portions of blue-stained sapwood and discolored inner bark tissues were removed from larval feeding galleries in three *P. sylvestris* and four *P. thunbergiana* trees. The tissue pieces were surface-disinfested in 0.5% sodium hypochlorite solution for 3 min, rinsed in sterile distilled water, and plated on 2% MEA. All plates were incubated at 25 C for 2 wk.

Seedling inoculations. Four- to 6-yr-old *P. thunbergiana* seedlings were obtained from the Department of Public Works Nursery, Falmouth, MA. They were potted in 2-gal plastic containers filled with a 1:2:2 (soil:peat:sand) mix. Three-year-old *P. sylvestris* seedlings obtained from Musser Forest, Indiana, PA, were potted in 1-gal plastic containers with the same soil mix. All plants were kept outdoors and watered as needed.

Inoculations were conducted outdoors in the summer of 1984. Eighty trees of each species were divided into four treatment groups of 20 trees each and inoculated with sterile 2% MEA or one of the three fungi most commonly isolated from *D. terebrans* (*L. terebrantis, Ceratocystis ips* (Rumb.) C. Moreau, and *Leptographium procerum* (Kendrick) Wingfield (= *Verticicladiella procera* Kendrick)). The lower stem was surface-disinfested with 70% ethanol, and two vertical incisions were cut on opposite sides of the stem. The two wounds were each about 1 cm long and 3–5 mm wide and were made to produce a flap of bark under which inoculum (agar pieces from the margins of 8- to 10-day-old colonies or sterile 2% MEA) was placed. The bark was then replaced over the inoculum and the wounds wrapped with Parafilm. The wounded area constituted 30–45% of the stem circumference.

Xylem pressure potential (P) and diffusive resistance (R_l) were measured weekly on both 1-yr-old and current-year needles of eight trees in each treatment (two or three measurements for each age of foliage per seedling). After 8 wk, the monitored trees were harvested. The remaining 12 trees in each treatment were periodically harvested (one or two trees in each treatment every 4–6 days) after P and R_l were measured. Stems of harvested trees were dissected and the percentage of the stem circumference showing inner bark discoloration was recorded. Sections of apparently healthy and discolored inner bark and xylem tissues were aseptically transferred to 2% MEA for microbial isolations.

Water relations measurements. The pressure-chamber technique was used to

measure P (18). Individual needle fascicles were removed from each seedling, and the fascicle sheath was removed to expose the conducting tissue of the fascicle (17). The fascicle was inserted into the holding plate of a portable pressure chamber (PMS Instrument Co., Corvallis, OR). Xylem fluid exudation from the fascicle end was observed while under pressure through a stereomicroscope mounted on an adjustable arm. Three needle fascicles were measured for each seedling and needle age, and the readings (in bars) were averaged.

R_l was measured with a diffusive resistance porometer (Model LI-60, Lambda Instruments Corp., Lincoln, NE). Two needles (one needle fascicle) were placed in a sensor with an aperture of 1 × 2 cm. Three pairs of needles were measured for each seedling. The data were corrected for differences in temperature, and the mean was calculated.

All measurements were taken in full sun from 7:00 to 10:00 A.M. to minimize variation in P and R_l caused by weather conditions. Preliminary measurements showed little variation in P or R_l during this period.

RESULTS

Field isolations. *L. terebrantis* was recovered from all 40 adult *D. terebrans*. *L. procerum* and *C. ips* were recovered from 20 and 10 insects, respectively. There were no differences in fungal recovery on different media, although perithecia of *C. ips* failed to develop on the selective medium so that transfer to 2% MEA was necessary for identification. Colonies of undetermined *Sporothrix* sp. and *Graphium* sp. were isolated from 25 of 40 insects. Both genera are anamorphs of *Ceratocystis* sp., but their relationship to *C. ips* recovered from *D. terebrans* was not determined.

Only *L. terebrantis* was isolated from blue-stained sapwood. Brown discolored inner bark tissues adjacent to galleries contained *L. terebrantis, C. ips*, and *L. procerum*. Fruiting structures of these fungi were found emerging from the walls of larval feeding galleries. The distribution of fungi in tissues was similar in both *Pinus* species.

