Components of Antagonism and Mutualism in *Ips pini*–Fungal Interactions: Relationship to a Life History of Colonizing Highly Stressed and Dead Trees

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ABSTRACT Efforts to describe the complex relationships between bark beetles and the ophiostomatoid (stain) fungi they transport have largely resulted in a dichotomous classification. These symbioses have been viewed as either mutualistic (i.e., fungi help bark beetles colonize living trees by overcoming tree defenses or by providing nutrients after colonization in return for transport to a host) or antagonistic (i.e., fungi compete for a limited resource and reduce brood development with no apparent benefit to the beetle). We investigated several components of one beetle–fungus interaction. Specifically, we addressed whether beetle entry into, and development within, a host tree vary with the degree of colonization by ophiostomatoid fungi. *Ips pini* (Say) transports several species of ophiostomatoid fungi, the most common being *Ophiostoma ips* (Rumbold) Nannfeldt, in the process of colonizing its host, *Pinus resinosa* Aitman. We introduced this fungus 0, 3, 7, and 10 d before beetle entry to characterize its effects on *I. pini* colonization and development. This sequence allowed quantification of temporal effects and comparison of results with other systems. Fungal growth was greatest when inoculated before beetle colonization. Fungal colonization reduced beetle entry into logs, but increased brood production. Mate capture was not significantly affected by fungal growth. The benefits imparted by *O. ips* to its beetle vector during brood development are compared with results from other systems. This difference may in part be related to the exploitation of highly stressed and dead trees, rather than vigorous hosts, by *I. pini*.

KEY WORDS bark beetles, *Ophiostoma ips*, ophiostomatoid fungi, symbiosis, insect–fungal interaction

BARK BEETLES, ESPECIALLY THOSE that colonize conifers, are intimately associated with a broad range of fungi, including basidiomycetes, ascomycetes, and various asexual fungi (Paine et al. 1997). Some of these relationships are clearly mutualistic: fungi benefit from transport to a new host, and beetles benefit from assistance in nutrition (Barras 1973, Six and Paine 1998, Ayres et al. 2000) or pheromone production (Brand et al. 1975, 1976; Brand and Barras 1977; Byers and Wood 1981; Leufven et al. 1984). Some fungi provide dietary nitrogen, amino acids, and possibly sterols to developing larvae after adult beetles colonize the host (Hodges et al. 1968, Barras and Hodges 1969, Bridges 1981, Ayres et al. 2000, Six 2003). Such activities can increase brood production (Six and Paine 1998). Many bark beetles possess specialized mycangia that house fungi for transport, suggesting a long shared evolutionary history (Six and Paine 1999; Six 2003).

Relationships of bark beetles with other fungi are less clear. Ophiostomatoid fungi (*Ophiostoma*, *Ceratocystis*, and *Ceratocystiopsis* spp., and their *Graphium* and *Leptographium* anamorphs) are almost exclusively transported on the beetle's exoskelton, including pit and sac mycangia (Whitney and Farris 1970, Harrington 1993, Paine and Hanlon 1994). These fungi are sometimes viewed as important agents that assist their vectors in killing trees (Christiansen 1985, Krokene and Solheim 1998). Most ophiostomatoid fungi, however, lack high levels of virulence, play a less significant (if any) role in tree killing (Bridges et al. 1985), and are confined within defensive lesions when inoculated into live trees (Shrimpton 1973; Raffa and Berryman 1982, 1983; Cook et al. 1986; Paine and Stephen 1987; Cook and Hain 1988; Klepzig et al. 1991; Lieutier et al. 1993; Raffa and Smalley 1995; Paine et al. 1997). Moreover, establishment of the ophiostomatoid fungus *Ophiostoma minus* (Hedgcock) H. & P. Sydow within logs before introduction of southern pine beetles (*Dendroctonus frontalis* Zimmerman) greatly reduces brood production (Barras 1970). This negative effect is sufficiently pronounced that the incidence of *O. minus* is inversely related with *D. frontalis* population densities (Lombardero et al. 2000, 2003). A third

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possibility is that ophiostomatoid fungi are not virulent, but rather assist beetles during colonization by reducing host plant defenses or obstructing water transport (Hemingway et al. 1977, Raffa and Berryman 1983, Leufven 1991, Paine et al. 1997). However, once the host is killed, these same fungi may act as antagonists either directly (e.g., by capturing resources; Yearian et al. 1972, Bridges and Perry 1985, Robins and Reid 1997) or indirectly (e.g., by outcompeting beneficial fungi; Klepzig and Wilkens 1997, Klepzig 1998). A complete characterization of a bark beetle–fungus relationship, as with other symbioses, may require evaluation of their interaction at several points in time (Callaway and Walker 1997).

