Mutualism and antagonism – Ecological interactions among bark beetles, mites and fungi. Klepzig, K.D., J.C. Moser, M.J. Lombardero, M.P. Ayres, R.W. Hofstetter, and C.J. Walkinshaw. 2001. Reprinted from: Pp. 237-267 *In* Biotic interactions in plant-pathogen associations (eds. M.J. Jeger and N.J. Spence). CAB International.

Abstract. Symbiosis may be defined as: "the acquisition and maintenance of one or more organisms by another that results in novel structures and (or) metabolism". Relationships among symbiotic organisms may change over time and ranges of resources. Other organisms may indirectly facilitate or interfere with these relationships. Interactions among bark beetles and their associated fungi and mites are complex examples of the manner in which symbioses change and are indirectly affected by other organisms. These complex relationships have been extensively studied in the southern pine beetle (SPB), a bark beetle that kills healthy living trees through mass colonization. The SPB is consistently associated with three main fungi. Two of these fungi (*Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. A.) are carried in a specialized structure (mycangium) in female SPB. The third fungus is carried phoretically on the exoskeleton. Both *C. ranaculosus* and *Entomocorticium* sp. A are also carried by phoretic mites of SPB. Due to the effects of these fungi on SPB larval development, their competitive interactions have significant implications. The two mycangial fungi provide nutrition to developing larvae, while the phoretic fungus interferes with larval development. These interactions appear to be mediated by phoretic mites which have mutualistically symbiotic relationships with the SPB associated fungi they vector. The multiple interdependencies in this system provide novel opportunities for control of, and further research on, this damaging forest pest complex.

Introduction. Insect-fungal complexes provide challenging and fascinating systems for the study of biotic interactions between plants, plant pathogens, insect vectors and other associated organisms. The types of interactions among these organisms (mutualism, antagonism, parasitism, phoresy, etc.) are as variable as the range of organisms involved (plants, fungi, insects, mites, etc.). We focus on bark beetles and their associated organisms, in particular, on the relationship between the southern pine beetle and its associates in coniferous trees of the southern United States. We begin, however, with an attempt to clearly define the terms we use to describe these relationships.

Symbiosis. Zook (1998) stated that "Defining symbiosis has become something of a life science cliché, an act of verbal, and often verbose, masochism". Nevertheless, before exploring the manners in which closely associated organisms can interact, we must attempt to arrive at some basic definitions. Perhaps the most widely used, and perhaps widely debated, definition of symbiosis comes from Frank and Debary who defined the term as the "Living together of unlike organisms". That definition is useful in that it manages to avoid placing any values on the interaction between organisms (mutualism is not implied here). However, this definition is also vague enough that it might encompass all manner of close relationships between unlike organisms that we might not view as at all symbiotic (e.g., a goldfish and a frog in a bowl, a mouse in a cow barn)**.** A more specific definition, which is value neutral and still broad enough to encompass the variety of symbiotic organisms, is: "the acquisition and maintenance of one or more organisms by another that results in novel structures and (or) metabolism" (Zook, 1998) (we have added the "or" to indicate our belief that the existence of modified structures **o r** metabolism is sufficient to qualify as symbiosis).

The continuum from mutualism to antagonism - Intersymbiont interactions. Even with a clearly stated and acceptable definition of symbiosis, problems arise in the classification of interactions between organisms. In particular, attempts to classify a specific relationship as being strictly competitive, or strictly mutualistic, may be frustrated by seemingly contradictory evidence. One group of researchers finds that a particular organism is more successful in the presence of another. Other research may indicate that the two organisms compete for resources and even actively defend against one another. In this case, one might ask, what is the true nature of this relationship? Are the organisms mutualists, or antagonists? Often a satisfactory answer can be arrived at by careful consideration of the developmental and/or resource state being considered in the attempt to classify the relationship. In effect, many studies of symbiotic relationships consider only a limited range of time (or resource conditions). Within a specific window in time it is often possible to characterize a relationship as being primarily mutualistic or antagonistic. However, as noted by Callaway and Walker (1997) most (if not all) studies examining competition and/or facilitation do not measure a long enough period of time. Relationships among closely associated, even symbiotic, organisms may change over the developmental cycles of the organisms (time) as well as over ranges of available nutrient and energy sources (resources). In addition, other organisms may indirectly effect a relationship between two organisms. These third, or even fourth, organisms may become an integral part of the manner in which the two original organisms interact, facilitating and/or interfering as time and resources change (Callaway and Walker, 1997).

