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Reciprocal interactions between the bark beetle-associated yeast *Ogataea pini* and host plant phytochemistry

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Abstract: Here we report the first experiments testing reciprocal effects between the bark beetle-associated yeast, *Ogataea pini*, and phytochemicals present in tree tissues (*Pinus ponderosa*). We tested two hypotheses: (i) tree phytochemicals mediate *O. pini* growth and (ii) *O. pini* affects chemical composition of plant tissues. We tested six monoterpenes on *O. pini* biomass growth in vitro and found that most monoterpenes inhibited *O. pini* growth; however mean *O. pini* biomass increased 21.5% when treated with myrcene and 75.5% when treated with terpinolene, relative to control. *Ogataea pini* was grown on phloem tissue ex vivo to determine whether *O. pini* affected phloem chemistry. Monoterpene concentrations declined in phloem over time, but phloem colonized by *O. pini* had significantly different concentrations of monoterpenes at two periods than phloem with no yeast. After 7 d, when *O. pini* was present, concentrations of the monoterpene Δ-3-carene was 42.9% lower than uncolonized phloem and concentrations of the monoterpene terpinolene was 345.0% higher than uncolonized phloem. After 15 d phloem colonized by *O. pini* had 505.4% higher concentrations of α-pinene than uncolonized phloem. These experiments suggest that *O. pini* responds to phytochemicals present in host tissues and the presence of *O. pini* might alter the chemical environment of phloem tissues during the early stages of beetle development. The interactions between *O. pini* and phytochemicals in pine vascular tissues might have consequences for the bark beetle that vectors *O. pini*, *Dendroctonus brevicomis*.

Key words: bark beetle, *Dendroctonus brevicomis*, monoterpene, *Ogataea pini*, phytochemistry, *Pinus ponderosa*, symbiosis, yeast

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INTRODUCTION

Davis et al. (2010) found that the ascomycetous yeast *Ogataea pini* (Saccharomycetales: Saccharomycetaceae) was present in the fungal transport structures (thoracic mycangia) of 56% of female western pine beetle *Dendroctonus brevicomis* LeConte. This frequency of association has been consistent for at least 50 y and across large geographic regions (Shifrine and Phaff 1956). The fidelity of this association implicates *O. pini* as a consistent symbiont in the *D. brevicomis* system. *Ogataea pini* establishes rapidly when compared with the filamentous fungal symbionts of *Dendroctonus* beetles and could disproportionately influence the incipient stages of tree colonization relative to slower growing filamentous fungi (Six and Wingfield 2011). Although a frequent association between *O. pini* and *D. brevicomis* originally was demonstrated by Shifrine and Phaff (1956) nearly 60 y before, the role played by *O. pini* in the *D. brevicomis* community remains poorly understood.

Experimental research regarding yeast species in bark beetle symbiont assemblages thus far has been restricted solely to the effects of volatile organic compounds produced by yeasts (Hunt and Borden 1990, Adams et al. 2008, Davis et al. 2010). Here we report the first test of interactions between the yeast *O. pini* and phytochemicals that are present in the primary host tree, ponderosa pine (*Pinus ponderosa*), of the western pine beetle, *D. brevicomis*. *Dendroctonus brevicomis* colonizes the vascular tissue of ponderosa pine (Wood 1982). Oleoresin exudation is a primary physiological response when healthy pine vascular tissues are damaged by invading organisms (Raffa and Smalley 1995). Thus *O. pini* vectored by the beetle must survive in a phytochemically saturated environment. The microbial associates of some *Dendroctonus* beetles are known to be highly sensitive to chemical variation in the environment (Paine and Hanlon 1994; Hofstetter et al. 2005, 2007), and the phytochemical variation previously described in host pine trees (Latta et al. 2000, 2003) creates a potential interface for interactions to occur between host tree tissues and *O. pini*.