The initial demonstrations of ophiostomatoid fungi reducing bark beetle brood production were conducted with *D. frontalis*, at a time when the predominant paradigm was of tree-killing fungi introduced by beetle vectors (Mathre 1964), in part modeled on the Dutch elm disease system (Sinclair and Campana 1978). Although these studies demonstrated fungal-to-insect antagonism to insects, the host material (logs) was inoculated substantially in advance of beetle introductions (Barras 1970). In nature, fungi and beetles arrive simultaneously. Therefore, we sought to consider temporal effects (and thus extent of fungal colonization) on beetle colonization and success.

The pine engraver, *Ips pini* (Say), occurs transcontinentally across North America (Lanier 1972) and colonizes most species of pine within its range (Wood 1982). In the Great Lakes region, *I. pini* is the most significant pest of mature red pine (*Pinus resinosa* Aitman) plantations (Klepzig et al. 1991, Erbilgin and Raffa 2002). These beetles show a pronounced preference for highly stressed, windblown, or dead trees (Schenk and Benjamin 1969). Males initiate colonization, release aggregation pheromones that attract both sexes, and mate with up to three females (Schenk and Benjamin 1969, Robins and Reid 1997). Each male constructs a nuptial chamber, from which females construct galleries 5–25 cm in length within which they lay eggs. Densities of male galleries range from 1.5 to 3.0 chambers per square decimeter (Poland and Borden 1994). The pine engraver does not possess glandular mycangia (Six 2003), but it does transport several fungal species (Furniss et al. 1995), of which the blue-staining *Ophiostoma ips* (Rumbold) Nannfeldt is the most predominant in Wisconsin (Klepzig et al. 1991).

The objective of this study was to quantitatively describe several components of the relationship between *I. pini* and its associate *O. ips.* Specifically, we sought to 1) assess the effect of *O. ips* on host acceptance by *I. pini* and 2) determine the effect of *O. ips* on reproduction of *I. pini.*

Materials and Methods

Test beetles were collected from a colony maintained at the University of Wisconsin-Madison. The colony originated from beetles collected in a red pine plantation in Sauk County, Wisconsin, and is maintained by supplying beetles with fresh red pine logs on a weekly basis and incorporating wild beetles several times each summer. Voucher specimens were deposited in the Insect Research Collection (University of Wisconsin, Madison, WI). The isolate of *O. ips* used in this study was isolated in pure culture in 2002 from a red pine recently killed by *I. pini.* Thomas Harrington (Department of Plant Pathology, Iowa State University, Ames, IA) confirmed our identification and deposited a subculture of this isolate in his reference collection (acquisition number C1927).

Bioassays. To assess the effects of *O. ips* on the development and brood production of *I. pini*, we cut 40, 16-dm^2 red pine logs from three (≈20.6 cm in diameter at breast height) trees. We waxed the ends of each log to prevent desiccation and randomly assigned each log to one of eight treatments. We mass inoculated 20 logs with *O. ips* by removing a 7-mm-diameter piece of bark with a no. 3 cork borer, adding a 3-mm-diameter piece of malt extract agar (MEA) colonized by *O. ips*, and replacing the bark over the hole. Each log received 20 inoculations arranged in four equidistant, longitudinal rows of five each. The remaining 20 logs were used as controls and were treated similar to those in the fungal treatment except that no fungus or MEA was placed into the mechanical wound. Each log was placed in a 19-liter rearing can, which was lined with two paper towels, equipped with two 237-ml emergence jars, and covered with black cloth under a wire mesh lid. The rearing room was held at 21–26°C, 45% RH and under constant light.