Fungal Interactions. Fungi utilizing the same resource may interact in at least three broadly defined ways (Rayner and Webber, 1984). For example, two fungal species may interact mutualistically (in which each facilitates the success of the other), neutralistically (in which each has no discernible effect on the other), or competitively (in which each tries to utilize the resource at the expense of the other). Competitive interactions may be detrimental to either species, and may be further subdivided into primary resource capture and secondary resource capture. In primary resource capture the interacting fungi compete to gain access and influence over uncolonized resource. At this point, the fungi are not directly challenging one another. However, as the fungi colonize the available resource, they may eventually come into direct contact with one another. The two directly interacting fungi may now engage in defense against one another (e.g., antiobiosis), they may intermingle with no discernible effects on one another, or they may attempt to engage in secondary resource capture (in which one fungus attempts to colonize the resource already held by the other). These fungal interactions may be of particular importance when they occur between species which are symbiotically linked to other species. The fungal associates of bark beetles have been extensively studied not only due to their effects on trees, but also as integral parts of complex systems of interacting organisms.

Bark beetles, mites and fungi.

The biology and ecology of bark beetle-fungal interactions have been extensively studied, and well reviewed elsewhere (Malloch and Blackwell, 1993; Paine *et al.*,, 1997). The interactions among insects that infest the bark, phloem and outer xylem of trees, and fungi that possess varying degrees of virulence within these tissues are complex. Fungi may be carried within specialized cuticular structures termed mycangia (Fig. 1), or externally in simple pits or on the exoskeleton. The roles of the associated fungi in the beetle life cycles may be differentiated by the manner in which they are vectored. Fungi carried within mycangia tend to be mutualists of the beetles, those carried externally are more likely to be tree pathogens, or wood-staining fungi. There is substantial taxonomic diversity among the fungi vectored by bark beetles, but many fall within the ascomycete genera *Ophiostoma* or *Ceratocystis* [the term ophiostomatoid is frequently used to refer to this group of fungi (Malloch and Blackwell, 1993)]. The details of the interactions among the many species of beetles and fungi vary extensively, making broad generalization problematic (Paine *et al.*,, 1997). We will concentrate, below, on the system which we study and which provides examples of the basic types of beetle-fungal interactions.

The southern pine beetle system. Although insect-fungus-mite interactions are important to several bark beetle species, these complex relationships have been extensively studied in the southern pine beetle (SPB). *Dendroctonus frontalis* Zimmermann (Coleoptera:Scolytidae) is among the most damaging of North American forest insects (Thatcher *et al.*, 1980; Drooz 1985; Price *et al.*, 1992). The SPB is considered a primary bark beetle, in that is essentially an obligate parasite (Raffa *et al.*, 1993) that attacks and kills healthy living trees through mass colonization by conspecifics (Paine *et al.*, 1997). Reproductive female beetles initiate attacks on host trees by boring entrance holes through the rough outer bark of southern pines, creating a nuptual chamber

(Fig. 2) and releasing a pheromone to attract more beetles to the tree (Kinzer *et al.*, 1969). The host tree attempts to repel the attack primarily through the release of preformed (constitutive) resin (Hodges *et al.*, 1979; Lewinsohn *et al.*, 1991a,b; Nebeker *et al.*, 1993; Ruel *et al.*, 1998). If enough SPB attack the tree in this manner, the tree's resin system is overcome, the beetles are able to complete development and the tree dies (essentially from disruption of water flow within the vascular system) (Fig. 3). Once the female beetle has mated, she begins chewing ovipositional (egg) galleries within the inner bark and phloem of the tree (Thatcher, 1960; Payne, 1983). As she does so, the female SPB inoculates several fungi into the phloem tissue (Bramble and Holst, 1940). Although many fungi have been associated with galleries of SPB in pine phloem, three have been the focus of most SPB-fungal research, and appear to have the most significant impacts on the SPB life cycle: *Ophiostoma minus* (Hedgc.) H. and P. Sydow, *Ceratocystiopsis ranaculosus* Perry and Bridges, and *Entomocorticium* sp. A (an undescribed basidiomycete, formerly referred to in the literature as isolate SJB122).

