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Competition between a biological control fungus, *Ophiostoma piliferum*, and symbionts of the southern pine beetle

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Abstract: A colorless isolate of *O. piliferum* was paired in a series of competitive interactions with three fungal symbionts of *Dendroctonus frontalis*, the southern pine beetle. Two of these fungi, *Ceratocystiopsis ranaculosus* and *Entomocorticium sp. A*, are considered to be mutualists of the southern pine beetle. The third fungal symbiont, *O. minus*, is considered to be an antagonist. I found strong evidence of differential competition between *O. piliferum* and all three symbionts. In primary and secondary resource capture contests on an artificial medium *O. piliferum* outcompeted all three fungi. In inoculations of natural substrate, *O. piliferum* outcompeted the two mutualists but did not outcompete *O. minus*. The ability of *O. piliferum* to outcompete beetle mutualists on both artificial and natural substrates indicates promise for this fungus as a biological control agent of the southern pine beetle. However, it may not be able to always prevent colonization by *O. minus* and the resultant discoloration of colonized wood.

Key Words: Cartapip, *Ceratocystiopsis ranaculosus, Dendroctonus frontalis, Entomocorticium, Ophiostoma minus*, SJB122.

INTRODUCTION

Interactions among fungi may be divided into three categories: mutualistic, neutralistic, and competitive (Rayner and Webber, 1984). Competitive interactions may be further subdivided into primary resource capture (interacting fungi compete to gain influence over a resource but do not directly challenge one another) and secondary resource capture (combative interactions). I am studying such interactions among a complex of fungi associated with the southern pine beetle (SPB), *Dendroctonus frontalis*, the most damaging insect pest of southern pines. In the process of chamber and gallery construction within the inner bark of host trees (Nelson, 1934), SPB commonly inoculate several associated microbes into their tree hosts (Bramble and Holst, 1940). I have limited my studies to the three major associates, among many, of SPB. Female beetles carry two fungi, *Ceratocystiopsis ranaculosus* and *Entomocorticium sp. A.* [*E. sp. A.* (Hsiau, 1996) formerly SJB122 (Barras and Perry, 1972)] within a specialized fungus bearing mycangium. These mycangial fungi ramify throughout the galleries of developing larvae and produce large ambrosia type spores which the SPB larvae are presumed to consume for their nutritional benefits (Barras and Perry, 1972; Barras, 1973; Goldhammer et al., 1990; Coppedge et al., 1995). These two fungi are considered to be mutualists of SPB.

Ophiostoma minus is carried phoretically on the exoskeleton of both male and female SPB and by an associated mite (Bridges and Moser, 1983; Rumbold, 1931). This fungus, once inoculated by attacking beetles into the tree, causes a characteristic, economically deleterious, blue-stain in wood. Although *O. minus* may assist SPB in killing the tree (Basham, 1970; Bramble and Holst, 1940; Caird, 1935; Mathre, 1964a; Nelson, 1934), it eventually competes with the SPB for uncolonized host tissue Barras 1970; Franklin, 1970) and is considered to be an antagonist of SPB.

Because bark beetles and their associates (Bridges and Moser, 1983) serve as the only effective means by which stain fungi can gain access to new host tissue (Dowding, 1969), fungi must compete for access to host tissue colonized by these insects. In previous work (Klepzig and Wilkens, 1997), we considered competitive interactions among the three major associates of the SPB, and found evidence for differential competition with the SPB antagonist outcompeting the SPB mutualists. In this study I sought to test the hypothesis that these slow growing mutualistic fungi might be vulnerable to competition from another rapidly growing, but nonstaining, fungus.

