Effects of symbiotic bacteria and tree chemistry on the growth and reproduction of bark beetle fungal symbionts

A.S. Adams, C.R. Currie, Y. Cardoza, K.D. Klepzig, and K.F. Raffa

Abstract: Bark beetles are associated with diverse assemblages of microorganisms, many of which affect their interactions with host plants and natural enemies. We tested how bacterial associates of three bark beetles with various types of host relationships affect growth and reproduction of their symbiotic fungi. Fungi were exposed to volatiles from bacteria in an arena that imposed physical separation but shared airspace. We also exposed fungi to vapors of the host compound, α-pinene, and to combinations of bacterial volatiles and α-pinene. Bacterial volatiles commonly stimulated growth of *Leptographium procerum* (W.B. Kendr.) M.J. Wingf. and *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & Wingf., which are symbiotic fungi of *Dendroctonus valens* LeConte and *Dendroctonus ponderosae* Hopkins, respectively. They less commonly stimulated growth of *Ophiostoma ips* (Rumbold) Nannf., which is associated with *Ips grandicollis* Eichhoff. Some bacteria inhibited *L. procerum*, *Ophiostoma montium* (Rumbold) von Arx (another associate of *D. ponderosae*), and *O. ips*. Bacteria greatly stimulated spore production of symbionts of *D. valens* and *D. ponderosae*. α-Pinene strongly affected some of these relationships, causing amplification, reduction, or reversal of the interactions among the bacteria and fungi. Our results show that some bacteria associated with bark beetles directly affect fungal symbionts and interact with tree chemistry to affect fungal growth and sporulation. The strongest effects were induced by bacteria associated with beetles adapted to attacking living trees with vigorous defenses, and on fungal reproductive structures.

Résumé : Les scolytes sont associés à divers assemblages de microorganismes dont plusieurs influencent leurs interactions avec les plantes hôtes et les ennemis naturels. Nous avons testé de quelle façon les bactéries associées à trois scolytes qui ont différents types de relation avec leur hôte influencent la croissance et la reproduction de leurs champignons symbiotiques. Les champignons ont été exposés aux substances volatiles produites par les bactéries dans un espace où ils étaient physiquement séparés mais partageaient le même espace aérien. Nous avons aussi exposé les champignons à des vapeurs d'α-pinène produit par l'hôte et à des combinaisons de substances volatiles produites par les bactéries et d'α-pinène. Les substances volatiles produites par les bactéries ont couramment stimulé la croissance de *Leptographium procerum* (W.B. Kendr.) M.J. Wingf. et de *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., les champignons symbiotiques associés respectivement à *Dendroctonus valens* LeConte et *D. ponderosae* Hopkins. Ils ont moins fréquemment stimulé la croissance d'*Ophiostoma ips* (Rumbold) Nannf. associé à *Ips grandicollis* Eichhoff. Certaines bactéries ont inhibé *L. procerum*, *Ophiostoma montium* (Rumbold) von Arx, un autre associé de *D. ponderosae*, et *O. ips*. Les bactéries ont fortement stimulé la production de spores des symbiotes de *D. valens* et *D. ponderosae*. L'α-pinène a grandement influencé ces relations, incluant l'augmentation, la diminution et l'inversion. Nos résultats montrent que certaines bactéries associées à des scolytes influencent directement les symbiotes fongiques et interagissent aussi chimiquement avec les arbres pour influencer la croissance et la sporulation des champignons. Les effets les plus prononcés ont été provoqués par les bactéries associées aux scolytes capables d'attaquer les arbres vivants dotés de mécanismes de défense vigoureux et observés sur les structures reproductrices des champignons.

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Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are both major economic pests and important ecological disturbance agents in forest ecosystems. Most bark beetles vector symbiotic fungi, usually within the Ophiostomatales genera *Ceratocystiopsis*, *Grosmannia*, and *Ophiostoma* and their anamorphs *Leptographium* and *Pesotum* (Upadhyay 1993). The relationship between the beetle and fungus is mutualistic in a number of systems. Some symbiotic fungi are consumed by beetles (Harrington 2005) and may benefit their nutrition by concentrating nitrogen (Ayres et al. 2000) or

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producing sterols (Bentz and Six 2006). In other systems, the symbionts may be weakly pathogenic to the host tree (Strobel and Sugawara 1985), play a role in exhausting tree defensive chemistry at high densities (Raffa and Berryman 1983), and (or) may synthesize detoxification enzymes (DiGuistini et al. 2007). In all cases, fungi benefit from their symbiosis with bark beetles through transport to otherwise inaccessible host trees (Six 2003; Six and Klepzig 2004).

The life cycles of bark beetles and their symbiotic fungi are tightly linked and are largely ectosymbiotic. Beetles carry fungal propagules to new host trees in their guts (Adams and Six 2007), on their exoskeleton, in specialized invaginations of the cuticle termed mycangia (Six 2003), and within nematode-containing structures termed nematangia, (Cardoza et al. 2006*b*). The fungi are inoculated into the host tree as adults construct egg galleries, and the fungi colonize phloem tissues in close proximity to broods during larval feeding. Following beetle eclosion, sticky fungal spores lining the pupal chamber secure the continuity of the symbiosis by ensuring transmission to the next host tree colonized by the beetle. The stability (success) of this ectosymbiotic relationship is potentially compromised by external influences, such as temperature (Six and Bentz 2007), water availability (Klepzig et al. 2004), and tree chemistry (Raffa and Smalley 1995; Klepzig et al. 1996). Additionally, fungi of bark beetles are vulnerable to competition from other microbes colonizing the subcortical environment of the host tree, such as fungal antagonists (Klepzig et al. 2001; Cardoza et al. 2006*a*) and bacterial endophytes (Adams et al. 2008).

Although less studied, bacteria have been isolated from bark beetle egg and larval galleries (Whitney 1971), oral secretions of adult beetles (Cardoza et al. 2006*a*), mycangia (Scott et al. 2008), whole beetles, and excreta of adult beetles (Bridges 1981; Scott et al. 2008). The frequent isolation of bacteria in association with bark beetles and their symbiotic fungi suggests that bacterial–fungal interactions are likely, but relatively few have been studied. *Micrococcus* sp. isolated from larval galleries of *Dendroctonus ponderosae* Hopkins stimulated growth of the symbiotic fungus *Ophiostoma montium* (Rumbold) von Arx, whereas the same bacterium inhibited growth of another fungal symbiont, *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & Wingf. (Adams et al. 2008). Several bacteria isolated from oral secretions of *Dendroctonus rufipennis* (Kirby) inhibited growth of several fungi that are detrimental to beetle development (Cardoza et al. 2006*a*). Further, a *Streptomyces* sp. colonizing galleries and mycangia of *Dendroctonus frontalis* Zimmermann selectively inhibited the beetle antagonistic fungal associate, *Ophiostoma minus* (Hedge.) H. & P. Sydow, whereas it had less effect on growth of its mutualistic fungus, *Entomocorticium* sp. A (Scott et al. 2008).

This study focused on interactions between bacteria and fungi isolated from three species of bark beetles selected for varying relationships with host trees: *Dendroctonus valens* LeConte, *D. ponderosae*, and *Ips grandicollis* Eichhoff. *Dendroctonus valens* is a solitary bark beetle that colonizes lower stems. In its native range, *D. valens* prefers trees weakened by disease or stress (Klepzig et al. 1991), and colonization typically does not kill the tree. In the late 1990s, *D. valens* invaded parts of China, colonizing a new host (*Pinus tabuliformis* Carr.) on which it functions as a primary mortality agent (Yan et al. 2005). A dominant fungal associate of *D. valens* is *Leptographium procerum* (Kendrick) Wingfield (Klepzig et al. 1995), although the impact of *L. procerum* on development of *D. valens* is unknown. This fungus is considered weakly pathogenic to the host tree (Wingfield 1986).

Dendroctonus ponderosae is an eruptive species that kills trees by mass attack. The physiological vigor of selected trees varies with beetle population phase. When populations of the stem-colonizing *D. ponderosae* are low, attack by *D. valens* and other lower-stem colonizers can predispose hosts to colonization. Once populations of *D. ponderosae* increase, it can kill healthy trees, often on a scale of many thousands of hectares. *Dendroctonus ponderosae* is closely associated with two fungi: *G. clavigera* and *O. montium* (Six 2003). Both fungi are considered nutritionally beneficial (Bentz and Six 2006; Bleiker and Six 2007), and feeding on these fungi may be essential to development of broods (Six and Paine 1998). Additionally, heavy inoculation of trees with *O. montium* caused 20-year-old *Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm. ex S. Wats. (Pinaceae) to wilt (Strobel and Sugawara 1985), and inoculation of mature *P. contorta* with varying densities of *G. clavigera* caused host induced defense to decline in a dose-dependent manner (Raffa and Berryman 1983), suggesting these weak plant pathogens may aid beetles in overcoming host defenses (Lee et al. 2006).