Before the bioassay, we surface sterilized all beetles with modified White's solution (Barras 1972). We added 20 male and 40 female beetles to each rearing can the same day (0 d), and 3, 7, or 10 d after fungal inoculation. Parental beetles entering the emergence jars during the first week were returned to the cans. Once progeny began to occur in the emergence jars, we collected, counted, and sexed progeny every 48–72 h. We also measured length and pronotal width for a subset of beetles from each collection date. Once emergence was complete (see Results), we removed the bark from each log and counted the number of male and female colonizers and females per male entrance site. We also measured gallery length. We replicated each treatment five times.

We quantified the growth of *O. ips* by periodically and destructively sampling a separate set of logs that were treated and colonized with beetles under the above-mentioned conditions. At 5, 15, and 30 d postinoculation or mechanical control, we collected four randomly located 100-cm^2 subsamples of bark from each log. We also included a full control, which was neither administered fungal inoculations nor mechanical wounds, nor colonized with beetles. This experiment was divided into three blocks with each treatment being replicated three times, except for the 15-d harvest, which was sampled twice. The complete design was [(two treatments (fungal inoculation, mechanical wound) * four beetle release times) + 1 control treatment] * three sampling dates * three blocks(reps) = 81 logs. We used a digital planimeter

(model 1250-1, Numonics Corp., Lansdale, PA) to measure the percentage of bark stained with ophiostomatoid fungi.

Statistical Analysis. Bark beetle colonization and development data and fungal growth data were analyzed using two-way analysis of variance (ANOVA) (PROC MIXED; Littell et al. 1996). For each analysis, each variable was tested for normality and homogeneity of variances by graphical analysis of residuals. For beetle size, we considered log from which beetle originated as a random effect. No transformations were required for beetle size (length or width); however, numbers of male and female colonizers were squared and all other variables were square root transformed to satisfy ANOVA assumptions. Transformations were not necessary for fungal growth data. F statistics were computed for all main effects with degrees of freedom for error assigned using the Satterthwaite approximation (Milliken and Johnson 1984, Littell et al. 1996). Significant treatment effects were further investigated by means of pairwise comparisons using multiple *t*-tests. Means and standard errors (before any transformation) were calculated using the PROC MEANS procedure and are reported for each fungal treatment × time combination. All reported differences among treatments are significant at $P < 0.05$, unless otherwise stated.

Fungal growth data from the digital planimeter were fit against time since inoculation by using Sigma Plot. Separate fits were conducted for each period before beetle introduction. We tested relationships by using linear, exponential, and power models, determined the best goodness-of-fit, and report the appropriate equation for each time since inoculation.

Results

Beetle Size and Development. Beetles were of similar size across all treatments (length: $F_{1,12} = 1.45$, $P = 0.252$; width: $F_{1,12} = 2.05$, $P = 0.178$). Across all treatments, females (3.72 ± 0.03 mm) were 3% shorter than males (3.85 ± 0.04 mm) ($F_{1,\ 8} = 5.71$, $P = 0.044$). Pronotal width measured 1.02 ± 0.01 and 1.04 ± 0.01 mm for females and males, respectively ($F_{1,\ 8} = 1.70$, $P = 0.228$). Progeny adults began to emerge at 22 d, with a median of 30.6 d and a maximum of 48 d.

Beetle Performance. The presence of fungi and the time of beetle introduction independently and interactively altered insect performance (Figs. 1 and 2). The magnitude of the response differed among the variables studied. The presence of *O. ips* reduced female colonization by 16% and caused a marginally significant ($P = 0.065$) reduction of male colonization (12%) relative to those exposed to noninoculated logs (Fig. 1a and b). Colonization was reduced by 47% in females and 49% in males when exposed to logs 10 d after fungal inoculation relative to those introduced simultaneously (Fig. 1a and b). Neither fungal inoculation nor degree of fungal colonization affected mate capture, with males mating with an average of 1.43 ± 0.03 females across all treatments (Fig. 1c).