Ophiostoma piliferum is transported to new host material by bark beetle vectors (Perry, 1991) where it causes a grey to black stain within infected conifer sapwood (Seifert, 1996). A colorless isolate of *O. piliferum* (marketed as Cartapip™ by Clariant Corporation, Charlotte, North Carolina) has been used to degrade pitch in wood chips (Blanchette et al.,

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1992). This isolate can tolerate the allelochemicals found in pine phloem (Klepzig et al., 1996) without staining the wood. Using this isolate, I sought to answer the following questions regarding its suitability as a biocontrol agent of SPB and its fungal associates: (i) Does differential competition occur between *O. piliferum* and *O. minus, E. sp. A.,* and *C. ranaculosus*?, and (ii) What are the primary and secondary resource capture capabilities of *O. piliferum* relative to *O. minus, E. sp. A.,* and *C. ranaculosus*?

MATERIALS AND METHODS

Fungal cultures.—I obtained a white mutant isolate of *O. piliferum* (Cartapip™ 97, Clariant Corporation, Charlotte, North Carolina). All the other fungi used in this study were isolated in 1995 from adult SPB collected from pine plantations in Louisiana (Klepzig and Wilkens, 1997) and maintained on agar slant cultures at 0 C. All four isolates used in these experiments have been deposited in the culture collections of Tom C. Harrington, Iowa State University, Ames, Iowa, and Harold H. Burdsall, USDA Forest Service, Forest Products Laboratory, Madison, Wisconsin.

Differential competition.—I used a modified de Wit replacement series (Klepzig and Wilkens, 1997) to determine if differential competition occurred among *O. piliferum* and *O. minus, E. sp. A,* and *C. ranaculosus.* I introduced varying proportions of inoculum onto plates of MEA (Difco Laboratories, Detroit, Michigan). Disks (0.5 cm diam) of colonized agar were aseptically removed from actively growing colonies of each of two fungal species and inoculated onto plates of MEA in ratios of 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0. Fungal disks were randomly placed within a 4 × 5 cm grid on the plates such that there were a total of 20 disks per plate. Each inoculum proportion treatment was replicated 10 times per fungal comparison. All plates were sealed with Parafilm® and incubated at 20 C in darkness. Because of the different morphologies of the various fungal colonies, I was able to trace the areas occupied by each fungus one wk after inoculation (2 wk after inoculation for *O. piliferum* vs. *E. sp. A*). The areas occupied by each fungus were then measured with a digital planimeter.

I compared the areas occupied in the various treatments and plotted the mean area occupied by each fungus against its initial inoculum proportion. In a de Wit replacement series, the absence of differential competition is indicated by a linear relationship between final representation in the population and initial inoculum proportion for each of the competing organisms. Differential competition is indicated when there is a significant positive deviation from linearity for one organism and a significant negative deviation from linearity for the other. I tested for deviations from linearity in the relationships between area colonized by each species and its initial inoculum proportion using a previously described model (Wilson and Lindow, 1994) in which an analysis of variance (ANOVA) is performed on log transformed means. I also calculated relative crowding coefficients (RCC) (Novak et al., 1993) for all the fungi in pairwise comparisons. The RCC of a species, e.g. *O. piliferum,* relative to another species, e.g. *O. minus,* may be calculated using the following formula:

RCC = [(area occupied by *O. piliferum* at 1:1)/(area occupied by *O. minus* at 1:1)]/[(area occupied by *O. piliferum* at 1:0)/(area occupied by *O. minus* at 1:0)]

An RCC of 1 indicates equal competition between species. An RCC greater than one would indicate, in this case, that *O. piliferum* is outcompeting *O. minus,* and an RCC of less than one would indicate the opposite (Novak et al., 1993).

Primary resource capture.—I quantified the primary resource capture capabilities of *O. piliferum* relative to *O. minus, C. ranaculosus,* and *E. sp. A* by exposing uncolonized substrate (MEA) to mixed fungal inoculum using techniques described previously (Klepzig and Wilkens, 1997). Mycelial plugs (0.5 cm diam) of fungi were inoculated at opposite edges of a plate of MEA. I challenged *O. piliferum* with *O. minus, E. sp. A,* or *C. ranaculosus* in pairwise combinations on plates of MEA (n=10, for all combinations). All plates were sealed with Parafilm, inverted, and incubated at 20 C in darkness. After 3-d intervals, and at 2-d intervals for 24 d thereafter, the areas occupied by each fungus were traced on the bottom of each plate. At the termination of the experiment, these areas were traced, as describe above, and measured with a digital planimeter. I calculated and compared the mean areas occupied by each fungus in each treatment using the Least Squares Means Procedure in SuperANOVA (Abacus Concepts, 1989).