Colonization of a host by either *D. valens* or *D. ponderosae* is often followed by stem-colonizing beetles that are associated with highly stressed trees, termed "secondary" bark beetles. *Ips grandicollis* commonly attacks and kills trees initially attacked by *D. valens* and vectors the symbiotic fungus *Ophiostoma ips* (Rumbold) Nannfeldt. *Ophiostoma ips* is also commonly found with other *Ips* species, including *Ips pini* (Say) (Klepzig et al. 1991). Any benefit of the fungus to beetles appears to be context dependent, as fungal infection may aid adults in colonizing the tree; however, feeding on phloem heavily colonized by the fungus is detrimental to development of larval stages (Kopper et al. 2004).

Host terpenes, particularly monoterpenes, are major deterrents to tree colonization by bark beetle – fungus complexes (Bohlmann et al. 2000). Monoterpenes have been shown to reduce the growth of some symbiotic fungi (Raffa et al. 1985; Klepzig et al. 1996). However, *G. clavigera* and *Entomocorticium* sp. A appear adapted to host monoterpenes, at least at the concentrations present in constitutive tissue, as growth is stimulated by their presence in vitro (Bridges 1987; Paine and Hanlon 1994). This may be due to the presence of cytochrome P-450 enzymes, which have been detected in *G. clavigera* (DiGuistini et al. 2007). These enzymes are involved in detoxification of plant chemical defenses in other systems (van den Brink et al. 1998). Currently, we have no information on how host tree compounds affect interactions between fungi and bacteria associated with bark beetles. Further, we do not know if these interactions vary among symbionts associated with beetles of different life histories.

The objectives of this study were to (*i*) determine how

Table 1. GenBank accession numbers for bacteria isolated from *Dendroctonus valens*, *Dendroctonus ponderosae*, and *Ips grandicollis*.

Beetle source	Closest match in GenBank	Sequence length (base pairs)	Score (%)	Acc. No. of match	Isolate	Acc. No. of isolate
D. valens	*Pantoea ananatis*	864	98	DQ365569.1	αB	EU580464
	Pantoea sp.	933	98	DQ849043.1	αC	EU58065
D. ponderosae	*Pseudomonas fluorescens*	713	99	EF424136.1	DPFF1a	EU476021
	Pectobacterium cypripedii (type strain)	870	99	EF122434.1	DPLF5	EU588723
I. grandicollis	*Chryseobacterium orangei*	702	97	EF204451.2	IgHE	EU580466
	Staphylococcus xylosus	941	98	DQ089746.1	IgHI	EU580467

Note: Sequences generated in this study and used for basic local alignment search tool searches in GenBank resulted from polymerase chain reaction amplification of the ribosomal RNA 16S region.

bacteria affect the major fungal symbionts of their beetle hosts, (*ii*) determine how host chemistry affects these interactions, and (*iii*) determine if interactions between bacterial and fungal symbionts of bark beetles are specific to associates of each beetle species.

Materials and methods

Isolation of microorganisms

Dendroctonus valens were collected from under the bark of naturally attacked, living *Pinus resinosa* Ait., near Spring Green, Wisconsin (43°09′N, 89°59′E). *Dendroctonus ponderosae* were collected from under the bark of naturally attacked *P. contorta* near Butte, Montana. *Ips grandicollis* were collected from under the bark of naturally colonized *P. resinosa* logs in Black River Falls State Park, Wisconsin (44°15′N, 90°40′E). Symbiotic fungi associated with each beetle were obtained by rolling adults on malt extract agar (MEA; Difco, Sparks, Maryland). *Grosmannia clavigera* and *O. montium* used in this study were obtained from K. Bleiker (Bleiker and Six 2007). All fungi were identified to species using morphological characteristics (Upadhyay 1981; Grylls and Seifert 1993).

Bacteria were isolated from each beetle using several techniques. Isolates from *D. valens* were obtained by crushing adults in phosphate buffered saline, pH 7.4 (Sigma-Aldrich Co., St. Louis, Missouri), and plating dilutions of the samples on water agar. Culture dishes were amended with α-pinene (98% purity; Sigma-Aldrich) using filter paper soaked with 1 mL of α-pinene placed in the lid of each culture dish. Isolates from *D. ponderosae* were obtained from mouthparts of larval and adult beetles using methods described in Cardoza et al. (2006*a*). Isolates from *I. grandicollis* were obtained by crushing adults in phosphate buffered saline, pH 7.4, and plating dilutions of the sample on 10% tryptic soy agar (TSA; Difco). Two relatively common isolates from each beetle species were selected to be used in bioassays. Five of these isolates are closely related to known associates of other species of bark beetle (Delalibera et al. 2007; Cardoza et al. 2009).

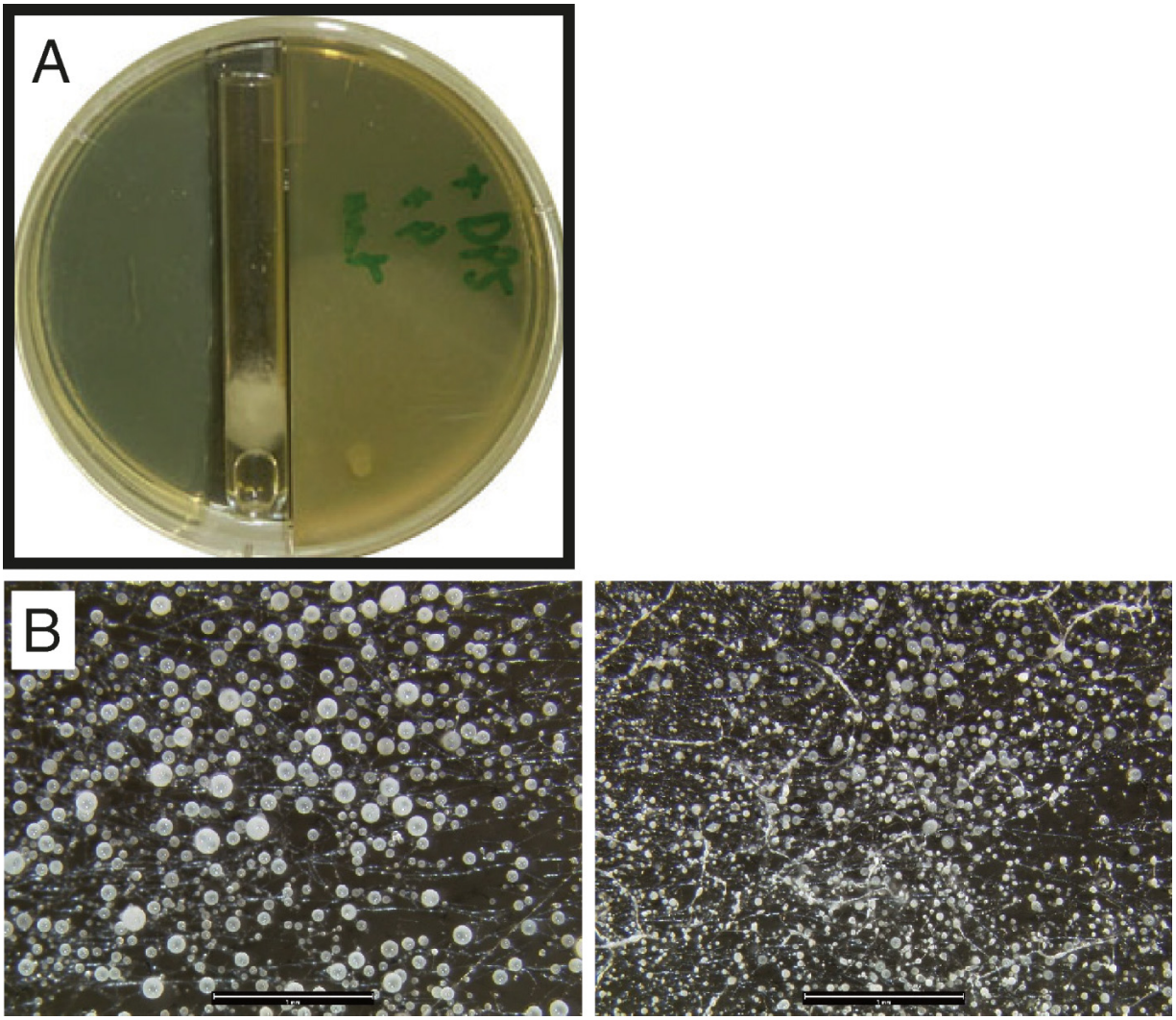
All bacteria were identified by direct sequencing of regions V5 of the rRNA 16S gene. The primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (based on the *Escherichia coli* numbering system) were used in polymerase chain reaction (PCR) reactions to amplify partial ribosomal RNA (rRNA) gene sequences as previously described (Holben et al. 2002), with the exception that PCR amplicons were used in direct sequencing reactions rather than for cloning. Sequencing of PCR products was performed by the University of Wisconsin Biotechnology Center. Sequences were deposited in GenBank under the accession numbers provided in Table 1. NCBI similarity scores were obtained using a basic local alignment search tool search of sequences previously deposited in GenBank (www.ncbi.nlm.nih.gov).

