Effects of Diterpene Acids on Components of a Conifer Bark Beetle–Fungal Interaction: Tolerance by *Ips pini* and Sensitivity by Its Associate *Ophiostoma ips*

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ABSTRACT Conifer resin and phloem tissue contain several phytochemical groups, composed primarily of monoterpenes, diterpene acids, and stilbene phenolics. The effects of monoterpenes and phenolics on stem-colonizing bark beetles and their associated microorganisms have been studied to some extent, but the roles of diterpene acids are largely unknown. Diterpene acids are known to have substantial feeding deterrent and growth inhibiting effects on a variety of insect groups and are known to inhibit a variety of fungi. We tested three diterpene acids present in red pine, *Pinus resinosa*, at various concentrations, on several life history components of the bark beetle *Ips pini* and the fungus *Ophiostoma ips*. No diterpene acid affected the host acceptance behavior or larval survival of *Ips pini*. In contrast, abietic acid and isopimaric acid strongly inhibited spore germination of *O. ips*, and abietic acid strongly inhibited mycelial growth. The levels of inhibition observed were higher than with any previous assays of monoterpenes or phenolics in this system. These results support the view that conifer defenses against bark beetle–fungal complexes are multifaceted, with all three phytochemical groups being important to *P. resinosa*, but each with varying relative activity against the beetles and fungi.

KEY WORDS host defense, conifer, diterpene acids, bark beetles, *Ips*, *Ophiostoma*

CONIFERS ARE WELL DEFENDED against subcortical insects and pathogens, possessing several traits that can reduce their likelihood of establishment (Lewinsohn et al. 1991, Nebeker et al. 1992, Franceschi et al. 1998, Hudgins and Franceschi 2004). For example, *Pinus* spp. have well-developed resin ducts that can physically cleanse wounds by expelling invaders and sealing off damaged tissue (Berryman 1972, Nebeker et al. 1992). These resins are comprised of a diverse array of chemicals, including terpenoids and phenolics, which are insecticidal and fungistatic at high concentrations (Cobb et al. 1968, Raffa et al. 1985). The compounds present in this resin undergo both quantitative and qualitative changes during inducible responses elicited after attack (Raffa and Berryman 1983, Christiansen 1985, Werner and Illman 1994, Martin et al. 2002).

Of the compounds present in conifer resin, monoterpenes are the most studied. Several of these monoterpenes are attractive, repulsive, or toxic to bark beetles and inhibitory or toxic to their associates (Langenheim 1994). They are also involved in pheromonal communication by bark beetles as synergists, inhibitors, and precursors. Phenolics have also been shown to inhibit beetle tunneling, fungal germination, and fungal growth (Klepzig et al. 1995). Diterpene acids occur in similar molar amounts as monoterpenes (Johnson and Croteau 1987, Bonello and Pearce 1993, Bonello et al. 2003), but their biological activities are not as well studied. Diterpenes are known to be important in several insect systems. For example, high diterpene acid concentrations deter sawfly larvae from current season's needles, allowing them to feed on mature needles only (Ikeda et al. 1977, Schuh and Benjamin 1984). Additionally, Sitka spruce that are resistant to white pine weevil produce more diterpene acids than do susceptible trees (Tomlin et al. 1996). Little is known about the role these diterpene acids may have against bark beetles and their associated fungi, however.

Bark beetles (Curculionidae: Scolytinae) develop in the subcuticular tissue of trees. Most species inhabit dead or dying trees (Wood 1982). The beetles that attack living conifers (primarily *Dendroctonus*, *Ips*, and *Scolytus*) are able to overcome defenses by engaging in pheromone-mediated mass attacks. However, even these "primary" tree-killing species typi-

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cally avoid well-defended trees, based largely on avoidance of certain token stimuli (Wallin and Raffa 2004).