Secondary resource capture.—I also quantified the ability of *O. piliferum* to colonize substrate that had already been colonized by the SPB-associated fungi. Again using previously described methods (Klepzig and Wilkens, 1997), one mycelial plug of *O. piliferum* was placed near the center of a one wk old colony of *O. minus* in each of ten 9-cm petri plates of MEA. This process was repeated with *E. sp. A.* and *C. ranaculosus* in place of *O. minus.* I then conducted the same experiment in reverse by placing a mycelial plug of *E. sp. A., C.ranaculosus,* or *O. minus* near the center of a one wk old colony of *O. piliferum* (n=10,

for all combinations). All plates were sealed with Parafilm® and the initial areas occupied by each fungus in each pairwise combination measured. The plates were then inverted and incubated at 20 C in darkness. The areas occupied by each fungus at 1 and 2 wk after inoculation were measured as describe above. I calculated and compared the mean areas occupied by each fungus at 0, 1 and 2 wk into the experiment using the Least Squares Means Procedure in SuperANOVA (Abacus Concepts, 1989).

Primary resource capture—natural substrate.—I used freshly harvested loblolly pine (*Pinus taeda* L.) logs in a modified version of the primary resource capture experiment described above. Each of ten logs (approximately 36 cm long and 12-20 cm diam), from each of four trees, were inoculated with pairwise combinations of *O. piliferum* and the three SPB associated fungi described above, for a total of three treatments (*O. piliferum* vs. *O. minus, O. piliferum* vs. *E. sp. A* and *O. piliferum* vs. *C. ranaculosus*). At each inoculation site, the log was wounded twice to the depth of the xylem surface. Each wound was then inoculated with one of two fungi and covered. After 2 wk the resulting lesion on the phloem surface was aseptically exposed, as described below. Each lesion site was surface sterilized with a 1% NaOCl solution, the length of the discolored area was measured, and samples were collected for isolation of the inoculated fungi. Samples were collected from 1 cm on both sides of each of the two wound inoculation sites, the top and bottom edges of the discolored area, and 1 cm beyond the top and bottom edges of the discolored area. Each sample was aseptically divided and placed on benomyl malt agar selective for the two mycangial fungi (Ross et al., 1997) and cycloheximide malt agar, selective for *O. minus* (Harrington, 1992). The benomyl agar consisted of 15 mg/mL malt extract (Difco Laboratories, Detroit, Michigan), 15 mg/mL agar (Difco Laboratories, Detroit, Michigan), 4 μg/mL benomyl (50WP, Rigo Co., Buckner, Kentucky), and 100 μg/mL streptomycin sulfate (ICN Biomedicals, Inc., Aurora, Ohio). The cycloheximide agar consisted of 25 mg/mL malt extract (Difco Laboratories, Detroit, Michigan), 10 mg/mL agar (Difco Laboratories, Detroit, Michigan), 200 μg/mL cycloheximide (ICN Biomedicals, Inc., Aurora, Ohio), and 100 μg/mL streptomycin sulfate (ICN Biomedicals, Inc., Aurora, Ohio). All plates were sealed with Parafilm® and incubated at 20 C in darkness. After 2 wk, I examined all plates for fungal growth and determined the extent to which each of the four fungi had grown within the logs. I calculated and compared the mean extent of growth for each of the fungi within the competitive pairings using the procedures described above. I analyzed the data using a replicated randomized block design in which the mean squares $(MS)_{\text{Treatment X Tree}}$ was used in place of the MS_{Error} in calculating the F-ratio for determination of significant treatment effects, because tree was considered a random effect.

