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Competition and Coexistence In a Multi-partner Mutualism: Interactions Between two Fungal Symbionts of the Mountain Pine Beetle In Beetle-attacked Trees

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Abstract Despite overlap in niches, two fungal symbionts of the mountain pine beetle (*Dendroctonus ponderosae*), *Grosmannia clavigera* and *Ophiostoma montium*, appear to coexist with one another and their bark beetle host in the phloem of trees. We sampled the percent of phloem colonized by fungi four times over 1 year to investigate the nature of the interaction between these two fungi and to determine how changing conditions in the tree (e.g., moisture) affect the interaction. Both fungi colonized phloem at similar rates; however, *G. clavigera* colonized a disproportionately larger amount of phloem than *O. montium* considering their relative prevalence in the beetle population. High phloem moisture appeared to inhibit fungal growth shortly after beetle attack; however, by 1 year, low phloem moisture likely inhibited fungal growth and survival. There was no inverse relationship between the percent of phloem colonized by *G. clavigera* only and *O. montium* only, which would indicate competition between the species. However, the percent of phloem colonized by *G. clavigera* and *O. montium* together decreased after 1 year, while the percent of phloem from which no fungi were isolated increased. A reduction in living fungi in the phloem at this time may have significant impacts on both beetles and fungi. These results indicate that exploitation competition occurred after a year when the two fungi

colonized the phloem together, but we found no evidence of strong interference competition. Each species also maintained an exclusive area, which may promote coexistence of species with similar resource use.

Introduction

The effect of interspecies interactions in shaping ecological communities is well known. Closely related species that co-occur in a habitat and have similar resource requirements may be expected to compete with each other. Competition occurs when one species negatively affects another by consuming a common limited resource (exploitation or indirect competition) or when one species controls access to a limited resource (interference or direct competition) [18]. Competition often results in competitive exclusion with the victor being the competitor that can maintain itself on the lowest level of the common resource [8]. Interspecies-interaction research has focused on competition between plants growing at high densities and animals competing for the same resource; comparatively less is known about interspecific interactions involving fungi, including competition [36].

Interactions among fungi may affect other organisms in the community through multi-trophic interactions. For example, many phloophagous bark beetles (Coleoptera: Curculionidae, Scolytinae) have associations with ophiostomatoid fungi (Ascomycota) that are considered mutualistic [12, 39]. The fungi are transported by the beetles to otherwise inaccessible habitats. The beetles may receive one or more benefits from their fungal associates, such as nutritional supplementation, conditioning of host tree tissues for brood development (e.g., chemical changes), or aid in overwhelming tree defenses (reviewed in [27]). Bark

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beetle–fungal associations are not always mutualisms and can also include commensalisms and antagonisms, in addition to being context dependent [3, 20]. Mutualistic and antagonistic interactions with fungi may affect beetle fitness and potentially influence beetle population dynamics [4, 5, 14, 15, 25, 37]. For example, the southern pine beetle (*Dendroctonus frontalis* Zimm.) receives nutritional benefits from two fungal associates, although one species provides greater benefit to the beetle than does the other [5, 6, 9]. A third fungal associate of the southern pine beetle that is also associated with phoretic mites adversely affects brood survival [3]. Because fungal associates may differentially affect beetle fitness, interactions among the fungi (e.g., competition) may indirectly affect beetle fitness by influencing the distribution of fungi within a tree and their availability to beetles.

The fungal associates of bark beetles share the same resources within a tree, a limited substrate and easily assimilable nutrients present in the phloem [35]. Species co-occurring on the same resources are likely to compete, and the species that can live on the lowest level of a resource is predicted to exclude its competitors over time (competitive exclusion) [8]. Laboratory studies have demonstrated that the fungal associates of bark beetles can compete and that the dominant competitor usually is the species with the fastest intrinsic growth rate (Bleiker and Six, in review) [21, 22]. However, growth of bark beetle-associated fungi may also be affected by other factors, such as the water potential (Bleiker and Six, in review) [22] and phloem chemistry [13] of the tree and the presence of other microorganisms (e.g., yeasts and bacteria) [2, 10]. Previous studies examining competitive interactions among bark beetle fungal associates have been conducted on artificial media in the laboratory under controlled conditions. However, in trees, conditions change radically over the life cycles of both the beetles and fungi, which could potentially affect the outcome of fungal interactions. Therefore, field studies are needed to determine how fungi actually grow and interact in trees over time.

We examined interactions between two ophiostomatoid fungi, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield (previously *Ophiostoma clavigerum*) and *Ophiostoma montium* (Rumbold) von Arx, in trees colonized by the mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins). Numerous microorganisms are associated with the MPB; however, *G. clavigera* and *O. montium* are the only filamentous fungi consistently isolated from its mycangia (structures of the integument specialized for transporting fungi) [4, 38, 45, 46]. The fungi are inoculated into tree tissues by parent beetles as they construct egg galleries in the phloem (reviewed in [32]). Fungal spores are transported on the exoskeleton of MPB adults as well as in mycangia, which

are located on the maxillary cardines [38, 45]. Developing larvae ingest phloem and fungal hyphae as they construct feeding galleries, and teneral (young, sexually immature) adults often feed on spores that commonly line pupal chambers prior to emergence [1, 4, 23, 37, 45]. Fungal spores are acquired on the exoskeleton and in mycangia of teneral adults during pre-emergence feeding [37, 45].

Both fungi appear to enhance beetle fitness through nutritional supplementation, but the two fungi are not equal in their effects. Evidence indicates that while *G. clavigera* confers the greatest fitness benefits to the MPB, having *O. montium* as an associate is better than having no fungal associate [4, 37]. Larval preference for phloem colonized by both fungi together compared to phloem colonized by only one fungus indicates that there may be a benefit of feeding on both fungi together [4]. *G. clavigera* and *O. montium* differ in their environmental tolerances, and having two mutualists may reduce the chance that the beetle is left without the benefits of a partner if the environment is unfavorable for one associate [40]. Given the potential importance of *G. clavigera* and *O. montium* in the diet of the MPB and their effects on beetle fitness, fungal growth in trees and interactions between the fungi may have significant implications for developing beetles.