Ophiostoma minus, the causal agent of the "blue stain", often found in the xylem and phloem of SPB infested wood is an ascomycetous fungus (Fig. 4a) carried phoretically on the SPB exoskeleton (Rumbold, 1931; Bridges and Moser, 1983) and by phoretic mites (Bridges and Moser, 1983), which we will discuss in detail below. Early research into the SPB-fungi system focused on the putative role of *O. minus* as a tree killing pathogen (Nelson and Beal, 1929; Nelson, 1934; Caird, 1935; Bramble and Holst, 1940; Mathre, 1964; Basham, 1970). However, the fungus is apparently not necessary for tree death to occur (Hetrick, 1949; Bridges, 1985; Bridges *et al.*, 1985). Although artificial inoculations of southern pines with *O. minus* do cause resinosis (Fig. 4b) and tissue damage (Fig. 4c), they do not result in mortality of mature trees (Nelson, 1934; Cook *et al.*, 1986; Cook and Hain, 1987; Parmeter *et al.*, 1992; Ross *et al.*, 1992; Nevill *et al.*, 1995; Popp *et al.*, 1995). It seems probable that *O. minus*, in concert with SPB tunneling, hastens tree death (Paine *et al.*, 1997). The benefits of this relationship to the fungus are clearer. Bark beetles and their arthropod associates serve as the only effective means by which stain fungi gain access to new host tissue (Dowding, 1969). Thus, at the early stages of attack, the SPB-*O. minus* relationship may be categorized as mutualistic, although the frequency with which these organisms are associated does not necessarily imply this (Harrington, 1993). Subsequent research has focused on the impacts of *O. minus* on SPB larval development. As SPB eggs hatch within the niches the female has created in the pine phloem, the fungi she inoculated begin growing and colonizing the tissue as well. Within this community of organisms patches of *O. minus* develop (Fig. 5). When these areas of heavy colonization by the blue stain fungus overlap areas within which the developing larvae are feeding, the SPB almost always suffers. Although much of the evidence has been circumstantial, higher levels of phloem colonization with *O. minus* are correlated with reduced developmental success - inhibited egg production, slower larval growth and development, even larval mortality (Fig. 6) (Barras, 1970; Franklin, 1970). In addition, overall levels of *O. minus* within SPB infestations have been negatively correlated with SPB population increase (Bridges, 1985). The relationship here seems simple. The more blue stain that is present, the less SPB reproductive success will occur (Lombardero *et al.*, 2000). At the time of larval development, *O. minus* appears to be a competitor and antagonist of SPB (Barras, 1970). The mechanism of this antagonism, however, has remained unclear. Some have

speculated that *O. minus* leaves the phloem nutrient impoverished and deprives the developing larvae of necessary sustenance (Hodges *et al.*, 1968; Barras and Hodges, 1969; Barras, 1970). As such, it has also been suggested that the beneficial roles of the two other major fungal associates of SPB consists largely of outgrowing or outcompeting *O. minus* and keeping this blue stain fungus out of SPB larval galleries (Bridges and Perry, 1985).

The antagonism of SPB larvae by *O. minus*, which at first seemed contradictory to the pattern seen between *O. minus* and attacking SPB adults, may be partially explained when the interactions of SPB with its two other significant fungal associates are examined. Each female SPB possesses a prothoracic structure specialized for transporting fungi (Fig. 7). This mycangium consists of paired invaginations of the exoskeleton each of which has one pore-like ventral opening and contains two types of secretory cells (Barras and Perry, 1972; Happ *et al.*, 1971). Within each side of the mycangium, the female SPB is able to maintain a pure culture of either *C. ranaculosus* (a hyaline ascomycete) (Fig. 8a) (Barras and Taylor, 1973) or *Entomocorticium* sp. A, formerly referred to in the literature as SJB122 (Fig. 9a) (Barras and Perry 1972; Happ *et al.*, 1976). This slow growing fungus is an amber colored basidiomycete whose sexual stage remains undescribed, but which appears to belong in this genus *Entomocorticium* (Hsiau, 1996). Each female may carry either one (rarely both) of the two fungi, or no fungi, in either of the two mycangial pouches (Bridges, 1985). Although it seems likely that the majority of inoculation of mycangial fungi into pine phloem occurs later (Barras, 1975) perhaps during oviposition, the relative virulence of these two fungi in healthy trees has also been investigated. Inoculations of both *C. ranaculosus* and *Entomocorticium* sp. A invariably result in smaller amounts of tree damage [e.g., resinous lesions(Fig. 8b, 9b)] than do inoculations with *O. minus*(Cook and Hain, 1985; Paine *et al.*, 1987). However, both mycangial fungi do cause reactions, especially at the tissue and cellular level, that differ from those seen in response to mere mechanical wounding (Fig. 8c, 9c, 10 a and b). SPB mycangial fungi do not appear to be highly virulent in their pine hosts nor do they seem to assist in any meaningful way in tree killing. It seems more likely that the proper window in time to evaluate the role of the mycangial fungi in the SPB life cycle is post-mass attack. Once the tree's resistance has been overcome, as the female SPB deposits her eggs within the pine phloem, she may inoculate the area immediately surrounding the eggs with the contents of her mycangium. As the eggs hatch the early instar larvae begin feeding, constructing fine, sinuous galleries as they go (Payne, 1983). Eventually, the larvae cease moving forward and begin enlarging their feeding area to an obovate shape. It is within these "feeding chambers" that one can find luxuriant growth of either of the two mycangial fungi (Fig. 11). It is assumed that the mid to late instar larvae feed on fungal hyphae and spores, although due, in part, to difficulties in artificially rearing SPB, it has never been explicitly demonstrated. It appears extremely likely that larval SPB get the majority of their nutrition from the fungal growth within their feeding chambers rather than from the phloem itself. The mycangial fungi may, in fact, provide their most substantial benefits to SPB by concentrating dietary N for larvae (Fig. 12) (Ayres *et al.*, 2000). For the fungi, again, the advantages of association with SPB are clear. The fungi obtain a selective medium within which to grow as they are borne, protected and pure, to the next available resource (Happ *et al.*, 1971). The benefits to the beetle from these fungi appear obvious as well.

