Transmission of *Leptographium procerum* to Eastern White Pine by *Hylobius pales* and *Pissodes nemorensis* (Coleoptera: Curculionidae)

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ABSTRACT

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Two weevils, *Hylobius pales* and *Pissodes nemorensis*, were studied as potential vectors of the fungus *Leptographium procerum*, the causal agent of procerum root disease. Transmission of the fungus to living trees was determined by caging field-collected and artificially contaminated *H. pales* and *P. nemorensis* on eastern white pine (*Pinus strobus*) seedlings for 24 hr and by caging artificially contaminated *H. pales* on 5-yr-old trees for 5 days. Feeding by artificially contaminated and field-collected adults of both weevil species resulted in transmission of *L. procerum* to eastern white pine seedlings and by artificially contaminated *H. pales* to 5-yr-old trees. To determine if transmission of the fungus during oviposition leads to contamination of the brood, field-collected adults of *H. pales* were allowed to oviposit on fresh white pine bolts. Oviposition by field-collected *H. pales* resulted in colonization of all of the bolts and contamination by *L. procerum* of 100% of the weevils that emerged over two subsequent generations. This study demonstrates the ability of *H. pales* and *P. nemorensis* to transmit *L. procerum* to seedling eastern white pines and the ability of *H. pales* to transmit the fungus to 5-yr-old eastern white pines. The contamination of weevils with *L. procerum* in eastern white pine bolts suggests that weevils may become contaminated during brood development in trees killed by the fungus.

Procerum root disease, caused by the fungus *Leptographium procerum* (Kendrick) M. J. Wingfield (4,5), mostly affects species of the genus *Pinus*. This disease has been found killing trees in Christmas tree plantations (3,4,6,8,10, 11) and is associated with growth loss of ozone-sensitive trees (5). Unlike other root diseases with a distinct disease center, such as Armillaria root rot, trees infected by *L. procerum* usually are scattered among healthy trees, which suggests an association with insects. Two weevil species, *Hylobius pales* (Herbst) and *Pissodes nemorensis* Germar, have been implicated as possible vectors (5,8,12,14). Lewis and Alexander (8) found that 64% of weevils recovered from 10 Christmas tree plantations in Virginia were contaminated with *L. procerum*. In another study, 48% of *H. pales* in Wisconsin and 50% of *H. pales* in Michigan were contaminated with *L. procerum* (14). *H. pales* and *P. nemorensis* also are capable of transmitting the fungus to fresh eastern white pine bolts (8). Both weevil species feed on branches and roots of pines (1,2), and feeding by *H. pales* has been found on stems of eastern white pines (*Pinus strobus* L.) during routine inspections of Christmas tree plantations (10). The weevils may become contaminated with the fungus during brood development in trees killed by *L. procerum* or by inoculation of the fungus into healthy stumps during bark beetle oviposition suggested by Witcosky et al (16) for blackstain root disease caused by *L. wageneri* (Kendrick) M. J. Wingfield. Despite reports of the association of weevils with procerum root disease, the ability of either weevil species to transmit *L. procerum* to living trees has not been clearly demonstrated.

The objectives of this study were to determine if *H. pales* and *P. nemorensis* transmit *L. procerum* to eastern white pine seedlings and 5-yr-old trees and to determine if oviposition by adults contaminated with *L. procerum* into uninfected bolts results in contamination of the next generation of weevils.

MATERIALS AND METHODS

Seedling transmission study. In 1989 and 1990, adults of *H. pales* and *P. nemorensis* were collected from baited pit-fall traps located in 15 Christmas tree plantations in southwestern Virginia. Weevils were collected from April to September in 1989 and March to September in 1990. An assay showed that 87 and 74% of individuals of *H. pales* and 21 and 14% of *P. nemorensis* were contaminated with *L. procerum* in 1989 and 1990, respectively (10). After the assay, both weevil species were placed together with fresh pine twigs in a 20 × 40 × 10 cm plastic box.

To establish the ability of *H. pales* to transmit *L. procerum* during feeding, weevils were randomly separated into two groups of 20—one group was contaminated artificially by allowing the weevils to walk for 30 s across the surface of malt-extract agar (MEA) in a petri dish containing conidia and conidiophores of *L. procerum*, and the second group received no treatment. Weevils were caged separately in polystyrene cups (296 cm^3) with lids for 24 hr on 2-yr-old eastern white pine seedlings growing in 2-L plastic pots. To prepare each cage, a hole was made in the center of the cup and lid to accommodate the seedling's stem, and both cup and lid were cut along one side to allow placement around the stem. The cup was placed around the stem at the soil line and taped together with masking tape. Modeling clay was placed around the stem at the bottom of the cup to keep the weevil from escaping through cracks between the cup and the stem. After the weevil was placed in the cup, the lid was secured on the cup and sealed with modeling clay around the stem.