In a study conducted on artificial media, Bleiker and Six (in review) observed reduced growth of both *G. clavigera* and *O. montium* when they were growing together compared to when they were growing alone, indicating interspecific competition. Bleiker and Six (in review) also investigated the effects of water potential on the growth of both species by amending media with potassium chloride or sucrose to create a range of water potentials. On potassium chloride-amended media, growth of both species decreased as water potential decreased (−0.4 through −3.3 MPa). On sucrose-amended media, growth of *G. clavigera* also declined as water potential decreased; however, growth of *O. montium* remained relatively constant regardless of water potential. The solute used to amend the media confounded the effect of water potential. Thus, the authors were not able to infer how changes in phloem moisture in beetle-attacked trees might affect interactions between *G. clavigera* and *O. montium*.

It is difficult to determine how the fungi may interact in trees from laboratory experiments because conditions, e.g., chemical defenses, water relations, and presence of other organisms, change rapidly in trees after beetle attack. Therefore, we examined colonization rates and the relative prevalence of *G. clavigera* and *O. montium* in trees attacked by the MPB in the field over the 1-year life cycle of the beetle. We also correlated phloem moisture, which may affect fungal growth, to the relative prevalence of the two fungi in trees.

Methods

The prevalence of *G. clavigera* and *O. montium* in the phloem of lodgepole pine trees (*Pinus contorta* var. *latifolia* Dougl. Ex Loud.) successfully attacked by the MPB was sampled over the 1-year life cycle of the beetle. In early June 2003, prior to the beetle's flight, a small stand of mostly healthy (un-attacked) mature lodgepole pine trees was selected on the DeBorgia Divide outside of Thompson Falls in western Montana (47°29'0" N and 115°15'11" W; elevation 1,510 m). The MPB population in the area had been at outbreak levels for a number of years, and MPB-caused mortality was evident in most stands of mature lodgepole pine in the area. Given the high beetle pressure and the dwindling supply of host trees in the area, we predicted that the MPB would colonize a high percentage of the remaining healthy trees in this stand in summer 2003.

Anticipating attack by the MPB, 60 mature lodgepole pine trees were numbered and monitored for boring dust (indicating successful MPB attack) starting on 10 June 2003. Each tree was checked every 3 to 4 days, and the day of attack (time zero, T0) was recorded as the date when the tree was first observed to be mass attacked (i.e., high density of attacks initiated on the lower bole). Over the course of the summer, 43 of the 60 trees were mass attacked by the MPB. Fifteen trees, representing the full range of attack dates, were selected for sampling. Wildfires in the area temporarily blocked access to the site from 12 to 26 August; however, observations made on 26 August (and after) revealed that no successful attacks were initiated after 11 August. Trees were grouped into three categories based on time of mass attack: early-season-attacked trees (two trees, attacked on 26 June); mid-season-attacked trees (eight trees attacked between 10 July and 28 July); and late-season-attacked trees, (five trees attacked on 11 August). We sampled each tree at four different times: 2–4 weeks after attack [T1, eggs and early instar larvae (first or second instars) were the most common life stages observed]; 9–11 weeks after attack prior to over-wintering [T2, late instar larvae (third or fourth instars) were the most common life stage observed]; 42–47 weeks after attack (T3, prepupae or pupae were the most common life stages observed); and 52–57 weeks after attack just prior to brood emergence (T4, teneral adults were the most common life stage observed).

At each sample time, a 15×15 cm square bark sample was removed at four target heights (1, 2, 3, and 4 m) from a randomly chosen aspect on the bole of each of the 15 trees for a total of 240 bark samples. Bark samples were staggered just above or below the target height in order to accommodate the four samples taken over time. Prior to removing the bark sample, the rough outer bark flakes were rubbed off, and the area was sprayed with 95% ethyl alcohol. Bark samples were removed with a chisel that had been sprayed with 95% ethyl

alcohol. The exposed sapwood was sprayed with pruning seal to reduce moisture loss. Each bark sample was placed in a plastic bag that was sealed and stored in a cooler or refrigerator for up to 2 days before isolations were made. Using sterile technique, 16 phloem plugs were removed from each bark sample on a 4×4 cm grid for a total of 3,840 phloem samples over the course of the study. Samples were removed with a 3-mm sterile diameter cork borer just below the phloem-cambium surface in order to exclude any contaminants acquired while handling the sample.

Each phloem sample was placed on 2% malt extract agar amended with 100 ppm cycloheximide [11] in a Petri dish. Cycloheximide allows for the growth of *Ophiostoma* and *Grosmannia* species while inhibiting the growth of ubiquitous, non-symbiotic fungi from the environment (e.g., *Trichoderma* and *Penicillium* spp.), which can quickly overrun cultures. Cultures were grown at approximately 22 °C for a minimum of 8 weeks before colonies were identified using morphological characteristics (hyphae, asexual and sexual structures) [44]. Pieces of autoclaved pine twigs were added to the cultures after the initial identifications were made because the fungi, especially *O. montium*, sporulate more readily on pine twigs than on artificial media, greatly facilitating identification. Subcultures were also made to facilitate identification. The cultures were re-examined after an additional 4 to 6 weeks. Most cultures of ophiostomatoid fungi also contained yeasts and likely bacteria. Because it was difficult to determine if any cultures containing *G. clavigera* and/or *O. montium* were free of other microorganisms and other microorganisms were not the focus of the study, we classified cultures with respect to the ophiostomatoid fungi. We calculated the percent of phloem colonized by *G. clavigera* only (C only), *O. montium* only (M only), both fungi growing together (CM), as well as the percent of phloem colonized by ophiostomatoid fungi (total Oph.; previous three categories summed). The percent of cultures from which only yeasts were isolated (yeast only; no ophiostomatoid fungi present) and from which no microorganisms were isolated (i.e., no live microorganisms in sample) was also calculated.

Phloem moisture was measured at each of the four sample times (T1, T2, T3, and T4), as well as on the sample date when mass attacks were first identified (T0). A 2.5 cm diameter arch punch was used to remove a phloem plug at 1.5, 2.5, and 3.5 m on the tree bole. The phloem plugs were immediately placed in small, individual plastic bags and transported on ice to the laboratory. The average percent dry weight phloem moisture content was calculated for each tree using the oven dry weight method and the formula: [(wet weight-dry weight)/dry weight]×100. Phloem samples were dried for 48 h at 60 °C prior to taking the dry weight.

