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ORIGINAL ARTICLE

Invasive bark beetle-associated microbes degrade a host defensive monoterpene

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Abstract Conifers respond to herbivore attack with defensive chemicals, which are toxic to both insects and their associated microorganisms. Microorganisms associated with insects have been widely reported to metabolize toxic chemicals, which may help both microorganisms and host insects overcome host conifer defense. *Dendroctonus valens* LeConte, an introduced exotic pest from North America to China, has killed millions of healthy pines. Alpha-pinene is the most abundant defensive monoterpene in Chinese *Pinus tabuliformis*. Although microorganisms associated with *D. valens* have already been investigated, little is known about their bioactivities when encountering host defensive monoterpenes. In this study, we evaluated the influences of different concentrations of α-pinene to *D. valens* and the three most frequently isolated yeasts and bacteria of *D. valens*, and further assayed microorganisms' capabilities to degrade α-pinene. Results showed that the gallery lengths and body weight changes of bark beetles were significantly affected by 6 mg/mL and 12 mg/mL of α-pinene applied in media compared to controls. The tolerance of experimental microorganisms to α-pinene varied depending on the microbial species. Two out of three yeast strains and all three bacterial strains degraded 20%–50% of α-pinene compared to controls in 24 h *in vitro*. The microorganisms capable of α-pinene degradation *in vitro* and their tolerance to high levels of α-pinene suggested that *D. valens*-associated microorganisms may help both microorganisms and the bark beetle overcome host α-pinene defense.

Key words α-pinene; associated microorganisms; *Dendroctonus valens*

Introduction

Many plants could rapidly respond to herbivore attack with defensive chemicals, which is a significant barrier for both insect herbivores and their associated microorganisms to successfully colonize plants (Raffa & Smalley, 1995; Kessler & Baldwin, 2002; Mithöfer & Boland, 2012). Insects harbor a large array of microbes, many of them have been reported to be involved in detoxification of defensive chemicals, which may compromise a plant's responses and be beneficial to both microorganisms and their host insects (Engel & Moran, 2013; Hansen & Moran, 2014; Mason *et al.*, 2014).

The attacking behavior of bark beetles (Coleoptera: Curculionidae: Scolytinae) causes host pines to produce significantly elevated levels of defensive chemicals, including monoterpenes (Miller *et al.*, 1986; Leufvén & Birgersson, 1987), which are toxic and even lead to the death of bark beetles (Smith, 1963; Byers, 1981; Phillips & Croteau, 1999; Seybold *et al.*, 2006). Bark beetles are associated with a large range of microorganisms, which have been shown to be affected by host defensive chemicals (Klepzig *et al.*, 1996; Hofstetter *et al.*, 2005; Adams *et al.*, 2011), and in turn some

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microorganisms can assist bark beetle-microorganism complexes to overcome host pine defense by reducing toxic chemicals (Boone *et al.*, 2013; Raffa, 2014; Cheng *et al.*, 2015). For example, *Dendroctonus ponderosae*-associated microbes have been shown to help the complexes reduce host defensive monoterpenes by culture-dependent and culture-independent techniques (Adams *et al.*, 2013; Boone *et al.*, 2013; Raffa, 2014).