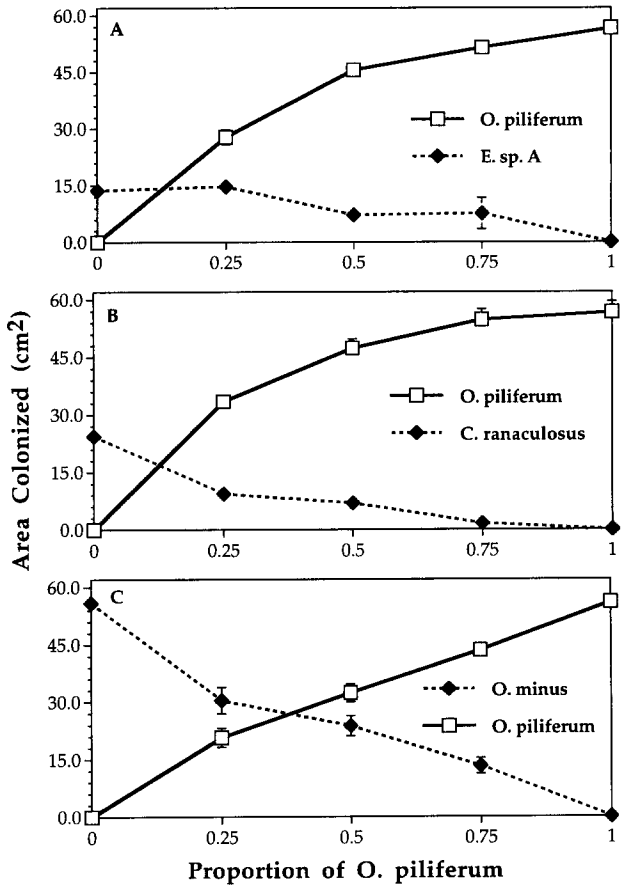

FIG. 1. De Wit replacement series. A. Mean area (with standard error bars) of malt extract agar colonized by *O. piliferum* and A. *E. sp. A* 2 wk after inoculation. B. *C. ranaculosus* 1 wk after inoculation. C. *O. minus* 1 wk after inoculation. In the absence of differential competition an "x" shaped pattern is seen, with the area colonized by both species directly proportional to the proportion of each species in the initial population.

RESULTS

Differential competition.—There was strong evidence of differential competition between *O. piliferum* and the two mycangial fungi of the SPB (FIG. 1). This pattern was not seen in any of our experiments. *Ophiostoma piliferum* easily outcompeted, and grew much faster than, both *E. sp. A* and *C. ranaculosus* in de Wit replacement series on MEA (FIG. 1A, B). In addition, both of these competitive interactions exhibited significant deviations from linearity [*O. pil-*