Beetles containing *Entomocorticium* sp. A are more fecund, heavier, and have higher lipid contents, than those containing *C. ranaculosus*. In turn, beetles containing *C. ranaculosus* tend to be more fit than those whose mycangia contain no fungi (Bridges, 1985; Goldhammer *et al.*, 1990, Coppedge *et al.*, 1995). Thus, the two mycangial fungi can be considered to be nutritional mutualists of SPB.

Attempts to adequately describe the complexity of SPB-fungal ecology must, in addition, involve consideration of the mites associated with both the beetle and the fungi. The SPB is associated with, and may transport from tree to tree, over 57 species of mites (Moser and Roton, 1971; Moser *et al.*, 1971;1974). The SPB associated acarofauna includes parasitic, predatory, fungivorous and omnivorous species. Only a few species within this complex are truly phoretic, in which the mite is transported on the external surface of the beetle and does not undergo feeding or ontogenesis during this period of transport (Lindquist, 1969; Smiley and Moser, 1974). In particular, phoretic mites within the genus *Tarsonemus* have been the focus of most of the limited amount of research conducted in bark beetle-mite interactions (Moser and Roton, 1971; Smiley and Moser, 1974; Moser, 1976; Bridges and Moser, 1983; Moser and Bridges, 1986). We have concentrated on three mite species *Tarsonemus ips* Lindquist, *Tarsonemus krantzii* Smiley and Moser, and *Tarsonemus fusarii* Cooreman. All three of these mites are common SPB associates (though *T. fusarii* is less common and seemingly more of a generalist than the other two species). *Tarsonemus ips*, *T. krantzii*, and *T. fusarii* are all phoretic on SPB, obtaining transport to new, suitable host material with no – directly – discernible deleterious effects on the beetle. However, all three mites have shown at least the potential to impact the SPB-fungus-tree interaction. All three mites possess sporothecae, which are specialized, flap-like structures of the integument (Fig. 13). In *T. ips* and *T. krantzii*, these sporothecae have been found, relatively frequently, to transport ascospores of *O. minus*(Bridges and Moser, 1983; Moser, 1985) and *C. ranaculosus*(Moser *et al.*, 1995). Despite the possibilities raised by these circumstances, nothing more was known about the relationships between the mites and their associated fungi, nor about the implications of these interactions to the beetle-fungus relationship.

Ecological interactions in the SPB community.

We have attempted to unravel the complex ecological interactions among tree killing bark beetles, fungi, and mites using SPB as our study organism. Taking a reductionist approach, we have considered the manner in which SPB associated fungi compete with one another and thus facilitate or interfere with the success of SPB. We have also considered the role of mites as indirect facilitating, and/or interfering, agents in fungus-insect-tree host interactions.

Fungal competition. The three major SPB associated fungi, *O.* minus, *Entomocorticium* sp. A, and *C. ranaculosus*, compete for the rare and ephemeral resource of uncolonized pine phloem (Klepzig and Wilkens, 1997). It is likely that, in doing so, these fungi follow the previously stated model of primary resource capture, followed by direct interaction, which can lead to defense, and/or secondary resource capture. Likewise, the degree to which these fungi differentially compete can be quantified. The de Wit replacement series has been used extensively to study plant competition, and is being increasingly accepted as an analytical tool for microbial competition (Adee *et al.*,