The red turpentine beetle, *Dendroctonus valens* LeConte (Scolytinae), a secondary pest in its native North America, has caused mortality of more than ten million healthy pines in central areas of Northern China after it first appeared in Shanxi Province in 1999 (Sun *et al.*, 2013). α-pinene is one of the most abundant compounds in host conifers' volatile monoterpenes in China (Chen *et al.*, 2006; Xu *et al.*, 2014), and the chemical would induce an increase in the numbers of lysosomes and mitochondria in midgut cells, which suggests α-pinene is toxic to *D. valens* (Lípez *et al.*, 2011). Associated yeasts and bacteria of *D. valens* have already been investigated (Lou *et al.*, 2014; Xu *et al.*, 2015). Three yeasts (*Candida piceae*, *Cyberlindera americana* and *Candida oregonensis*), and three bacteria (*Serratia* sp., *Pseudomonas* sp. and *Rahnella aquatilis*) are most frequently isolated species associated with *D. valens* (Lou *et al.*, 2014; Xu *et al.*, 2015). Since insect-associated microbes are widely involved in the detoxification of host defensive chemicals, we postulated *D. valens*-associated microorganisms may be involved in the degradation of α-pinene, which benefit both *D. valens* and their associated microorganisms. In this study, we assayed the drilling and feeding behaviors of *D. valens* at different concentrations of α-pinene. We compared the *in vitro* antibiotic activities of the monoterpene to *D. valens*-associated yeast and bacterial strains at three concentrations, then the capabilities of chemical degradation for these microorganisms were measured and compared.

Materials and methods

Insects and microbial strains

Adult beetles were captured during the dispersal phase using traps baited with the standard *D. valens* lure [(+)-α-pinene: (−)-β-pinene: (+)-3-carene = 1 : 1 : 1] in the Tunlanchuan Forestry Station (37°48′N, 111°44′E, average elevation 1400 m), west of Gujiao City, Shanxi province in June 2014. Sexes of bark beetles were distinguished by listening for stimulation produced by males (Xu *et al.*, 2014). Log bolts (≥30 cm) were cut into 0.5 m lengths and three pairs of adult beetles were introduced for each bolt, then bolts were placed in plastic boxes (40 cm diameter, 50 cm height) at room temperature. Beetles were collected as they emerged successfully from infested bolts.

Three yeast strains including Y3 (*Candida piceae* KF142551), Y17 (*Cyberlindera americana* KF142570), Y86 (*Candida oregonensis* KF142582) and three bacterial strains including B326 (*Serratia* sp. KJ781959), B330 (*Pseudomonas* sp. KJ781935) and B35 (*Rahnella aquatilis* KJ781939) were obtained from the Institute of Zoology, Chinese Academy of Sciences, Beijing, China and maintained at 4°C.

Chemicals

(+)-α-pinene (98% purity), (−)-β-pinene (98% purity), (+)-3-carene (97% purity), heptyl acetate (≥98% purity), and dimethyl sulfoxide (99.5% purity), used for all experiments were purchased from Sigma Aldrich (Shanghai, China).

Drilling and feeding behaviors of D. valens *at different concentrations of host defensive compound α-pinene*

To make phloem medium, *Pinus tabuliformis* phloem was freeze-dried, ground and autoclaved to sterilize and remove volatile monoterpenes, as previously described (Wang *et al.*, 2013). Three grams of agar (NewProbe, Beijing, China) was mixed with 90 mL boiling distilled water and 6 g ground phloem (Wang *et al.*, 2013), then α-pinene was added into medium after cooling to about 50°C (final concentration of α-pinene: 0, 6, 12 mg/mL, respectively). About 2.5 mL of phloem medium was then poured into each glass tube (1 mm diameter, 5 mm height) and dried for 12 h.

Adult beetles (45♀, 45♂) were randomly chosen and separated into three groups (each group: 15♀, 15♂) and weighed. After that, they were individually introduced into the media. The beetles in α-pinene-free phloem media were set as a control group and those in 6 mg/mL and 12 mg/mL α-pinene phloem media were set as treatment groups. After 6 h feeding at room temperature, feeble or dead beetles were discarded, and the remaining vigorous beetles were weighed again and the lengths of their galleries were measured. Gallery lengths for those beetles that failed to enter the media were treated as zero.