Effect of bacteria and α-pinene volatiles on growth of fungi

We tested the treatment effects of each bacterium, the host tree monoterpene α-pinene, and the combination of each bacterium and α-pinene on each fungus. The growth arena for this assay was a 100 mm diameter, divided Petri dish (Fisher Scientific) (Fig. 1A). MEA (1.5% *m/v*) was poured on one side of each dish for growing fungi and 10% TSA was poured on the opposite side of each dish for growing bacteria. A strip of TSA approximately 1 cm wide was removed along the division of each dish. Fungus inoculum for bioassays consisted of plugs from the leading edge of fungus culture growing on MEA. One plug of agar (3 mm diameter) and hyphae was removed from the culture and placed fungus side down onto the MEA in each divided Petri dish. The fungus inoculum was placed approximately 1 cm from the division of the dish, and approximately 1 cm away from the exterior edge of the dish. For the bacterial treatment, each bacterium was applied in monoculture. Each bacterium was grown to stationary growth phase in 10% tryptic soy broth and 20 μL evenly spread on the TSA using a Drigalski spatula. For the α-pinene treatment, a glass test tube containing 1 mL of α-pinene, which was absorbed in cotton, was placed into the 1 cm wide gap made by the removal of TSA. All dishes were sealed with PTFE thread seal tape (Malaysia), inverted, and stored at room temperature in the dark.

For each fungus–treatment combination, each assay ended after the fungus in one dish grew to cover 75% of the diameter of the dish. Mycelial growth (linear) of each fungus was measured from the point of inoculation to the leading edge of the hyphae, parallel to the division of the dish. The number of clusters of conidiophores was measured in the 1 mm^2 area at 3 cm from the inoculation point of the fungus (for *G. clavigera* and *L. procerum* only). Clusters of conidiophores, rather than individuals, were counted because ex-

Fig. 1. (A) Bioassay arena. The Petri dish has a central plastic division that separates the medium but does not meet the lid of the dish. A strip of agar was removed to allow placement of a test tube for α-pinene treatment. Each bacterium was inoculated evenly over the tryptic soy agar (left half of the dish) for bacterial treatment, and each fungus was inoculated on one edge of the malt extract agar (right half of the dish). Conidia and conidiophore measurements were made 1 cm from the inoculation point of the fungus, in the location parallel to the dish divider. (B) Digitized image of conidiophores of *Leptographium procerum* grown with α-pinene (left) and with *Pantoea* sp. isolate αB and α-pinene (right). Each sphere is a mass of conidia produced by one or more conidiophores. Scale bar = 1 mm.



pelled masses of conidia often joined two to four conidiophores together. The Automontage software (Syncroscopy, Frederick, Maryland) was used to digitally visualize conidiophores, and actual counts were made using Adobe Photoshop version 8.0 software (Adobe Systems Inc., San Jose, Califonia) (see Fig. 1B). The number of conidia was counted in an 8 mm diameter area 3 cm from the inoculation point. The 8 mm diameter agar plug was removed using a cork borer and forceps, placed in 1 mL PBS, and vortexed. Conidia in the wash were counted using a hemacytometer (Hausser Scientific, Horsham, Pennsylvania) and a Leica DM LB2 microscope (Selangor, Malaysia). All combinations of treatments were replicated seven times for each bioassay.

To determine the direct effect of α-pinene on bacteria, 10 μL of culture of each bacterium in tryptic soy broth was inoculated into 1 mL of tryptic soy broth in 24-well cell culture plates (Corning Inc., Corning, New York). Wells were amended with α-pinene to equal approximately 0%, 1%, and 5% concentrations. These concentrations of α-pinene fit within the lower range of α-pinene concentrations in constitutive and induced host trees (Lieutier et al. 1991). Individual wells were sealed using VIEWseal pressure sensitive adhesive (Greiner Bio-one, Germany). Three replicates of each culture were shaken at medium speed (500 r/min) and orbital directionality, held at 24 °C, and optical density was measured every hour for 23 h using a DXT 880 Multimode Detector (Beckman Coulter, Corona, Calfornia) at absorbance of 595 nm.

Statistical analysis

Fungal responses to bacteria, α-pinene, and the combination of bacteria and α-pinene were tested independently using one-way ANOVA (JMP_{IN} version 4.0.2, SAS Institute Inc., Cary, North Carolina). Transformations of the data were used as needed to meet the requirements of normality.

Table 2. Results of one-way ANOVA testing for effects of bacteria, α-pinene, and the combination of bacteria and α-pinene on the growth, conidiophore production, and conidia production of the predominant fungal symbionts of three bark beetle species.

	Growth				Conidiophore production				Conidia production			
		Treatment				Treatment				Treatment		
Fungus and bacterium	Control (mean (SE))	df	*F*	*P*	Control (mean (SE))	df	*F*	*P*	Control (mean (SE))	df	*F*	*P*
Leptographium procerum												
Pantoea sp. isolate αB	34.7 (0.7)	3,22	23.9	<0.0001	27.4 (4.5)	3,17	24.3[c]	<0.0001	9.2 (2.3)	3,18	29.9	<0.0001
Pantoea sp. isolate αC	41.4 (0.3)	3,24	29.2	<0.0001	49.7 (2.7)	3,24	38.6[a]	<0.0001	39.0 (2.0)	3,16	48.8[b]	<0.0001
Pseudomonas sp.	31.9 (1.4)	2,23	20.6[a]	<0.0001	19.5 (2.5)	3,15	13.5[b]	<0.001	3.7 (1.7)	3,13	8.8[a]	<0.01
Pectobacterium sp.	47.9 (0.3)	3,21	27.2[a]	<0.0001	23.0 (3.2)	3,21	58.9	<0.0001	28.4 (2.5)	3,19	25.2[c]	<0.0001
Chryseobacterium sp.	43.4 (0.2)	3,24	216.2	<0.0001	28.1 (2.7)	3,23	15.0	<0.0001	17.4 (0.9)	3,20	37.0[c]	<0.0001
Staphylococcus sp.	44.1 (0.5)	3,23	2.4	0.09	38.1 (3.8)	3,23	31.3[c]	<0.0001	57.2 (8.2)	3,12	7.1	<0.01
Grosmannia clavigera												
Pantoea sp. isolate αB	40.1 (0.2)	3,24	70.8	<0.0001	35.0 (2.3)	3,24	10.6	0.0001	23.6 (3.0)	3,20	15.0	<0.0001
Pantoea sp. isolate αC	40.9 (0.1)	3,24	35.3[a]	<0.0001	54.9 (2.8)	3,24	36.3	<0.0001	36.7 (2.2)	3,16	21.4[c]	<0.0001
Pseudomonas sp.	44.5 (0.4)	3,19	18.3	<0.0001	36.3 (2.5)	3,19	49.1[b]	<0.0001	45.5 (4.9)	3,15	20.0[c]	<0.0001
Pectobacterium sp.	41.3 (0.3)	3,22	17.1	<0.0001	39.8 (5.5)	3,22	30.1	<0.0001	13.1 (2.7)	3,16	61.0	<0.0001
Chryseobacterium sp.	42.1 (0.2)	2,23	43.6[a]	<0.0001	39.7 (2.9)	3,23	27.3[c]	<0.0001	34.8 (8.2)	3,16	41.2	<0.0001
Staphylococcus sp.	45.2 (0.2)	3,23	16.6	<0.0001	38.7 (3.0)	3,23	7.9	<0.001	48.7 (3.9)	3,20	7.3	<0.01
Ophiostoma montium												
Pantoea sp. isolate αB	46.0 (0.2)	3,24	9.4	0.0003					1.8 (0.6)	3,24	2.8[c]	0.06
Pantoea sp. isolate αC	46.6 (0.3)	3,24	3.4[a]	0.04					2.2 (0.2)	3,23	30.8[c]	<0.0001
Pseudomonas sp.	48.9 (0.5)	3,23	4.2	0.02					0.7 (0.4)	3,23	6.6[c]	<0.01
Pectobacterium sp.	45.7 (0.4)	3,21	11.2	0.0001					3.0 (0.6)	3,21	3.7[a]	<0.05
Chryseobacterium sp.	47.4 (0.4)	3,23	4.4	0.01					3.5 (0.7)	3,16	5.4[b]	<0.01
Staphylococcus sp.	47.4 (0.3)	3,24	0.7	0.57					4.5 (0.4)	3,16	5.8	<0.01
Ophiostoma ips												
Pantoea sp. isolate αB	46.1 (0.1)	3,23	53.8[a]	<0.0001								
Pantoea sp. isolate αC	41.4 (0.2)	3,23	46.4[a]	<0.0001								
Pseudomonas sp.	42.9 (0.4)	3,24	26.1[a]	<0.0001								
Pectobacterium sp.	48.8 (0.4)	3,22	43.7[a]	<0.0001								
Chryseobacterium sp.	41.9 (0.3)	3,24	30.6[a]	<0.0001								
Staphylococcus sp.	45.2 (0.2)	3,24	24.9[a]	<0.0001								

