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Competitive Interactions among Symbiotic Fungi of the Southern Pine Beetle

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The southern pine beetle, a damaging pest of conifers, is intimately linked to three symbiotic fungi. Two fungi, *Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. A, are transported within specialized structures (mycangia) in the beetle exoskeleton and are mutualists of the beetle. A third fungus, *Ophiostoma minus*, is transported externally on the beetle exoskeleton (phoretically) and is an antagonist of the beetle. This study examined competitive interactions among these three fungi. The results of de Wit replacement series and primary and secondary resource capture assays with these fungi provide strong evidence for differential competition between the phoretic and mycangial fungi. *O. minus* was the most able to capture both uncolonized and colonized resources. *Entomocorticium* sp. A and *C. ranaculosus*, although equal to one another in competitive abilities, differed in their ability to compete with *O. minus*. *Entomocorticium* sp. A was able to maintain space free of *O. minus* to a much greater degree than was *C. ranaculosus*. The outcome of such competitive interactions may have significant impacts on the biology of this ecologically and economically important beetle.

The southern pine beetle (SPB), *Dendroctonus frontalis* Zimmermann, the most damaging insect pest of southern pines, must kill its tree host in order to complete development. Reproductive adults bore holes through the outer bark, mate, and construct ovipositional galleries within the inner bark and phloem of host trees (19). In the process, beetles inoculate several associated microbes into the phloem and xylem of their tree hosts (7). In particular, three fungi are commonly and intimately associated with the SPB. Female beetles carry two fungi, *Ceratocystiopsis ranaculosus* Perry and Bridges and *Entomocorticium* sp. A. *Entomocorticium* sp. A (17) is an undescribed basidiomycete formerly referred to only by its isolate designation, SJB122 (5). Both of these fungi are carried within a specialized invaginated secretory structure within the prothorax of the female SPB (5). These fungi are inoculated by ovipositing females into the inner bark and phloem of host trees. Therein, they ramify throughout the galleries of developing larvae and produce large spores similar to those of the ambrosia fungi (5). SPB larvae are presumed to obtain nutritional benefits by consuming these fungi (4, 5, 12). Because these mycangial fungi are associated with increased beetle fecundity (4, 15), weight, ratio of population increase (8), size, and lipid content (12), they are generally considered to be mutualists of the SPB. A third fungus, *Ophiostoma minus* (Hedgc.) H. and P. Sydow, is not found within the mycangium but is carried phoretically on the exoskeleton by the SPB and a closely associated tarsonemid mite (9, 23). This fungus is also inoculated by attacking beetles into tree hosts, where it fruits heavily and causes a characteristic blue staining in the inner bark, phloem, and xylem. Initially, the fungus may assist the SPB in killing the tree by reducing water conduction through the phloem (6, 7, 11, 18, 19), and in this regard, it may be considered a mutualist of the SPB. However, eventually, *O. minus* competes with the SPB for uncolonized host tissue. Beetle development is severely negatively affected in areas of host tissue that are heavily infected with this fungus. These blue-stained areas are characterized by inhibited SPB egg production, larval growth, and larval development (3, 14). For these reasons, *O. minus* is considered to be an antagonist of the SPB.

The SPB is vitally important to its associated fungi. Bark beetles and their arthropod associates (9) serve as the only effective means by which stain fungi can gain access to new host tissue (13). As SPB larvae feed and develop within the phloem, their galleries and pupal chambers serve as sites of abundant fruiting by these fungi (5). In this way, and via attachment to spore-bearing mites phoretic on SPB (9), the fungi greatly facilitate transmission to new tree hosts by emerging SPB. Competition for tree tissue is likely to be of paramount importance in the life cycle of all three fungi. Likewise, the outcome of such competitive interactions may drastically affect the dynamics of the attack process and subsequent reproductive success of the SPB. Despite this, little is known of how *O. minus*, *C. ranaculosus*, and *Entomocorticium* sp. A interact with one another in their quest for this common resource.

Fungal interactions may be broadly divided into three categories, mutualistic, neutralistic, and competitive (21). In this study, we focused on competitive interactions that are detrimental to either or both interacting species and may be further subdivided into primary resource capture and secondary resource capture (combative interactions). In primary resource capture, interacting fungi compete to gain "initial access to and influence over an available resource" (21) but do not directly challenge one another. In this system, *O. minus*, *C. ranaculosus*, and *Entomocorticium* sp. A are engaged in competition for a rare and ephemeral resource, uncolonized phloem. Continued colonization almost inevitably leads to direct contact between interacting fungi. In such cases, several scenarios are possible. Each fungus may defend (via antibiosis or other means) its already colonized resource, one fungus may challenge another and engage in secondary resource capture (21) (as in replacement), or the two fungi may merely intermingle with one another, with no discernible deleterious effects to either.