Bark beetles are closely associated with a group of ascomycete fungi known as ophiostomatoid or "stain" fungi (*Ophiostoma* and *Ceratocystis* spp. and their anamorphs *Graphium* and *Leptographium*). These fungi can be transported phoretically on the beetle's exoskeleton (Whitney and Farris 1970, Harrington 1993, Paine and Hanlon 1994) or internally (Furniss et al. 1995). Their relationships with their bark beetle vectors are complex and only partially understood (Klepzig 1998). Some fungal associates exert markedly negative effects on beetles, at least during their development phase (Barras 1970, Six and Paine 1998, Kopper et al. 2004). Some can facilitate beetle nutritional physiology (Ayres et al. 2000). Some species, such as *Ceratocystis polonica* (Siemaszko) Moreau, can kill trees, even without its beetle vector *Ips typographus* L., and therefore facilitate host colonization (Horntvedt et al. 1983, Christiansen 1985, Krokene and Solheim 1998). However, this high level of virulence seems to be an exception, because most ophiostomatoid fungi are confined within defensive lesions when inoculated into healthy trees (Shrimpton 1973, Raffa and Berryman 1983, Paine and Stephen 1987, Cook and Hain 1988, Klepzig et al. 1991, Lieutier et al. 1993, Raffa and Smalley 1995). The inability to kill trees does not preclude the possibility that some ophiostomatoid fungi function as co-factors, i.e., organisms that alter or disrupt host plant defenses in manners that render the host suitable for beetle establishment (Hemingway et al. 1977, Raffa and Berryman 1983, Leufvén 1991, Paine et al. 1997). Regarding diterpene acids, *Ophiostoma piliferum* is known to be able to degrade diterpene acids and is even efficient enough to be employed as a biopulping agent (Blanchette et al. 1992). However, it is not known whether this trait is common to all stain fungi or if degradation of diterpene acids influences bark beetle performance.

The pine engraver [*Ips pini* (Say)] is an important pest of red pine, *Pinus resinosa* (Aitman), in the Lake States region (Klepzig et al. 1991). This beetle attacks trees under physiological stress. Males initiate colonization and attract conspecifics through the production of aggregation pheromones. During colonization, *I. pini* inoculates the tree with several species of fungi, of which *Ophiostoma ips* (Rumbold) Nannfeldt is predominant in Wisconsin (Klepzig et al. 1991).

The objectives of this study were to determine the effects of diterpene acids present in conifers on (1) *I. pini* adult host acceptance behavior, (2) *I. pini* larval survival, (3) *O. ips* germination, and (4) *O. ips* mycelial growth. We chose abietic, dehydroabietic, and isopimaric acids because they are present in conifers and are known to exhibit biological activity against insects and fungi. The compounds we selected also represent the two major subclasses of diterpene acids: abietic and dehydroabietic acid in the abietic subclass and isopimaric acid in the pimaric acid subclass. The concentrations of acids used in this study represent a range because there is little information on diterpene acid content on a per gram of plant tissue basis.

Materials and Methods

Beetle Bioassays. Test beetles were collected from a colony maintained on fresh *P. resinosa* logs on the University of Wisconsin-Madison campus. Detailed rearing procedures are discussed in Wallin and Raffa (2000). Wild beetles are introduced into this colony several times per year. Voucher specimens were deposited in the University of Wisconsin Insect Research Collection.

The beetle entry bioassay was modified after those designed by Klepzig et al. (1996) and Wallin and Raffa (2000). The assay unit consisted of an 11 by 35-mm plastic petri dish into which we added a mixture of *P. resinosa* phloem, agar, and water. Phloem was freeze-dried, ground through a Wiley mill (0.5-mm screen), and autoclaved before use. Treating phloem in this manner removes volatile monoterpenes below levels detectable by gas liquid chromatography (Wallin and Raffa 2000). Bacto-agar (2 g; Difco, Detroit, MI) was dissolved in boiling double distilled water (60 ml), and phloem (4 g) was added to this mixture. Abietic, dehydroabietic, and isopimaric acids (Orchid-Helix Biotech, Vancouver, British Columbia, Canada) were dissolved in acetone (3 ml) and added to the molten agar mixture to yield the appropriate percentage by mass (milligrams diterpene acid per milligrams of media times 100). Controls consisted of acetone alone and no solvent. Medium was poured into petri dishes to a thickness of 4 mm, allowed to set with lids for 24 h at 24°C, and dried for 4 h without lids in a laminar flow hood at 24°C. Each amended agar disk was removed from the base of the petri dish and placed in the inverted lid, so that there was a 2-mm gap surrounding the medium. One male *Ips* was placed in this gap, and the arena was sealed with parafilm to maintain moisture. The assays were conducted in a growth chamber at 24°C in the dark for 5 h. Arenas were scored to determine if the beetles entered the media. Beetles that appeared feeble during the early stages of the assay, as evidenced by an inability to remain standing even if righted (a condition that did not vary among treatments), were excluded. Each treatment contained a minimum of 22 replicates.