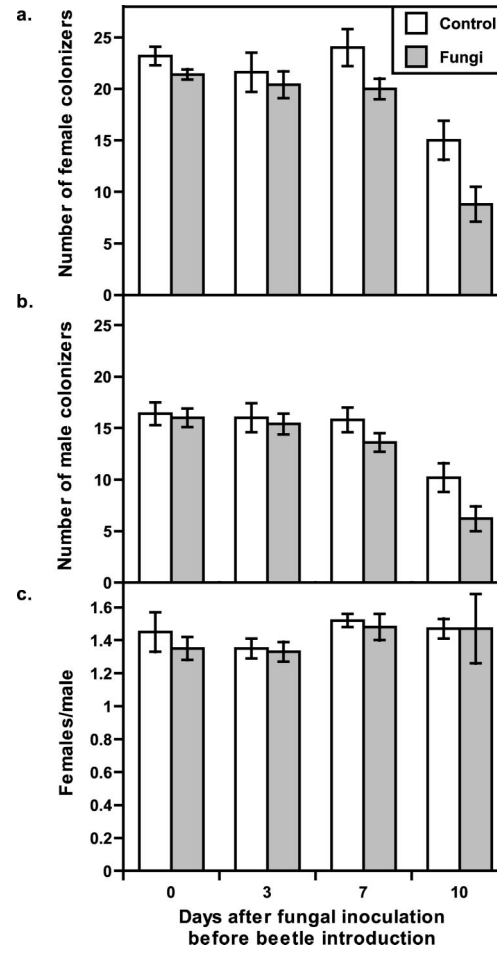

Fig. 1. Effect of *O. ips* on colonization and mate capture of *I. pini*. Error bars indicate ±1 standard error. *P* values indicate results of two-way ANOVA. (a) Number of female colonizers (fungi: $F_{1,32} = 9.39$, $P = 0.004$; time: $F_{3,32} = 17.21$, $P = <0.001$; fungi × time: $F_{3,32} = 0.58$, $P = 0.634$). (b) Number of male colonizers (fungi: $F_{1,32} = 3.65$, $P = 0.065$; time: $F_{3,32} = 14.73$, $P = <0.001$; fungi × time: $F_{3,32} = 0.39$, $P = 0.762$). (c) Females per male (fungi: $F_{1,32} = 0.43$, $P = 0.517$; time: $F_{3,32} = 1.04$, $P = 0.388$; fungi × time: $F_{3,32} = 0.05$, $P = 0.985$).

Parental gallery length, which correlates positively with brood production (Aukema and Raffa 2002), was marginally affected by the fungal treatment and significantly affected by the time between fungal inoculation and beetle infestation (Fig. 2a). Parent beetles in logs colonized by *O. ips* tended ($P = 0.097$) to construct longer galleries (8%) than did those reared in noninoculated logs. Beetles colonizing logs 10 d after they were inoculated with *O. ips* constructed galleries 41% shorter than did those colonizing logs inoculated simultaneously with *O. ips*.

The effect of fungal inoculation on brood production depended on the length of time *O. ips* was able to colonize the log before beetle colonization (fungi × time interaction; Fig. 2b). The most striking difference was noted for logs colonized by with beetles 3 d after

