Interactions of *Hylastes* Species (Coleoptera: Scolytidae) with *Leptographium* Species Associated with Loblolly Pine Decline

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ABSTRACT *Hylastes* spp. (Coleoptera: Scolytidae) were evaluated as potential vectors of *Leptographium* spp. fungi. Bark beetles were trapped from stands of loblolly pine, *Pinus taeda* L., exhibiting a range of decline symptoms in central Alabama. Under controlled conditions, field-collected adult *Hylastes salebrosus* Eichoff (Coleoptera: Scolytidae) and *Hylastes tenuis* Eichoff (Coleoptera: Scolytidae), which had been surface-sterilized and inoculated with *Leptographium terebrantis* Barras & Perry and *Leptographium serpens* (Goid.) Wingfield, transmitted the fungi into 100% of wounded and unwounded loblolly root sections with which they were confined. None of the sterilized and uninoculated beetles transmitted any *Leptographium* spp. to roots. Significantly more *H. salebrosus* and *H. tenuis* brood emerged from roots infected with *Leptographium* species than from sterile roots, indicating an enhancement of *Hylastes* reproduction.

KEY WORDS blue-stain, symbiosis

LOBLOLLY PINE, *Pinus taeda* L., decline symptoms have been observed with increasing frequency at various locations in Alabama, Louisiana, and South Carolina within 30–50-yr-old stands (Campbell and Copeland 1954; Lorio 1966; Brown and McDowell 1968; Oak and Tainter 1988; Hess et al. 1999a, b; Eckhardt 2003). The symptoms associated with loblolly pine decline are nonspecific and common to decline diseases in general, including littleleaf disease (Lorio 1966). They include short chlorotic needles, sparse crowns, reduced radial growth and, eventually, death (Lorio 1966; Hess et al. 1999a, b). Root systems of declining trees exhibit high rates of mortality and infection with the vascular stain fungi *Leptographium procerum* (Kendrick) Wingfield, *Leptographium terebrantis* Barras & Perry, *Leptographium lundbergii* Lagerb. & Melin, and *Leptographium serpens* (Goid.) Wingfield (Eckhardt 2003).

Ophiostomatoid fungi are consistently associated with bark beetle species (Paine et al. 1997), yet their roles in the life cycles of these insects remain poorly understood. Initial research on stain fungi–bark beetle interactions focused on the role of the beetles as vectors of these fungi, but pathogenicity tests of bark beetle-associated fungi have produced mixed results. Some stain fungi are capable of killing seedlings (Rane and Tattar 1987), whereas others are associated with the death of mature trees when accompanied by mass wounding and inoculation (Mathre 1964, Horntvedt et al. 1983). However, most inoculation experiments result in restricted host defensive reactions (Shrimpton 1973; Raffa and Berryman 1982, 1983a,b; Cook and Hain 1986, 1988; Paine and Stephen 1987; Raffa 1991; Lieutier et al. 1993, Raffa and Smalley 1995; Paine et al. 1997). Harrington (1993) has concluded that most bark beetle-associated stain fungi are not pathogenic and that, at most, they might weaken trees by lowering host resistance.

The ecological relationships of most stain fungi and bark beetles are unclear. In a few extensively studied systems, it seems that these fungi either have little effect on their insect hosts, or reduce their reproductive success (Barras 1970, Yearian et al. 1972, Klepzig and Wilkens 1997, Robins and Reid 1997, Klepzig et al. 2001) by reducing brood production and/or causing larval avoidance of stained regions. There also is evidence that, rather than killing trees, the stain fungi may reduce the exposure of colonizing beetles to plant defensive chemicals to tolerable levels (Hemingway et al. 1977, Christiansen and Horntvedt 1983, Raffa and Berryman 1983a). Based on this body of evidence, Raffa (1995) proposed that the net impact of ophiostomatoid fungi on their bark beetle vectors may vary with the conditions of the host, ranging from negative in dead logs to positive in healthy, well-defended trees. A direct test of this model is difficult with aggressive beetles because mass attacks are required to colonize trees, and the beetles cannot develop without