Our objectives were to test the effects of chemical components of oleoresins of *Pinus ponderosa* on *O. pini* isolated from *D. brevicomis* and to determine whether the presence of *O. pini* affects the secondary chemistry of tree vascular tissues. We tested two hypotheses: (i) host monoterpenes mediate the

growth of *O. pini* and (ii) the presence of *O. pini* in phloem (vascular tissue) influences chemical composition of phloem. We tested the effects of six monoterpenes on *O. pini* biomass growth in vitro and found that some monoterpenes inhibited *O. pini* growth but two monoterpenes stimulated growth. We also tested the effect of *O. pini* on the monoterpene composition of phloem tissue in ex vivo assays. We found that monoterpenes degrade over time in phloem tissue but inoculation with *O. pini* significantly affected concentrations of monoterpenes in phloem tissues over time relative to yeast-free phloem.

MATERIALS AND METHODS

Isolation of O. pini *from* D. brevicomis.—*Ogataea pini* was isolated from the fungal transport structures (mycangia) of live *D. brevicomis* females as described in Davis et al. (2010). Live *D. brevicomis* were collected from Lindgren funnel traps baited with enhanced western pine beetle pheromone lures (Synergy Semiochemicals Corp, P130 Lot No. WPP10416). Traps were placed in forested areas dominated by ponderosa pine (*Pinus ponderosa*) in northern Arizona, USA, at 2100–2200 m. Beetles were live-trapped and placed individually into clear, size 0 gelatin capsules (Torpac, Lot No. 1100049271) and stored in the lab in dark environmental chambers at 5 C until used for microbial isolations. Beetles were held fewer than 24 h before dissection, and only live female beetles were dissected. We dissected only female beetles because males do not exhibit mycangia in this species. All insects used for isolation of *O. pini* were collected 18 May 2009–18 Jun 2009.

Effect of monoterpenes on Ogataea pini *growth.*—We chose to assay biomass of *O. pini* instead of optical density (OD) or colony forming units (CFUs) because of the high repeatability and reduced variability of biomass measurements. In addition biomass provides a direct estimate of colony size and performance. Before experimental assays the growth function for *O. pini* biomass was determined in 1.5% malt extract broth (MEB; Difco, Lot No. 7306921). This was done so that the effect of monoterpene treatments could be assessed experimentally at a single time. We chose to use liquid media because it is consistently and easily separated from yeast biomass. The pH of MEB media was 4.7 ± 0.2, and the pH of the natural substrate (ponderosa pine phloem) was 4.5 (Elkinton et al. 1981). Three isolates of *O. pini* were chosen randomly and stock cultures were created by inoculating 100 mL MEB with 10 µL *O. pini* cell culture in sterile Erlenmeyer flasks. Flasks were placed on a platform shaker (Excella E1) at 15% maximum speed (70 rpm.) and incubated in the dark 24 h at 25 C. Sterile scintillation vials containing 10 mL MEB were inoculated with 100 µL volumes of each stock culture (dilution factor of 10^{-2}). Seven vials were made for each *O. pini* isolate, and vials were incubated in the dark at 25 C on the platform shaker, 0, 1, 2, 4, 8, 24 and 48 h. This was replicated twice for each isolate. After incubation vials containing *O. pini* culture and MEB were centrifuged at 20 000 rpm (22 C, Sorvall RC2-B) 5 min (modified from Zheng et al. 2005). A pipette was used to remove the supernatant from the cell pellet left in each vial. We washed yeast cells with purified deionized water, centrifuged vials at 20 000 rpm and again removed the supernatant from the cell pellet. Vials were placed in drying ovens at 70 C for 24 h. Total biomass growth for each replicate at each period was determined by subtracting the vial weight before inoculation from the vial weight following removal from drying ovens. Vials were accurately weighed to 1×10^{-2} mg. Voucher specimens of *O. pini* were preserved in 20% glycerol/80% malt extract broth (Difco, Lot No. 7306921) and placed in −80 C storage freezers in the Center for Microbial Genetics and Genomics in Flagstaff, Arizona, USA (Davis et al. 2010).