Note: The control values (with no bacteria and no α-pinene) for each fungus in each treatment are given. Relative changes from controls due to each treatment are shown in Figs. 2–4.

[a]Data were square-root transformed prior to statistical analysis.

[b]Data were log transformed prior to statistical analysis.

[c]Data were rank transformed prior to statistical analysis.

Because each assay was analyzed independently, the appropriate transformation method for each analysis was determined separately (see Table 2 for specific transformation used). Post hoc comparisons were analyzed using Tukey's mean separation test.

Chi-square tests of independence (StatView version 4.57, Abacus Concepts, Inc., California) were used to test the null hypothesis that interactions between bacteria and fungi are independent of their respective beetle associations. Contingency tables were constructed with columns grouping (*i*) interactions between bacteria and fungi isolated from the same beetle species and (*ii*) bacteria and fungi isolated from different beetle species and rows categorizing three possible outcomes of the interaction compared with controls: less than, no difference, or greater than. This test was conducted on the outcomes of the effects of bacteria and the combination of bacteria and α-pinene.

To assess bacterial tolerance to α-pinene, we compared growth during the exponential phase. Absorbance values of each bacterium growing in broth amended with various α-pinene concentrations were log transformed. The time period with the most linear log absorbance values was selected based on r^2 values (StatView version 4.57). During this period of exponential growth, the doubling time of each culture was calculated using methods described by Middelbeek et al. (1992). Generation time was then calculated as the difference in absorbance values at the beginning and end of the exponential growth phase divided by the $\log_2$ of the duration (number of hours) of the exponential growth phase (Middelbeek et al. 1992). The Wilcoxon or Kruskal–Wallis test (JMP_{IN} version 4.02) was used to test the null hypothesis that α-pinene does not affect the growth of each bacterium.

Results

Isolation and identification of microorganisms

The most frequently described fungal associates of each beetle were isolated and used in this study: *L. procerum* from *D. valens*, *G. clavigera* and *O. montium* from *D. ponderosae*, and *O. ips* from *I. grandicollis*.

Bacteria were isolated from all three bark beetle species. The two bacteria from each beetle species selected for fungal bioassays were two *Pantoea* sp. (isolates αB and αC) isolated from *D. valens*, *Pseudomonas* sp. and *Pectobacterium* sp. isolated from *D. ponderosae*, and *Chryseobacterium* sp. and *Staphylococcus* sp. isolated from *I. grandicollis* (Table 1).

Effects of bacteria and α-pinene on fungal growth and reproduction

Because of the large number of permutations we evaluated and the complexity of these relationships, we simplified data presentation by showing the proportional change from the control for each two- or three-way interaction (Figs. 2–4). Each of these figures shows multiple sets of plots arising from two bacteria associated with each of the three beetles, tested in combination with four fungi (one from *D. valens*, two from *D. ponderosae*, one from *I. grandicollis*). These plots are labeled alphabetically to facilitate description in the text. Each plot includes the effect of the bacterium, α-pinene, and their combination. If desired, the absolute values of fungal growth or reproductive structures in the treatment assays (Figs. 2–4) can be calculated using the control values in Table 2.

Effects on mycelial growth

Effects of bacteria on mycelial growth

Bacteria associated with D. valens

Both *Pantoea* sp. isolates αB and αC from *D. valens* stimulated the *D. valens* symbiont *L. procerum* by 15% and 6%, respectively (Figs. 2A and 2B). Similarly, growth of *G. clavigera* was stimulated by both *Pantoea* sp. isolates αB and αC by 5% and 3%, respectively (Figs. 2G and 2H). In contrast, neither isolate affected growth of *O. montium* (Figs. 2M and 2N), whereas *Pantoea* sp. isolate αB inhibited growth of *O. ips* by 5% (Fig. 2S), and *Pantoea* sp. isolate αC stimulated growth of *O. ips* by 5% (Fig. 2T).

Bacteria associated with D. ponderosae

In the *D. ponderosae* complex, we observed two effects of beetle-associated bacteria on fungal growth. These were a 6% inhibition of *O. montium* by *Pectobacterium* sp. (Fig. 2P) and a 39% stimulation of *L. procerum* by *Pseudomonas* sp. (Fig. 2C).

Bacteria associated with I. grandicollis

Neither bacterium from *I. grandicollis* affected growth of the *I. grandicollis* symbiont, *O. ips* (Figs. 2W and 2X). However, *Chryseobacterium* sp. from *I. grandicollis* inhibited growth of *L. procerum* by 4% (Fig. 2E) and of *O. montium* by 5% (Fig. 2Q). This *Chryseobacterium* sp. stimulated growth of *G. clavigera* by 3% (Fig. 2K). *Staphylococcus* sp. did not significantly affect the growth of any fungus (Figs. 2F, 2L, 2R, and 2X).