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Table 1. Sources of *Leptographium* isolates used for vector inoculation studies

Isolate	Isolate no.	Collection site	Host source
L. serpens	LOB-1-00-308	Talladega National Forest, Shoal Creek Ranger District, Alabama	Root insect (*H. salebrosus*) from infected *P. taeda*
L. serpens	LOB-1-00-532	Talladega National Forest, Shoal Creek Ranger District, Alabama	Root insect (*H. tenuis*) from infected *P. taeda*
L. terebrantis	LOB-1-00-312	Talladega National Forest, Oakmulgee Ranger District, Alabama	Root insect (*H. salebrosus*) from infected *P. taeda*
L. terebrantis	LOB-1-00-805	Talladega National Forest, Oakmulgee Ranger District, Alabama	Root insect (*H. tenuis*) from infected *P. taeda*

killing their hosts. However, less aggressive beetles (e.g., *Hylastes*) associated with compromised hosts are more easily studied. *Hylastes* spp. are root feeding bark beetles that typically attack unhealthy, declining, wounded, or even dead pines (Wood 1982; Klepzig et al. 1991, 1995; Jacobs and Wingfield 2001) and have been associated with decline diseases in pines (Klepzig et al. 1991, 1995; Jacobs and Wingfield 2001). Therefore, the purpose of this experiment was to determine the effectiveness of the vector and to clarify the roles (mutualistic, antagonistic, or otherwise) that *Leptographium* spp. play in the development of these beetles.

Materials and Methods

Fungal Isolation. Pitfall traps (adapted from Klepzig et al. 1991) for capturing crawling insects were used continuously for an 8-wk period on 15 plots (10 asymptomatic and five symptomatic) during spring 2000, to allow for best chance of capturing the emergence period of most bark beetles (Drooz 1985). One trap was placed at the center of each subplot for each of the 15 plots (three traps per plot). These traps consisted of 20-cm sections of 10-cm-diameter polyvinyl chloride plastic drain pipe with eight entrance holes equally spaced around the pipe circumference at one end. The interior of each trap was coated with a thin layer of liquid Teflon (Northern Products, Woonsocket, RI) to prevent the escape of the captured insects. Both ends were capped with removable plastic lids, and two holes were drilled in the bottom lid for drainage. The traps were buried, leaving entrance holes slightly above ground level. Each trap was baited with two 8-ml glass vials, one containing 95% ethanol and one containing steam distilled southern pine turpentine (Hercules), and two cut pine stems ≈5 cm in length by 2 cm in diameter. Trapped insects were collected weekly and placed in sterile polyethylene specimen cups and refrigerated at 4°C for no more than 3 d. These insects were identified and rolled nondestructively across 2% malt extract agar (MEA) and MEA containing 800 mg/liter cycloheximide and 200 mg/liter streptomycin sulfate (CSMA) (Hicks et al. 1980). Plates were incubated at 25°C under fluorescent lighting (460 μmol m^{-2} s^{-1}) for 2 wk and examined for fungal growth. Single-spore isolations were made and grown on MEA under a 12-h photoperiod (460 μmol m^{-2} s^{-1}) and placed on silica gel (Dhingra and Sinclair 1995) for long-term storage at 4°C for later identification to species. Cultures were then plated on MEA and grown in the dark for comparison to species described in Jacobs and Wingfield (2001). After identification, representative isolates were sent to M.J. Wingfield (FABI, Pretoria, South Africa) for confirmation.

Vector Study. Loblolly pine roots ≈5–7 cm in diameter (6.4-cm-diameter mean) were removed from 35- to 40-yr-old healthy pines at the Palustris Experimental Forest (Rapides Parish, LA) and tested for the presence of *Leptographium* spp. by plating root tissue on MEA and CSMA (Hicks et al. 1980) and visual examination. Only roots that were negative for *Leptographium* spp. were used in these experiments.