Phloem moisture was also measured on un-attacked (control) trees using the methods described above for

attacked trees. Most of the trees originally selected as controls were subsequently attacked by beetles and are hereafter referred to as “eventually attacked trees.” New, un-attacked control trees were selected to replace the controls that were attacked; however, many of these trees were also subsequently attacked. As a result, phloem moisture was only measured in six true control trees (referred to as “never attacked”); one tree was sampled at three different sample times over the summer, one tree was sampled twice, and the other four trees were only sampled at one sample time each. These nine measurements of phloem moisture for trees that were never attacked spanned the entire attack period from 26 June to 11 August 2003. Phloem moisture was also measured in ten trees that were eventually attacked; all trees in this category were only sampled once (because they were mass attacked by the next sample time). Sample times again spanned the entire attack period.

Data Analysis

In order to meet the assumptions of normality, the arcsine (or angular) transformation [41] was applied to the percent of phloem colonized by fungi. Transformed data were used in all statistical tests. Back transformed means and least squares (LS) means are reported and used in graphs. Standard errors (SEs) were calculated from back-transformed confidence intervals (± 1 SE), which were calculated from the transformed means. Phloem moisture data did not require transformation to meet the assumptions of the analyses.

A repeated measures multivariate analyses of variance (MANOVA) was used to test for variation in the percent of phloem colonized by *G. clavigera* and *O. montium* among sample heights (1, 2, 3, and 4 m) and fungi isolated (C only, M only, and CM). All interactions, including those with time, were included in the analysis. Because sample height and interactions involving sample height were not significant (see “Results”), data for the samples were summed by tree so that sample height could be removed from the analyses. This decreased the number of factors and excluded four-way interactions in the analysis and provided a better estimate of the percent of phloem colonized by the fungi at each sample time (now calculated from 64 phloem samples per tree). A repeated measures MANOVA was then used to test for the effects of time of attack (early-, mid-, and late-season-attacked trees), fungal species isolated, and their interaction on the percent of phloem colonized by *G. clavigera* and *O. montium*. All interactions, including those with time, were included in the analysis. Significant multivariate results were followed by univariate ANOVAs conducted using a general linear model (GLM) approach. Significant *F* tests were followed by Tukey–Kramer’s honestly significant difference (HSD) test, which is a conservative post hoc test appropriate for uneven sample sizes [41].

The Pearson product-moment correlation coefficient (Pearson’s *r*) was used to test for a correlation between the percent of phloem colonized by *G. clavigera* only and *O. montium* only at the same sample time. The percent of phloem colonized by both fungi growing together was also correlated with the percent of phloem colonized by *G. clavigera* only and *O. montium* only at each sample time.

To determine how phloem moisture in un-attacked trees varied over the course of a season, a two-way ANOVA was used to test for variation in phloem moisture with sample date (seven dates over the course of the summer). Phloem moisture measurements from control trees (never attacked or eventually attacked) and attacked trees at T0 were included in the analysis. Tree group (never attacked, eventually attacked, or attacked at T0) was included as a second factor in the ANOVA. The effects of time of attack (early-, mid-, and late-season-attacked trees), fungal species, and their interaction on phloem moisture in the 15 attacked trees was examined using a repeated measures MANOVA. Significant multivariate results were followed by ANOVAs conducted using a GLM approach. Significant *F* tests were followed by Tukey–Kramer’s HSD test.

Pearson’s *r* was used to test for an association between phloem moisture at one sample time and phloem moisture at the previous sample time in attacked trees. Pearson’s *r* was also used to test for a relationship between the amount of phloem colonized by *G. clavigera* only, *O. montium* only, or both fungi together and phloem moisture at the same and preceding sample times.

Significance was set at $P \leq 0.05$, and all analyses were conducted using JMP™ 5.1 (SAS Institute, Cary, NC, USA).

Results

Attack Period

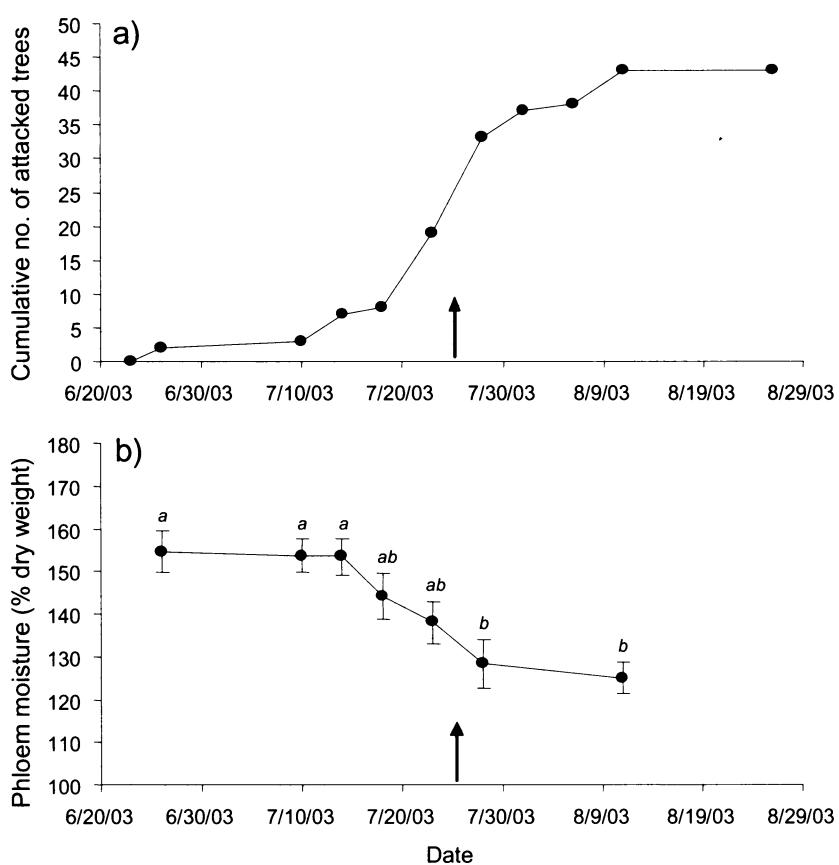
MPB initiated mass attacks on 43 of the 60 mature lodgepole pine trees that were monitored during the summer of 2003 (Fig. 1a). MPB mass attacked trees between 23 June and 11 August; however, most new attacks were found on 23 and 28 July (11 and 14 trees, respectively; Fig. 1a). Thus, the peak attack period for the MPB likely occurred around this time (Fig. 1a).