We experimentally tested the effect of monoterpenes on *O. pini* growth with the above technique, except that 15 randomly chosen isolates were used and three subsamples were performed for each isolate. Treatment consisted of amending vials containing 10 mL MEB/100 µL *O. pini* stock culture with monoterpenes at a ratio of 5% by volume. Monoterpene concentration can be 0.5–30% of phloem mass (Klepzig et al. 1995, Raffa and Smalley 1995) and 20–40% in resin (Klepzig et al. 1995, Hofstetter et al. 2005), and test chemical concentrations of 5% have been used in studies of similar ecological systems (Wallin and Raffa 2000, Eckhardt et al. 2009). Controls consisted of 100 µL *O. pini* stock culture/10 mL MEB with no monoterpenes added. Each replicate was incubated 30 h in the dark on a platform shaker (70 rpm) at 25 C. Because monoterpenes are insoluble in MEB continuous agitation of growth media ensured that monoterpenes were distributed homogenously in growth media. Relative biomass was determined by dividing the biomass for each individual replicate treated with monoterpenes by the mean control biomass for each isolate. Monoterpenes used in the experiment are listed below and were obtained from these commercial sources: ACROS Organics (New Jersey): (1R)-(+) α-pinene (98%), (1S)-(−) β-pinene (98%), stabilized Δ-3-carene (90%), stabilized myrcene (90%), (+) limonene (97%); and Fluka (Japan): terpinolene (> 85%).

The effect of O. pini *on phloem chemistry.*—*Ogataea pini* was grown ex vivo on phloem collected from *P. ponderosa* to determine whether the presence of *O. pini* in phloem tissue affects phytochemical (monoterpene) composition over time (Klepzig et al. 1995). Phloem tissue was collected by felling a healthy ponderosa pine in the same stand from which *D. brevicomis* were obtained, scraping off the bark with a draw blade and peeling the phloem from the tree with sterile filet knives and placing it in sterile airtight bags. Phloem tissue was collected from the same position along the bole of a single tree. Phloem tissue was taken to the lab and punched into 2.5 cm diam disks with a sterilized No. 16 punch (C.S. Osborne, Texas). The phloem was approximately 3 mm thick. Phloem disks were inoculated directly with 10 µL *O. pini* cell culture and placed on water agar in 60 × 15 mm Petri dishes (Fisherbrand) with the side of the disk containing cell culture inverted so as to be facing downward into the water agar. A total of 76 disks were cut, 28 inoculated with *O. pini* and 48 that were not inoculated (control).

Twenty of the inoculum-free disks were analyzed at 0 d. After 7 d half the inoculated phloem disks (n = 14) and half the control disks (n = 14) were removed from the water agar and cut into 0.5 × 0.5 mm squares with a sterile razorblade under laminar flow (Klepzig et al. 1995, Raffa and Smalley 1995). An extract of each phloem disk was prepared for gas chromatography by submerging the cut phloem tissues in 10 mL hexane in a 20 mL glass vial. Each vial was allowed to incubate at room temperature 24 h. This was repeated with the remaining replicates at 15 d. Vials were frozen at −20 C until analyzed by gas chromatography.

Gas chromatography conditions.—One gram of each extract was dissolved in hexane (GC grade, > 99.0%) to a total volume of 2.0 mL. Subsequently 50 μL dissolved extract again was diluted to 5 mL with GC grade hexane and 50 μL 9480 mg/L fenchone, the internal standard, was added. The same concentration of fenchone also was added to the standards. Six calibration standards, each with increasing concentrations of monoterpenes (α-pinene, β-pinene, myrcene, carene, limonene and terpinolene), were prepared along with two check standards. All standards were created from a stock solution that contained highly pure monoterpenes dissolved in GC-grade hexane. All samples and standards were transferred to 2 mL vials with ivory PTFE/red rubber septa.