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TABLE 1. Ratios of inocula (0.5-cm-diameter MEA disks) used in de Wit replacement series with *O. minus* versus *Entomocorticium* sp. A

Proportion of *O. minus*	Proportion of *Entomocorticium* sp. A	No. of disks	
		O. minus	*Entomocorticium* sp. A
0	1.0	0	20
0.2	0.8	4	16
0.4	0.6	8	12
0.6	0.4	12	8
0.8	0.2	16	4
1.0	0	20	0

We are specifically interested in the microbial community dynamics that occur within trees that have succumbed to attack by the SPB-microbial complex. By either providing, or denying, nutrition for developing larvae, microbial interactions directly affect SPB reproduction and development. Our primary goals in this study were to define the competitive interactions and elucidate the competitive hierarchies (25) that occur among *O. minus*, *C. ranaculosus*, and *Entomocorticium* sp. A in a controlled setting, on a semidefined medium. We quantified, on artificial media, (i) the extent to which differential competition occurs, (ii) the primary resource capture capabilities, and (iii) the secondary resource capture capabilities, for *O. minus*, *C. ranaculosus*, and *Entomocorticium* sp. A. We also used loblolly pine (*Pinus taeda* L.) logs to quantify the primary resource capture capabilities of these three fungi in a natural substrate.

MATERIALS AND METHODS

Fungal cultures. We isolated all the fungi used in this study from adult SPBs collected from pine plantations in Louisiana. We isolated *O. minus* by crushing adult SPBs on a selective medium. This medium contains cycloheximide, which inhibits the growth of many fungi but is not inhibitory towards fungi in the genera *Ophiostoma* and *Leptographium* (16). Both *Entomocorticium* sp. A and *C. ranaculosus* were isolated by aseptically dissecting mycangia from surface-sterilized adult SPBs (5) and placing the mycangia pieces on 2% malt extract agar (MEA). We then allowed all isolates to grow for 1 to 2 weeks and obtained pure cultures by transferring hyphal tips of each species to sterile plates of unamended MEA. At least 20 isolates of each species were grown on MEA and compared for colony morphology and approximate growth rate. We selected one representative isolate—*O. minus* (isolate W9518.OM), *Entomocorticium* sp. A (isolate W955.B), and *C. ranaculosus* (isolate W951.C)—and used this isolate for all experiments described below. All three isolates used in these experiments have been deposited in the culture collections of Tom C. Harrington, Iowa State University, Ames, and Harold H. Burdsall, Forest Products Laboratory, USDA Forest Service, Madison, Wis.

Differential competition. We used a substitutive design to determine if differential competition occurs among the three major fungal associates of the SPB. The de Wit replacement series has been used extensively to study competition among plants (24) but has only rarely been used in examining such interactions among microbes (2, 26). In de Wit replacement series with microorganisms, potentially competing microbes are introduced onto a substrate at varying proportions of inoculum. The population size of each organism at the termination of the experiment is determined as a function of inoculum proportion. In the absence of differential competition, the relationship between population size and inoculum proportion is expected to be linear for each organism. 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. An analysis of variance (ANOVA) is performed on log-transformed means to test for deviations from linearity in the relationships between area colonized by each species and its initial inoculum proportion (26).

We introduced varying proportions of inoculum onto plates of MEA. We chose MEA as a standard, semidefined, readily available medium suitable for this type of controlled, repeatable, preliminary study. We pitted the fungi against one another in a series of pairwise combinations. An example of the ratios of inocula that we used in our modified de Wit replacement series is provided in Table 1. The same initial proportions of inocula were used in the other pairwise combinations; only the fungal species used varied. We aseptically removed disks (0.5 cm in diameter) from actively growing colonies of each fungus and inoculated them onto plates of MEA in the designated ratios. Fungal disks were randomly placed within a grid (4 by 5 cm) 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. We sealed all plates with Parafilm and incubated them at 20°C in the dark. Two weeks after the inoculation, we traced the areas occupied by each fungus on sheets of transparent plastic. These areas were later measured with a digital planimeter. We compared the areas occupied in the various treatments with the areas expected to be occupied by the same fungi grown in isolation and plotted the mean area occupied by each fungus against its initial inoculum proportion. We tested for departure of this relationship from linearity by a previously described model (26). We also calculated relative crowding coefficients (RCC) (20) for all three of the fungi in pairwise comparisons. In our case (because we did not utilize a 1:1 ratio), we defined the RCC of species A to B as [(area occupied by fungus A at 0.4:0.6)/(area occupied by fungus B at 0.6:0.4)]/[(mean occupied by fungus A at 1:0)/(area occupied by fungus B at 1:0)]. An RCC of 1 indicates equal competition between species, an RCC greater than 1 indicates that species A is outcompeting species B, and an RCC less than 1 indicates that species B is outcompeting species A (20).