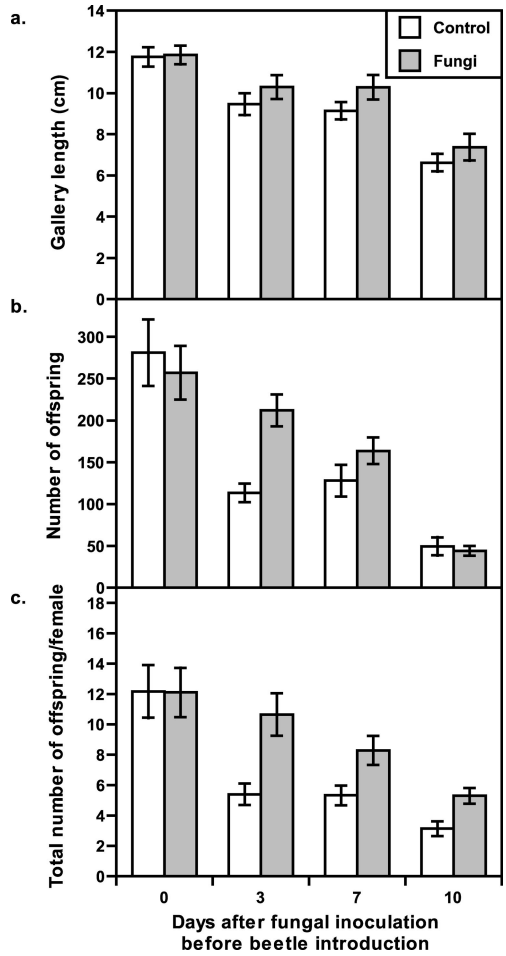

Fig. 2. Effect of *O. ips* on gallery length and progeny production of *I. pini*. Error bars indicate ±1 standard error. *P* values indicate results of two-way ANOVA. (a) Gallery length (fungi: $F_{1,32} = 2.76$, $P = 0.097$; time: $F_{3,32} = 25.40$, $P = <0.001$; fungi × time: $F_{3,32} = 0.21$, $P = 0.889$). (b) Total number of progeny (fungi: $F_{1,32} = 3.97$, $P = 0.055$; time: $F_{3,32} = 47.50$, $P = <0.001$; fungi × time: $F_{3,32} = 3.33$, $P = 0.032$). (c) Offspring per female (fungi: $F_{1,32} = 15.82$, $P = <0.001$; time: $F_{3,32} = 21.15$, $P = <0.001$; fungi × time: $F_{3,32} = 2.27$, $P = 0.099$).

inoculation with *O. ips*, with the number of progeny 87% higher in the fungal than control treatment. We found no significant differences between treatments for beetles introduced 0, 7, or 10 d after fungal inoculation. Overall across treatments, fungal inoculation of logs increased the number of progeny per female by 40%. Moreover, colonization by *O. ips* tended ($P = 0.099$) to ameliorate the effects of log age, with females producing similar numbers of progeny when introduced simultaneously (0 d), but >50% more progeny when introduced 3, 7, or 10 d after fungal inoculation, relative to their respective control treatments (Fig. 2c).

Larval galleries and pupal chambers containing living insects occurred in the same area as occupied by *O. ips*. We could not determine the sequence of colonization. Joint occupation of tissue usually occurred when the beetles were third instar, pupa, or callow adults, which corresponded to the time period when the logs were most colonized by *O. ips*. We were unable to determine whether specific instars that were feeding in the areas colonized or uncolonized by *O. ips* differed in their survival to adulthood.

Table 1. Growth rate equations for *O. ips* after inoculation into *Pinus resinosa* logs

Days before beetle introduction	Equation ($y = ax^b$)	*F*	*P*
0 d	$y = (0.067)\ x^{1.88}$; $R^2 = 0.77$	19.64	0.004
3 d	$y = (0.027)\ x^{2.15}$; $R^2 = 0.85$	32.81	0.001
7 d	$y = (0.019)\ x^{2.29}$; $R^2 = 0.96$	136.55	< 0.001
10 d	$y = (0.003)\ x^{2.90}$; $R^2 = 1.00$	1,327.41	< 0.001

Time (days) is the dependent variable and fungal growth (square centimeters) is the independent variable

Fungal Growth. As expected, logs receiving fungal inoculations had significantly more area colonized by *O. ips* than did logs in the mechanical wound or complete control treatments (Table 1). This difference was evident by at least day 15. Fungal growth was nonlinear and best described by exponential equations at all times (Table 2). The relationship between area colonized and time since introduction was significant for this model for all time intervals before beetle introduction. Both the value of the exponent and the correlation with time since inoculation seemed to increase with time interval before beetle introduction. Attempts to exclude *O. ips* were largely successful during the relevant period of this experiment, as larval development is typically completed by 10–15 d (Schenk and Benjamin 1969). However, some contamination still occurred.

Table 2. Area stained by *O. ips* (square centimeters) in bark samples harvested from *P. resinosa* logs at three different time periods

Treatment	No. of days before beetle introduction	Time of Log Harvest: 5 d	15 d	30 d
Fungal inoculation	0 d	0[a]	11.64 ± 2.21bc	40.40 ± 5.44ef
	3 d	0[a]	6.80 ± 1.04b	41.83 ± 5.21ef
	7 d	0[a]	9.70 ± 1.30bc	46.38 ± 3.10fg
	10 d	0[a]	7.10 ± 0.76b	52.50 ± 5.08g
Mechanical wound	0 d	0[a]	0.075 ± 0.075a	32.63 ± 8.68de
	3 d	0[a]	0[a]	27.57 ± 5.18d
	7 d	0[a]	0[a]	28.34 ± 3.37d
	10 d	0[a]	0[a]	28.37 ± 8.10d
Control	na	0[a]	0[a]	16.71 ± 7.95c

Letters indicate differences in fungal growth within and among harvest times. Means followed by the same letter are not significantly different at the $P \leq 0.05$ level. Means comparisons are adjusted for all terms in the model.

na, not applicable.