1990; Snaydon, 1991; Wilson and Lindow, 1994; Klepzig and Wilkens, 1997; Klepzig, 1998) but see a cautionary note in Newton *et al.,* (1998). In using this technique with microbes, varying proportions of inoculum of potentially competing microbes are introduced onto a substrate. In the case of competing fungal hyphae, this may consist of inoculating substrate (e.g., agar medium, pine billets) with varying numbers of agar disks colonized with hyphae of one fungal species. The initial inoculum of one species is increased with each replicate as the initial inoculum of the other species is decreased. The population size (in the case of fungal hyphae, the area colonized) is determined at the end of the experiment as a function of initial population size (in this case, the percentage of each species in the original population). If no differential competition is occurring between the two species, it is expected that there will be a close to one-to-one linear relationship between proportion of the fungus in the initial inoculum and its representation (area colonized) in the final population (Fig. 14) (Wilson and Lindow, 1994). Differential competition between two fungi is indicated when there is a significant positive deviation from linearity for one and a significant negative deviation from linearity for the other. To determine this, the areas colonized by each fungus at the end of the experiment are recorded, and the means are calculated and log transformed. An ANOVA is performed on the transformed means to test for deviations from linearity in the relationships between final area colonized and initial inoculum proportion, for each fungal species. Specifically, pairwise competitions between *O. minus*, *Entomocorticium* sp. A., and *C. ranaculosus* can be conducted to determine the degree to which differential competition occurs among these frequently co-occurring fungi vectored by the same beetle. From laboratory experiments, it is absolutely clear that differential competition occurs amongst these three fungi (Klepzig and Wilkens, 1997). In all three pairwise comparisons, there were significant deviations from linearity in the relationships between initial and final population representation in the competing fungi (Fig. 15). The clearly superior competitor, at least on the artificial media used, was *O. minus* whose rapid growth rate and aggressive resource capture tactics overwhelmed the two mycangial fungi at even the lowest levels of *O. minus* inoculum (Fig. 16). The mycangial fungi were rapidly outcompeted by *O. minus* for the available substrate. *Entomocorticium* sp. A. and *C. ranaculosus*, however, were very similar in their relative competitive abilities, and the graph of their de Wit replacement series reflected this (Fig. 15c). There is even the appearance of the classic 'x'-shaped pattern in the data, which would (but for the skewing in the favor of *C. ranaculosus*) suggest a lack of differential competition.

Beyond the determination of the existence of differential competition with SPB associated fungi, is the question of the outcomes of competition among these fungi. Which, for example, of the SPB fungi is best able to hold on to colonized substrate in the face of a concerted secondary resource capture effort by another fungal species? We have measured the relative primary and secondary resource capture capabilities of these three fungi. When the three SPB associated fungi are forced to compete one-on-one on both artificial medium (malt extract agar) and natural substrate (loblolly pine billets), *O. minus* invariably comes out the victor in primary resource capture. Due again, in no small part, to its relatively rapid growth rate, *O. minus* can quickly colonize and gain control of substantially more of the available territory (uncolonized agar as well as pine phloem) than can either of the two mycangial fungi (Fig. 17). As is the case in considering differential competition, the two mycangial fungi are approximately equal

competitors for the capture of primary resource. However, due to its higher growth rate, *C. ranaculosus* is significantly able to outcompete *Entomocorticium* sp. A.

Once the primary resource capture phase of the one-on-one competition is over, however, and the direct confrontations begin, the SPB associated differ in their competitive abilities in interesting ways. When *O. minus* grows into the same area of substrate as *C. ranaculosus*, aerial hyphae begin developing at the colony margins of both species. Within a short while (about 48 hours), however, the heavily melanized hyphae of *O. minus* have grown over the margins of the hyaline *C. ranaculosus* colonies and begun the process of secondary resource capture (Fig. 17b). By the 11^{th} day of competition between these two fungi, *C. ranaculosus* colonies have most often been completely overgrown by *O. minus*. The competition between the fast growing *O. minus* and the slow growing, amber colored, floccose basidiomycete, *Entomocorticium* sp. A unfolds in a much different fashion. Although, due to the slow growth rate of *Entomocorticium* sp. A, *O. minus* is able to capture a great deal of uncolonized substrate before it reaches the basidiomycete, the direct interaction of these two fungi slows *O. minus* drastically. Very slightly before the growing hyphae of *O. minus* reach the *Entomocorticium* sp. A colony margins, they slow in growth rate. There is little to no development of the aerial hyphae seen in the *O. minus*/*C. ranaculosus* interaction. The *O. minus* colony, if it grows further, grows around the *Entomocorticium* sp. A colony, never growing over the basidiomycete and never accomplishing any secondary resource capture (Fig. 17a). This dramatic limitation on the growth and further spread of *O. minus* suggests either very close range diffusion of antibiotics from *Entomocorticium* sp. A to *O. minus*, or localized nutrient depletion by *Entomocorticium* sp. A such that *O. minus* cannot develop further in substrate which has been colonized by *Entomocorticium* sp. A. These same patterns of competitive interactions also hold true within loblolly pine billets.