A Hewlett-Packard 5890 Series II Gas Chromatograph (GC) with a 6890 series auto-sampler injector and a 100-vial tray was used for the analysis of the extracts. The system uses on-column injection and a flame ionization detector (FID). A J&W Scientific capillary GC column was used. It is a DB-1 column, 30 m long, with 0.25 mm internal diameter and film 0.25 μm thick. The column was heated to 300 C for 1 h before the start of analysis to remove residues. These parameters were used: Helium flow through column: 20 mL/min, Hydrogen for FID: 30 ml/min, compressed air for FID: 370 mL/min, and nitrogen (FID auxiliary gas): 10 mL/min. The injector was kept at 275 C and the detector at 300 C for the entire run.

SRI Instruments peak simple model 202 data system and software was used to acquire the FID signal from the GC and draw chromatograms. Monoterpenes were identified by retention time. This software identifies each peak within a defined window of expected retention time. Calibration curves were created for each monoterpene by plotting the corrected peak area (area of the monoterpene peak divided by the area of the fenchone peak for that sample) versus the known monoterpene concentration for each calibration standard. This calibration curve was used to determine the monoterpene concentration in each diluted sample. A duplicate and a check standard were analyzed every 10 samples, and all results were found to be repeatable. The original mass of the sample extract and dilution factor were accounted for so that results are reported as milligrams of each monoterpene per milliliter extract.

Statistical analyses.—All statistical analyses were performed with the software JMP 8.0 (SAS Institute), and all tests incorporate a type I error rate of $\alpha = 0.05$. *Ogataea pini* growth rates were log-transformed to conform to assumptions of normality before hypothesis testing. The growth curve for *O. pini* was established by fitting a logarithmic growth function to the transformed response of *O. pini* biomass to time (h).

The effect of host monoterpenes on *O. pini* isolates was tested with one-way ANOVA with monoterpene treatment (n = 6) as a fixed effect on the response of mean *O. pini* biomass (mg) after 30 h. A Tukey HSD test was used to separate means. To analyze variation in monoterpene concentrations between phloem colonized by *O. pini* and uncolonized phloem a nested one-way ANOVA was used with *O. pini* presence as the treatment, nested by period (7 d, 15 d) on the response of mean concentration (mg/mL) of the six monoterpenes. We did not include the results from the 20 disks that were analyzed at 0 d in the statistical analysis because tissues analyzed at 0 d were not inoculated with *O. pini*. However the monoterpene concentrations from disks at 0 d are included in the display of results for baseline comparison because monoterpene concentrations decline over time.

RESULTS

Effect of monoterpenes on O. pini *growth.*—From our preliminary biomass growth assays *O. pini* achieved maximum biomass in 10 mL MEB at 30 h after inoculation into MEB at a dilution factor of 10^{-2}, and this relationship followed a logarithmic growth function (FIG. 1). As a result *O. pini* was incubated 30 h during experiments testing the growth response of *O. pini* to host monoterpenes.

Amendment of MEB with host monoterpenes significantly affected biomass growth of 15 isolates of *O. pini* after 30 h (whole model: $F_{5,249} = 23.39$, $P < 0.0001$). Four of the six monoterpenes inhibited *O. pini* growth relative to control (no monoterpenes): media amended with (1R)—(+) α-pinene reduced *O. pini* biomass growth by 53.6% on average; (1S)—(−) β-pinene reduced *O. pini* biomass growth by 54.4% on average; Δ-3-carene reduced *O. pini* biomass growth by 37.7% on average; and (+) limonene inhibited growth of *O. pini* by 41.9% on average. In contrast amendments with myrcene and terpinolene stimulated *O. pini* biomass growth by 21.5% and 75.5% relative to control respectively (FIG. 2).

The effect of O. pini *on phloem chemistry.*—Monoterpene concentrations declined rapidly in phloem tissues over time; however tissues inoculated with *O. pini* had significantly different concentrations of some monoterpenes after both 7 d and 15 d. The presence of *O. pini* in phloem tissues significantly affected the concentration of two monoterpenes after 7 d and only one monoterpene at 15 d (refer to TABLE I for summary of ANOVA results). After 7 d concentration of the monoterpene Δ-3-carene was on