Effects of α-pinene on mycelial growth

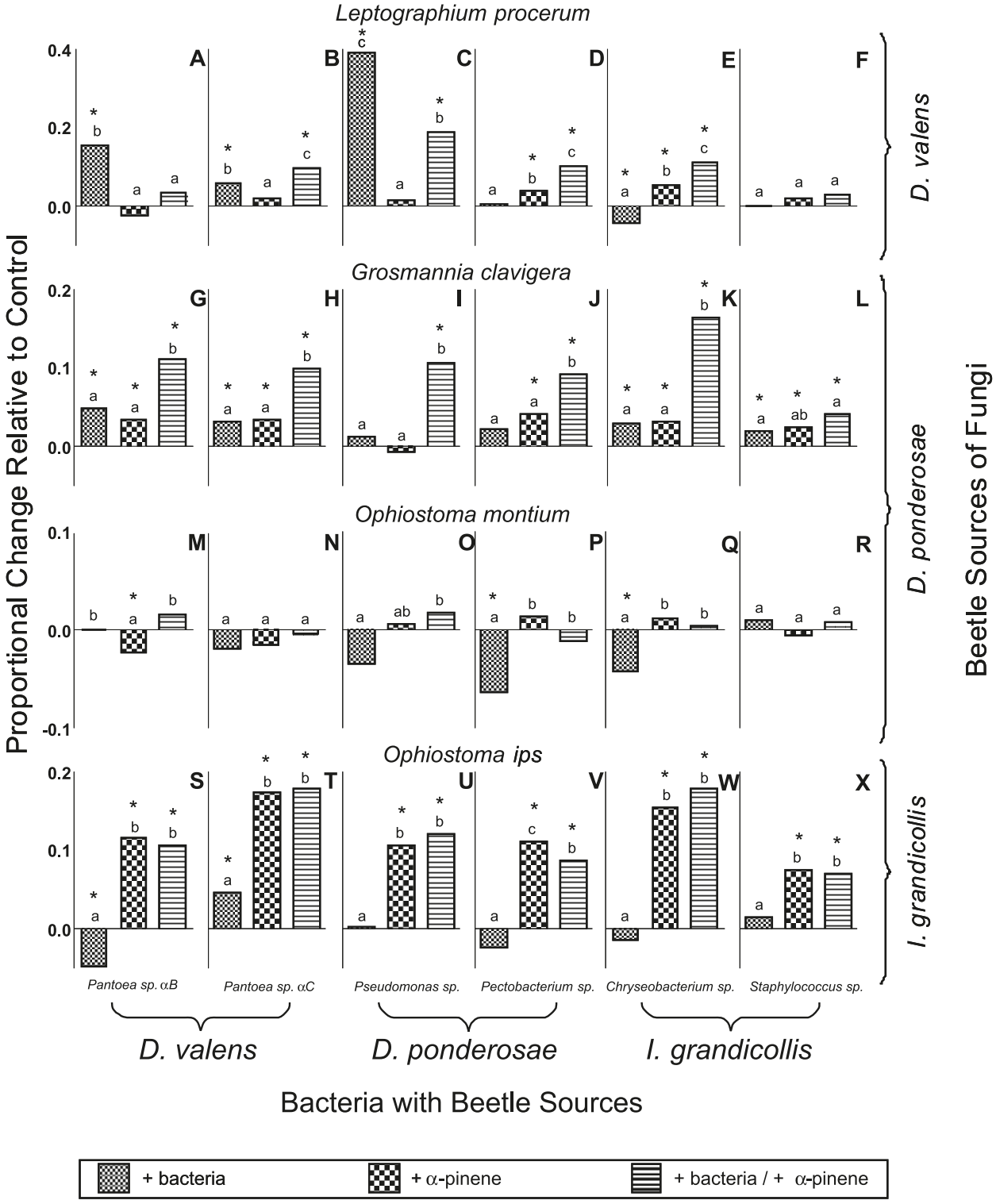
Growth of *L. procerum* was not affected by α-pinene in four assays (Figs. 2A, 2B, 2C, and 2F) and was weakly stimulated in two assays by 4% and 5% (Figs. 2D and 2E). Growth of *G. clavigera* was weakly stimulated by α-pinene in five assays varying from 2% to 4% stimulation (Figs. 2G, 2H, 2J, 2K, and 2L) and was not affected in one assay (Fig. 2I). Growth of *O. montium* was not affected by α-pinene in five assays (Figs. 2N–2R) and was weakly inhibited in one by 2% (Fig. 2M). Growth of *O. ips* was stimulated by α-pinene in all six assays varying from 7% to 17% stimulation (Figs. 2S–2X).

Effects of bacteria and α-pinene on mycelial growth

Bacteria associated with D. valens

The combination of α-pinene and *Pantoea* sp. isolate αC resulted in the greatest growth of *L. procerum* compared with all other treatments, with a stimulatory effect of 9% (Fig. 2B). Growth of *L. procerum* did not differ between the control and the combination of α-pinene and *Pantoea* sp. isolate αB. However, growth with this combination was significantly less than that of *L. procerum* in the presence of isolate αB alone (Fig. 2A). Growth of *G. clavigera* was stimulated by the combination of α-pinene and each *Pantoea* sp. isolate: αB and αC stimulated growth by 11% and 10%, respectively. This growth was significantly greater than with

Fig. 2. Proportional change in mycelial growth relative to controls of *Leptographium procerum*, *Grosmannia clavigera*, *Ophiostoma montium*, and *Ophiostoma ips* due to bacteria, α-pinene, and both bacteria and α-pinene. Bars with the same letter are not significantly different from each other (Tukey's test, $P > 0.05$). Bars with asterisks are significantly different from the controls.



either α-pinene or each bacterium alone in each assay (Figs. 2G and 2H). Growth of *O. montium* was not affected by the combination of α-pinene and either *Pantoea* sp. isolate (Figs. 2M and 2N), but growth with α-pinene and isolate αB was significantly greater than with α-pinene alone (Fig. 2M). Growth of *O. ips* in the combination of α-pinene and each bacterium from *D. valens* was significantly greater than the control. However, this growth was not statistically different than with α-pinene alone in either assay (Figs. 2S and 2T).

Bacteria associated with D. ponderosae

The combination of α-pinene and *Pseudomonas* sp. stimulated growth of *G. clavigera* by 11%, which was significantly greater than growth with either α-pinene or the bacterium alone (Fig. 2I). Similarly, the combination of α-pinene and *Pectobacterium* sp. stimulated growth of *G. clavigera* by 9% and was significantly greater than growth with either α-pinene or the bacterium alone (Fig. 2J). Growth of *O. montium* was not affected by the combination of α-pinene and either bacterium from *D. ponderosae* (Figs. 2O and 2P). The growth of *O. montium* with each of these bacteria and α-pinene was statistically equivalent to that with α-pinene alone, and growth with α-pinene and *Pectobacterium* sp. was significantly greater than growth with the bacterium alone (Fig. 2P). Growth of *L. procerum* was stimulated by 19% in the combination of α-pinene and *Pseudomonas* sp., which was statistically greater than growth with α-pinene alone and less than with the bacterium alone (Fig. 2C). Growth of *L. procerum* was stimulated by 10% in the combination of α-pinene and *Pectobacterium* sp., which was significantly greater than with either α-pinene or the bacterium alone (Fig. 2D). The combination of α-pinene and each bacterium from *D. ponderosae* stimulated growth of *O. ips* by 12% for α-pinene and *Pseudomonas* sp. and by 9% for α-pinene and *Pectobacterium* sp. (Figs. 2U and 2V). The combination of α-pinene and *Pseudomonas* sp. was not statistically different from that with α-pinene alone (Fig. 2U) and the combination of α-pinene and *Pectobacterium* sp.; however, it was statistically less than with α-pinene alone (Fig. 2V).

Bacteria associated with I. grandicollis

Growth of *O. ips* was stimulated by the combination of α-pinene and *Chryseobacterium* sp. and *Staphylococcus* sp. by 18% and 7%, respectively (Figs. 2W and 2X). In both assays, this growth was significantly greater than in the control and with each bacterium alone. However, the combination of α-pinene and each bacterium was not statistically different from that with α-pinene alone. Growth of *L. procerum* was stimulated by 11% with the combination of α-pinene and *Chryseobacterium* sp., which was significantly greater than growth with either α-pinene or this bacterium alone (Fig. 2E). The combination of α-pinene and *Staphylococcus* sp. did not affect growth of *L. procerum* (Fig. 2F). The combination of α-pinene and *Chryseobacterium* sp. stimulated growth of *G. clavigera* by 16%, which was statistically greater than growth with either α-pinene or this bacterium alone (Fig. 2K). The combination of α-pinene and *Staphylococcus* sp. stimulated *G. clavigera* by 4%, which was not statistically different than growth with either this bacterium or α-pinene alone (Fig. 2L). Growth of *O. montium* was not affected by the combination of α-pinene and either bacterium from *I. grandicollis* (Figs. 2Q and 2R). The addition of α-pinene negated the inhibitory effect of *Chryseobacterium* sp. (Fig. 2Q).

Effects on fungal reproductive structures

Effects of bacteria on fungal reproductive structures

Bacteria associated with D. valens

Pantoea sp. isolate αB from *D. valens* stimulated conidiophore production of the *D. valens* associate, *L. procerum*, by 110% (Fig. 3A) and its conidia production by 240% (Fig. 4A). *Pantoea* sp. isolate αC did not affect conidiophore (Figs. 3B) or conidia production (Fig. 4B) of *L. procerum*. Isolate αB stimulated production of conidiophores of *G. clavigera* by 89% (Fig. 3G) but did not affect conidia production (Fig. 4G). *Pantoea* sp. isolate αC did not affect conidiophore (Fig. 3H) or conidia production (Fig. 4H) of *G. clavigera*. Neither *O. montium* nor *O. ips* produced conidiophores in these bioassays. Although *Pantoea* sp. isolate αB did not affect *O. montium* conidia production (Fig. 4M), αC stimulated *O. montium* conidia production by 550% (Fig. 4N). *Ophiostoma ips* did not produce conidia in these bioassays.