Environmental (abiotic) factors may also alter the intensity and nature of competitive interactions (Callaway and Walker 1997). Temperature drastically affects growth rates in all three SPB associated fungi (Fig. 18). Of particular note are the differences in the manner in which the three fungi respond to varying temperatures. *Ophiostoma minus* seems particularly adaptable to a range of temperatures; its range of optimal temperatures for growth is wider, and its minimum growth temperature lower, than are the same variables for either of the two mycangial fungi. This may be due, in part, to the protected manner in which the mycangial fungi are transported (within a mycangium) and cultivated (within the galleries of successful SPB) relative to *O. minus* (which is transported on beetle and mite exoskeletons, and inoculated by SPB attacking living trees). Nutrient levels within phloem may also impact growth and competitive interactions among SPB fungi.

Several implications for SPB and its pine host arise from the interactions described above. It is apparent that *O. minus* is best equipped to capitalize on the uncolonized phloem available in the early stages of SPB attack in pines. Not only does this aggressive fungus grow more rapidly than the two mycangial fungi, it is also more tolerant of pine allelochemicals than *Entomocorticium*sp. A (Bridges, 1987). This, of course, may be advantageous to the beetle and, especially if *O. minus* does assist in killing the tree, disadvantageous to the tree. As tree resistance is overcome, and the female beetles begin inoculating the mycangial fungi into the phloem, the aggressive saprophytic (for the tree is essentially dead at this point) characteristics of *O. minus*

become a disadvantage for SPB. At this point, the female needs to establish colonies of either *Entomocorticium* sp. A or *C. ranaculosus* in the vicinity of the larvae, and far enough away from growth of *O. minus* for the fungi to become established and serve as a larval food source. At this point the differences between the two mycangial fungi come into focus. One of the fungi, *C. ranaculosus*, grows marginally faster than the other, but – once *O. minus* reaches it – does not seem capable of defending this territory enough to allow larval development (Klepzig and Wilkens, 1997). This fungus, especially when considered with its apparent relative inferiority as a larval nutritional substrate (Bridges, 1983; Goldhammer *et al.,* 1990; Coppedge *et al.,* 1995) would seem to be of less value as a symbiont than *Entomocorticium* sp. A. *Entomocorticium* sp. A, while slower growing than *C. ranaculosus*, is definitely capable of growing and providing nutrition for SPB larvae, even when surrounded by *O. minus*. The key to larval success, then, may be establishing a thriving culture of *Entomocorticium* sp. A soon enough, or far enough away, that it can grow without interference from *O. minus* (recalling that when these two fungi compete for uncolonized substrate, *O. minus* wins). In this sense, as well as in the nutritional sense, *Entomocorticium* sp. A is apparently the superior of the two mycangial fungi.

Here we find the SPB system posing another conundrum. The question might be stated thus "If *O. minus* is antagonistic to SPB larvae, and *C. ranaculosus* is of only moderate (or negative) value as a symbiont, why are these fungi so consistently associated with the beetle? Where is the selection pressure for maintaining fungal relationships of dubious value?" Recalling that "the success of species in a community is affected not only by direct interactions between species, but also by indirect interactions among groups of species (Miller, 1994, as cited in Callaway and Walker, 1997), the phoretic mites of SPB seem deserving of consideration.

Mite-fungus interactions. As described above, both *T. ips* and *T. krantzi* possess sporothecae, within which they carry spores of *O. minus* and/or *C. ranaculosus*(Bridges and Moser, 1983; Moser, 1985; Moser *et al.,* 1995). Neither of these mites have ever been found to transport *Entomocorticium* sp. A. Until recently, however, the nature of the relationship between these tarsonemid mites and the fungi they apparently vector into pine phloem, remained undescribed. When cultures of *T. ips*, *T. krantzi*, and *T. fusarii* are initiated on pure cultures of the three major SPB fungal associates, reproduction occurs, but the results vary in a manner that helps explain the questions raised by the fungal competition research described above (Lombardero *et al.,* 2000). All three mites can successfully reproduce, and their offspring thrive (larval survival to first reproduction has been conservatively estimated at 90%), on colonies of *O. minus*(Table 1) . Indeed, colonies of all three mites species have positive growth rates when feeding upon new hyphal growth of the fungal species they transport (Table 2) – *O. minus* and *C. ranaculosus*. However, none of the three mites had significant population growth when feeding on the one fungus they do not transport, *Entomocorticium* sp. A. When *T. fusarii* colonies were established on two other fungal species (which are commonly vectored by other bark beetles, but only occasionally associated with SPB), the colonies reproduced successfully on *Leptographium terebrantis* Barras and Perry but not on *Ophiostoma ips* (Rumbold) Nannf.. Field observations showing that over ten times the number of tarsonemid mites are found within patches of *O. minus* infested phloem vs. other areas,

further the case for a symbiotic association of these two organisms (Lombardero *et al.*, 2000).