To test if diterpene acids affect larval survival, we exposed larvae to the same treatments as described above. Larval survival assays were conducted in 96-well tissue culture plates (Falcon, Franklin Lakes, NJ). Phloem agar disks (6 mm diameter) were cut from the petri dishes with agar at various concentrations of diterpene acids, as described earlier, using a no. 2 cork borer and randomly assigned to the tissue culture plate. One second- or third-instar larva was added to each well and monitored daily for 5 d, noting if the larva fed and/or was alive. Larvae that appeared lethargic during the early stages of the assay (which did not vary among treatments) were excluded. Each treatment contained a minimum of 26 replicates.

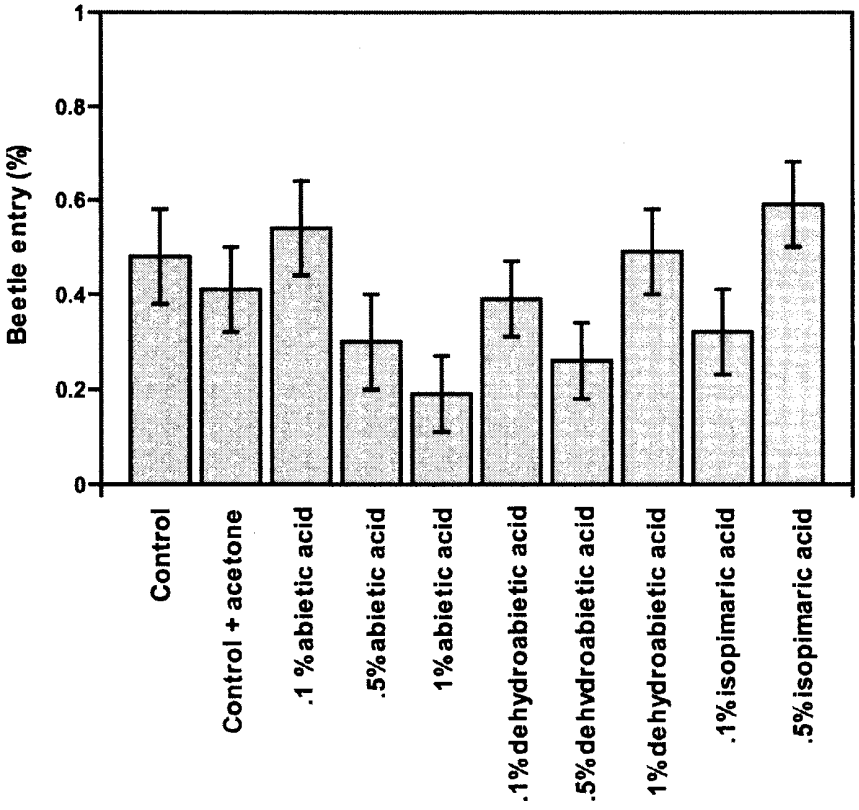

Fig. 1. Entry rates by adult male *I. pini* into phloem-based agar amended with diterpene acids. Assays were conducted for 5 h of darkness in 11-mm petri dishes. Bars indicate ±SE. *P* values indicate results of one-way ANOVA ($F_{9,18}$ = 1.81, P = 0.134).

The adult entry and larval survival assays were each set up as a completely randomized block design. We used the control + acetone treatment as our baseline for data analysis. Data were analyzed using a generalized linear mixed model (PROC MIXED, glimmix macro). Analysis of variance (ANOVA) was used to determine treatment effects, with diterpene acid level as a fixed effect and block as a random effect. We report means and SEs for each treatment as reported by PROC MEANS.

Fungal Bioassays. We used a single clone of *O. ips,* which was isolated in pure culture from a red pine recently killed by *I. pini.* Thomas Harrington (Iowa State University, Ames, IA) confirmed our identification and deposited a subculture in his collection (acquisition no. C1927).

We modified the bioassay of Klepzig et al. (1996) to determine the effects of diterpene acids on fungal germination. We amended 2% malt-extract agar with abietic, dehydroabietic, or isopimaric acid by dissolving them in acetone and adding them to the molten agar mixture to yield the appropriate percentage by mass (milligrams diterpene acid per milligram of media times 100). We applied three levels of diterpene acids, 0.1, 0.5, and 1.0% of diet fresh weight, except that no 1.0% isopimaric acid treatment was used because of insolubility. Control treatments consisted of blanks and solvent alone. Amended agar was added to 11 by 35-mm plastic petri dishes and allowed to cool for 24 h. Spores were obtained by placing mucilaginous conidial masses in a test tube containing 2% malt extract broth. The test tubes were continuously aerated until a ring of conidia formed (» 14 d). A spore suspension of 200,000 spores/ml was prepared and added to the petri dishes in 35-µl aliquots. Spores were spread across each plate using a glass rod and were incubated for 24 h at 20°C before counting germination. A total of 15 petri dishes were used for each treatment.