Bacteria associated with D. ponderosae

Pseudomonas sp. and *Pectobacterium* sp. from *D. ponderosae* stimulated conidiophore production of *G. clavigera* by 52% and 77%, respectively (Figs. 3I and 3J), neither bacterium affected conidia production of this fungus (Figs. 4I and 4J). *Pseudomonas* sp. stimulated conidia production of *O. montium* by 330% (Fig. 4O). *Pectobacterium* sp. had no effect (Fig. 4P). *Pseudomonas* sp. and *Pectobacterium* sp. stimulated conidiophore production of *L. procerum* by 490% and 85%, respectively (Figs. 3C and 3D). *Pseudomonas* sp. stimulated conidia production of *L. procerum* by 1300% (Fig. 4C), and *Pectobacterium* sp. had no effect (Fig. 4D).

Bacteria associated with I. grandicollis

Neither bacterium from *I. grandicollis* affected conidiophore production of *L. procerum* or *G. clavigera* (Figs. 3E 3F, 3L, and 3K). Similarly, neither bacterium from *I. grandicollis* affected conidia production of *L. procerum* (Figs. 4E and 4F) or *O. montium* (Figs. 4Q and 4R), and *Chryseobacterium* sp. did not affect conidia production of *G. clavigera* (Fig. 4K). *Staphylococcus* sp. stimulated conidia production of *G. clavigera* by 40% (Fig. 4L).

Effects of α-pinene on fungal reproductive structures

Production of conidiophores of *L. procerum* was stimulated by α-pinene in five assays, varying from 90% to 220% (Figs. 3A, 3B, 3D, 3E, and 3F). In three of these five assays, production of conidia was also stimulated by 30%–100% (Figs. 4B, 4D, and 4E). Production of conidiophores by *G. clavigera* was stimulated by α-pinene in all six assays by 76%–150% (Figs. 3G–3L), and production of conidia was stimulated in five assays by 33%–210% (Figs. 4G, 4H, 4J, 4K, and 4L). Production of conidia by *O. montium* was not affected by α-pinene in five assays (Figs. 4M, 4O–4R) and was inhibited in one by 55% (Fig. 4N).

Effects of bacteria and α-pinene on asexual fungal reproductive structures

Bacteria associated with D. valens

Conidiophore production of *L. procerum* treated with the combination of α-pinene and *Pantoea* sp. isolates αB and αC was stimulated by 750% and 330%, respectively (Figs. 3A and 3B). Conidia increased by 470% and 200%, respectively (Figs. 4A and 4B). In each case, this treatment combination resulted in the greatest number of both conidio-

Fig. 3. Proportional change in number of clusters of conidiophores relative to controls of *Leptographium procerum* and *Grosmannia clavigera* produced when growing with bacteria, α-pinene, and both bacteria and α-pinene. Bars labeled with the same letter are not significantly different from each other (Tukey's test, $P > 0.05$). Bars with asterisks are significantly different from the controls.

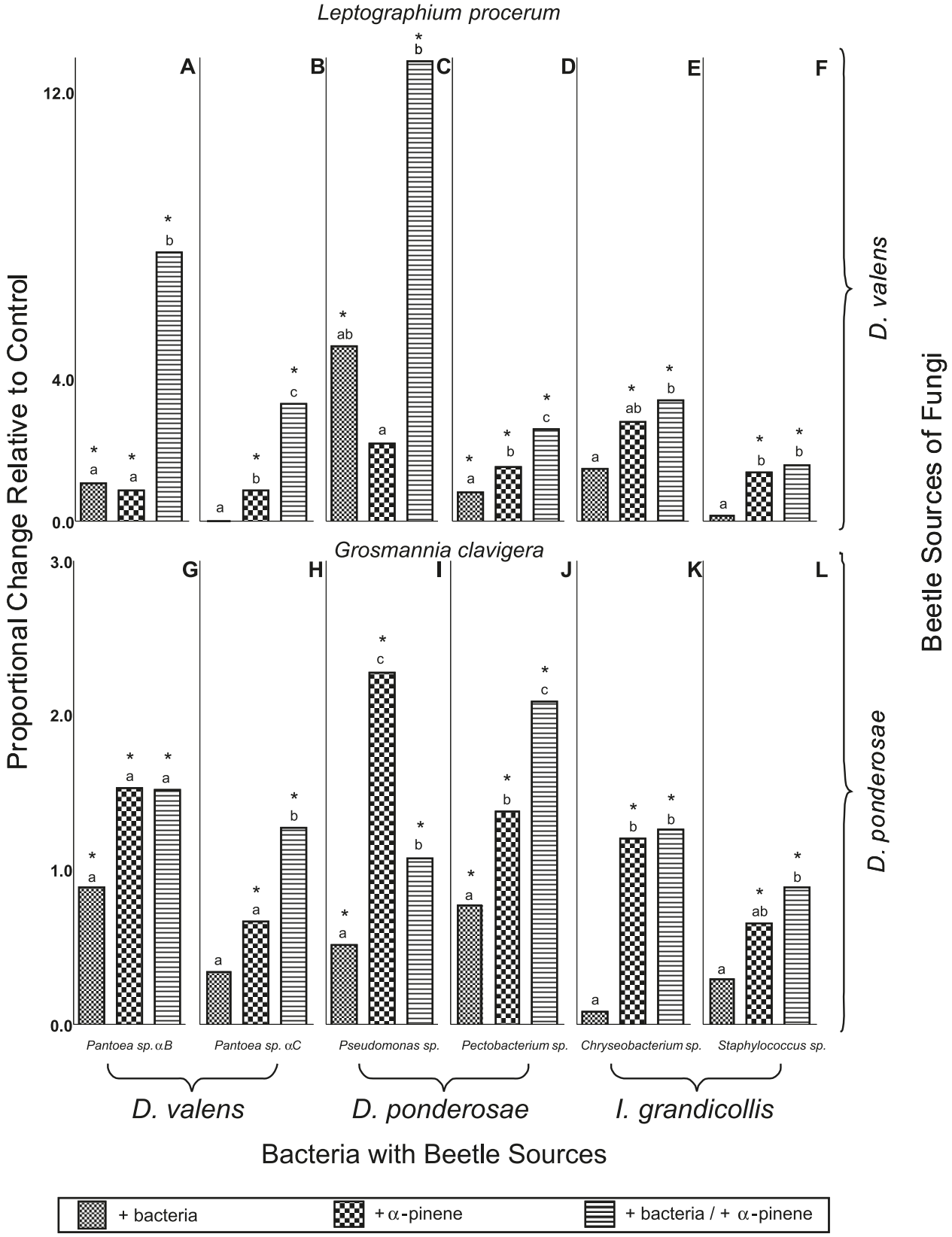
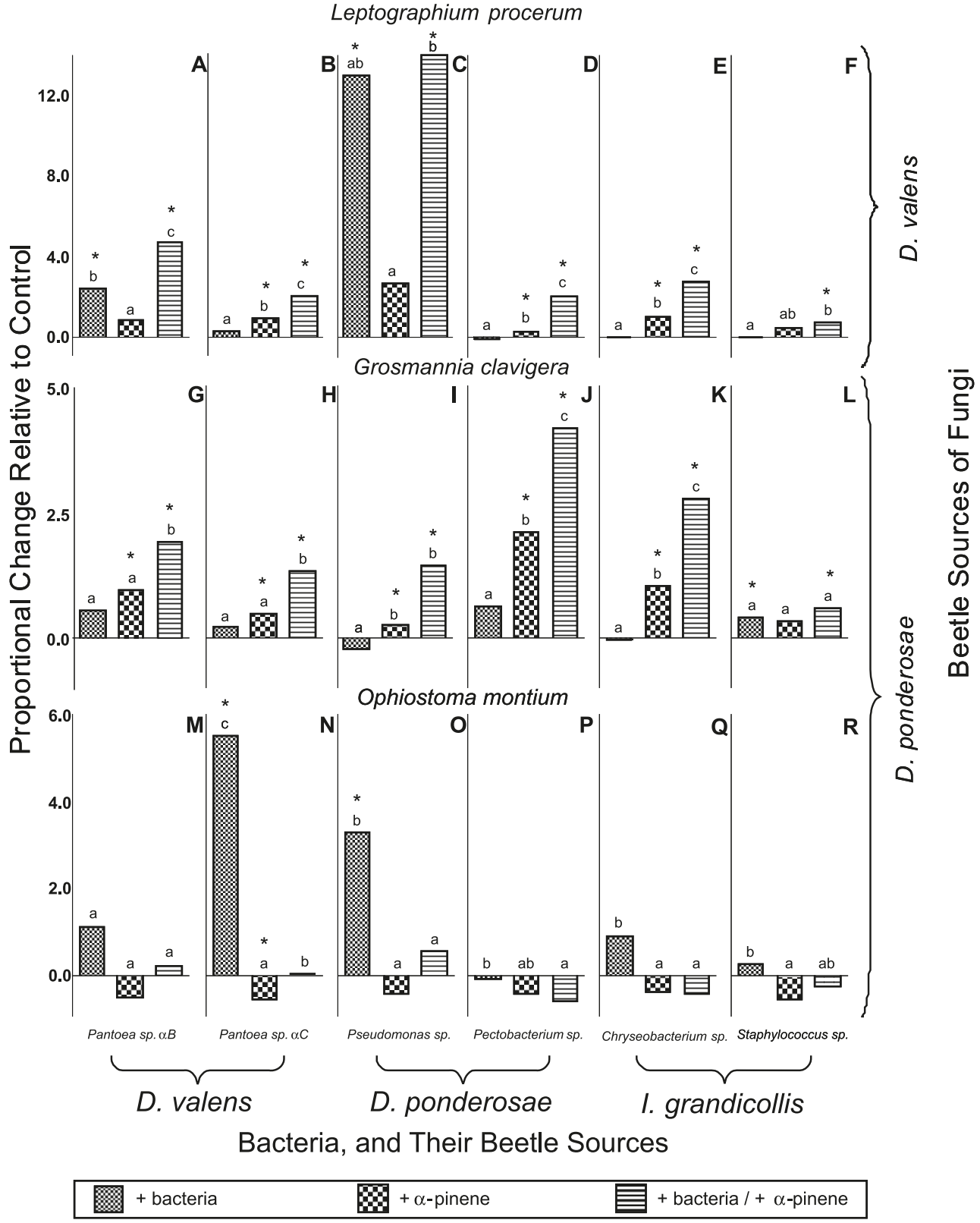