A total of 24 *Leptographium*-free root sections were cut 30 cm in length (adapted from Six and Paine 1998). Twelve root sections were drilled with entrance holes to facilitate and/or induce entry by the beetles, and 12 roots were left undrilled. Twenty-four roots (12 drilled and 12 undrilled) were divided per beetle species, *Hylastes salebrosus* Eichoff and *Hylastes tenuis* Eichoff. The severed ends of each root were dipped in paraffin to retard desiccation, and the root sections then were buried under moist, sterilized sand in plastic boxes (one root segment per box either drilled or undrilled). One hundred and twenty *H. salebrosus* and 120 *H. tenuis* were collected in pitfall traps (adapted from Klepzig et al. 1991), surface-sterilized with commercial bleach, ethanol, and distilled water solution [10:10:80 (vol:vol)] for 1 min, and gently rolled on CSMA to verify absence of viable *Leptographium* spp. propagules. Sixty *H. salebrosus* and 60 *H. tenuis* then were placed on growing cultures of *L. terebrantis* or *L. serpens* (Table 1) for ≈12 h to allow inoculum acquisition. Sixty inoculated and 60 uninoculated adults of each species were introduced into the plastic boxes containing the root sections (five males and five females per species per box) and covered with cheesecloth to prevent escape. Boxes were kept at 25°C under a photoperiod of 8:10 (L:D) h (56 μmol m^{-2} s^{-1}), and moistened with distilled H_2O every other day. Root sections were visually examined every 2–3 d to detect adult entrance. After 9 wk, roots were stripped to visually determine adult mating, larval survival, staining, and presence of fungal fruiting structures. Parent adult insects were removed and not included in determinations of brood production. Samples from galleries of inoculated and sterilized beetles were plated on CSMA and MEA to determine