Phloem Moisture

Unattacked Trees and Attacked Trees at the Day of Mass Attack (T0)

Trees that were not attacked during the 2003 season had higher phloem moisture than trees that were sampled prior to being attacked but which were eventually attacked later

Figure 1 Effect of time on **a** the cumulative number of lodgepole pine trees mass attacked by the mountain pine beetle, and **b** phloem moisture (% dry weight). Arrows indicate peak attack time for the MPB beetle at the site. Phloem moisture was measured on non-attacked trees, but many of the sample trees were subsequently attacked by the MPB and had to be replaced by new trees. As a result, phloem moisture measurements include six trees that were never attacked by the mountain pine beetle: one tree was sampled at three different sample times, one tree was sampled at two different sample times, and four trees were sampled at only one sample time. Ten trees were sampled either prior to being attacked or on the day they were attacked (T0, see text). Sample sizes (n) for phloem moisture at each date in the order they occur on the x -axis are 4, 7, 5, 3, 4, 3, and 8 trees. For phloem moisture, LS means (\pm SE) are shown, and dates with the same letter are not significantly different at $P \leq 0.05$



in the season or trees that were sampled in the very initial stage of mass attack (T0; $F_{2,25}=5.92$, $P=0.008$; Fig. 2). Phloem moisture decreased in all trees during the last 2 weeks of July ($F_{6,25}=8.61$, $P<0.0001$; Fig. 1b). Phloem moisture was higher in trees sampled on or before 14 July 2003 than in trees sampled on or after 28 July 2003; trees sampled on 18 and 23 July were not different from trees sampled earlier or later in the season (Fig. 1b).

Attacked Trees

Phloem moisture of trees mass attacked by the MPB varied with sample date ($F_{4,9}=603.93$, $P<0.0001$) but not with time of mass attack (trees attacked early-, mid-, or late-season; $F_{2,12}=0.17$, $P=0.85$; Fig. 3). Overall, phloem moisture decreased rapidly after the initial mass attack, dropping over 40% in the first 2–4 weeks (between T0 and T1; Fig. 3). Although phloem moisture decreased rapidly in all trees following attack, differences among early-, mid-, and late-season-attacked trees varied over time (significant interaction, $\lambda_{8,18}=0.07$, $P=0.0008$; Fig. 3). Late-season-attacked trees had lower phloem moisture when mass attacks were first initiated (T0) compared to trees that were attacked early or mid-season ($F_{2,36}=19.61$, $P<0.0002$; Fig. 3), likely because phloem moisture decreases in trees over the summer. At sample times T1 and T2, phloem

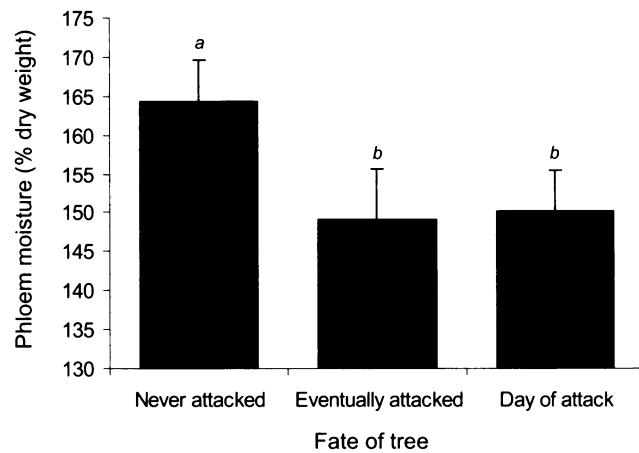


Figure 2 Phloem moisture (% dry weight) of lodgepole pine trees that were not attacked by the mountain pine beetle (never attacked), trees that were sampled prior to being attacked (eventually attacked), and trees that were sampled before and on the day of attack. The “never attacked” category includes nine measurements on six different trees: one tree was sampled at three different sample times, one tree was sampled at two different sample times, and four trees were sampled at only one sample time. Ten “eventually attacked” and 15 “day of attack” trees were sampled at one sample time each. LS means (\pm SE) are shown so that variation due to sample date is removed. Tree classes with the same letter are not significantly different at $P \leq 0.05$

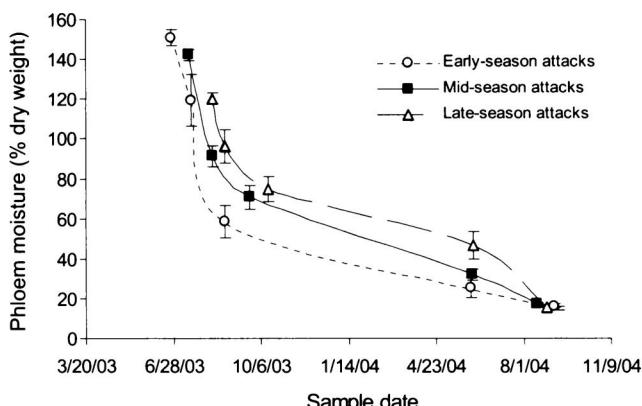


Figure 3 Phloem moisture (% dry weight) of lodgepole pine trees successfully mass attacked by the mountain pine beetle at different times in the summer: early-, mid-, and late-season. Phloem moisture was sampled five times over the 1-year life cycle of the beetle: the day of mass attack (T0); 2–4 weeks after attack (T1, most brood were eggs or early instar larvae); 9–11 weeks after attack prior to overwintering (T2, most brood were late instar larvae); 42–47 weeks after attack (T3, most brood were prepupae or pupae); and 52–57 weeks after attack prior to emergence (T4, most brood were teneral adults). For each type of attacked tree (early-, mid-, or late-attack), the symbols on the line correspond to T0 through T4

moisture did not vary with time of attack ($F_{2,36}=2.36, P=0.14$ and $F_{2,36}=0.77, P=0.48$, respectively). At T3, time of attack was significant ($F_{2,36}=3.88, P=0.05$): Phloem moisture was 25% in early-season-attacked trees, 32% in mid-season-attacked trees, and only 47% in late-season-attacked trees; however, no pairwise comparisons were statistically different. At the last sample time, there was little variation in phloem moisture, and it did not vary among trees attacked early-, mid-, and late-season ($F_{2,36}=2.71, P=0.11$).