In vitro *antibiotic activities of α-pinene at three concentrations to* D. valens*-associated yeasts and bacteria*

Yeast strains Y3 (*Ca. piceae*), Y17 (*Cy. americana*), Y86 (*Ca. oregonensis*) and bacterial strains B326

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(*Serratia* sp.), B330 (*Pseudomonas* sp.), B35 (*R. aquatilis*) were used for the study. The bacterial strains were grown in M9 minimal medium (Savithiry *et al.*, 1998), and yeast strains were grown in Liquid Sabouraud Medium (Leufvén *et al.*, 1984). After adjusting culture optical cell densities at 600 nm (OD_{600}) to 0.5, strains were diluted 1 : 100 into 4 mL media containing 150 ng/μL, 450 ng/μL, 600 ng/μL of α-pinene for each strain ($n = 5$). After incubations (12 h for bacteria, 24 h for yeasts) at 30°C, the OD value of each tube was measured at 600 nm. Controls with no α-pinene and equal amounts of dimethyl sulfoxide (DMSO) were completed for each strain using identical conditions.

Degradation of α-pinene by D. valens*-associated yeasts and bacteria*

Yeast strains Y3 (*Ca. piceae*), Y17 (*Cy. americana*), Y86 (*Ca. oregonensis*) and bacterial strains B326 (*Serratia* sp.), B330 (*Pseudomonas* sp.), B35 (*R. aquatilis*) were incubated overnight. A dilution of 1 : 100 of each strain was made when cultures were adjusted to an OD_{600} of 0.5. After incubation (12 h for bacteria, 24 h for yeasts) at 30°C, α-pinene dissolved in DMSO was added to a 4 mL suspension (final α-pinene concentration 500 ng/μL) and shaken for further 24 h. A suspension containing equivalent monoterpene with equal amount of autoclaved yeast or bacterial strains was run as a control in the same manner for each group. All solutions ($n = 5$) were extracted with hexane containing an internal standard (heptyl acetate) and then stored for the chemical analysis.

Chemical analysis

Extracts (2 μL) were injected splitless into a gas chromatography – mass spectrometer (GC-MS: Agilent 6980N GC coupled 5973 mass selective detector) equipped with an HP5-MS capillary column (0.25 mm internal diameter × 60 m; Agilent Technologies, Inc., Palo Alto, CA, USA), and the column temperature was programmed from an initial temperature of 50°C for 1 min, then increased by 5°C/min to 100°C, by 3°C/min to 130°C, and by 20°C to 320°C and held for 2 min. Components of the extracts were identified by comparing retention times and mass spectra with authentic standards and those in the NIST02 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA). Quantification was performed using an internal standard (heptyl acetate) that was added to each sample.

Statistical analysis

Prior to analysis, we tested all variables for normality with the Kolmogorov-Smirnov test and homogeneity of group variances with Levene's test. In comparisons of gallery lengths and body weight changes among different treatments, means of cases were tested using independent-samples *t*-test, one-way analysis of variance (ANOVA) test followed by Bonferroni test (equal variances), or Welch's ANOVA test followed by Dunnett's T3 test (unequal variances). In comparisons of OD_{600} values of each strain among different concentrations, data were analyzed using one-way ANOVA test followed by Bonferroni test (equal variances) or Welch's ANOVA test followed by Dunnett's T3 test (unequal variances). In α-pinene degradation experiments, data were analyzed using independent-samples *t*-test. Differences between two groups were considered as significant when $P < 0.05$. All data were analyzed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA) for Windows, and figures were drawn using Origin 8.5 (Origin Lab Corporation, Northampton, MA, USA).