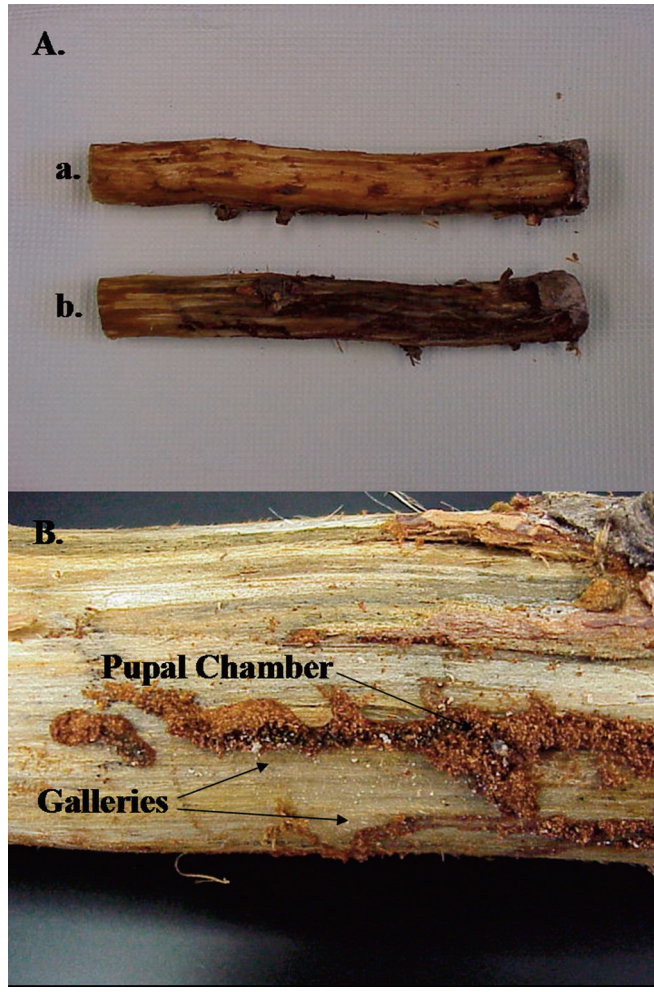

Fig. 1. (A) *P. taeda* root sections infested with *H. tenuis* root beetles. (a) Surface-sterilized, uninoculated beetles. (b) Beetles inoculated with *Leptographium* spp. Note extensive staining. (B) Root section showing *L. terebrantis* introduced by *H. salebrosus* growing in galleries and pupal chambers.

the presence or absence of *Leptographium* spp. and other fungi.

Data Analysis. A total of eight treatments were used, factorial combination, drilling versus insect versus fungi. The entire experiment was replicated twice. Number of emerging brood, pupae, and larvae were compared among treatments by using analysis of variance (ANOVA). Mean separations were conducted using Tukey's W. All analyses were performed using SAS (SAS Institute 2001).

Results

Fungal Recovery. *H. salebrosus* and *H. tenuis* both vectored *L. terebrantis* and *L. serpens* to 100% of the roots into which they were introduced. Neither fungus was found in root sections infested with sterilized vectors. *L. terebrantis* and *L. serpens* were also recovered from 100% of *H. salebrosus* and *H. tenuis* adults emerging from, and larvae feeding within, infested root sections. These root sections also exhibited the extensive staining typical of *Leptographium* infection (Fig. 1A). Fungi were recovered from entrance and exit holes, galleries, and pupal chambers (Fig. 1B) of roots receiving inoculated vectors but not from roots receiving sterile vector controls.

Insect Recovery. Because beetle reproductive success significantly differed by species of *Hylastes*, ($F = 86.56$; df = 1, 22; $P < 0.0001$), the two insect species were considered separately in subsequent analyses.

Neither the creation of wound courts ($F = 0.16$; df = 1, 22; $P < 0.07$) nor the species of *Leptographium* used ($F = 0.03$; df = 1, 22; $P < 0.87$), significantly affected the reproductive success of *H. salebrosus* (Fig. 2A). However, the presence of *Leptographium* spp. on *H. salebrosus* adults did significantly increase their reproductive success ($F = 691.74$; df = 1, 22; $P < 0.0001$); brood production was ≈2.5 times higher in *Lep-*