Primary resource capture. In an effort to quantify primary resource capture capability, we used an artificial medium to measure the relative competitive saprophytic abilities of *O. minus*, *C. ranaculosus*, and *Entomocorticium* sp. A. This experiment involved the exposure of uncolonized substrate (MEA) to mixed fungal inoculum and analysis of the resulting colonization patterns. In this type of assay, "organisms achieving dominance in the early stages of colonization [are] regarded as possessing high competitive ability" (21). We exposed uncolonized MEA plates to a mixture of mycelial disks of *O. minus*, *C. ranaculosus*, and *Entomocorticium* sp. A and measured the areas colonized by each fungus. In the first experiment (no spatial separation), the competing fungi were inoculated onto the center of the plate, directly adjacent to one another. In the second experiment (spatial separation), the competing fungi were inoculated at opposite

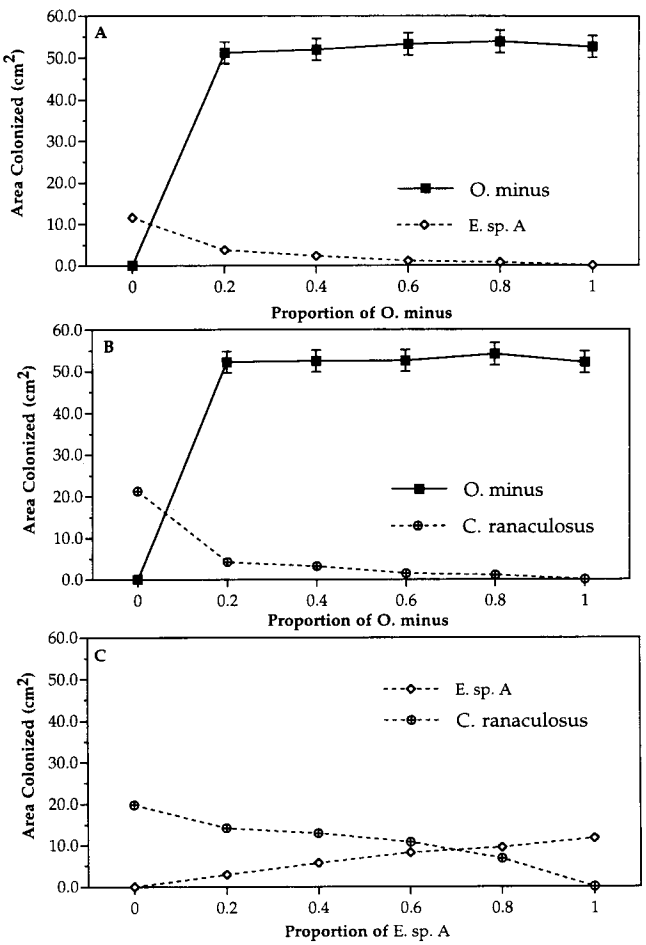


FIG. 1. Replacement series graphs for SPB fungal associates. Values are mean (with standard error bars) total areas colonized by each fungus versus proportions of inoculum 1 week after inoculation onto artificial medium. (A) *O. minus* versus *Entomocorticium* sp. A; (B) *O. minus* versus *C. ranaculosus*; (C) *Entomocorticium* sp. A versus *C. ranaculosus*. $n = 10$ for all combinations.

TABLE 2. ANOVA statistics for test for deviation from linearity in relationship between two competing fungal species in a de Wit replacement series[a]

Comparison and value group	Source	df	Sum of squares	Mean square	*F*	*P*
O. minus vs *Entomocorticium* sp. A						
Area occupied by *O. minus*	Proportion	5	24.66	4.93	28,442.03	0.0001
	Residual	54	0.009	0.0001		
Area occupied by *Entomocorticium* sp. A	Proportion	5	19.98	3.99	156.78	0.0001
	Residual	54	1.38	0.03		
O. minus vs *C. ranaculosus*						
Area occupied by *O. minus*	Proportion	5	24.73	4.95	80,168.95	0.0001
	Residual	54	0.003	0.00006		
Area occupied by *C. ranaculosus*	Proportion	5	26.06	5.21	646.26	0.0001
	Residual	54	0.44	0.008		
Entomocorticium sp. A vs *C. ranaculosus*						
Area occupied by *Entomocorticium* sp. A	Proportion	5	6.06	1.21	83.14	0.0001
	Residual	53	0.77	0.01		
Area occupied by *C. ranaculosus*	Proportion	5	26.54	5.31	1,568.66	0.0001
	Residual	53	0.18	0.003		

[a] ANOVA was performed on adjusted areas [log(area occupied by fungus + 0.5) − log(initial inoculum proportion + 0.5)]. A significant *P* value indicates significant differences in the adjusted means and a significant deviation from linearity.

edges of the plate, such that the fungi had room to grow into uncolonized substrate before coming in contact with one another.

No spatial separation. We removed mycelial plugs (0.5 cm in diameter) from actively growing colonies of *O. minus*, *Entomocorticium* sp. A, and *C. ranaculosus* and placed them facedown in the center of 9-cm plastic petri plates of MEA in the following combinations (each combination on five petri plates, in all cases): one disk of *O. minus*, one disk of *Entomocorticium* sp. A, one disk of *C. ranaculosus*, one disk of *O. minus* plus one disk of *Entomocorticium* sp. A, one disk of *O. minus* plus one disk of *C. ranaculosus*, and one disk of *O. minus* plus one disk of *Entomocorticium* sp. A plus one disk of *C. ranaculosus*. We sealed all plates with Parafilm and inverted and incubated them at 20°C in the dark. After 3 days, and at 2-day intervals for 24 days thereafter, we measured two colony diameters at right angles to one another for each fungus. We calculated and compared the mean colony sizes at each time for each fungus in each treatment by the least-squares means procedure in ANOVA (1).