Results

The influence of α-pinene on gallery lengths and body weight changes of bark beetles are summarized in Figure 1. The boring lengths by female and male *D. valens* in control treatments were 2.04 ± 0.24 cm and 1.65 ± 0.25 cm, respectively, which were significantly higher than gallery lengths in 6 mg/mL and 12 mg/mL of α-pinene medium treatments. No significant differences existed for gallery lengths of both sexes between 6 mg/mL and 12 mg/mL of α-pinene medium (Fig. 1A,B). Changes in body weight of all tested adult beetles were measured (Fig. 1C,D). The body weight changes in control groups for both sexes were significantly larger than beetles in the other two treatments, and no statistical differences existed between 6 mg/mL and 12 mg/mL of α-pinene media (Fig. 1C,D). There were no significant differences in changes of body weight and gallery lengths between male and female at three different treatments (changes of body weight: control, $t = 0.31$, $P > 0.05$; 6 mg/mL α-pinene treatment, $t = 0.79$, $P > 0.05$; 12 mg/mL α-pinene treatment, $t = 0.40$, $P > 0.05$; changes of gallery length: control, $t = 1.13$, $P > 0.05$; 6mg/mL α-pinene treatment, $t = 2.12$, $P > 0.05$; 12 mg/mL α-pinene treatment, $t = 1.57$, $P > 0.05$).

The effects of α-pinene at four concentrations, 0, 150, 450 and 600 ng/μL on the growth of three yeast strains Y3 (*Ca. piceae*), Y17 (*Cy. americana*), Y86 (*Ca. oregonensis*) and three bacterial strains B326 (*Serratia* sp.), B330

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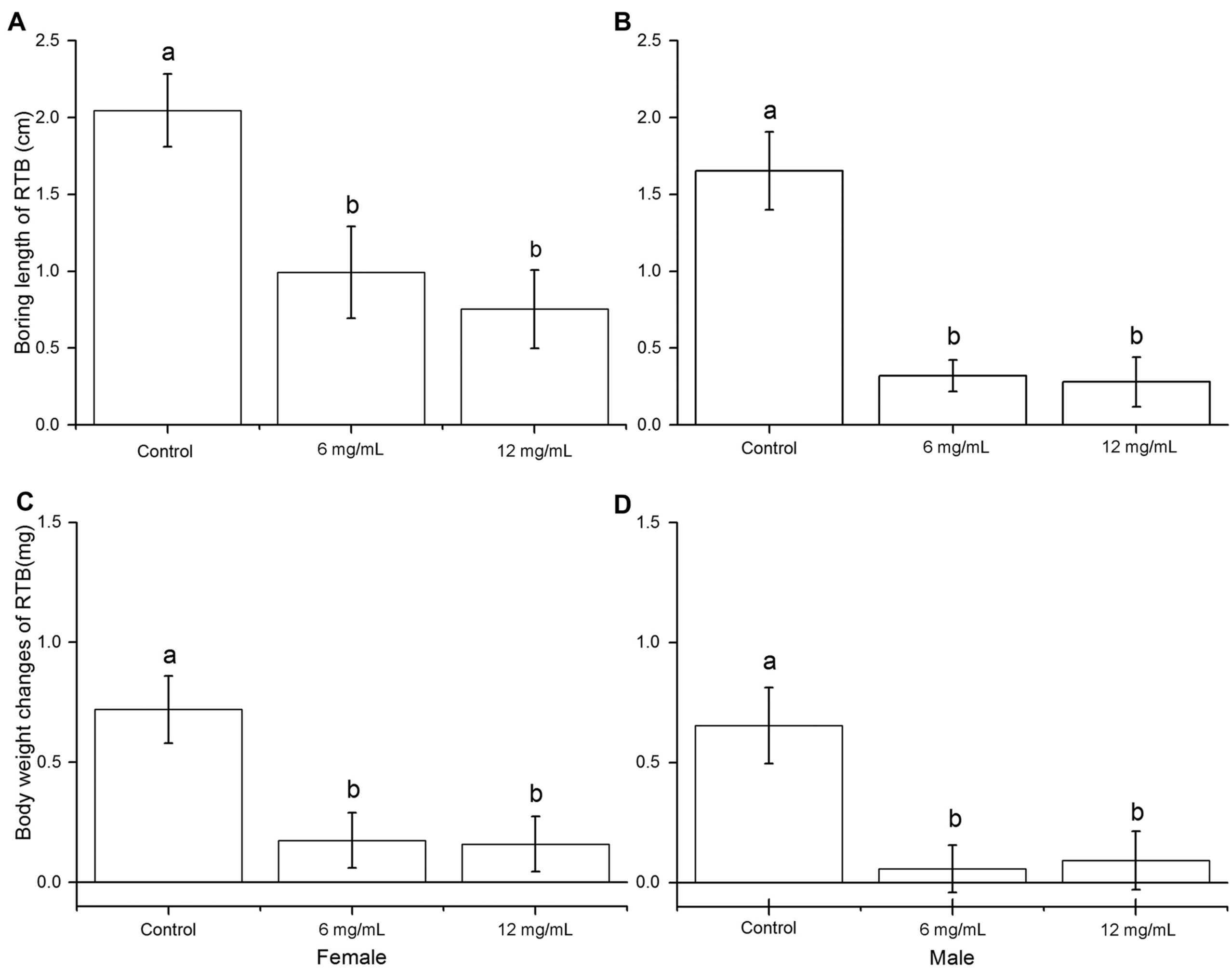