Phloem moisture at each of the four sample times was not correlated with the previous sample time (T0–T1, $r=0.38, P=0.17$; T1–T2, $r=0.34, P=0.22$; T2–T3, $r=0.39, P=0.15$; T3–T4, $r=-0.02, P=0.95$).

Fungal Colonization in Attacked Trees

G. clavigera, *O. montium*, and yeasts were the most common microorganisms isolated from the phloem of MPB-attacked trees. The percent of phloem colonized by these fungi was similar in early-, mid-, and late-season-attacked trees (Fig. 4). The percent of phloem colonized by *G. clavigera* and/or *O. montium* is described below with the results of the repeated measures MANOVA. Yeasts were the only microorganisms isolated from approximately 13% of the phloem samples at T1; however, the number of samples containing only yeasts dropped to near 0% at later sample dates when much of the phloem was colonized by ophiostomatoid fungi (in addition to yeasts much of the time; Fig. 4). No microorganisms were isolated from over 50% of phloem samples at T1 (Fig. 4). However, the percent of samples containing only un-colonized phloem dropped dramatically by T2 when most phloem was already colonized by ophiostomatoid fungi.

The percent of phloem colonized by the ophiostomatoid fungal associates of the MPB did not vary with sample height on the bole of the tree ($F_{3,168}=0.07, P=0.97$), and there was no sample height by fungus interaction ($F_{6,168}=1.06, P=0.39$). Thus, isolations made from the four bark samples were combined by tree for each sample time, and the percent of phloem colonized was calculated from a total of 64 phloem samples per tree at each sample time. Fungus species isolated and sample time were significant factors in

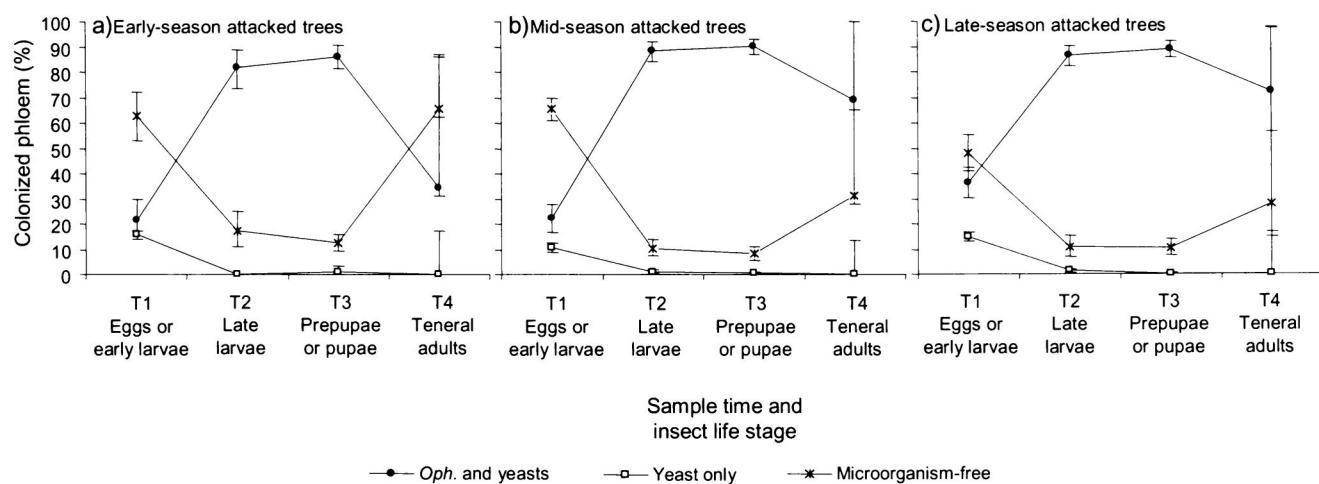


Figure 4 Percent of phloem colonized by ophiostomatoid fungi (*G. clavigera* and *O. montium* alone or in combination) and yeasts, yeast only, or microorganism-free in lodgepole pine trees attacked by the mountain pine beetle. Trees were attacked **a** early-, **b** mid-, or **c** late-

season ($n=2, 8$, and 5 trees, respectively) and were sampled four times over the 1-year life cycle of the mountain pine beetle (T1 through T4), corresponding to different life stages of the insect. Back-transformed means (\pm SE) are shown

this analysis (results for these factors are reported below for the test using data summarized at the tree level).

The percent of phloem colonized by ophiostomatoid fungi varied over time ($F_{3,34}=36.67, P<0.0001$; Fig. 4). The percent of phloem colonized by ophiostomatoid fungi increased sharply between T1 and T2, remained relatively constant between T2 and T3, and decreased between T3 and T4 (Fig. 4). The percent of phloem colonized by ophiostomatoid fungi did not vary with time of attack ($F_{2,36}=1.31, P=0.28$) but did vary by fungus ($F_{2,36}=13.96, P<0.0001$; Figs. 4 and 5). The interaction between sample time and time of attack did not affect the percent of phloem colonized by ophiostomatoid fungi ($F_{6,68}=1.50, P=0.19$). The interaction between fungus and time of attack had a significant effect ($\lambda_{6,68}=6.25, P<0.0001$), and the three-way interaction among sample time, time of attack, and fungus on phloem colonized by ophiostomatoid fungi was marginally significant ($\lambda_{12,90}=0.57, P=0.06$). At T1, the percent of phloem colonized did not vary with fungus ($F_{2,36}=0.42, P=0.66$), and there was no interaction between time of attack and fungus ($F_{4,36}=1.96, P=0.12$). At T2, there was more phloem colonized by hyphae of both fungi together or *O. montium* only than by *G. clavigera* only. The fungus by time of attack interaction approached significance ($F_{4,36}=2.41, P=0.07$), likely due to the relatively large percentage of phloem colonized by both fungi in late-season-attacked trees (Fig. 5c). At T3, there was more phloem colonized by both fungi together than by either fungus alone ($F_{2,36}=23.94, P<0.0001$); however, this was only true for early- and late-season-attacked trees (significant interaction $F_{4,36}=6.12, P=0.0007$; Fig. 5). At T4, the percent of phloem colonized did not vary with fungus or

the fungus by time of mass attack interaction ($F_{2,36}=0.23, P=0.79$ and $F_{4,36}=0.15, P=0.96$, respectively).