Seedling inoculations. Seven of eight *P. sylvestris* seedlings and six of eight *P. thunbergiana* seedlings inoculated with *L. terebrantis* and monitored weekly died within 8 wk of inoculation. Symptoms began as chlorosis of the current-year needles and developed 10–32 days (mean = 25 days) after inoculation in *P. sylvestris* and 17–30 days (mean = 24 days) after inoculation in *P. thunbergiana*. The inner bark of these necrotic seedlings was completely discolored, and the xylem was blue-stained in a zone extending 2–8 cm above and below the inoculation wounds. *L. terebrantis* was recovered from discolored inner bark

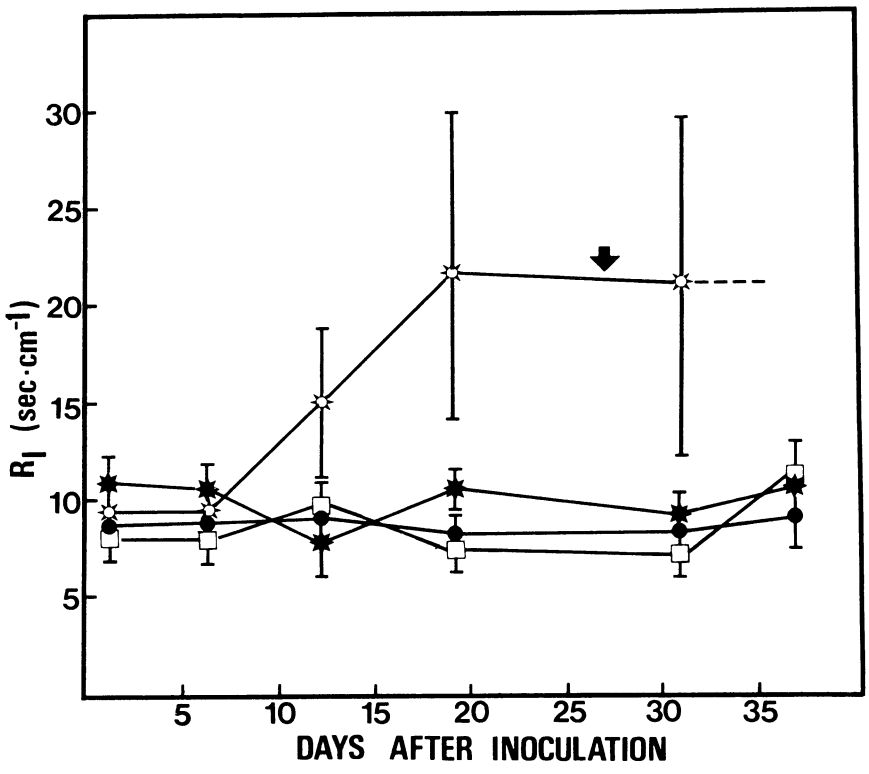

Fig. 3. Mean diffusive resistances (with standard errors) of 1-yr-old foliage of *Pinus sylvestris* seedlings inoculated with sterile malt agar (closed circle), *Leptographium terebrantis* (open star), *L. procerum* (closed star), and *Ceratocystis ips* (open square). Arrow indicates average date of symptom onset. Each point is the mean of eight seedlings.