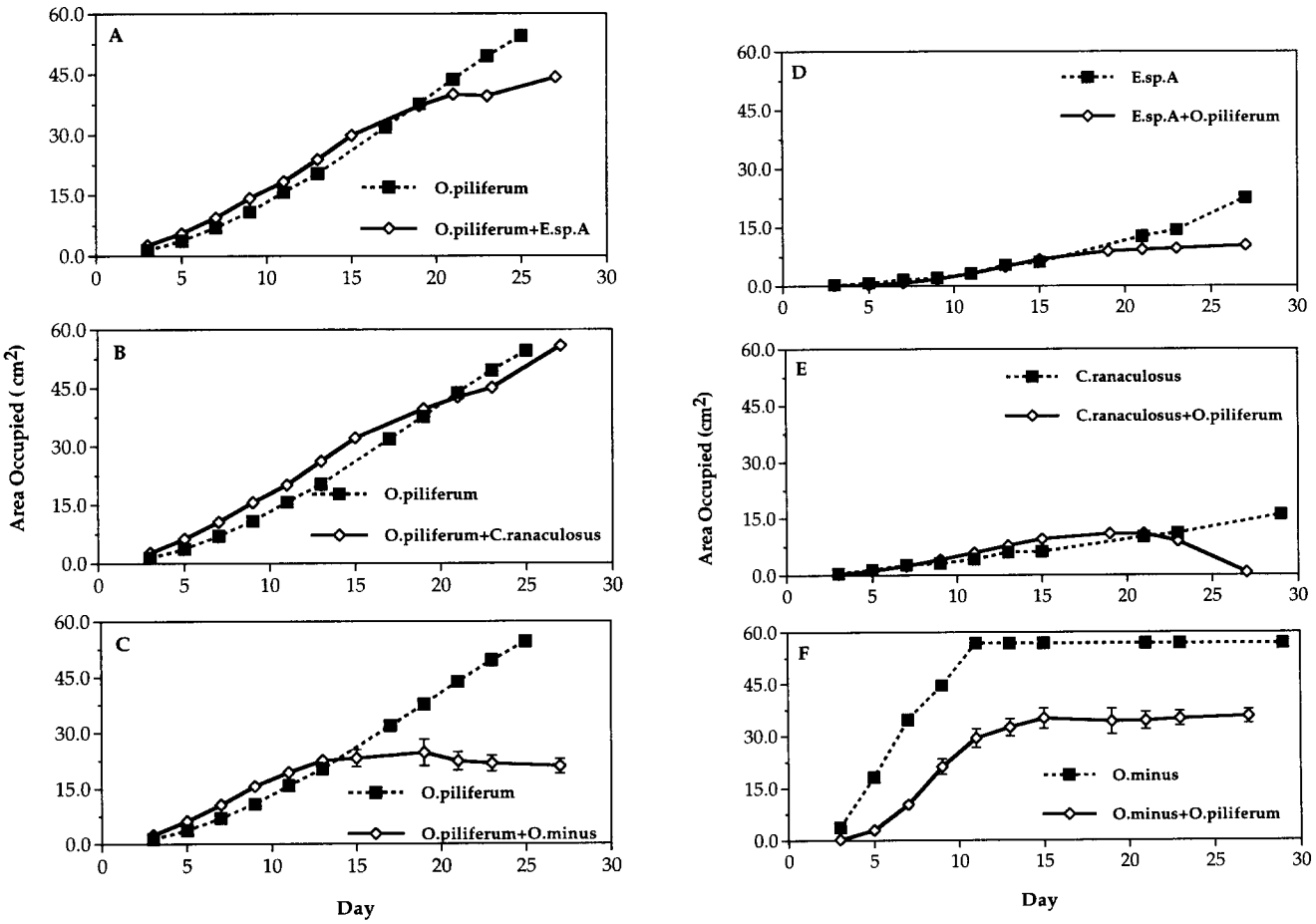

FIG. 2. Primary resource capture by *O. piliferum* and SPB fungal symbionts on malt extract agar. Mean area, bars = SE. A.–C. Occupied by *O. piliferum* growing alone and in competition with A. *E. sp. A*, B. *C. ranaculosus*, and C. *O. minus*. D.–F. Three fungi alone and in competition with *O. piliferum*. D. *E. sp. A*. E. *C. ranaculosus*. F. *O. minus*.

iferum (F=4.78, P = 0.006) vs. *E. sp. A* (F=2.74, P = 0.05), and *O. piliferum* (F=6.32, P = 0.002) vs. *C. ranaculosus* (F=29.98, P = 0.0001)]. Relative crowding coefficients calculated for these interactions (RCC for *O. piliferum* vs. *E. sp. A* = 1.53, and RCC for *O. piliferum* vs. *C. ranaculosus* = 2.93) also indicated a strong competitive advantage for *O. piliferum*. The de Wit replacement series indicated only slight differential competition between *O. piliferum* and *O. minus* (FIG. 1C). Likewise, the growth rates of these two fungi were similar. However, analysis of the relationship between the area colonized and proportion of initial inoculum revealed significant departures from linearity for *O. piliferum* (F=7.05, P = 0.0008) vs. *O. minus* (F=4.35, P = 0.01). The two fungi had captured equal amounts of substrate when *O. piliferum* represented only 40% of the initial inoculum. In addition, the RCC for *O. piliferum* vs. *O. minus* (1.36) indicated a slight competitive advantage for *O. piliferum*.