Spatial separation. We removed mycelial plugs (0.5-cm diameter) from actively growing colonies of *O. minus*, *Entomocorticium* sp. A, and *C. ranaculosus* and placed them facedown at opposite edges of 9-cm plastic petri plates of MEA in the combinations described above ($n = 10$ plates in all cases), such that there was an approximately 8-cm-wide area of uncolonized agar between the two disks. We sealed all plates with Parafilm and inverted and incubated them at 20°C in the dark. After 3 days, and at 2-day intervals for 26 days thereafter, we measured the largest diameter of each fungus. We calculated and compared mean colony sizes at each time for each fungus in each treatment by the least-squares means procedure in ANOVA (1).

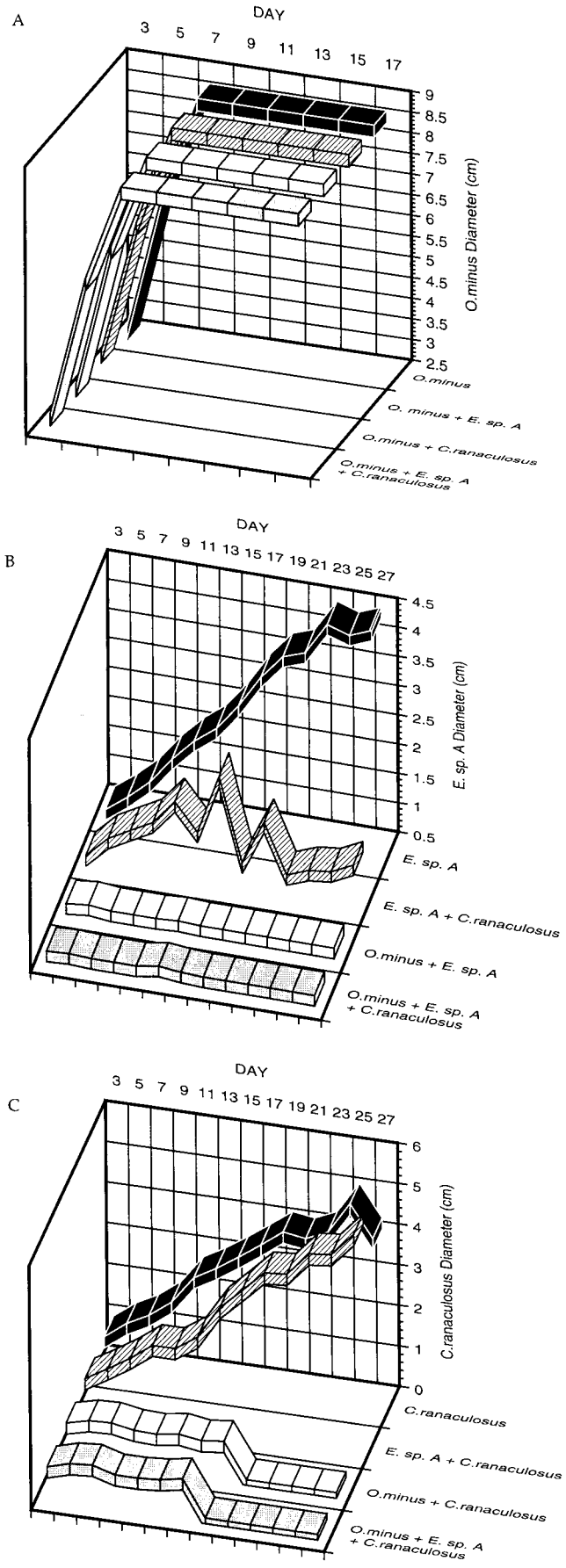
Secondary resource capture. Although measuring competitive saprophytic ability allowed some quantification of competitive interactions among fungi, it did not account for what occurred once a resource had become fully colonized. We wished to quantify the ability of *O. minus* to colonize substrate once it had been colonized by the two mycangial fungi. We used an artificial medium to examine the secondary resource capabilities of these three fungi. One mycelial plug of *Entomocorticium* sp. A was placed at the growing edge, and one was placed near the center of a 1-week-old colony of *O. minus* in each of 10 9-cm petri plates of MEA. This process was repeated with mycelial plugs of *C. ranaculosus* in an additional 10 plates of *O. minus*. One week later, we placed one mycelial plug of *O. minus* at the growing edge and one near the center of a 2-week-old colony of *Entomocorticium* sp. A in each of 10 9-cm petri plates of MEA. We repeated this process with mycelial plugs of *O. minus* in another 10 plates of *C. ranaculosus*. We sealed all plates with Parafilm and inverted and incubated them at 20°C in the dark. After the fungi within the plates had been allowed to interact for 1 week, we traced the areas colonized by each fungus within each plate onto transparent sheets of plastic. These traced areas were later measured with a digital planimeter. The mean colony area at the beginning and end of the experiment for each fungus in each treatment was calculated and compared by the least-squares means procedure in ANOVA (1).