Fig. 1 Gallery lengths and body weight changes of *Dendroctonus valens*, feeding on control, 6 mg/mL and 12 mg/mL concentrations of α-pinene in phloem media. The data were analyzed using one-way analysis of variance (ANOVA) test (gallery lengths of female, $F_{2,39} = 7.31$, $P < 0.01$; body weight changes of female, $F_{2,39} = 6.63$, $P < 0.01$; body weight changes of male, $F_{2,39} = 6.70$, $P < 0.01$) or one-way Welch's ANOVA test (gallery lengths of male, $F_{2,22.9} = 12.22$, $P < 0.001$). *Post hoc* pairwise comparisons were done using Bonferroni test or Dunnett's T3 test, respectively. Labels with different letters are significantly different at $P = 0.05$. (A) The boring length of female beetles; (B) the boring length of male beetles; (C) the body weight changes of female beetles; (D) the body weight changes of male beetles.

(*Pseudomonas* sp.), B35 (*R. aquatilis*) associated with *D. valens* are shown in Figure 2A and B, respectively. In general, α-pinene exerted a significant reduction on the growth of Y17 (*Cy. americana*), Y86 (*Ca. oregonensis*), B35 (*R. aquatilis*) and B330 (*Pseudomonas* sp.), a significant promotion on the growth of B326 (*Serratia* sp.), and a slight promotion on the growth of Y3 (*Ca. piceae*) even if not significantly, at concentrations 150, 450 and 600 ng/μL of α-pinene compared to control (Fig. 2). As for Y86 (*Ca. oregonensis*), B35 (*R. aquatilis*), B326 (*Serratia* sp.) and B330 (*Pseudomonas* sp.), the OD_{600} values had no significant changes among 150, 450 and 600 ng/μL concentrations of α-pinene (Fig. 2). The growth of Y17 (*Cy. americana*) at 450 ng/μL and 600 ng/μL were significantly reduced compared to those in 150 ng/μL of α-pinene suspension (Fig. 2).

The degraded percentages of α-pinene by *D. valens*-associated yeast strains and bacterial strains, including Y3 (*Ca. piceae*), Y17 (*Cy. americana*), Y86 (*Ca. oregonensis*), B326 (*Serratia* sp.), B330 (*Pseudomonas* sp.) and B35 (*R. aquatilis*) are presented in Figure 3, which showed that two yeast strains (Y3 [*Ca. piceae*], Y17 [*Cy.*