There were no significant correlations between the percent of phloem colonized by *G. clavigera* only and *O. montium* only at any sample time (Table 1). Similarly, there were no significant correlations between the percent of phloem colonized by *G. clavigera* only and both fungi together at any sample time (Table 1). The percent of phloem colonized by *O. montium* only and both fungi together was negatively correlated at T2 and T3 (Table 1).

Phloem Moisture and Fungal Colonization

The percent of phloem colonized by *G. clavigera* only was not correlated with phloem moisture at the same or preceding sample time (Table 2). Correlations between the percent of phloem colonized by *O. montium* only and phloem moisture were only significant at T1 when higher phloem moisture corresponded to a decrease in the percent of phloem colonized by *O. montium* (Table 2). The only significant correlation between phloem moisture and the percent of phloem colonized by both *G. clavigera* and *O. montium* together was when the percent of phloem colonized at T4 was positively correlated with phloem moisture at T3 (Table 2). There were two significant correlations between the total percent of phloem colonized by ophiostomatoid fungal associates of the MPB and phloem moisture. These were a negative correlation between phloem colonized at T2 and phloem moisture at T1 and a positive correlation between the total percent of phloem colonized at T4 and phloem moisture at T3 (Table 2).

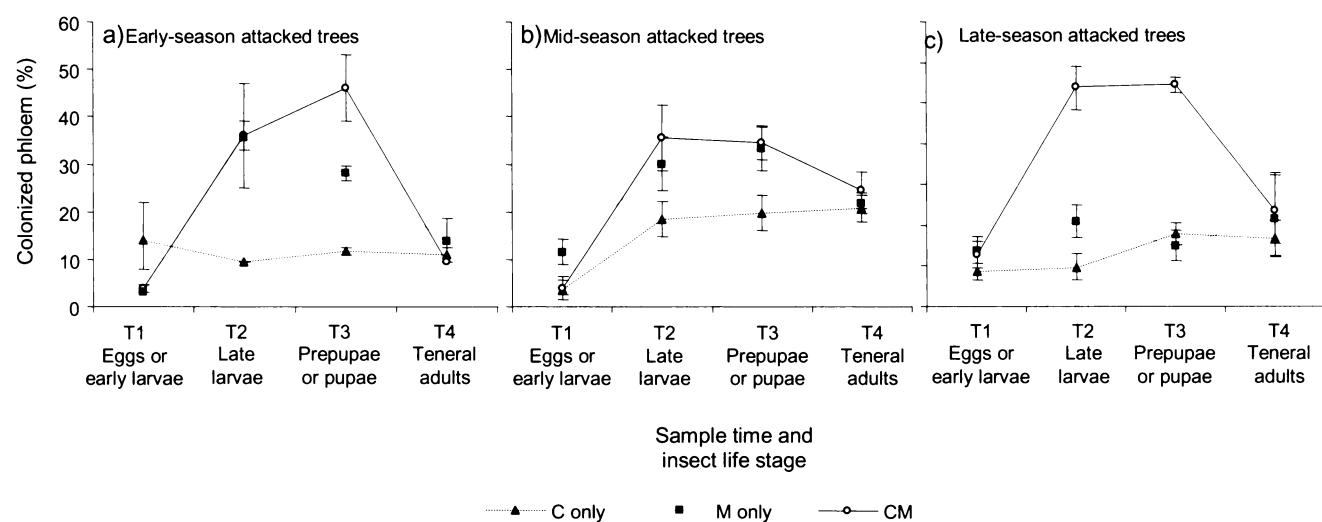


Figure 5 Percent of phloem colonized by *G. clavigera*-only (C only), *O. montium*-only (M only), or both fungi (CM) growing together in the phloem of lodgepole pine trees attacked by the mountain pine beetle. Trees were attacked (a) early-, (b) mid-, or (c) late-season ($n=$

2, 8, and 5 trees, respectively) and were sampled four times over the 1-year life cycle of the mountain pine beetle (T1 through T4), corresponding to different life stages of the insect. Back-transformed means (\pm SE) are shown

Table 1 Pearson's correlation coefficients (r) for comparisons of the percent of phloem colonized by either *G. clavigera* only, *O. montium* only, or both fungi growing together at four sample times in 15 lodgepole pine trees attacked by the mountain pine beetle

	Sample time and insect life stage			
	T1 Eggs/early larvae	T2 Late larvae	T3 Prepupae/pupae	T4 Teneral adults
<i>G. clavigera</i> only/ <i>O. montium</i> only	0.22	-0.25	-0.29	0.25
<i>G. clavigera</i> only/both fungi together	0.26	-0.29	-0.27	0.04
<i>O. montium</i> only/both fungi together	0.20	-0.69*	-0.70*	0.42

* $P \leq 0.05$

Discussion

The multiple fungal associates of bark beetles coexist in a highly variable and dynamic environment. Each fungus must not only survive in this rapidly changing habitat but also sporulate in pupal chambers of the beetles at the time when new adults are pre-emergence feeding so that spores can be acquired in the mycangia and disseminated to the next habitat (tree). By colonizing more phloem, a fungus will be able to sporulate in more pupal chambers and be acquired by more beetles, increasing the number of spores that are transmitted to new trees by beetles. Changes within a tree over time (e.g., moisture, chemistry, nutrient levels, and temperature) may have significant effects on fungal growth and survival. Thus, the distribution of *G. clavigera*

and *O. montium* within a tree, as well as interactions between them, may change over time. Given the differential effects of *G. clavigera* and *O. montium* on MPB fitness [4, 37], the distribution of each fungus, as well as interactions between the fungi, may ultimately impact the MPB by affecting how many beetles are exposed to each fungus.