Primary resource capture—natural substrate. We used freshly harvested loblolly pine (*P. taeda* L.) logs in a modified version of the primary resource capture experiment described above. For each replicate, we harvested one loblolly pine tree (approximately 15 to 20 years old) from a site in Rapides Parish, near Pollock, La. We cut 10 logs (approximately 36 cm long and 12 to 20 cm in diameter) from each tree and transported them to the laboratory. We shaved the rough outer bark from each log, sterilized the surface of each log with a 20% bleach solution, and pounded four nails into the bottom of each log (to serve as legs upon which the logs could be stood on end). The logs were all stored in this fashion on a countertop in a dark room at 26°C. Within 24 h, we inoculated each log with pairwise combinations of the three fungal isolates described above, for a total of three treatments (*O. minus*-*Entomocorticium* sp. A, *O. minus*, and *Entomocorticium* sp. A-*C. ranaculosus*). Three additional treatments, involving pairings with another fungus [*Ophiostoma piliferum* (Fries) H. and P. Sydow], were part of a separate study and will not be discussed further here. For each treatment, we made two circular wounds to the xylem surface, spaced 2 cm apart, vertically on the log surface, by using a 1-cm-diameter sterile cork borer. Each of the wounds was inoculated with one of two competing fungi. After both wounds had been inoculated, we covered the wound sites with labeled duct tape and allowed the fungi to grow within the logs for 2 weeks.

We removed the tape and, as aseptically as possible, shaved away the inner bark such that the phloem surface was exposed. We surface sterilized the exposed phloem with a 20% bleach solution, measured the length of the discolored area, and collected subsamples for isolation of the inoculated fungi. Because these fungi grow mainly vertically when inoculated into trees (7) and bolts (3), we concentrated our subsampling on tissues which were located varying distances vertically from the inoculation site. For each inoculation treatment, we used a flame-sterilized scalpel to collect a subsample 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 subsample was aseptically divided into two equal pieces. We placed one piece of each subsample on benomyl malt agar, selective for the two mycangial fungi (22), and we placed the other piece on cycloheximide malt agar, selective for *O. minus* (16). We sealed all plates with Parafilm and incubated them in the dark in a 20°C growth chamber for 2 to 3 weeks. We then examined all plates for growth of the three fungi and determined the extent to which the fungi had grown within the logs. This experiment was repeated four times with 10 logs each from four different trees from the same site described above. We calculated and compared the mean extent of growth for each of the fungi within the competitive pairings by the procedures described above. The data was analyzed with a replicated randomized block design in which the $MS_{treatment \times tree}$ was used in place of the MS_{error} in calculating the F ratio for determination of significant treatment effects.

RESULTS

Differential competition. There was strong evidence of differential competition between *O. minus* and *C. ranaculosus* and between *O. minus* and *Entomocorticium* sp. A (Fig. 1A and B). Even the addition of a small proportion of *O. minus* inoc-



ulum to plates containing *C. ranaculosus* or *Entomocorticium* sp. A inocula resulted in complete, or nearly complete, colonization by *O. minus*. The point at which *O. minus* and either of the two mycangial fungi had colonized equal areas was in between the 0 and 20% *O. minus* inoculum levels. Analysis of the relationship between area colonized and proportion of inoculum revealed a significant departure from linearity for the interaction between *O. minus* and *Entomocorticium* sp. A and between *O. minus* and *C. ranaculosus* (Table 2). In contrast, competition between *Entomocorticium* sp. A and *C. ranaculosus* appeared to be closer to equal (Fig. 1C). The areas colonized by each fungus closely paralleled the proportions of their inocula. The point at which these two species colonized equal areas, however, was near the 70% *Entomocorticium* sp. A inoculum level. Analysis of the relationship between area colonized and proportion of inoculum revealed this slight, but significant, competitive advantage for *C. ranaculosus* (Table 2).

RCC calculated from the de Wit replacement series data revealed significant differences in competitiveness as well. The RCC for *O. minus* versus *Entomocorticium* sp. A (10.56) indicated that *O. minus* greatly outcompeted *Entomocorticium* sp. A. The RCC (14.64) for the *O. minus*-versus-*C. ranaculosus* interaction indicated that *O. minus* greatly outcompeted *C. ranaculosus* as well. The RCC for the *Entomocorticium* sp. A-versus-*C. ranaculosus* interaction (0.89) indicated that, while the two mycangial fungi are very close to being equal competitors, *C. ranaculosus* did slightly outcompete *Entomocorticium* sp. A.