To establish a standard to evaluate the efficiency of the weevils to transmit the fungus, 20 eastern white pine seedlings were inoculated directly with *L. procerum*. A 5 × 10 mm piece of bark was removed with a scalpel, and a similar-sized block of MEA colonized by the fungus was inserted and wrapped with Parafilm to prevent contamination. Twenty additional seedlings were inoculated with sterile MEA blocks as controls.

All trials were replicated three times in 1989—twice in July and once in September. The seedlings were held in a greenhouse for 6 mo or until disease symptoms developed. On examination, the foliage and roots of each seedling were removed, and the portion of the stem fed on by the weevil was surface-sterilized in a 10% sodium hypochlorite solution for 10 min. The bark, including the cambium, fed on by the weevil was removed and plated onto cyclohexamide-amended 1.5% malt extract agar (AMA) (9). The xylem portion of the stem was cut into 1-cm sections and plated separately on AMA. Inoculated

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and control seedlings also were debarked and the bark plated onto AMA. Total lesion length was measured, and four 1-cm sections from above and below the inoculation point were plated sequentially from the inoculation point onto AMA. After 3 wk, the isolation plates were examined for *L. procerum*.

The study was repeated in 1990 with several changes made. Only 10 seedlings were used in each treatment, the sterile MEA dish from control inoculations was examined for possible contamination by *L. procerum* after 3 wk, and seedlings were examined after 3 mo in the greenhouse. Trials with *H. pales* were replicated five times—three times in May and twice in June 1990. In addition, trials were conducted with *P. nemorensis* and replicated three times—twice in May and once in June 1990. For trials with *P. nemorensis*, only seven seedlings were used per treatment.

Differences between treatments were analyzed by calculating the chi-square value for each trial using a 4×2 contingency table. Where treatment interaction was significant, the treatments were analyzed using a 2×2 contingency table.

Transmission to 5-yr-old trees. To test the ability of *H. pales* to transmit *L. procerum* to older eastern white pines, individuals of *H. pales* were contaminated artificially with the fungus and caged on six 5-yr-old trees. The trees were approximately 1.25–1.75 m high and spaced 1.8 × 1.8 m apart in a 0.25-ha Christmas tree plantation on the Virginia Polytechnic Institute and State University campus. Weevils were contaminated with the fungus as described and two weevils were caged per tree at the root collar for 5 days. Cages consisted of 10 × 10 × 8 cm plastic containers with lids that enclosed the root collar of each tree. Any space between the cage and the stem was sealed with modeling clay.

To evaluate the efficiency of the weevils in the transmission of *L. procerum*, four 5-yr-old pines were inoculated with the fungus by removing a bark plug with a 10-mm cork borer from the four cardinal directions around the base of each tree, approximately 10 cm above the ground. An equivalent sized plug of MEA colonized by *L. procerum* was inserted into the holes and the area was wrapped with Parafilm. Four other trees were control-inoculated using sterile MEA plugs, and another four trees were retained as untreated controls.

A detailed examination of the trees was made after 5 mo. The trees were delimbed and topped 1 m aboveground before excavation of the stump. In the laboratory, the root collar area of each tree was surface-sterilized with 70% EtOH and the bark, including the cambium, from the areas fed on by the weevils was removed and plated onto AMA. The debarked area was surface-sterilized with 70% EtOH and sapwood samples were taken from the root collar with a 10-mm cork borer with preference given to areas with evidence of weevil feeding. Bark samples also were taken from trees inoculated with *L. procerum* and control-inoculated trees and plated onto AMA. Total lesion length was measured and isolations were taken from two of the four inoculation points made at the base of the trees. Four 1-cm samples from above and below the inoculation point were taken with a 10-mm cork borer and plated sequentially from the inoculation point onto AMA. Untreated control trees were debarked, and bark and sapwood samples were taken randomly from the area 10–15 cm above the root collar. The isolation plates were examined for conidiophores of *L. procerum* after 3 wk.

Brood development. The procedure of Speers and Cody (13) was modified to rear *H. pales*. Field-collected individuals of *H. pales* were caged in a clear plastic box (20 × 40 × 10 cm) together with three split billets (20 cm long and 5–8 cm in diameter) of eastern white pine. The weevils were allowed to oviposit on the billets for 1 wk and the billets were replaced weekly for 4 wk. When removed from the weevils, the billets were placed in another clear plastic box (20 × 40 × 10 cm) with moistened paper towels and incubated at 22–24 C. The billets were examined after 4 wk for *L. procerum* by culturing pieces of wood or by observing the fungus growing directly on the billets.