Ecological/economic implications. The web of complex relationships between mycangial fungi, phoretic fungi and phoretic mites associated with SPB, have significant implications to its life cycle and population dynamics of SPB. The possibility that *O. minus* assists SPB in killing tree hosts, or at least in overcoming tree resistance and/or conditioning the host tissue means that it may be vitally important that this fungus is present on the beetles or on their phoretic mites. Subsequently, the apparent dependence of developing larvae on vigorous growth of the mycangial fungi (especially *Entomocorticium* sp. A) demonstrates the importance of the presence of this fungus in the mycangium. If all three of the fungi are present within the network of SPB galleries, then the outcomes of fungal competions become extremely important. If *O. minus* is able to colonize the phloem around developing larvae, either because *Entomocorticium* sp. A has not yet become sufficiently established or because *C. ranaculosus* became established but was outcompeted by *O. minus*, larval development may be severely reduced. If *Entomocorticium* sp. A is outcompeted in the phloem by *C. ranaculosus*, the outcome may be similar, due to the relative inability of *C. ranaculosus* to exclude *O. minus*as well as its inferiority as a nutritional substrate. The success of the phoretic mites is linked similarly to the outcome of fungal competition for phloem. All three *Tarsonemus* species seem to be highly dependent upon the successful vectoring, inoculation and growth of the fungi they perform best on, *O. minus* and *C.ranaculosus*. The possibility also arises of exploiting the interdependencies between beetle and fungus as control or management options for SPB.

The negative effects of *O. minus* on SPB larval development could be seen as a positive, if one the aim was to lower SPB population levels. However, augmentation of *O. minus* levels in the field might be counterproductive if it resulted in greater amounts, or degrees of bluestained wood, which is of lesser economic value both as lumber and pulp (Seifert, 1993). This has led to investigations into the use of a similar fungus, marketed under the trade name of Cartapip (Clariant Corporation, Charlotte, NC USA). Cartapip is a colorless strain of *Ophiostoma piliferum* (Fries) H. and P. Sydow which has been used to degrade pitch in wood chips (Blanchette *et al.*, 1992) and outcompete blue stain fungi. This white fungus differentially competes with all three SPB associated fungi (Fig. 19), outcompetes the mycangial fungi (and to a lesser degree, *O. minus*) in primary resource capture (Fig. 20) (Klepzig, 1998). While Cartapip is not able to capture already colonized substrate from *Entomocorticium* sp. A or *O. minus*, it does show some promise as a possible biocontrol agent of SPB, by virtue of its ability to interfere with the symbiotic relationships between SPB and its fungi.

Conclusions. Relationships among symbiotic organisms may change over time and ranges of resources. Other organisms may indirectly facilitate or interfere with these relationships. Interactions among bark beetles and their associated fungi and mites are complex examples of the manner in which symbioses change and are indirectly affected by other organisms. These complex relationships have been extensively studied in the southern pine beetle (SPB), a bark beetle that kills healthy living trees through mass colonization. The SPB is consistently associated with three main fungi. Two of these fungi (*Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. A.) are carried in a

specialized structure (mycangium) in female SPB. The third fungus is carried phoretically on the exoskeleton. Both *C. ranaculosus* and *Entomocorticium* sp. A are also carried by phoretic mites of SPB. Due to the effects of these fungi on SPB larval development, their competitive interactions have significant implications. The two mycangial fungi provide nutrition to developing larvae, while the phoretic fungus interferes with larval development. These interactions appear to be mediated by phoretic mites which have mutualistically symbiotic relationships with the SPB associated fungi they vector. The multiple interdependencies in this system provide novel opportunities for control of, and further research on, this damaging forest pest complex.

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Figure 1. Southern pine beetle mycangium. A) Light micrograph of cross section of mycangium with fungal spores contained within. B) Scanning electron micrograph of mycangium. Mycangium has been partially dissected to reveal fungal spores contained within.

Figure 2. Southern pine beetle adults in pine phloem. The outer bark has been stripped away to reveal the male and female near the nuptial chamber, the female is beginning to create ovipositional gallery.