Primary resource capture—no spatial separation. In plates in which the inoculum disks were not spatially separated, *O. minus* rapidly outgrew both *C. ranaculosus* and *Entomocorticium* sp. A (Fig. 2). Mean colony diameters of *O. minus* increased to the maximum available area within 7 days (Fig. 2A). Mean colony diameters of *Entomocorticium* sp. A and *C. ranaculosus* were limited by *O. minus* (Fig. 2B). In the presence of *O. minus*, *Entomocorticium* sp. A did not capture any new space and did not grow; however, it did not lose any space to *O. minus* (Fig. 2B). *Entomocorticium* sp. A disks remained free of *O. minus* throughout the course of the experiment. Not only did *C. ranaculosus* colonies not increase in size when paired with *O. minus*, *O. minus* was able to capture space from *C. ranaculosus* (Fig. 2B). Within 17 to 19 days, *C. ranaculosus* disks had been completely overgrown by *O. minus*. In pairings of *Entomocorticium* sp. A and *C. ranaculosus*, *C. ranaculosus* enjoyed a slight competitive advantage (Fig. 2B and C). *Entomocorticium* sp. A growth was decreased in the presence of *C. ranaculosus* relative to growth in the absence of a competing fungus (Fig. 2B). However, *C. ranaculosus* growth in the presence of *Entomocorticium* sp. A was nearly equal to that of *C. ranaculosus* grown alone (Fig. 2C).

Primary resource capture—spatial separation. When inoculum disks were placed at opposite edges of the plates, with uncolonized substrate between them, *O. minus* outcompeted *C. ranaculosus* for the available uncolonized space in between the inoculum disks (Fig. 3A). In pairings with *Entomocorticium* sp. A, *O. minus* eventually grew around *Entomocorticium* sp. A and colonized the remaining empty area. This was seen during days 7 to 11 of the assay, during which growth of *O. minus* in the presence of *Entomocorticium* sp. A lagged behind that of *O. minus* growing alone (Fig. 3A). In both cases, *O. minus* was

FIG. 2. Primary resource capture capabilities of SPB fungal associates inoculated adjacent to one another. Values are mean colony diameters of *O. minus* (A) ($F = 0.86$; $P = 0.680$), *Entomocorticium* sp. A (B) ($F = 22.00$; $P = 0.0001$), and *C. ranaculosus* (C) ($F = 28.76$; $P = 0.0001$) when grown alone or in competition with other fungi. $n = 5$ for all combinations.

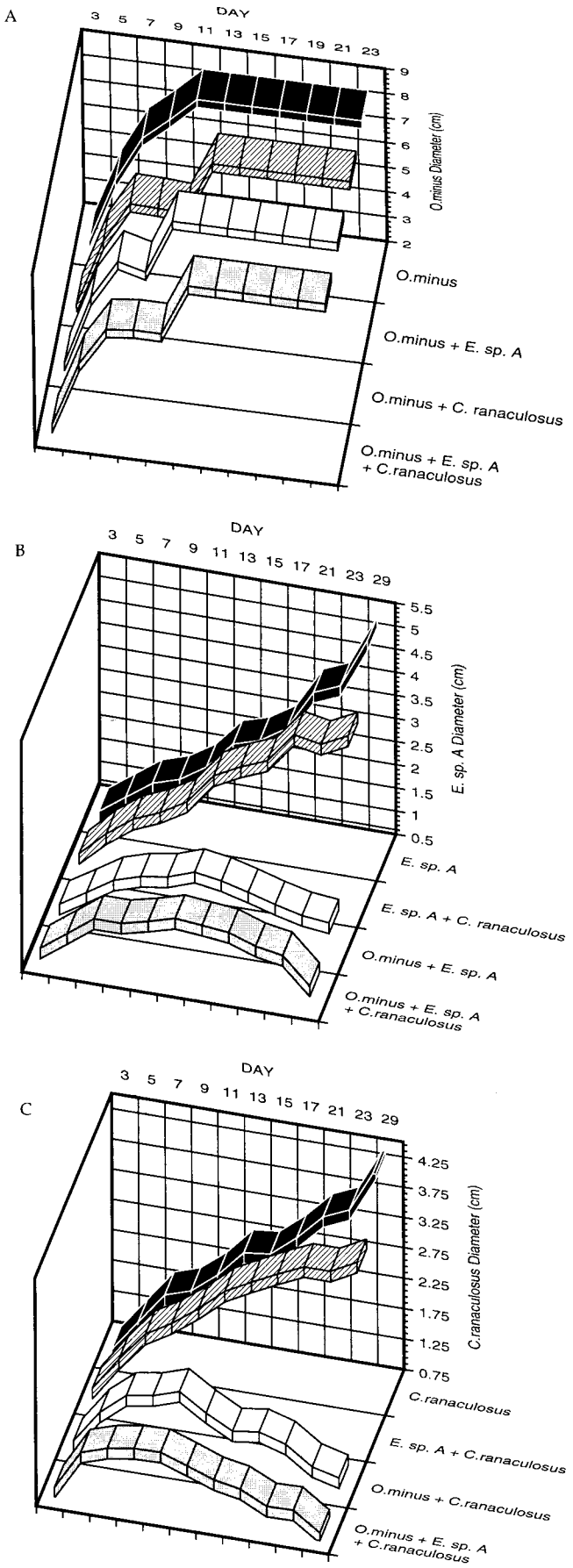


FIG. 3. Primary resource capture capabilities of SPB fungal associates inoculated opposite to one another with uncolonized medium in between. Values are mean colony diameters of *O. minus* (A) ($F = 40.11$; $P = 0.0001$), *Entomocorticium* sp. A (B) ($F = 17.81$; $P = 0.0001$), and *C. ranaculosus* (C) ($F = 56.63$; $P = 0.0001$) when grown alone or in competition with other fungi. $n = 10$ for all combinations.