Newly emerged adults were assayed for contamination with *L. procerum* by placing them on AMA plates for 24 hr. The weevils then were caged with pine billets using the technique described above and the billets were changed weekly for 3 wk. When removed from the weevils, the billets were placed in a clear plastic box (20 × 40 × 10 cm) with moistened paper towels and incubated at 22–24 C. After 4 wk, transmission of *L. procerum* was determined by observing conidiophores of the fungus growing directly on the billets. On emergence, the second generation weevils were placed on AMA to determine whether they were contaminated with *L. procerum*.

RESULTS

Seedling transmission study. For all trials in 1989, feeding by the artificially contaminated and field-collected *H. pales* resulted in the transmission of *L. procerum* to 92 and 58% of the eastern white pine seedlings, respectively (Table 1).

Individuals of *H. pales* fed on the seedlings by consuming the bark and cambium. The weevils did not feed by girdling the stem but fed in patches that sometimes continued for 1–2 cm vertically along the stem. Although none of the seedlings were killed, some were damaged considerably by weevil feeding. Several feeding wounds were resinous and not completely callused by the end of the test period, whereas others were completely callused over. *L. procerum* was recovered from all types of feeding wounds but not other portions of the stem.

Over the study period, 18% of the seedlings fed on by the field-collected weevils and 13% of those fed on by the artificially inoculated weevils died. The first seedling died 30 days after feeding occurred. All of the dead seedlings had obvious feeding damage, and the bark near the feeding damage was either pulpy or dried and adhered to the wood. In either case, the wood beneath the bark was stained brown and *L. procerum* was recovered from the dead seedlings.

In addition to *L. procerum*, the field-collected weevils also transmitted *Ophiostoma piceae* (Munch) Sydow & Sydow to 15% of the seedlings in the second replicate and to 75% in the third.

The wounds of seedlings inoculated agar colonized by *L. procerum* varied from being completely callused to callus production only at the lateral edges of the wound with a vertical lesion extending from 0.2 to > 2 cm in length. Five percent of the seedlings inoculated with *L. procerum* died before the end

Table 1. The number of seedlings from which *Leptographium procerum* was recovered after feeding by artificially infested or field-collected *Hylobius pales* and inoculation with agar colonized by *L. procerum* or sterile agar in 1989 and 1990

Year and trial	No. of trees tested	Artificially infested weevils	Field-collected weevils	Agar colonized by *L. procerum*	Sterile agar
1989					
1	20	20 a[z]	11 b	20 a	3 c
2	20	17 ab	16 b	20 a	10 c
3	20	17 a	7 b	20 a	6 b
1990					
1	10	10 a	5 b	10 a	0 c
2	10	10 a	8 b	10 a	0 c
3	10	9 ab	8 b	10 a	0 c
4	10	10 a	7 b	10 a	0 c
5	10	10 a	6 b	10 a	0 c

[z]Values followed by the same letter, within rows, are not significantly different ($P = 0.05$, chi-square test).

of the 6-mo incubation period. At the point of inoculation, the bark was pulpy or dry and adhered to the stem with a brown stain in the wood.

Wounds of the control-inoculated seedlings were completely callused, but *L. procerum* was recovered from between 15 and 50% of the plants (Table 1). The fungus was recovered from the region next to the inoculation point, suggesting that the inoculation instruments had become contaminated.

In 1990, for all trials, feeding by artificially contaminated and field-collected individuals of *H. pales* resulted in the transmission of *L. procerum* to 98 and 70% of the seedlings, respectively (Table 1).

In the three trials with *P. nemorensis*, artificially contaminated individuals transmitted *L. procerum* to 100% of the seedlings on which they fed. Field-collected *P. nemorensis* transmitted the pathogen to 14, 43, and 29% of the seedlings in trials 1–3, respectively.

Feeding wounds created by *P. nemorensis* were smaller than those caused by *H. pales*. Individuals of *P. nemorensis* fed by chewing a small hole in the bark, inserting their beaks, and consuming the cambium under the bark. Feeding was in patches along the seedling stem. After 3 mo, many of the feeding wounds were still resinous and yielded *L. procerum*.

Feeding by field-collected *H. pales* or field-collected *P. nemorensis* also resulted in transmission of *O. piceae* to 28 and 38% of the seedlings, respectively.

Wounds of seedlings inoculated with agar colonized by *L. procerum* varied from being completely callused to a vertical lesion extending from 0.2 to >2 cm in length. Only three of the seedlings inoculated with *L. procerum* died during the 3-mo incubation period.

L. procerum was not recovered from any of the control-inoculated seedlings or the MEA plates used for control inoculations. All wounds of the control seedlings were completely callused, and none of the seedlings died during the study.

Transmission to 5-yr-old trees. The artificially contaminated weevils fed at the root collars of all of the trees on which they were caged, and *L. procerum* was recovered from all of these trees. The initial feeding wounds occurred as ragged oval-shaped pits in the bark into the cambium and occasionally extended to the sapwood. Feeding wounds often were close together with one to three feeding pits per square centimeter. These wounds were similar to those observed on eastern white pines in Christmas tree plantations (10).