Figure 3. A pine tree mass attacked by southern pine beetle. Each of the numerous pitch tubes are an attempt by the tree to flood the beetles out of the tree through heavy resin flow.

Figure 4. *Ophiostoma minus*. A) Culture grown on malt extract agar. B) Tissue damage from inoculation of *Pinus taeda* with *O. minus* note heavy accumulation of tannins and related defense compounds in cambial tissue.

Figure 5. Areas of "bluestain", within southern pine beetle infested pine, due to infection with *O. minus*.

Figure 6. Southern pine beetle larval galleries within pine logs. Both logs infested with surface sterilized beetles, log on right was inoculated with *O. minus*, log on left was not inoculated. Larval development in *O. minus* infected logs was heavily reduced compared to uninfected logs.

Figure 7. Mycangium dissected from a female southern pine beetle. The head (above) and prothoracic legs (below) have been removed. Two streams of yeast-like spores of the fungi contained within the mycangium can be seen streaming from the pore-like openings of the structure.

Figure 8. *Ceratocystiopsis ranaculosus*. A) Culture grown on malt extract agar. B) Tissue damage from inoculation of *Pinus taeda* with *C. ranaculosus*, note only moderate accumulation of tannins and related defense compounds in cambial tissue.

Figure 9. *Entomocorticium* sp. A. A) Culture grown on malt extract agar. B) Tissue damage from inoculation of *Pinus taeda* with *Entomocorticium* sp. A, note only moderate accumulation of tannins and related defense compounds in cambial tissue.

Figure 10. Tissue damage due to mechanical wounding of *Pinus taeda*. Note lack of tannin accumulation and related defense compounds, and presence of callus growth, in cambial tissue.

Figure 11. Growth of mycangial fungi in southern pine beetle pupal chamber. Note sporulation.

Figure 12. Phloem N (%) in southern pine beetle infested phloem. "Good brood" = lack of bluestain, growth of mycangial fungi, abundant larval feeding galleries and pupal chambers; "Failed brood" = poor larval feeding and development, and lack of pupal chambers; "Blue stain" = abundant growth of *Ophiostoma minus* in larval gallery system, poor larval development; "No gallery" = no larval galleries present in area of sampling; all compared to "Uninfested trees" which contained no southern pine beetles. Bars followed by different letters are significantly different at $p<0.05$ level.

Figure 13. *Tarsonemus* sp. mites carrying A) crescent shaped spores of *Ophiostoma minus*, and B) tadpole shaped spores of *Ceratocystiopsis ranaculosus* within sporothecae (laterally located on the mite body).

Figure 14. DeWit replacement series diagram which would theoretically result if there was not differential competition between two co-occurring species.

Figure 15. DeWit replacement series diagrams resulting from competition between: A) *Ophiostoma minus* and *Entomocorticium* sp. A.; B) *O. minus* and *Ceratocystiopsis ranaculosus*; and C) *C. ranaculosus* and *Entomocorticium* sp. A. Standard errors are given about each mean.

Figure 16. Differential competition between fungi associated with southern pine beetle. 100% (right) and 80% (left) of mycancial fungus in the initial inoculum. A) *Ophiostoma minus* and *Entomocorticium* sp. A. Note that although *O. minus* is outcompeting *Entomocorticium* sp. A., that the original inoculum disks of the mycangial fungus are still uncolonized by *O. minus* ; B) *O. minus* and *Ceratocystiopsis ranaculosus.* Note that although *O. minus* is outcompeting *C. ranaculosus*, that the original inoculum disks of this mycangial fungus have been colonized by *O. minus.*

Figure 17. Secondary resource capture in competitive interactions between southern pine beetle associated fungi. A) *Ophiostoma minus* vs. *Entomocorticium* sp. A. Note that *O. minus* has not captured substrate already colonized by *Entomocorticium* sp. A. B) *O. minus* vs. *Ceratocystiopsis ranaculosus.* Note that *O. minus* has captured substrate already colonized by *C. ranaculosus.*

Figure 18. Effects of temperature on linear growth of southern pine beetle associated fungi (*Ophiostoma minus*, *Entomocorticium* sp. A, and *Ceratocystiopsis ranaculosus*) growing on malt extract agar.

Figure 19. DeWit replacement series diagrams resulting from competition between *Ophiostoma piliferum* (Cartapip) and: A) *Entomocorticium* sp. A.; B) *Ceratocystiopsis ranaculosus*; and C) *Ophiostoma minus*. Standard errors are given about each mean.

Figure 20. Differential competition between *Ophiostoma piliferum* (Cartapip) and *Ophiostoma minus*. Note the mutual ability of each species to keep the other from colonizing the substrate it holds.