able to eventually reach its maximum possible diameter (given the plate size). However, when growing edges of *O. minus* colonies came into contact with *Entomocorticium* sp. A (by day 7), they could not overgrow the colony and were able only to grow around it (by day 13). When growing colonies of *O. minus* came into contact with *C. ranaculosus*, however, they rapidly overgrew the established colony and, by day 11, had grown to their maximum extent (Fig. 3A). Similar trends are reflected in the colony diameters of *Entomocorticium* sp. A and *C. ranaculosus* when paired with *O. minus* (Fig. 3B and C). In contrast, the two mycangial fungi grew to the same extent and at approximately the same rates whether they were grown alone or with one another (Fig. 3B and C).

Secondary resource capture. Neither *C. ranaculosus* nor *Entomocorticium* sp. A could capture any space occupied by *O. minus*. *O. minus* captured more space from *C. ranaculosus* than from *Entomocorticium* sp. A. After 1 week of competition, the mean diameter of *O. minus* colonies introduced around and onto *Entomocorticium* sp. A colonies was significantly less than the diameter of *O. minus* colonies introduced into plates containing *C. ranaculosus* (Fig. 4A). Conversely, during the course of 1 week, *Entomocorticium* sp. A was able to grow to a much greater extent in the presence of a competing colony of *O. minus* than was *C. ranaculosus* (Fig. 4B).

Primary resource capture—natural substrate. The mean vertical growth of fungi inoculated into logs differed significantly according to tree ($P < 0.02$ for *Entomocorticium* sp. A and $P < 0.0001$ for *O. minus* and *C. ranaculosus*) and treatment ($P < 0.0001$ for all three fungi). *O. minus* was consistently able to colonize greater lengths of loblolly pine phloem than either *Entomocorticium* sp. A (Fig. 5A) or *C. ranaculosus* (Fig. 5B). The two mycangial fungi grew to a lesser extent, although *C. ranaculosus* typically outgrew *Entomocorticium* sp. A (Fig. 5C).

DISCUSSION

SPBs are typically much more successful in developing within tissue that is predominantly infected with the mutualistic fungi *C. ranaculosus* and *Entomocorticium* sp. A than are beetles developing within trees predominantly infected with the antagonistic fungus *O. minus* (3, 4). The nature and outcome of the competitive interactions among *O. minus*, *Entomocorticium* sp. A, and *C. ranaculosus* are thus vitally important to the SPB. Despite this, it was not previously known whether these fungi differentially competed. In this study, we have demonstrated, in artificial medium and natural substrate, that the three major symbionts of the SPB do compete for substrate. The victor in these competitive interactions was invariably *O. minus*. The SPB antagonist outgrew both SPB mutualists and would seem to be better able to capture uncolonized resources than either of these two mycangial fungi. This may be due in large part to the higher growth rate of *O. minus*. In the de Wit replacement series with these species, the mycangial fungi were only competitively equal to *O. minus* at the very lowest inoculum (<10%) levels of *O. minus*.

Given the chance to establish some colonized territory before encountering *O. minus*, both mycangial fungi were able to grow. Upon contact with the mycangial fungi, *O. minus* reacted in a species-specific manner. *O. minus* was fully able to capture

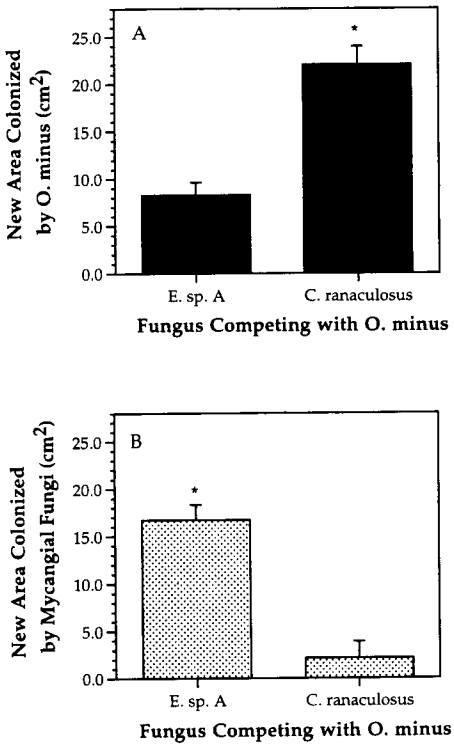


FIG. 4. Secondary resource capture capabilities of SPB fungal associates. Values are mean (with standard error bars) new areas (square centimeters) colonized by *O. minus* when inoculated near the center and at the edge of a 2-week-old *Entomocorticium* sp. A or *C. ranaculosus* colony (A) ($F = 34.20$; $P = 0.0001$) and *Entomocorticium* sp. A and *C. ranaculosus* when grown alone for 1 week and inoculated with *O. minus* at the center and the edge of the colony (B) ($F = 46.83$; $P = 0.0001$). $n = 10$ for all combinations.