Fig. 4. Proportional change in number of conidia relative to controls of *Leptographium procerum*, *Grosmannia clavigera*, and *Ophiostoma montium* produced when growing with bacteria, α-pinene, and both bacteria and α-pinene. Bars labeled with the same letter are not significantly different from each other (Tukey's test, $P > 0.05$). Bars with asterisks are significantly different from the controls.



phores and conidia. Combinations of α-pinene and *Pantoea* sp. isolates αB and αC also stimulated *G. clavigera* conidiophore production by 150% and 130%, respectively (Figs. 3G and 3H), and conidia production by 190% and 130%, respectively (Figs. 4G and 4H). Conidiophore production by *G. clavigera* with the combination of α-pinene and *Pantoea* sp. isolate αB was greater than with the bacterium alone but was not statistically different from the number of conidiophores produced with α-pinene alone (Fig. 3G). The combination of α-pinene and *Pantoea* sp. isolate αC with *G. clavigera* resulted in the greatest number of conidiophores (Fig. 3H), and the combination of α-pinene and either *Pantoea* sp. isolate with *G. clavigera* resulted in the greatest number of conidia (Figs. 4G and 4H). The combination of α-pinene and either *Pantoea* sp. isolate did not affect conidia production by *O. montium* (Figs. 4M and 4N). The number of conidia produced by *O. montium* with the combination of α-pinene and *Pantoea* sp. isolate αB was not statistically different from other treatments (Fig. 4M). However, the number of conidia produced with *Pantoea* sp. isolate αC was statistically intermediate to those with isolate αC alone and α-pinene alone (Fig. 4N).

Bacteria associated with D. ponderosae

The combination of α-pinene and *Pseudomonas* sp. stimulated conidiophore production of *G. clavigera* by 110%, which was less than the number of conidiophores produced with α-pinene alone but greater than that with the bacterium alone (Fig. 3I). This combination stimulated conidia production of *G. clavigera* by 140%, although this was not statistically different than conidia production with α-pinene alone (Fig. 4I). The combination of α-pinene and *Pectobacterium* sp. stimulated *G. clavigera* production of conidiophores by 210% (Fig. 3J) and conidia by 420% (Fig. 4J), both of which were statistically greater than with all other treatments. The combination of α-pinene and bacteria from *D. ponderosae* resulted in similar patterns of conidiophore and conidia production of *L. procerum*. The combination of α-pinene and *Pseudomonas* sp. stimulated production of conidiophores by 1300% (Fig. 3C) and conidia by 1400% relative to controls (Fig. 4C). This production was not statistically different than that with the bacterium alone. α-Pinene and *Pectobacterium* sp. stimulated *L. procerum* production of conidiophores by 260% (Fig. 3D) and conidia by 200% (Fig. 4D), both of which were greater than with all other treatments. Conidia production of *O. montium* was not affected by the combination of α-pinene and either bacterium from *D. ponderosae* (Figs. 4O and 4P), and conidia production with α-pinene and *Pseudomonas* sp. was less than with this bacterium alone (Fig. 4O).

Bacteria associated with I. grandicollis

The combination of α-pinene and *Chryseobacterium* sp. stimulated conidiophore production of *L. procerum* by 130% relative to controls. This was statistically greater than with the bacterium alone but not statistically different from α-pinene alone (Fig. 3E). This combination also stimulated production of conidia of *L. procerum* by 280%, which was statistically greater than that for all other treatments (Fig. 4E). The combination of α-pinene and *Staphylococcus* sp. also stimulated *L. procerum* conidiophore (Fig. 3F) and conidia production (Fig. 4F) relative to controls by 160% and 68%, respectively. However, these were not statistically different than conidiophores and conidia produced with α-pinene alone. The combination of α-pinene and *Chryseobacterium* sp. stimulated conidiophore production of *G. clavigera* by 130%. This was statistically greater than production with the bacterium alone but not different than with α-pinene alone (Fig. 3K). This combination stimulated production of conidia of *G. clavigera* by 220%, which was statistically greater than for all other treatments (Fig. 4E). The combination of α-pinene and *Staphylococcus* sp. also stimulated *G. clavigera* conidiophore production by 90% relative to controls. This was not statistically different than conidiophores produced with α-pinene alone (Fig. 3L). This combination stimulated conidia production of *G. clavigera* by 60% relative to controls. This effect was not statistically different than that with either α-pinene or the bacterium alone (Fig. 4L). The combination of α-pinene and either *Chryseobacterium* sp. or *Staphylococcus* sp. did not affect conidia production of *O. montium* (Figs. 4Q and 4R).

Direct effects of α-pinene on bacteria

α-Pinene did not affect the growth of the bacteria from *D. valens* or *D. ponderosae* (Table 3). In contrast, it strongly reduced the growth of both bacteria from *I. grandicollis*, of which neither grew to exponential phase within 23 h in the presence of α-pinene at either 1% or 5% concentration (Table 3). In the absence of α-pinene, *Chryseobacterium* sp. and *Staphylococcus* sp. reached the beginning of exponential growth at 4 and 14 h after inoculation, respectively.

Cross-system analysis

Effects of bacterial volatiles or the combined effects of bacteria and α-pinene on fungus growth were independent of the beetle species from which the fungus and bacteria were isolated (effect of bacteria, $\chi^2 = 3.9$, $P = 0.14$; effect of bacteria and α-pinene, $\chi^2 = 0.00$, $P = 1.00$). That is, the outcome of the interaction between bacteria and fungi is not dependent upon beetle species association.

Discussion

Volatiles from bark beetle associated bacteria commonly altered the growth and reproduction of bark beetle associated fungi in vitro. These effects included both stimulation and inhibition, depending on the particular bacterial–fungal combination, but stimulation was more common. Moreover, a host tree compound accentuated, reduced, negated, or even reversed these interactions depending on the particular combination. Given our experimental methodology and current knowledge of bacteria physiology, it is reasonable to predict that the bacterial compounds causing these effects include aromatic metabolites. Compounds such as alcohols, esters, hydrocarbons, isoprenes, ketones, sulfur compounds, and terpenoids are all known to be produced by some bacteria in vitro (Schöller et al. 1997, 2002).

Stimulation of fungi by bacteria may facilitate some beetle–fungal interactions. Overall, the effects of bacteria were much greater on the numbers of reproductive structures than on mycelial growth of fungi (Figs. 2–4). Fungal conidiophores are produced in high abundance along the walls of beetle galleries and pupal chambers and are necessary to

Table 3. Effect of α-pinene on the exponential growth phase of bacteria associated with bark beetles.