The fungal growth assay was conducted using the method of Klepzig et al. (1996). Molten MEA (1 ml) was added to a 7-ml scintillation vial followed by 50 µl of the desired diterpene acid stock solution to yield 0.1, 0.5, or 1.0% fresh weight of media (no 1.0% isopimaric treatment was used because of solubility problems). The vial was capped, agitated, and set horizontally. Once the agar solidified, the vial was uncapped to allow the acetone to evaporate. When acetone could no longer be detected in the vial (» 6 h), a 2-mm-diameter piece of MEA colonized by actively growing *O. ips* was added to the middle of the vial. Care was taken so that the mycelial side of the colonized MEA was placed on the surface of the medium. The vials were capped and incubated at 24°C in the dark. After 3 d, mean linear growth (mm) was calculated. Each treatment contained 10 replicates.

We used the control + acetone treatment as our baseline for data analysis for both *O. ips* assays. Data were ranked before statistical analysis (PROC

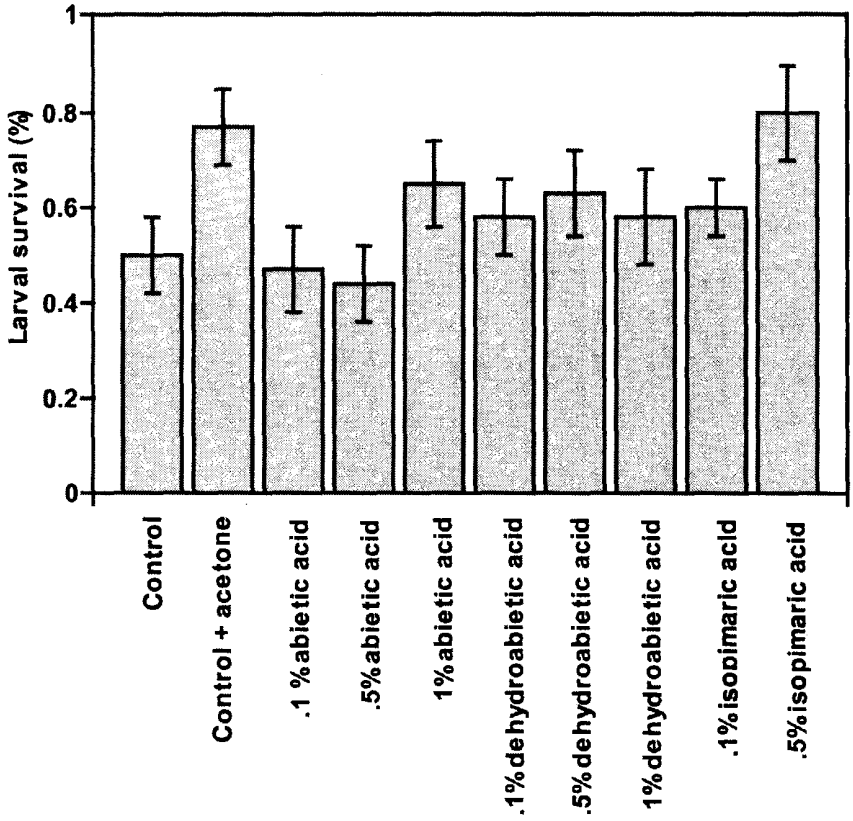

Fig. 2. Survival of *I. pini* larvae in phloem-based agar amended with diterpene acids. Assays were conducted for 5 d in 96-well tissue culture plates. Bars indicate ±SE. *P* values indicate results of one-way ANOVA ($F_{9,54}$ = 1.58, P = 0.145).

RANK), because of heterogeneity in the variance across treatments. Ranked data were analyzed using ANOVA (PROC MIXED; Littell et al. 1996) with means and SEs calculated using the PROC MEANS procedure.