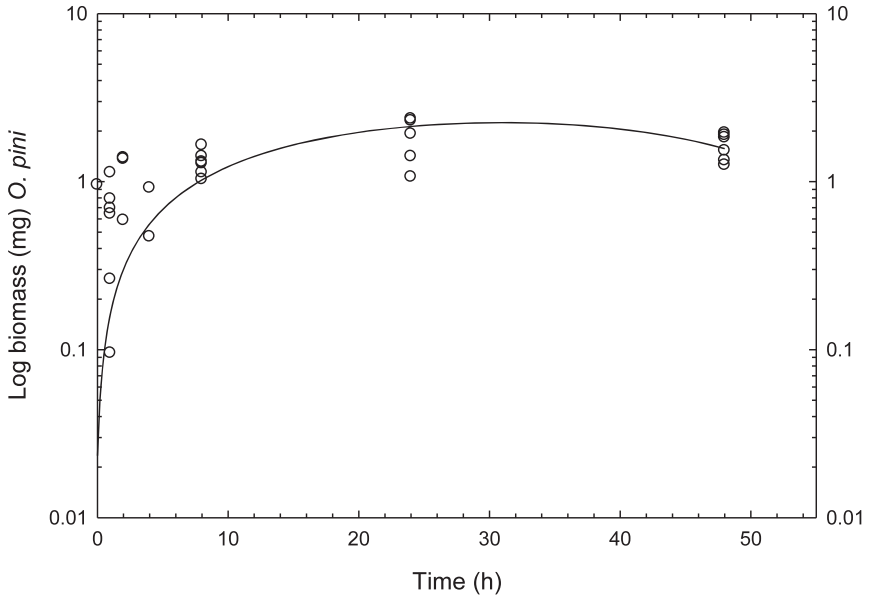

FIG. 1. Biomass increase of *O. pini* in 10 mL MEB (malt extract broth) over time. Maximum biomass (log-transformed) was achieved at 30 h after inoculation at a dilution factor of 10^{-2}, and the relationship significantly follows a logarithmic growth function ($F_{2,31} = 15.64$, $P < 0.0001$, $r^2 = 0.85$). Equation: Log (Biomass) = 0.478 + 0.078 × Time (h) −0.002 *(Time [h] −14.00)^2.

average 42.9% lower in phloem that was colonized by *O. pini* than phloem that was uncolonized and concentration of the monoterpene terpinolene was 345.0% higher in phloem colonized by *O. pini*. After 15 d only concentrations of the monoterpene α-pinene varied between colonized and uncolonized phloem and α-pinene was 505.4% higher in colonized phloem than uncolonized phloem.