Discussion

Ophiostoma ips had both positive and negative effects on I. pini.

The presence of *O. ips* significantly reduced female and tended to reduce male colonization. This suggests that beetles can detect whether fungi are already established in the host. In the field, fungal establishment is indicative of a host already colonized by some beetles. No antiaggregation pheromone has been reported in *I. pini*, which can experience high intraspecific competition in this limited resource. However, evidence of antiaggregation pheromones has been found in another fungus–coleopteran system, with odors from wood-decaying fungi repelling deathwatch beetles (Belmain et al. 2002). It is not known whether *I. pini* uses metabolites emitting from tissue colonized by *O. ips* to prevent crowding, but our data (Fig. 1a) are consistent with this mechanism.

Growth of *O. ips* had no effect on brood emergence when beetles and fungi were introduced simultaneously, which is consistent with Yearian et al. (1972). However, fungal presence increased brood emergence when fungi were inoculated into logs before beetle colonization. Moreover, fungal growth seemed to extend the suitability of logs for reproduction by *I. pini.* These results suggest that *O. ips* may maintain moisture or nutrient levels in the phloem, or outcompete harmful fungi (Bridges and Perry 1985, Klepzig and Wilkens 1997, Klepzig 1998). It is possible that *O. ips* provides *I. pini* with nutrients, but this could not explain why beetles introduced simultaneously with fungi did not outperform beetles in uninoculated logs.

The occurrence of some *O. ips* in the mechanical wound treatments toward the end of the study likely originated from the gut and frass of adult *I. pini* (Leach et al. 1934, Furniss et al. 1995). We did find some ophiostomatoid fungi contamination in the controls, but only after beetle development was mostly completed (Table 2).

Our study provides further evidence that impacts of microbial associates on bark beetle fitness should be viewed in terms of net rather than singular effects (Paine et al. 1997). Even the opposing trends identified here understate the complexity of these interactions. For example, the "antagonistic" component may have variable consequences depending on whether the perspective of previously entered or arriving beetles is considered. Likewise, the "mutualistic" effects on brood production represent averages and so could conceivably arise partly from released competition that benefits some beetles when others are inhibited by fungi. Further studies addressing the relative success of individual beetles are needed to resolve these issues.

Additional factors are likely to affect this interaction. For example, *I. pini* and *O. ips* experience a chemically different environment when they colonize live versus dead trees (Raffa and Smalley 1995). Moreover, fungal species vary in their competitive abilities (Klepzig and Wilkens 1997, Klepzig 1998) and in the presence of other microorganisms the *I. pini–O. ips* relationship could change. In addition, this study illustrates the value of testing for effects over time. For example, one's interpretation of effect of *O. ips* on *I. pini* colonization would differ depending on whether beetles were introduced 10 d after inoculation or simultaneously with the fungus (Fig. 1a and b).

These results also suggest system-specific differences. Our results are similar to those with *Dendroctonus ponderosae* Hopkins–*Ophiostoma clavigerum* (Robinson-Jeffrey & Davidson) Harrington, in that beetles produced more progeny when their ophiostomatoid fungi were present (Six and Paine 1998). However, they differ from the *D. frontalis–O. minus* and *D. ponderosae–O. montium* (Rumbold) von Arx systems, where the fungus reduced brood development (Barras 1970, Franklin 1970, Six and Paine 1998, Lombardero et al. 2000). Unlike bark beetles that colonize living and often healthy trees (e.g., *Ips typographus* L.), *I. pini* primarily colonizes highly stressed or dead hosts. Establishment of *O. ips* can reduce the rapid decline of host suitability for *I. pini* development as well as colonization by subsequent *I. pini* adults (Figs. 1a and b; 2b and c). During colonization of dead hosts, there is little to no benefit incurred to pioneers by subsequent arrivers (Raffa 2001). *O. ips* may conceivably have the added benefit of reducing competition by other phloeophagous insects and microorganisms.

Because only a few bark beetle–ophiostomatoid fungus relationships have been studied in detail, identification of general patterns is not yet possible. However, the importance of the timing of fungal establishment is likely to be manifested in many systems and supports the notion that symbiotic relationships should be viewed in terms of net effects.

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