Diterpene Acid Analysis. We used a high-performance liquid chromatography (HPLC) method modified after Chow and Shepard (1996) to quantify the amount of diterpene acids remaining in MEA as a quality control measure. Amended diet was extracted in 4 ml HPLC grade methanol (Fisher, Milwaukee, WI) for 24 h. Extracts were filtered through glass wool to remove particulates before HPLC analysis. Samples were analyzed using a Shimadzu LC-10AS HPLC fitted with an Alltech C-8 column (250 by 4.6 mm; Alltech Associates, Deerfield, IL) and a 20-µl sample loop. The UV-visible light detector (Shimadzu SPD-10a) was set at 220 nm. The mobile phase consisted of 65% acetonitrile (Fisher) /35% filtered double distilled water at 2 ml/min. Remaining diet was oven dried and weighed. Linear regression (r^2 < 0.98) was used to determine means of diterpene acids per milligram of media based on the standard curve of pure diterpene acids. Paired t-tests were used to determine if our extraction levels differed from initial target levels.

Results

Beetle Bioassays. These diterpene acids did not affect entrance behavior (Fig. 1), because entrance in the abietic acid, dehydroabietic acid or isopimaric acid treatments did not differ from the acetone control ($F_{3,6}$ = 2.48 P = 0.159; $F_{3,6}$ = 1.15: P = 0.401; $F_{2,4}$ = 0.70: P = 0.550, respectively). Additionally, these diterpene acids did not significantly affect larval survival (Fig. 2). Larvae in the abietic acid, dehydroabietic acid, or isopimaric acid treatments performed similar to those in the acetone control ($F_{3,18}$ = 1.82: P = 0.179; $F_{3,18}$ = 0.86: P = 0.479 $F_{2,12}$ = 1.52: P = 0.258, respectively). Survival across all treatments was 61%.

Fungal Bioassays. These diterpene acids had pronounced effects on germination by *O. ips* (Fig. 3; $F_{9,149}$ = 14.21, P = <0.001). The response differed among the compounds and concentrations applied. Germination was similar between the control and acetone control treatments. Abietic acid dramatically affected *O. ips* germination, reducing it by 39% in the 0.1%, 30% in the 0.5%, and 91% in the 1.0% treatments, relative to the acetone control. Germination in the dehydroabietic acid treatments was similar to the acetone control. However, at the 0.5% level, dehydroabietic acid significantly reduced germination by 26% relative to the control treatment. Isopimaric acid strongly reduced germination, with germination in the 0.1 and 0.5% treatments 43 and 74% lower than was observed in the acetone-only treatment, respectively.

Fungal growth was significantly influenced by the presence of these diterpene acids (Fig. 4; $F_{9,99}$ = 45.15, P £ 0.001). The magnitude of this response varied depending on the compound and concentration.