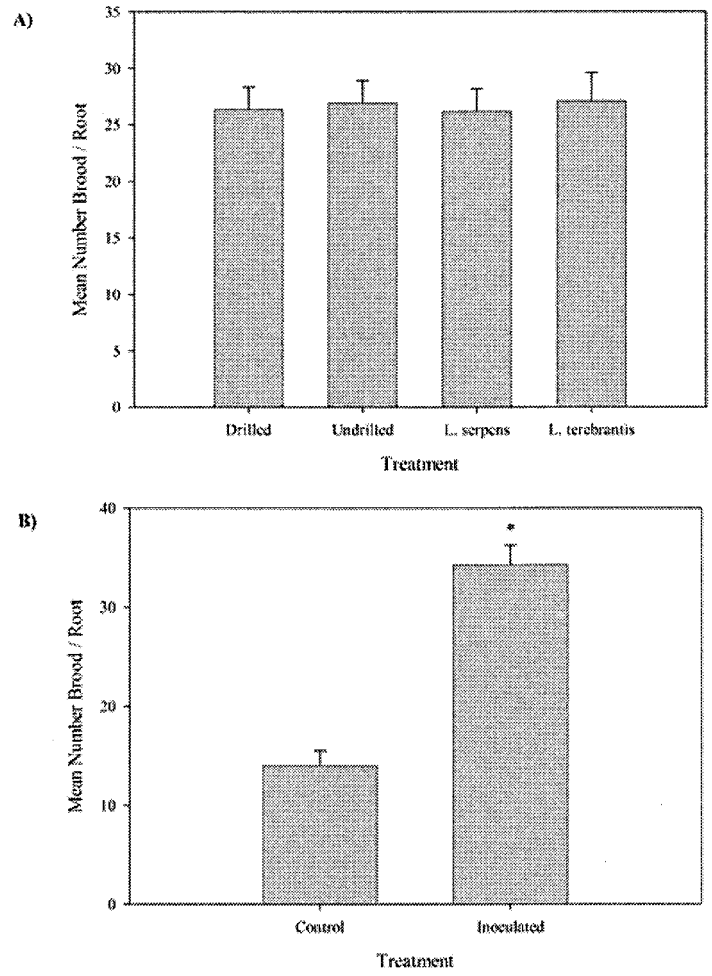

Fig. 2. (A) Effects of wound and fungal treatments on *H. salebrosus* brood production in *P. taeda* roots. Bars indicate standard error. (B) Effects of *Leptographium* spp. on *H. salebrosus* brood production in *P. taeda* roots. Bars indicate standard error. *, significant difference from control ($P < 0.0001$).

tographium vector-infected roots than in sterilized controls (Fig. 2B).

No significant difference in reproduction success on *H. tenuis* was seen between drilled or undrilled roots ($F = 0.17$; df = 1, 22; $P < 0.69$) or between the species of *Leptographium* used ($F = 0.05$; df = 1, 22; $P < 0.72$) (Fig. 3A). However, the presence of *Leptographium* spp. on *H. tenuis* adults did significantly increase their reproductive success ($F = 49.83$; df = 1, 22; $P < 0.0001$) by 19% (Fig. 3) over sterilized controls. Out of a subsample of 174 emerging *H. salebrosus* and 152 *H. tenuis*, 162 and 152 were females, respectively. These results coupled with those obtained by Klepzig (1994) with laboratory colonies of *H. porculus*, indicate the strong possibility of parthenogenic reproduction in this genus. *Wolbachia*, an endosymbiont, could also account for this observation. Infection by *Wolbachia* has been reported to cause cytoplasmic incompatibility, parthenogenesis, and feminization in arthropods (Bourtzis and O'Neill 1998) and recently has been reported in the coffee berry borer (Coleoptera: Scolytidae) (Vega et al. 2002).

Discussion

In this study, reproduction by two species of *Hylastes* was increased in the presence of *Leptographium* fungi. This is the first report that we are aware of in which stain fungi positively affected nonaggressive beetles. Raffa and Smalley (1995) reported that although phytopathogenic fungi can assist bark beetles in killing trees, trees respond to the presence of these fungi by accumulating allelochemicals to concentrations that adversely affect the beetle vector. It is possible under the conditions of this study that such an accumulation was prevented by the use of severed (versus intact) roots. Raffa (1995, 2003) suggested three possible, nonexclusive, mechanisms by which ophiostomatoid fungi could affect beetle populations: 1) Certain fungi may reduce host tree resistance