resources from territory colonized by *C. ranaculosus* but was not able to capture any territory from *Entomocorticium* sp. A. *Entomocorticium* sp. A was better able to maintain antagonist (*O. minus*)-free space in artificial medium than was *C. ranaculosus.*

Given the apparent difference in the abilities of *Entomocorticium* sp. A and *C. ranaculosus* to maintain antagonist-free space for their insect vector, the outcome of competitive interactions between these two species takes on special significance. *Entomocorticium* sp. A and *C. ranaculosus* were approximately equal competitors. However, in all of the cases in which these two mycangial fungi were paired, *C. ranaculosus* maintained a slight competitive edge. In the replacement series, the two fungi did not compete equally until *Entomocorticium* sp. A made up approximately 70% of the total inoculum. This might indicate that, in order to accrue the maximum benefits of carrying *Entomocorticium* sp. A, the SPB might have to carry a slightly higher spore load. However, comparable replacement series data are not yet available for these fungi under field conditions.

The exact roles of the three major fungal associates of the SPB in its life cycle have never been explicitly demonstrated. It has been hypothesized that *Entomocorticium* sp. A and *C. ranaculosus* may act as tree pathogens, provide nutrients to developing SPB (8), or exclude *O. minus* from areas of larval development (10). The results of this study suggest that *Entomocorticium* sp. A may be of more value to the SPB than is *C. ranaculosus* in at least one of these possible roles. *Entomocorticium* sp. A was consistently more effective at keeping *O. minus* out of colonized areas than was *C. ranaculosus*. If this trend holds true in SPB-infested tree tissue as well, *Entomocorticium* sp. A may be presumed to be a better protector of SPB larval development.

The microbial ecology of the SPB system, and that of many other insect-fungal complexes, remains poorly understood. In particular, the interactions of these fungi under a variety of conditions need to be studied. For example, in some cases, competitive interactions vary with levels of available nutrients (20). We hypothesize that the competitive interactions observed here may be altered by the many variables (e.g., N levels, levels of plant allelochemicals, and temperature) found within their natural habitat, as well as by genetic variation among fungal isolates. Future studies will concentrate on such interactions within host tissue, both pine logs and living trees, as well as on manipulative experiments to reveal the benefits and detriments of these symbiotic microorganisms to this destructive forest pest.

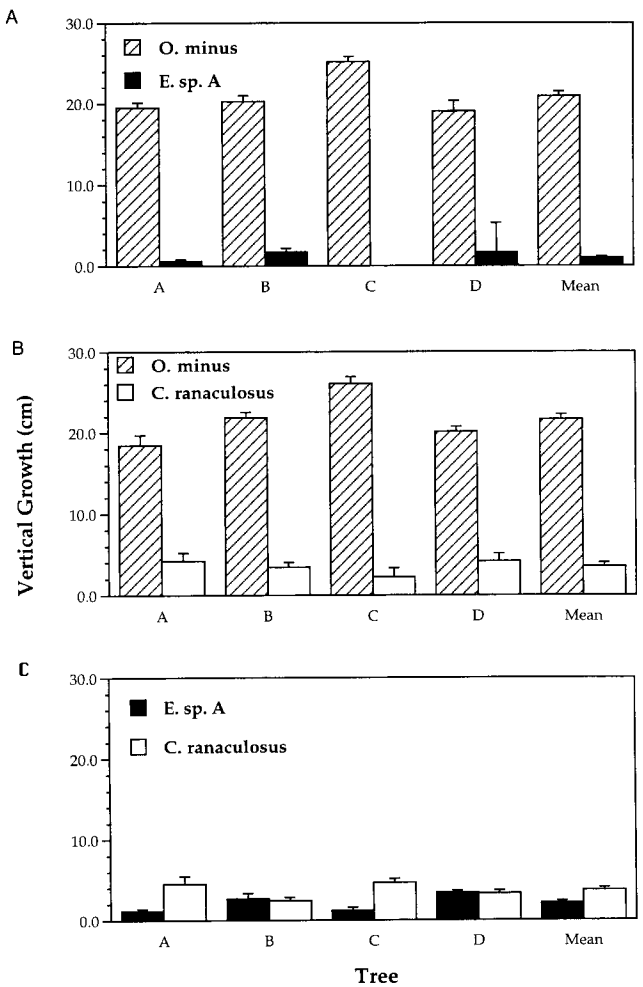


FIG. 5. Primary resource capture capabilities of SPB fungal associates inoculated apart from one another in uncolonized loblolly pine logs. Mean vertical lengths of growth after 2 weeks by each of the two competing fungi are given for each of the four loblolly pine trees (A to D), as are overall means, combining data for all trees. $n = 10$ for each tree. (A) *O. minus* versus *Entomocorticium* sp. A; (B) *O. minus* versus *C. ranaculosus*; (C) *Entomocorticium* sp. A versus *C. ranaculosus.*

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