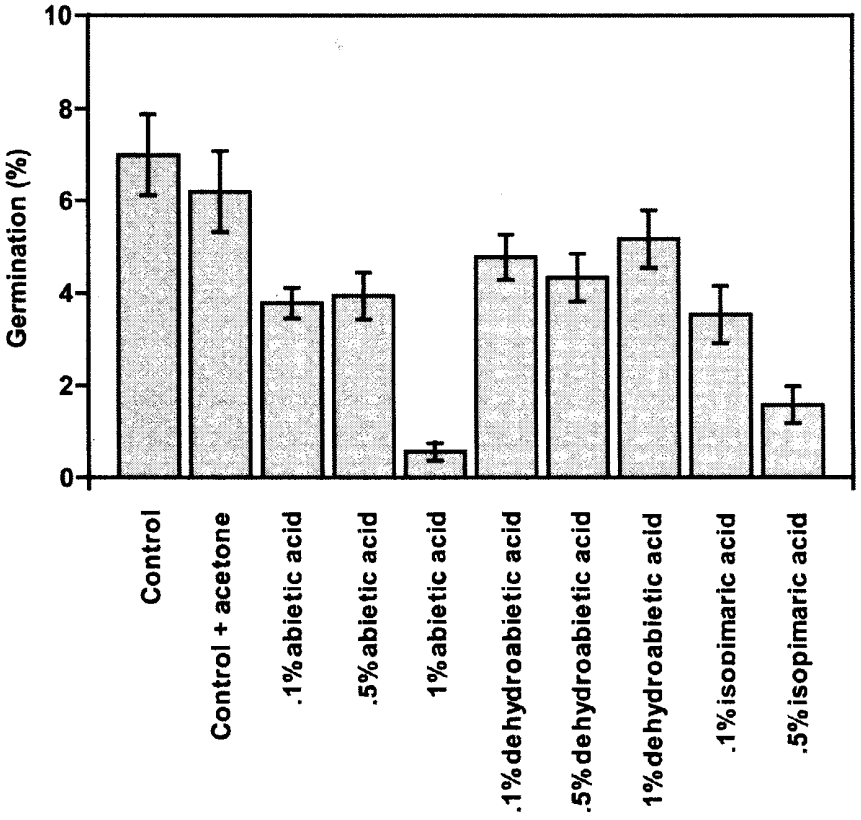

Fig. 3. Effect of diterpene acids on *O. ips* germination. Assays were conducted for 24 h. Bars indicate ±SE. *P* values indicate results of one-way ANOVA ($F_{9,149}$ = 14.21, *P* £ 0.001).

There was no difference between the control and the acetone control treatments. Abietic acid strongly reduced fungal growth, with fungi in the 0.1, 0.5, and 1.0% treatments growing 47, 74, and 84% more slowly than did those in the acetone-only treatment. Dehydroabietic acid significantly reduced growth relative to both control treatments, but this effect did not vary among the concentrations applied. Overall, dehydroabietic acid reduced growth by 24%. Isopimaric acid showed weak, and inconsistent, reductions in mycelial growth.

Media Extractions. The diterpene acid concentrations recovered from amended diet did not differ from our target levels (*P* values for abietic, dehydroabietic, and isopimaric acid were *P* = 0.085, *P* = 0.647, and *P* = 0.077, respectively).

Discussion

Overall, these three diterpene acids had pronounced effects on the predominant fungal associates of the pine engraver but little to no effects on the beetles themselves. Of the compounds tested, abietic acid and isopimaric acid were most inhibitory.

The effects of abietic acid and isopimaric acid on beetle-associated fungi are stronger than has been reported for other compounds. For example, 1% abietic acid reduced *O. ips* linear growth by 84% and its germination by 91%, and 0.5% isopimaric acid reduced germination by 75%. By comparison, the predominant monoterpene in *P. resinosa*, a-pinene, reduced the linear growth of *Leptographium terebrantis* Barras and Perry and *Leptographium procerum* (Kendrick) Wingfield by only 23 and 6%, respectively, even when applied at 100% saturated atmospheric conditions (Klepzig et al. 1996). The most inhibitory monoterpene was *g*-terpinene, which caused 54.5% reduction of L. terebrantis growth, but this compound occurs in only trace amounts (Raffa and Smalley 1995). Reductions in germination of *L. terebrantis* and *L. procerum* by a-pinene were 58 and 69%, respectively, and g-terpinene caused a 74% reduction on L. procerum. Similarly, the strongest monoterpene-induced growth reduction of *Trichosporium symbioticum* Wright, an associate of *Scolytus ventralis* LeConte, was only 45%, in response to myrcene (Raffa et al. 1985). Polar extracts of induced *P. resinosa* phloem tissue, which consist primarily of stilbene phenolics, likewise inhibited *L. terebrantis* linear growth by only 45%.

These results support the view that conifer defenses against bark beetle–microbial complexes are multifaceted within single host trees. Based on the available evidence in *P. resinosa*, monoterpenes seem to be the most active compounds against beetles, although they also have some fungistatic activity (Raffa and Smalley 1995, Klepzig et al. 1996, Wallin and Raffa 2000). Diterpene acids seem to be the most active compounds against fungi, markedly reducing both germination