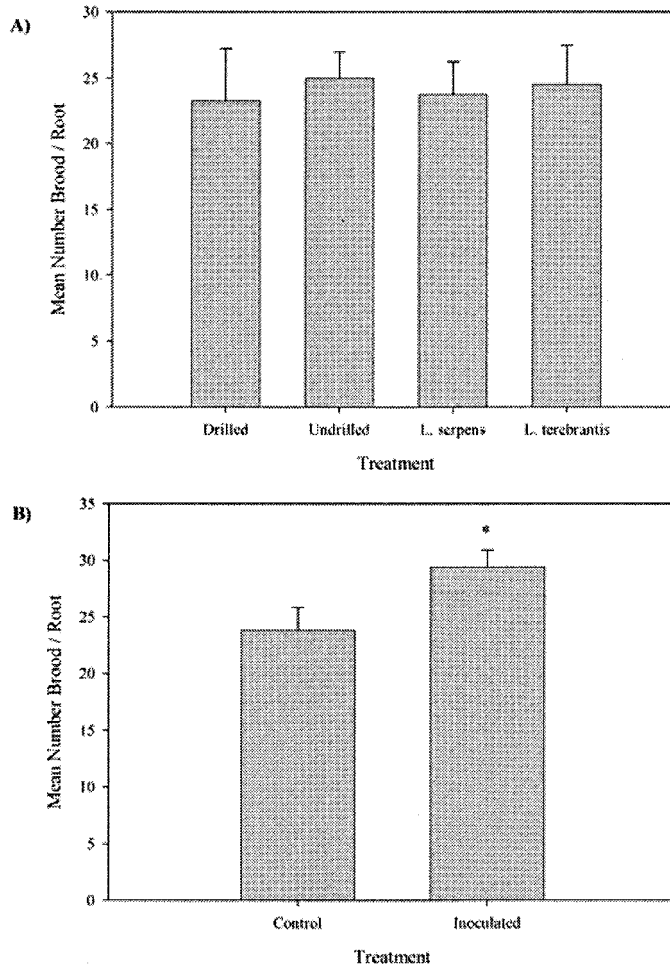

Fig. 3. (A) Effects of wound and fungal treatments on *H. tenuis* brood production in *P. taeda* roots. Bars indicate standard error. (B) Effects of *Leptographium* spp. on *H. tenuis* brood production in *P. taeda* roots. Bars indicate standard error. *, significant difference from control ($P < 0.0001$).

against bark beetles. For example, *Ophiostoma piliferum* is used as a biopulping agent, primarily due to its ability to degrade diterpene acids (Blanchette et al. 1992), which have allelochemical properties. 2) Some fungi may compete with developing larvae for host nutrients or otherwise interfere with brood development (Barras 1970, 1973; Ayres et al. 2000). The nitrogen content of phloem is ≈0.38% in healthy loblolly pine; therefore, bark beetles must concentrate dietary nitrogen by 16–26-fold (Hodges and Lorio 1969). Ayres et al. (2000) noted regions of high N concentration associated with colonies of mycangial fungi, perhaps because the hyphae of mycangial fungi extract N from phloem and concentrate it into the feeding chamber. In contrast, N concentrations were lower where *O. minus*, a blue stain fungus, grew. 3) Some fungi may compete with other fungi that either facilitate brood development (e.g., mutualistic mycangial fungi) or with those that compete with developing beetle larvae (antagonistic nonmycangial fungi) (Klepzig and Wilkens 1997).

The first mechanism offers the best explanation for the results presented here. Certainly, it did not seem that the *Leptographium* species competed with the insects within roots (mechanism 2). In this study, control roots did not contain contaminating fungi that could have affected results one way or the other (mechanism 3). Even in the root sections we used, levels of allelochemicals might be sufficient to interfere with *Hylastes* feeding and development in the absence of fungi. It is, perhaps, most likely that the *Leptographium* species inoculated into the roots increased beetle success by making the host material more suitable for their insect vectors, either by detoxification of the host chemistry, or by the production of metabolic by-products that the beetles found useful or nutritive. Further research is needed to determine whether one or both of these mechanisms contribute to the results we observed.

The vectoring of the two root fungi most commonly associated with loblolly pine decline by the two most common root-feeding bark beetles provides additional

evidence for the involvement of these bark beetle–fungus complexes in this disease syndrome. Both beetles were able to transmit fungi to wounded as well as unwounded roots. In addition, beetles of closely related species may be attracted to wounds or host volatiles associated with wounds (Rudinsky and Zethner-Moller 1967, Owen 1985, Witcosky et al. 1987, Phillips 1990, Klepzig et al. 1991, Hobson et al. 1993). Collectively, these data strengthen the putative role of *H. salebrosus* and *H. tenuis* as agents of loblolly pine decline.

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