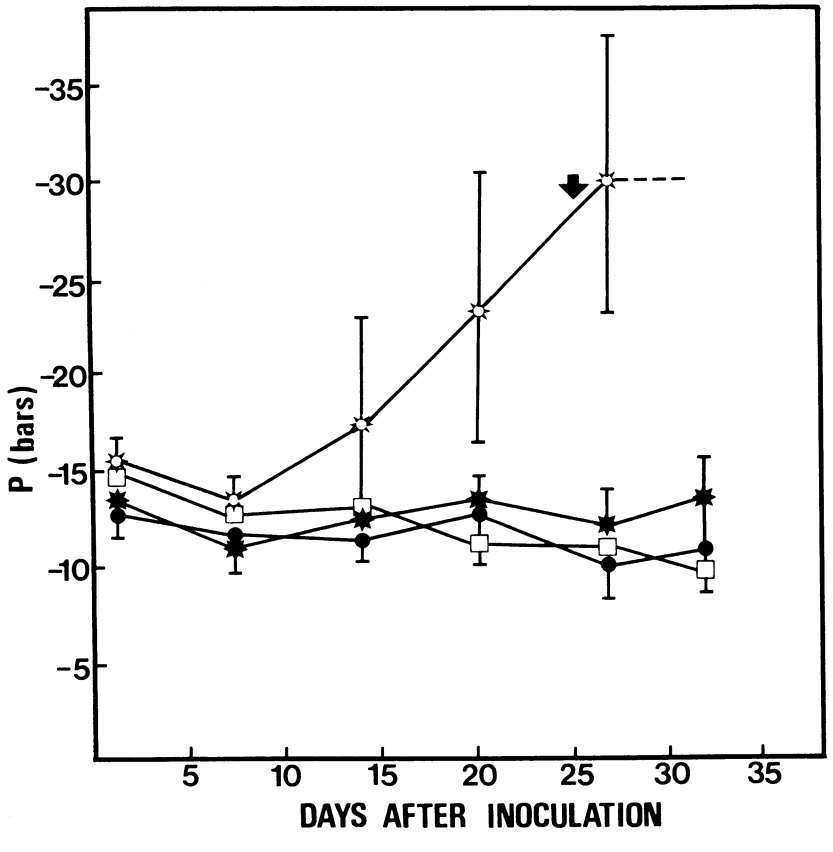

Fig. 4. Mean xylem pressure potentials (with standard errors) of 1-yr-old foliage of *Pinus sylvestris* seedlings inoculated with sterile malt agar (closed circle), *Leptographium terebrantis* (open star), *L. procerum* (closed star), and *Ceratocystis ips* (open square). Arrow indicates average date of symptom onset. Each point is the mean of eight seedlings.

and blue-stained xylem but not from unstained xylem tissue adjacent to the blue-stained areas.

The three asymptomatic seedlings in the *L. terebrantis* treatment showed resin-soaked, discolored areas in the inner bark and xylem at the points of inoculation after 8 wk. *L. terebrantis* was recovered from the remnant inoculum at the sites.

Seedlings of both *Pinus* species inoculated with *L. procerum, C. ips*, or sterile 2% MEA failed to develop foliar symptoms after 8 wk. Resin-soaked, discolored inner bark and xylem tissues were visible 0.2–0.3 cm around fungal inoculation sites. The discoloration was similar to that found around inoculation sites in asymptomatic, *L. terebrantis*-inoculated seedlings. Inoculated fungi were recovered from the inoculation sites. Discoloration and resinosis in seedlings inoculated with sterile 2% MEA were restricted to inoculated wound sites.

P became more negative and R_1 increased before symptom development in seedlings of both species inoculated with *L. terebrantis* (Figs. 1–4). Data from current-year and 1-yr-old foliage were similar; therefore, data from 1-yr-old foliage are presented. Changes in P and R_1 for the treatment as a group occurred 10–14 days before foliar symptom development. Standard errors reflect the seedling-to-seedling variability in the onset of changes in P and R_1. Figure 5 presents R_1 and P data from three *P. thunbergiana* seedlings inoculated with *L. terebrantis*. P and R_1 of trees inoculated with *C. ips* or *L. procerum* did not differ from levels found in control trees (Figs. 1–4).

Trees of both species, inoculated with sterile 2% MEA, *L. procerum*, or *C. ips* and harvested periodically, demonstrated no significant increase in percent stem discoloration during the experiment. The average percent stem discoloration of seedlings inoculated with sterile 2% MEA, *L. procerum*, and *C. ips* was 40, 52, and 45%, respectively, in *P. sylvestris* and 34, 50, and 33%, respectively, in *P. thunbergiana*. There were no significant differences in percent stem discoloration, R_1, or P among these treatments.

In trees inoculated with *L. terebrantis* and harvested periodically, discoloration reached 100% of the stem circumference. Seedlings of both species with 100% stem discoloration also had $R_1 > 35$ and P < -40 in both current-year and 1-yr-old foliage. This occurred before foliar symptom development in six *P. thunbergiana* seedlings and five *P. sylvestris* seedlings harvested 11–25 days after inoculation. All trees of both species with 100% inner bark discoloration, without the development of foliar symptoms, did not show any blue stain in the xylem. *L. terebrantis* was reisolated from the discolored inner bark tissues but not from the unstained xylem of these trees.