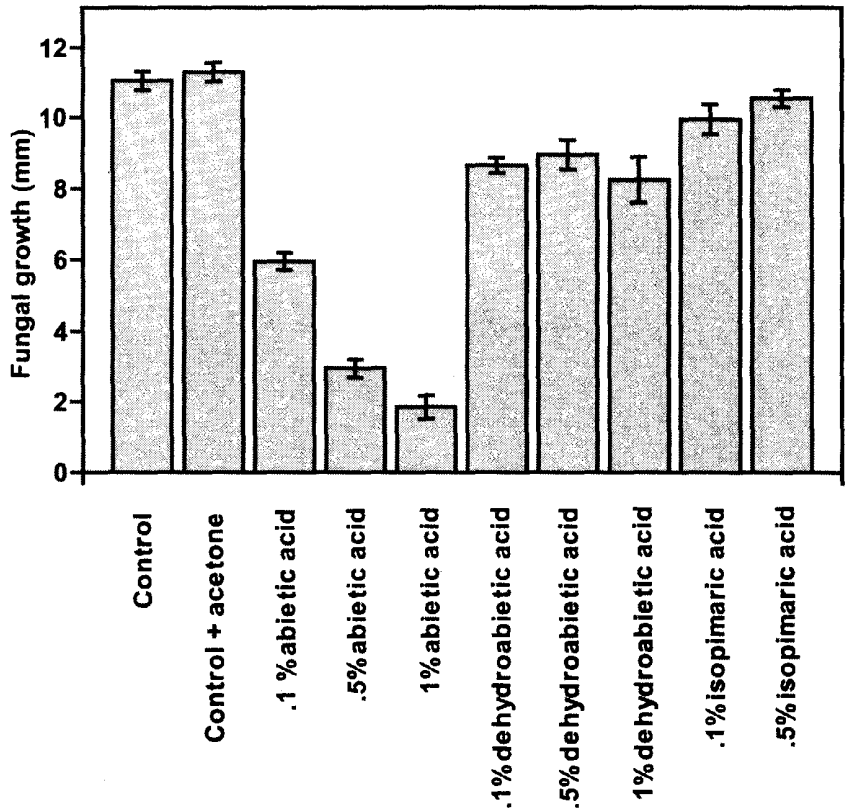

Fig 4. Effect of diterpene acids on *O. ips* growth (3 d). Bars indicate ±SE. *P* values indicate results of one-way ANOVA ($F_{9,99}$ = 45.15, *P* £ 0.001) .

and mycelial growth (Figs. 3 and 4). The absence of diterpene acid effects on beetle behavior or survival (Figs. 1 and 2) seems to reflect substantial tolerance by these beetles, because many other insects, such as conifer sawflies, show substantial feeding deterrence and developmental inhibition by these compounds (Ikeda et al. 1977). Our observation that diterpene acids had no effect on beetle behavior should be taken with some caution, however, because the data suggest a potential effect of abietic acid obscured by experimental variation. Overall, phenolics tend to have intermediate activity against both beetles and fungi (Klepzig et al. 1996). Among the chemical groups present in *P. resinosa* phloem, monoterpenes and diterpene acids are highly inducible and phenolics are weakly inducible (Raffa and Smalley 1995, Klepzig et al. 1996).

It is not yet possible to infer how such different relative activities of monoterpenes, diterpene acids, and phenolics on the beetles and their associated fungi affect overall host susceptibility or beetle population dynamics. Previous studies have shown that effects of *O. ips* on *I. pini* reproduction can be quite subtle, with variable and sometimes contrasting effects arising from colonization density, temporal sequence, and beetle life stage (Robins and Reid 1997, Kopper et al. 2004). For example, the highly antifungal activity of diterpene acids could potentially enhance *I. pini* reproduction by removing the competitive actions of *O. ips* or enhance overall host resistance against initial establishment by the overall beetle–fungal complex. Future research on the roles of associated fungi across the full time course of these interactions is needed to resolve those questions.

Development of controlled bioassays that emulate natural conditions is a major challenge to our understanding of endophytic herbivores. These experiments provide two new tools. Previous studies of phytochemical effects on bark beetles have concentrated almost entirely on adults, although some experiments have been conducted with eggs (Raffa and Berryman 1983). No studies have been conducted with larvae, yet this is obviously a critical stage in the interaction. Endophytic insects in general, and bark beetles in particular, have very fragile larvae, because they normally do not encounter the external environment. In this new assay, larval survival averaged **60.2%** across all treatments. Hopefully this method will prove useful for studying other bark beetle species and host compounds. Second, amending denatured phloem with host compounds has proven an effective method for evaluating host acceptance behavior, with species from three genera proving sensitive to host tissue status (constitutive versus induced), root condition (infected versus healthy), and beetle age, experience, lipid content, heredity, and population phase (Klepzig et al. 1996, Wallin and Raffa 2000, 2002, 2004). However, this method is time-consuming because of the large amounts of phloem (and hence trees) required and expensive because of the costs of synthetic com-

pounds. We miniaturized this assay in this study, thereby reducing the phloem and synthetic materials needed by 61%. The beetles behaved normally under these conditions, but entry into control medium was lower (45 versus 80% in the previous larger arenas). Thus, additional work is needed to improve the practicality and cost-effectiveness of reliable host acceptance assays.

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