Primary resource capture.—In the one-on-one challenge on MEA, *O. piliferum* outcompeted all three SPB associates for access to uncolonized substrate. The growth of *O. piliferum* was only slightly reduced in the presence of the two mycangial fungi (FIG. 2A, B) and the fungus was rapidly able, perhaps due to its faster growth rate, to capture most of the uncolonized medium. *Ophiostoma piliferum* reduced the growth of both mycangial fungi, and the area colonized by either of these two fungi was less than 25% of the area colonized by *O. piliferum* (FIG. 2D, E). Growth of *O. piliferum* was substantially reduced when it was grown in competition with *O. minus* (FIG. 2C). Although *O. minus* was able to colonize substrate at a rate almost equal to that of *O. piliferum*, its own growth was substantially reduced when it was grown in competition with *O. piliferum* (FIG. 2F).

Secondary resource capture.—None of the fungi were able to capture a significant amount (> 0.1 cm^2) of substrate from *O. piliferum*, nor was *O. piliferum* able

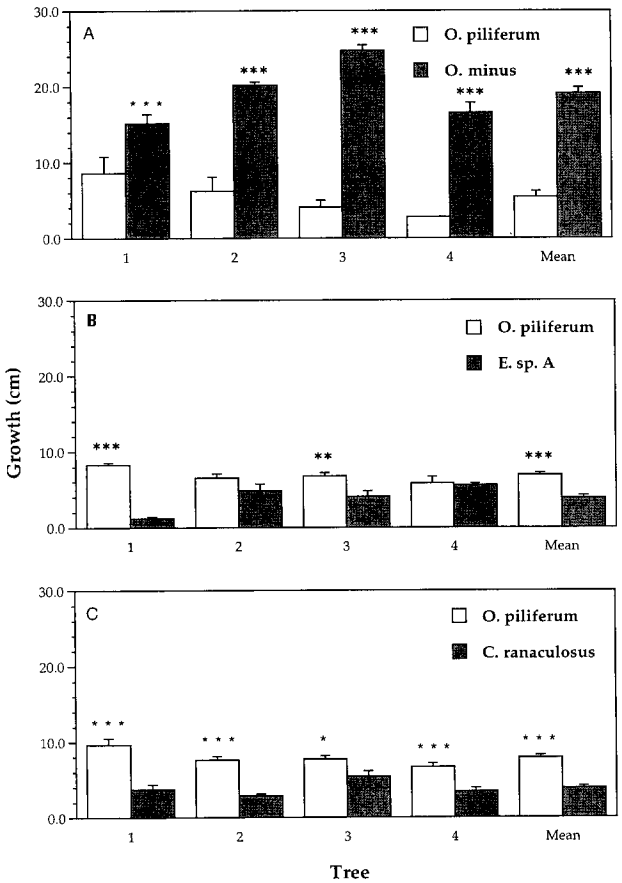

FIG. 3. Primary resource capture by *O. piliferum* and SPB fungal symbionts inoculated into loblolly pine logs from four trees (1–4). Mean area, bars = SE. A.–C. A. Occupied by *O. piliferum* and *O. minus* growing in competition for 2 wk. B. Occupied by *O. piliferum* and *E. sp. A* growing in competition for 2 wk. C. Occupied by *O. piliferum* and *C. ranaculosus* growing in competition for 2 wk. Asterisks indicate significant differences between treatments. * = $P < 0.06$, ** = $P < 0.01$, *** = $P < 0.0001$.

to capture substantial territory from established colonies of any of the SPB fungal associates.