O. montium and *G. clavigera* colonized the phloem of trees at similar rates, except for the two early-attacked trees where *O. montium* colonized the phloem faster than *G. clavigera* (Fig. 5a). However, the inoculation rates of the two fungi may have varied [39]. In summer 2003, the year that the trees were attacked, 88% of MPBs caught in flight traps at the site carried only *O. montium*, while 11% carried only *G. clavigera*; less than 1% of beetles carried both fungi (Thompson Falls site, [40]). Assuming that the fungal

Table 2 Pearson's correlation coefficients (r) for comparisons between the percent of phloem colonized by fungi at four sample times and phloem moisture at the same and preceding sample time in 15 lodgepole pine trees attacked by the mountain pine beetle

Colonized phloem	Phloem moisture				
	T0 Day tree attacked	T1 Eggs/early larvae	T2 Late larvae	T3 Prepupae/ pupae	T4 Teneral adults
<i>G. clavigera</i> only					
T1	-0.24	0.05			
T2		0.08	0.10		
T3			-0.13	0.02	
T4				-0.12	0.19
<i>O. montium</i> only					
T1	-0.42	-0.50*			
T2		-0.10	-0.29		
T3			-0.06	-0.24	
T4				0.27	0.28
<i>G. clavigera</i> and <i>O. montium</i> growing together					
T1	-0.47	0.01			
T2		-0.24	0.09		
T3			0.42	0.53	
T4				0.71*	0.30
Total <i>G. clavigera</i> and <i>O. montium</i>					
T1	-0.44	-0.20			
T2		-0.59*	-0.26		
T3			0.49	0.46	
T4				0.62*	0.31

* $P \leq 0.05$

complement carried by dispersing beetles at the site reflects inoculation rates for each species of fungus into trees. *O. montium* may have initially colonized a larger proportion of phloem because it was inoculated into trees at a rate eight times higher than *G. clavigera*. In turn, *G. clavigera* appears to have colonized a disproportionately large amount of phloem compared to *O. montium*. For example, in mid-season-attacked trees, *G. clavigera* colonized about half of the amount of phloem as *O. montium* (Fig. 5b), despite being inoculated at a lower rate. *G. clavigera* is considered to be a more virulent pathogen with a higher tolerance for low oxygen conditions than *O. montium* and thus may be better adapted for growing in living, defended tree tissues [42, 43]. This may have allowed *G. clavigera* to initially grow more rapidly than *O. montium* in tree tissues.

Growth of *G. clavigera* would also be predicted to be faster than *O. montium* in trees during much of the summer at the study site based on average ambient temperatures. *G. clavigera* grows faster than *O. montium* on artificial media at temperatures below 22 °C; however, *O. montium* grows faster than *G. clavigera* at temperatures above 27 °C [31, 37, 43]. The two fungi grow at similar rates on artificial media between 22 and 27 °C, and growth of both species slows as temperatures approach 0 °C. In 2003, average temperatures for the last week of June and for the months of July and August in the nearby town of Thompson Falls never exceeded 23.2 °C (NOAA: <http://www.ncdc.noaa.gov>). Actual site temperatures were likely slightly cooler because the site was 725 m higher in elevation than Thompson Falls. In early-season-attacked trees, the percent of phloem colonized by both fungi together increased between T2 in late August and T3 the following June (Fig. 5a), while phloem colonized by *O. montium* only decreased, and phloem colonized by *G. clavigera* remained the same. Cooler average monthly temperatures in September and October (average monthly temperatures at Thompson Falls were 16.4 and 10.8 °C, respectively) would have slowed growth of both fungi; however, *G. clavigera* still grows significantly faster than *O. montium* at 10 °C [31, 43]. This may have facilitated *G. clavigera*'s colonization of territory already occupied by *O. montium*, leading to a decrease in the amount of phloem colonized by *O. montium* only in early-season-attacked trees in the early fall. Growth of *O. montium* may have been minimal during this time, and thus any gains in territory may have been negligible. However, this interpretation requires some caution as only two early-season-attacked trees were available for sampling at the site. In mid- and late-season-attacked trees, the percent of phloem colonized by each fungus remained relatively constant between T2 and T3 (Figs. 5b and c) likely because temperatures were too cold in late September and mid-October for either fungus to grow substantially (average monthly temperatures fell below 0 °C in November).

Adams and Six [1] sampled fungi in phloem adjacent to different life stages of the MPB and found that *G. clavigera* was most commonly associated with pre- and post-wintering late instar larvae, supporting our assertion that *G. clavigera* may have had an advantage when temperatures were cooler. However, Adams and Six [1] also found that *O. montium* was most likely to be found in phloem adjacent to teneral adults, whereas our results indicate that the fungi colonize similar amounts of phloem in the tree at this time.

The results of this study raise the question of whether *G. clavigera* and *O. montium* actually compete in the phloem of beetle-attacked trees. Although there is likely substantial overlap in the niches of the two fungi, competitive exclusion of one fungus by the other did not occur over the 1-year time period. Furthermore, hyphae of the two fungi were apparently intermingled in the phloem, although the relative density of hyphae or the biomass of each species was not discernable. This is in contrast with the southern pine beetle system, where studies conducted in artificial media found that one of the mutualistic fungal associates was able to exclude the antagonistic fungal associate from its territory [21]. In addition, both mutualistic fungal associates of the southern pine beetle were unable to colonize territory already occupied by the antagonistic associate [21]. Interestingly, in the MPB system, both fungi may be considered mutualists of the beetle, and there may be a benefit to feeding on the two fungi together [4]. Our conclusions in this study are consistent with the results of Bleiker and Six (in review), which found that both *G. clavigera* and *O. montium* were able to colonize artificial media that was already occupied by the other species; however, growth of each species slowed when media colonized by the other species was encountered. In this study, the lack of a negative correlation between the percent of phloem colonized by *G. clavigera* only and *O. montium* only (Table 1) indicates that strong interference (direct) competition, such as deadlock-, combative-, or replacement-type interactions, did not occur between mycelia of these two species. It is interesting to note that the percent of phloem colonized by *O. montium* only was significantly negatively correlated with the percent of phloem colonized by both fungi together at T2 and T3. This supports our previous assertion that *G. clavigera* colonized areas already occupied by *O. montium*, likely during the fall. The decline in the percent of phloem colonized by both fungi together at T4 and the corresponding increase in phloem yielding no (live) fungi suggests that both species were adversely affected when growing together. This may be due to an added strain on resources, such as moisture (discussed below), which could be exacerbated when the two fungi co-occur. If a common, limiting resource is responsible for the decrease in phloem colonized by both fungi together and the corresponding

increase in the percent of phloem yielding no isolations of fungi, then the results of this study indicate exploitation (indirect) competition between *G. clavigera* and *O. montium*.