Wounds created to inoculate the cambium of trees with agar colonized by *L. procerum* were callused, but resin was actively flowing from all wounds after 5 mo. There was no discoloration of the sapwood generated after inoculation, but sapwood present at the time of inoculation was resinous vertically to 2–5 cm from the inoculation point. The fungus was recovered from all of the trees inoculated with *L. procerum* and up to 3 cm from the inoculation point, which indicated that the fungus was actively growing.

None of the trees on which *H. pales* had fed or were inoculated with *L. procerum* showed foliar symptoms of procerum root disease when harvested. This suggests that trees can be infected with *L. procerum* longer than 5 mo before visual symptoms develop.

Wounds of the control-inoculated trees were completely callused, although the wounds of two trees were still resinous. Resinous areas in the sapwood present at the time of control inoculation extended vertically 1–2 cm in length from the inoculation point. *L. procerum* was not recovered from any of the control-inoculated trees, nor was it recovered from any of the untreated controls.

Brood development. Over a 28-day period, 23 weevils emerged from the billets on which the field-collected *H. pales* had oviposited. The first weevils emerged 55 days after the first billets were removed. All of the weevils were contaminated with *L. procerum*. The fungus was observed sporulating on 11 of the 12 billets at feeding sites, in larval galleries, and in pupal chambers. Bark and wood cultures from the remaining billet yielded *L. procerum*.

Eleven weevils emerged from the billets oviposited on by the first generation colony. All of the second-generation weevils were contaminated with *L. procerum* and five also were contaminated with *O. piceae*.

DISCUSSION

This study demonstrates that *H. pales* and *P. nemorensis* can vector *L. procerum*. Field-collected individuals of *H. pales* and *P. nemorensis* and those artificially contaminated with *L. procerum* fed on eastern white pine seedlings and made wounds suitable for transmission of the pathogen. Artificially contaminated *H. pales* also transmitted *L. procerum* to 5-yr-old trees. The ability of the artificially contaminated *H. pales* to feed on the rough bark of 5-yr-old eastern white pines confirms field observations that the weevils will feed at the base of mature Christmas trees (10).

Field-collected individuals of *P. nemorensis* transmitted *L. procerum* to fewer seedlings than did field-collected *H. pales*, but this was expected as field-collected individuals of *P. nemorensis* were contaminated less frequently with the fungus (10).

The absence of foliar symptoms on the 5-yr-old pines also was expected. Wingfield (15) observed no foliar symptoms 12 mo after the inoculation of 15-yr-old eastern white pines with *L. procerum*, and Horner (3) noted similar findings 12 mo after inoculating sapling loblolly (*P. taeda* L.), Scots (*P. sylvestris* L.), and eastern white pines with the fungus. From work on a related study, we suspect a long latent period and that foliar symptoms only become apparent during the latter stages of disease development (11).

The following rules of proof for insect transmission of a plant pathogen have been proposed by Leach (7): 1) insects are commonly associated with diseased plants; 2) insects visit and create infection courts in susceptible hosts suitable for infection; 3) insects carry inoculum of the pathogen in the field; and 4) insects successfully transmit the pathogen under laboratory conditions. This study completes Leach's postulates for *H. pales* and *P. nemorensis* as vectors of *L. procerum* by fulfilling the fourth postulate and providing evidence for the second.

Other studies have shown that *H. pales* and *P. nemorensis* are associated with diseased trees (4,5,11). The recovery of *L. procerum* from individuals of *H. pales* and *P. nemorensis* collected in nature has been reported by several authors (5,8,10, 12). Individuals of *H. pales* and *P. nemorensis* commonly are found in Christmas tree plantations (1,2,6,8, 10,14), and feeding wounds created by adult *H. pales* resemble feeding wounds observed in the field (10). Thus, numerous opportunities exist for the insects to visit healthy plants under conditions suitable for transmission of the pathogen.

O. piceae also was transmitted to seedlings by field-collected *H. pales* and *P. nemorensis*. Transmission of this fungus by insects to eastern white pine or other species of pines has not been reported previously. Transmission of *O. piceae* occurred both separately and in conjunction with *L. procerum*.

Field-collected weevils carried sufficient *L. procerum* to inoculate breeding material during oviposition with the result that the emerging brood was contaminated with the fungus. Moreover, the emerging brood also carried sufficient inoculum to inoculate new breeding material and contaminate a second brood. Thus, two generations of weevils raised under controlled conditions from field-collected weevils were contaminated with *L. procerum* and were able to transmit the fungus. These results demonstrate that *H. pales* contaminated with *L. procerum* can inoculate uninfected breeding material, such as stumps and slash, and that the emerging brood can be contaminated with the fungus.

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