Primary resource capture—natural substrate.—The mean vertical growth of fungi inoculated into logs differed significantly according to tree (F=5.10, $P < 0.002$), treatment (F=172.57, $P < 0.0001$), and tree x treatment interaction (F=7.37, $P < 0.0001$). However, in considering means of data from all trees combined, *O. piliferum* was able to outgrow both mycangial fungi in natural substrate. After two wk it occupied more space in loblolly pine logs than did *E. sp. A* (6.9 vs. 3.8 cm^2, respectively) or *C. ranaculosus* (7.9 vs. 3.9 cm^2, respectively) (FIG. 3). In contrast, *O. minus* outgrew *O. piliferum* within natural substrate. After two wk of competition, *O. minus* occupied more phloem tissue within loblolly pine logs than was occupied by *O. piliferum* (19.1 vs. 5.4 cm^2, respectively). Many of the differences found in the pooled data were significant when data were analyzed according to tree as well (FIG. 3).

DISCUSSION

Ophiostoma piliferum is highly competitive with the fungal associates of SPB. This fungus readily out competed both of the mycangial mutualists on both artificial and natural substrates. Although *O. piliferum* outcompeted *O. minus* on an artificial medium, its competitive advantage was substantially less over this fungus than its advantage over the mycangial fungi. In addition, *O. minus* outcompeted *O. piliferum* on natural substrate. With this exception, the data from the experiment in loblolly pine logs appear to reflect the same trends seen on MEA. *Ophiostoma piliferum* competed closely with *O. minus,* and the two mycangial fungi were relatively inferior competitors on artificial medium. It should be noted that the outcome of many of these interactions may have largely been due to differences in fungal growth rates. In this as well as previous (Klepzig and Wilkens, 1997) work the faster growing fungi were better able to capture uncolonized substrate. However, differences in growth rate do not account for ability to maintain substrate free of invading fungi. In most cases, for example, *E.* sp. A grew slower than *C. ranaculosus* but was better able to defend its colonized space from *O. minus* (Klepzig and Wilkens, 1997) and *O. piliferum.*

It is likely that *O. piliferum* would affect the early stages of SPB attack of host trees. While *O. piliferum* can kill *Pinus taeda* when mass inoculated into seedlings (Basham, 1970), it is generally not considered to be pathogenic to mature trees (Mathre, 1964b). In this respect it may do less damage to host trees in the early stages of SPB attack than would *O. minus.* At the same time, *O. piliferum* may be capable of limiting *O. minus* growth and resulting in less overall staining of affected wood while not causing any significant losses in wood strength (Seifert, 1996). As SPB attacks proceed, the impacts of *O. piliferum* may increase.

One likely scenario for the use of *O. piliferum* in the field would involve inoculating a spore suspension of the fungus into trees as soon as it becomes apparent that the trees are being attacked (e.g., at the first sign of pitch tubes). If *O. piliferum* were introduced in relatively high doses and was able to infect host tissue either before or at the same time as SPB fungal associates did, it might be able to capture the uncolonized phloem first and prevent the subsequent establishment of these fungi. Any exclusion or limitation of growth of mycangial fungi would re-

sult in reduced larval nutrition, growth and emergence (Barras, 1970; 1973). This could effectively reduce SPB brood production from inoculated trees and ultimately slow the spread of an SPB infestation. However, it will likely be key for *O. piliferum* to get a head start, for it to serve as an effective biocontrol agent. In our studies *O. piliferum* was never able to capture already colonized substrate (fungi inoculated into substrate at the same time, or after, *O. piliferum* were likewise unable to compete effectively with this fungus).

Before we can evaluate the ability of *O. piliferum* to serve as a biological control agent of this insect-fungal complex we need to obtain more information regarding the basic ecology of these fungal interactions. Our future efforts will concentrate on determining ways in which abiotic factors such as the nutrient and allelochemical content of the host tree might affect fungal competition. Ultimately, we will focus on evaluations of the competitive abilities of this fungus in the field.

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