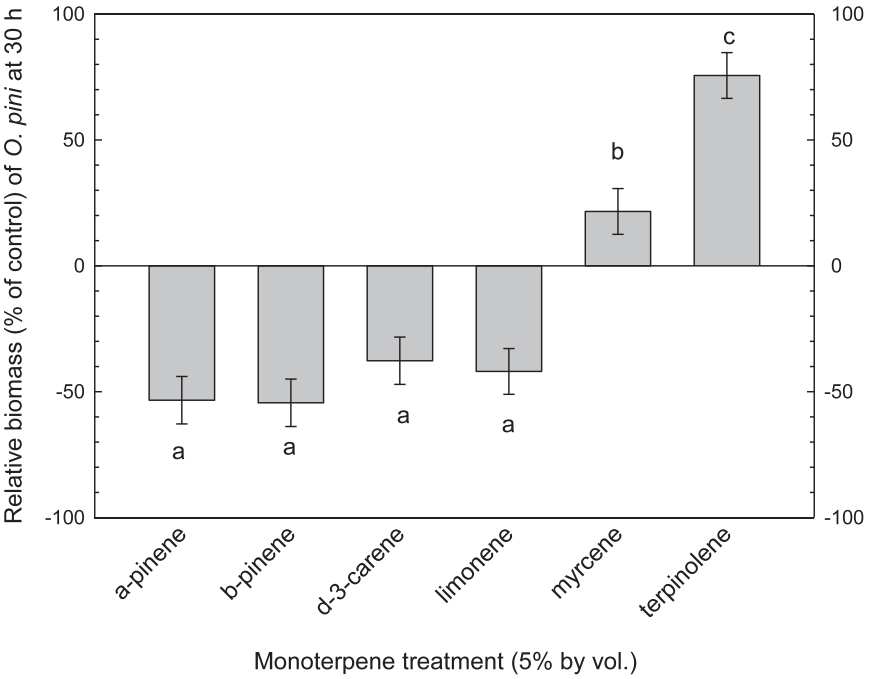

FIG. 2. The effects of six monoterpene amendments (5% by volume) to MEB on the mean biomass of *O. pini* at 30 h relative to mean control biomass of *O. pini* on MEB without monoterpenes (middle axis). The monoterpenes α-pinene, β-pinene, Δ-3-carene, and limonene inhibited *O. pini* biomass growth relative to control, but amendments with myrcene and terpinolene stimulated an increase in *O. pini* biomass. Bars represent one standard error.

TABLE I. Results of ANOVA testing the effect of *O. pini* (treatment) on monoterpenes present in the phloem of ponderosa pine at two time periods. Values are mean monoterpene concentrations (mg/ml) ± SE. Significant differences in monoterpene concentrations between treatment and control, within each period (7 d, 15 d), are highlited in boldface. The phloem tissues from 0 d were not inoculated with *O. pini* and so were not statistically analyzed for treatment effects

	Time								
		7 d				15 d			
Monoterpene	0 d	*O. pini*	Control	F	df	*O. pini*	Control	F	df
α-pinene	46.30 (± 4.55)	18.66 (± 3.87)	7.90 (± 5.19)	2.76	1, 26	**2.78 (± 0.51)**	**0.55 (± 0.72)**	6.31[a]	1, 25
β-pinene	1.05 (± 0.75)	0.05 (± 0.04)	1.04^{-17} (± 0.06)	0.54	1, 26	0.00	0.00	0.00	1, 25
Myrcene	10.80 (± 1.54)	1.00 (± 0.29)	0.80 (± 0.40)	0.16	1, 26	0.22 (± 0.10)	1.52^{-16} (± 0.15)	1.44	1, 25
Δ-3-carene	4.30 (± 0.19)	**1.33 (± 0.43)**	**3.10 (± 0.57)**	6.00[b]	1, 26	2.05 (± 0.23)	2.00 (± 0.16)	0.03	1, 25
Limonene	49.15 (± 6.63)	9.83 (± 2.05)	9.50 (± 2.76)	0.00	1, 26	2.94 (± 0.84)	0.88 (± 1.19)	1.96	1, 25
Terpinolene	0.35 (± 0.10)	**0.38 (± 0.11)**	**0.00 (± 0.15)**	4.02[b]	1, 26	0.00	0.00	0.00	1, 25

[a] $P < 0.01$.
[b] $P < 0.05$.

DISCUSSION

Effect of monoterpenes on O. pini *growth.*—Our in vitro experiments support the hypothesis that phytochemicals present in vascular tissues of ponderosa pine mediate the growth of *O. pini*. Also these experiments are the first to quantify biomass growth rate in any bark-beetle associated yeast (FIG. 1). We demonstrate here that over a fixed period the biomass of *O. pini* varies depending on which monoterpenes are present (FIG. 2). Although the majority of monoterpenes examined here (e.g. α-pinene, β-pinene, Δ-3-carene and limonene) inhibited the growth of *O. pini*, exposure to myrcene and terpinolene stimulated an increase in *O. pini* biomass growth compared to *O. pini* that was incubated in pure growth media. These experiments show that several ubiquitous monoterpenes can significantly affect *O. pini* growth and that these effects can vary.