Bacterium	α-Pinene concn. (%)	Intercept	Slope	F	P	r^2	Occurrence of exponential growth after inoculation (h)	Doubling time (h)			
								Median	90% CI	χ^2	P
Pantoea sp. isolate αB	0	–0.06	0.02	147.2	<0.0001	0.93	4–9	1.27	1.20–1.46	1.87	0.40
	1	–0.06	0.02	267.0	<0.0001	0.94	4–9	1.12	1.00–1.34		
	5	–0.05	0.01	476.4	<0.0001	0.96	4–11	1.90	1.09–1.99		
Pantoea sp. isolate αC	0	–0.07	0.02	117.8	<0.0001	0.86	4–10	1.34	0.92–1.75	0.69	0.71
	1	–0.05	0.01	166.5	<0.0001	0.87	4–12	1.33	0.97–2.18		
	5	–0.05	0.01	241.7	<0.0001	0.89	4–13	1.55	1.07–2.57		
Pseudomonas sp.	0	–0.05	0.01	452.7	<0.0001	0.95	3–11	1.54	1.32–1.65	4.36	0.11
	1	–0.04	0.01	1296.0	<0.0001	0.98	3–12	2.05	1.87–2.33		
	5	–0.06	0.02	1137.7	<0.0001	0.98	4–11	1.65	1.56–2.09		
Pectobacterium sp.	0	–0.07	0.01	455.1	<0.0001	0.98	5–10	1.26	1.13–1.56	0.56	0.76
	1	–0.07	0.01	694.7	<0.0001	0.97	5–11	1.36	1.20–1.88		
	5	–0.07	0.01	906.0	<0.0001	0.981	5–11	1.28	1.16–1.40		
Chryseobacterium sp.	0	–0.07	0.02	541.4	<0.0001	0.97	4–10	1.71	1.53–1.75	7.62	0.02
	1	0.00	0.00	8.2	<0.01	0.11	—	0			
	5	0.00	0.00	76.6	<0.0001	0.67	—	0			
Staphylococcus sp.	0	–0.37	0.02	104.3	<0.0001	0.79	14–23	1.35	1.25–1.54	7.62	0.02
	1	0.00	0.00	0.0	0.98	0.00	—	0			
	5	–0.01	0.00	78.5	<0.0001	0.65	—	0			

ensure that fungal spores come in contact with newly eclosed adult beetles and, hence, are transported to new host trees. This environment may be ideal for fungal growth because of its increased oxygen and lower moisture (Scheffer 1986) and location where fungi can be continually spread by beetle larvae. The gallery is also likely a location of bacterial concentration and metabolism because of deposits of frass, which can support extended microbial growth (Dillon and Dillon 2004). These volatiles might also inhibit competing fungi. For example, volatiles produced aerobically by bacteria can inhibit fungal growth (Bruce et al. 2003) and melanin production (Payne et al. 2000), a characteristic positively correlated with virulence of antagonistic fungi (Klepzig 2005).

The effects of each bacterium varied across different combinations of beetle–fungal associations. In general, fungal growth response to bacteria appears to be correlated with bark beetle biology, particularly the host tree environment in which they develop. This is illustrated by the summary of our results in Table 4. *Dendroctonus valens* completes development in the host tree without killing it. Thus, the microbial complex associated with *D. valens* needs to tolerate tree defense chemicals of its common hosts. We found that bacteria isolated from *D. valens* interact with α-pinene to create an environment that stimulates growth and reproduction of the *D. valens* fungus associate, *L. procerum* (Table 4).

Dendroctonus ponderosae commonly attacks living host trees and kills the tree. The environment within the host at the beginning of colonization is terpene rich (Raffa and Berryman 1983) and declines over time (Pettersson and Boland 2003). *Grosmannia clavigera* is predominant in larval galleries during middevelopment (Adams and Six 2007). At this stage, the microbial complex associated with *D. ponderosae* is exposed to a moderate level of terpenes. Growth of *G. clavigera* was stimulated in such an environment in vitro and even more so when exposed to the combination of α-pinene and bacteria (Table 4). Interestingly, α-pinene is a minor component of the chemical defense of *P. contorta* (Zavarin et al. 1969), the most common host of *D. ponderosae* and *G. clavigera*. *Ophiostoma montium* is abundant in pupal chambers of *D. ponderosae* (Adams and Six 2007). The environment within the pupal chamber likely is a site with low amounts of tree-synthesized volatiles, and we observed conidia production by *O. montium* to be greatest in this environment with bacteria in vitro. Even though growth of *O. montium* was not affected by the presence of bacteria, increased conidia production in the host tree may improve the chance that *O. montium* spores are acquired by a beetle vector prior to beetle emergence from the host tree.

In contrast to *D. valens* and *D. pondersae*, *I. grandicollis* commonly colonizes trees already declining in health and exhibiting depressed defenses. Thus, symbiotic bacteria associated with bark beetles that colonize weakened host trees are not likely adapted to growing in high-terpene environments. As expected, growth of the bacteria associated with *I. grandicollis* was suppressed by α-pinene in vitro. Interestingly, the growth of the dominant fungal associate of *I. grandicollis*, *O. ips*, was stimulated by α-pinene, which we would not have predicted. Future research is needed to resolve this trend and to determine if other bacteria associ-

Table 4. Summary of the statistically significant ($P < 0.05$) effects of bacteria, α-pinene, and bacteria and α-pinene on fungus growth and conidiophore and conidia production from each of the six fungus–bacterial combinations.

Beetle	Fungus	Effect of bacteria					Effect of α-pinene					Effect of bacteria + α-pinene				
Growth																
Dendroctonus valens	*Leptographium procerum*	+	+	+	0	–	0	0	0	0	+	0	0	+	+	+	+	0
Dendroctonus ponderosae	*Grosmannia clavigera*	+	+	0	0	+	+	+	+	0	+	+	+	+	+	+	+	0
	Ophiostoma montium	0	0	0	–	–	0	–	0	0	0	0	+	0	0	0	0	0
Ips grandicollis	*Ophiostoma ips*	–	+	0	0	0	0	+	+	+	+	+	0	0	0	–	0	0
Conidiophores																		
Dendroctonus valens	*Leptographium procerum*	+	0	+	0	0		+	+	0	+	+	+	+	+	+	0	0
Dendroctonus ponderosae	*Grosmannia clavigera*	+	0	+	0	0		+	+	+	+	+	0	+	–	+	0	0
Conidia																		
Dendroctonus valens	*Leptographium procerum*	+	0	+	0	0	0	0	+	0	+	0	+	+	+	+	+	0
Dendroctonus ponderosae	*Grosmannia clavigera*	0	0	0	0	0	+	+	+	+	+	0	+	+	0	+	+	0
	Ophiostoma montium	0	+	+	0	0	0	0	–	0	0	0	0	+	0	0	0	0

Note: –, Inhibitory; 0, no effect; and +, stimulatory. Categories are the result of pairwise comparisons between the treatments of bacteria and α-pinene compared with the control treatment and between the treatment of the combination of bacteria and α-pinene compared with growth in the α-pinene treatment.

ated with *I. grandicollis* are also suppressed by α-pinene and other terpenes.

We did not observe patterns consistent with the hypothesis that bacteria isolated from one beetle promote the growth of that beetle's dominant fungal associate while not affecting or even hindering the associates of other beetles. Promotion of a symbiont through protection from an antagonist was detected with a *Streptomyces* sp. and fungal associates of *D. frontalis* (Scott et al. 2008) and with several bacteria and a fungal associate of *D. rufipennis* (Cardoza et al. 2006*a*). However, we tested only a small proportion of the possible interactions in this system. Additionally, because of the large number of comparisons needed to test for interspecific trends, we necessarily tested only one isolate of each bacterium and fungus. Intraspecific variation of free-living bacterial and fungus phenotypes may be high, and such variation could affect the outcome of these interactions. Although this is the first study to detect potentially important interactions among tree chemistry, symbiotic ophiostomatoid fungi, and symbiotic bacteria, further research is necessary to determine if similar interactions occur in the full chemical profile of the host tree and to determine the frequency of association of these bacteria with the host beetle.

Because beetles may derive multiple costs and benefits from fungi, interactions between fungi and bacteria may impact beetle fitness. Airborne signals from bacteria isolated from three bark beetle species affected the growth and reproduction of beetle-symbiotic fungi. Further, we showed that a major phytochemical affecting beetle interactions with conifers, α-pinene, can alter this interaction. We propose that such interactions between bacteria, fungi, and host tree of the beetle are important for the overall symbiosis among bark beetles and fungi.

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