The effects of phloem moisture on fungal growth were strongest at the beginning and end of the 1-year life cycle of the MPB. Attack by the MPB occurred during the last week in July and corresponded with decreasing phloem moisture (Fig. 1). MPB either preferentially attacked or were more likely to successfully attack trees with lower levels of phloem moisture (Fig. 2). Low phloem moisture may be indicative of water stress in trees as the xylem (water-conducting tissue) and phloem are hydraulically connected [17]. Other studies have also found that the MPB attacks trees in the summer after moisture levels have declined [29]. Trees of low vigor, such as those under water stress, have been found to be less resistant to bark beetle and fungal colonization [e.g., 7, 26, 28–30]. Thus, beetles may preferentially attack water-stressed trees, or attacks on water-stressed trees may be more likely to succeed because of lowered tree defenses.

While it is widely accepted that the ability of water-stressed pines to physically ‘pitch out’ or expel attacking beetles is diminished, there may be another benefit to the beetle of attacking such trees. Fungal growth is inhibited in wood with a high moisture content (generally >120% dry weight) and a low oxygen content; optimal moisture content for fungal growth in wood is between 60% and 80% (reviewed in [35]). Thus, fungal growth may initially be inhibited in newly attacked trees, especially those with high moisture contents. Negative correlations between phloem moisture and fungal growth at early sample times support this hypothesis, although the effect was greater for *O. montium* than *G. clavigera* (Table 2). The fungi may be able to colonize the phloem of trees with lower initial moisture levels more rapidly than trees with higher moisture levels. In fact, the fungi were able to colonize more phloem in late-season-attacked trees, which had initial phloem moisture levels below 120% at T0, compared to early- and mid-season-attacked trees by T1, which had moisture levels in excess of 140% (Figs. 3 and 4).

Attacking trees with lower phloem moisture would promote faster colonization of the phloem by fungi and allow the beetles to receive benefits (e.g., nutritional supplementation or phloem detoxification) from their fungal symbionts sooner.

While fungal growth may have initially been inhibited by excessive phloem moisture (and limited oxygen), fungal survival may have ultimately been reduced by a shortage of phloem moisture at the end of the 1-year developmental period of the beetle. Moisture in wood becomes limiting for the growth and survival of ophiostomatoid fungi below 20% [19, 35]. Thus, moisture becomes limiting at the most critical time in the 1-year life cycle of the beetle—when

teneral adults are feeding and acquiring spores prior to emergence (Fig. 3). The overall drop in the percent of phloem colonized by ophiostomatoid fungi between T3 and T4 was a result of the decrease in phloem colonized by both fungi together and to a lesser extent of only *O. montium* (Figs. 4 and 5). Stronger positive correlations between phloem moisture and the percent of phloem colonized by both fungi together compared to each fungus alone late in the 1-year period supports moisture being most limiting when the two fungi colonized phloem together (Table 2). Both species ultimately appear to be negatively affected when one species invades territory already occupied by the other. A potential explanation may be that resource use is higher when the two fungi are growing together if the density of hyphae or fungal biomass per unit area increases when one fungus colonizes an area already occupied by the other species. Moisture may be a common limiting resource leading to strong indirect (exploitation) competition between the two fungi towards the end of the 1-year period.

Phloem moisture decreased rapidly in all trees once they were mass attacked by beetles (Fig. 3); however, it was not well-correlated between sample times (Table 2). This suggests that multiple factors, e.g., relative humidity, temperature, aspect or degree of exposure of tree bole, rate of beetle development, rate of fungal colonization, and proportion of the tree bole attacked, likely interact over time to determine the drying process. Optimal moisture levels for fungal growth (60–80% [35]) were reached by T2, which corresponds with the presence of late instar larvae—the life stages during which most of the total consumption and growth typically occurs in insects [34]. In this study, as in others, *G. clavigera*, *O. montium*, and yeasts were the most common microorganisms isolated from tissues of lodgepole pine trees attacked by the MPB, indicating a close association among these organisms [1, 19, 24, 46].

Our results indicate that, while exploitation competition occurred when the two fungi colonized phloem together towards the end of the 1-year life cycle of the beetle, interference competition was limited in this system. Species engaging in exploitation competition may coexist if each species can maintain an exclusive area or if there is some mechanism for partitioning the resource [33]. Interestingly, both fungi maintained almost the same amount of exclusive territory at the most critical time—the period when fungal spores are acquired by beetles prior to dispersal—despite fluctuations in area occupied throughout the year (Fig. 5). It is unknown if *G. clavigera* and *O. montium* partition resources in the phloem; however, they respond differently to potassium chloride compared to sucrose when the solutes are used to amend the water potential of media, suggesting that they may differ somewhat in their resource use or environmental tolerances (Bleiker and Six, in review).

Environmental variability may also promote coexistence of similar species by differentially affecting their growth rates and competitive abilities (e.g., [33]). Conditions in beetle-attacked trees change considerably over time, and fungi may be differentially affected by water availability, temperature, tree defensive chemicals, and the presence of other microorganisms (Bleiker and Six, in review) [2, 13, 15, 16, 20, 21, 25]. Differences in the temperature and oxygen tolerances of the two fungi may also lead to niche differentiation and promote coexistence of the two species [16, 33, 40, 43]. In fact, it has been demonstrated that the relative abundance of fungal associates carried by bark beetles varies with temperature [16, 40] as well as with population levels of phoretic mites and their fungal symbionts [15, 25]. Living in such a dynamic habitat, bark beetles may benefit from having multiple fungal symbionts that prosper under different environmental conditions [16, 40]. Resource partitioning, even on a fine scale, a constantly changing environment, and maintaining exclusive areas, may promote coexistence of species with overlapping niches and prevent competitive exclusion from occurring before the organisms are transported to the next habitat.

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