Ponderosa pines exhibit substantial polymorphism in monoterpene composition (Sturgeon 1979; Latta et al. 2000, 2003). At least four chemically distinct and geographically interspersed chemotypes exist in northern Arizona, where all *O. pini* isolates used in the present study were collected (Latta et al. 2000, 2003; Davis and Hofstetter unpubl data). Our experiments suggest that the variation in monoterpene composition between individual pine trees stands to substantially affect growth patterns of *O. pini*. Given that *O. pini* and other yeasts are known to affect the growth of filamentous fungal symbionts associated with *D. brevicomis* (Adams et al. 2008, Davis et al. 2010), the effect of host monoterpenes on *O. pini* may have indirect consequences for interactions among the fungal species assemblage vectored by the beetle.

The effect of O. pini *on phloem chemistry.*—Our ex vivo assays support the hypothesis that the presence of *O. pini* affects the concentration of phytochemicals present in the vascular tissues of ponderosa pine. We noted a rapid decrease in monoterpene concentrations of phloem disks over time; however inoculation with *O. pini* significantly affected the concentration of several monoterpenes at two periods. After 7 d concentration of the monoterpene Δ-3-carene was reduced by 42.9%, compared with phloem not colonized by *O. pini*, and the concentration of terpinolene was 345.0% higher on average in phloem colonized by *O. pini*. This result was unexpected because *O. pini* was found to produce Δ-3-carene in headspace (Davis et al. 2010). After 15 d concentration of the monoterpene α-pinene was 505.4% higher in phloem colonized by *O. pini*. No other monoterpene concentrations were affected by the presence of *O. pini*. Future studies addressing carbon assimilation by *O. pini* would be beneficial for linking a physiological mechanism to these effects.

Our studies show that the presence of *O. pini* in the phloem tissues of *P. ponderosa* affects concentrations of monoterpenes that are known to have a range of effects on nutritional filamentous fungi associated with *Dendroctonus* bark beetles (Paine and Hanlon 1994, Hofstetter et al. 2005). The presence of *O. pini* in phloem tissues might act to indirectly inhibit or enhance growth of filamentous fungi as a result of chemical changes to the phloem resource. The effect of *O. pini* on phloem chemistry was temporally variable so growth of nutritional filamentous fungi in phloem may shift over time relative to the chemical effect that *O. pini* has on phloem tissues. A change in monoterpene concentrations in host trees also could affect the colonization patterns of conspecific and

heterospecific beetles in a host tree (Hunt and Borden 1990, Byers 1995, Paine and Raffa 1997).

In other ecological systems colonization of plant tissues by insect-vectored yeast species has been shown to reduce the toxicity of compounds that are damaging to the insect vector. For example in the cactophilic *Drosophila*-yeast associations colonization of necrotic cactus tissues by yeasts was shown to reduce concentrations of 2-propanol and acetone, compounds that are otherwise toxic to *Drosophila* (Starmer and Aberdeen 1990). Also the colonization of necrotic cactus tissues by yeasts was found to concentrate proteins and novel sterols that contribute to the nutrition of fly larvae (Heed and Kircher 1965). These studies support the notion that insect-vectored yeasts might alter the chemical conditions of host plants in a way that benefits both the insect and the yeast. Symbiotic associations between insects and yeasts have ecologically significant effects, especially with tree-killing bark beetle species that are capable of drastically altering ecosystem properties when beetle populations reach high densities. Currently research investigating patterns of reciprocal interaction between plant chemistry and insect-vectored yeast species is scarce, aside from several seminal studies (e.g. Heed and Kircher 1965, Hunt and Borden 1990, Starmer and Aberdeen 1990), although insect-yeast symbioses appear to represent ecologically significant interactions.

The experiments reported here demonstrate two important patterns: (i) the bark beetle-associated yeast *O. pini* is likely responsive to variation in the monoterpene composition of host plants (pines) and (ii) the colonization of pine vascular tissues by *O. pini* appears to influence the concentration of three monoterpenes over time. The effects of shifting monoterpene concentrations may influence the suitability of pine vascular tissue as a habitat for the growth of nutritional filamentous fungi that mycophagous beetle larvae feed on. This pattern might have important ecological consequences for bark beetle populations. Further research regarding the effects of *O. pini* in the *D. brevicomis* symbiont assemblage is needed to complement our current understanding of the factors that mediate these ecologically important species complexes.

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