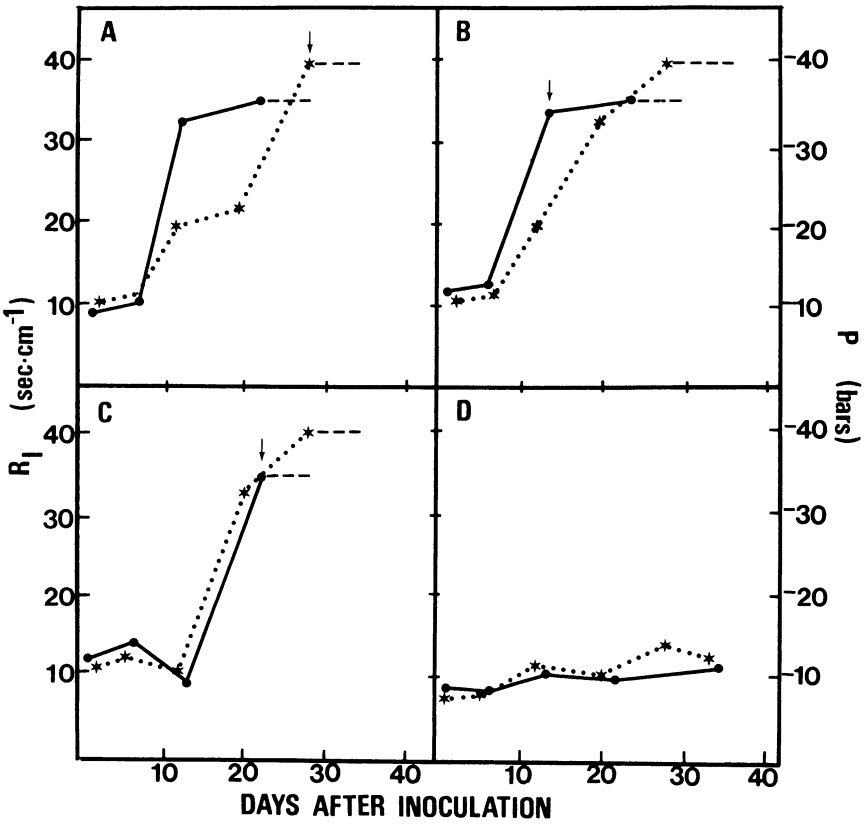

Fig. 5. Diffusive resistance (closed circle) and xylem pressure potential (closed star) of **(A–C)** three *Pinus thunbergiana* seedlings inoculated with *Leptographium terebrantis* and **(D)** one *P. thunbergiana* seedling inoculated with sterile malt agar. Each point is the mean of three measurements of 1-yr-old foliage. Arrows indicate date of symptom onset.

DISCUSSION

L. terebrantis was the primary blue-stain fungus associated with *D. terebrans* and was apparently responsible for the blue sapwood discoloration near *D. terebrans* galleries in *P. sylvestris* and *P. thunbergiana*. Adult insects also vector *L. procerum* and *C. ips*, but these fungi did not invade the sapwood extensively near larval feeding galleries. The microbial flora associated with *D. terebrans* is typical of bark beetles (5,13). *L. terebrantis, L. procerum*, and *C. ips* previously have been found associated with *Dendroctonus valens* Lec., a closely related bark beetle species (6,20).

Of the fungi tested in this study, only *L. terebrantis* killed *P. sylvestris* and *P. thunbergiana* seedlings. The pathogenicity of this fungus has been demonstrated in other *Pinus* species (6,20). *L. procerum* and *C. ips* also have been reported to be pathogenic to other *Pinus* species (2,3,11,14). The limited lesion development resulting from inoculation of these fungi may indicate that *L. procerum* and *C. ips* are weak pathogens of *P. thunbergiana* and *P. sylvestris*.

Symptom development in seedlings inoculated with *L. terebrantis* was similar to that in trees infested naturally with *D. terebrans* (8). In both cases, chlorosis in the youngest foliage was the first symptom observed. Mature trees became necrotic from 8 wk to a year after insect infestation (8), whereas seedlings in this study usually died in a shorter time. Results of this study suggest that *L. terebrantis* may play a role in the death of trees infested by *D. terebrans*.

L. terebrantis had colonized the xylem and inner bark of necrotic seedlings but was found only in discolored inner bark tissue of seedlings that had not yet developed symptoms, indicating that xylem invasion by this fungus occurs after invasion of the phloem and cambium. Discolored areas of inner bark adjacent to *D. terebrans* galleries are evident in recently attacked *P. sylvestris* and *P. thunbergiana* before blue stain develops in the sapwood (K. K. Rane and E. M. Bennett, *unpublished*). *L. terebrantis* has been isolated from these discolored zones. The pattern of *L. terebrantis* invasion in inoculated seedlings is similar to that observed in naturally infested trees.

Internal water stress developed in seedlings inoculated with *L. terebrantis*, as indicated by increased R_1 and more negative P. Seedlings showing internal water stress did not show blue-stained

xylem, nor was *L. terebrantis* isolated from the xylem, until foliar symptoms developed. Death of trees infested by bark beetles has been attributed to the blockage of xylem tracheids by resin (2) or aspiration of the tracheid pits by air or fungal hyphae (14,16). Recently, the death of Norway spruce (*Picea abies*) inoculated with *Ceratocystis polonica* has been attributed to sapwood colonization by the fungus (4,9). In our study, water stress appeared to develop in inoculated seedlings by disruption of the phloem and cambial tissues rather than by extensive invasion of the sapwood. Destruction of phloem and cambium has also been cited as the mechanism for pathogenicity of *C. dryocoetides*, a fungal symbiont of the bark beetle *Dryocoetes confusus* (15).

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