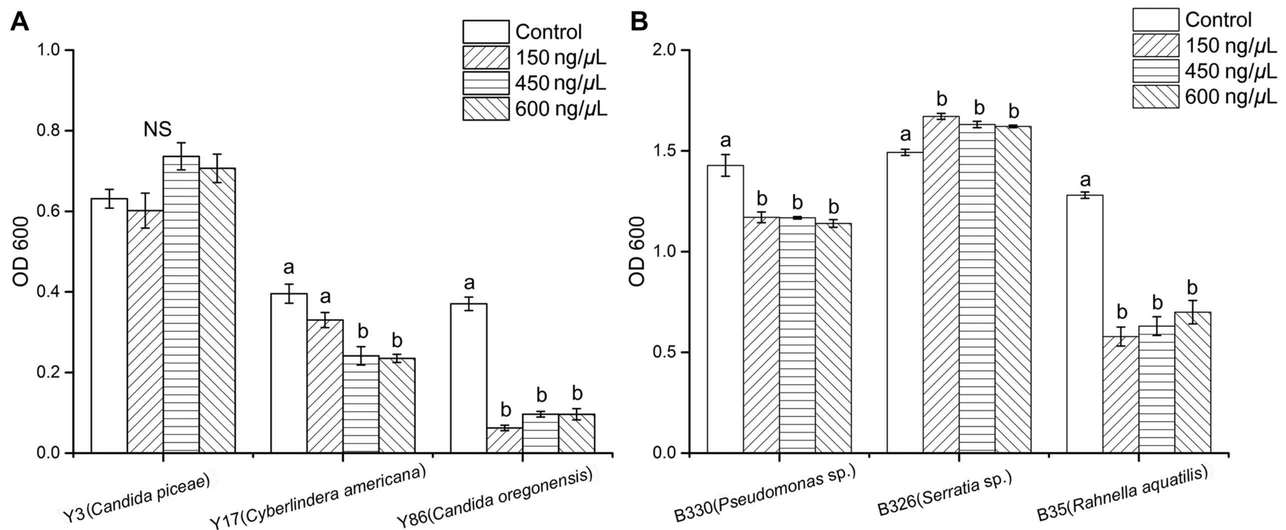

Fig. 2 Average values (± SE) of optical cell densities at 600 nm (OD_{600}) of three yeast strains and three bacterial strains, including Y3 (*Candida piceae*), Y17 (*Cyberlindera americana*), Y86 (*Candida oregonensis*), B326 (*Serratia* sp.), B330 (*Pseudomonas* sp.) and B35 (*Rahnella aquatilis*) at 0, 150, 450 and 600 ng/μL α-pinene concentrations, respectively. The data were analyzed using one-way analysis of variance (ANOVA) test (*Ca. piceae*, $F_{3,16} = 3.31$, $P < 0.05$; *Cy. americana*, $F_{3,16} = 15.33$, $P < 0.001$; *Ca. oregonensis*, $F_{3,16} = 144.81$, $P < 0.001$; *Serratia* sp. $F_{3,16} = 31.57$, $P < 0.001$; *R. aquatilis* $F_{3,16} = 53.26$, $P < 0.001$), or the one-way Welch's ANOVA test (*Pseudomonas* sp., $F_{3,7.28} = 7.17$, $P < 0.05$). *Post hoc* pairwise comparisons were done using Bonferroni test or Dunnett's T3 test, respectively. Labels with different letters are significantly different at $P = 0.05$.

Americana]) and all three bacterial strains (B35 [*R. aquatilis*], B326 [*Serratia* sp.] and B330 [*Pseudomonas* sp.]) significantly reduced α-pinene than control. Y3 (*Ca. piceae*) and B330 (*Pseudomonas* sp.), the most effective yeast strain and bacterial strain, decreased 44.92% ± 8.45% and 50.17% ± 1.29% of α-pinene applied to suspension than control. The amounts of α-pinene in tubes with Y86 (*Ca. oregonensis*) had no significant changes compared to those in tubes with equal amounts of autoclaved strains.

Discussion

We evaluated the influences of α-pinene to *D. valens* by assessing the drilling and feeding behaviors at three different concentrations of α-pinene, and the results suggested that α-pinene has a negative effect on *D. valens*. The results support research in other systems which documented that increased concentrations of α-pinene has a inhibitive effect on feeding behavior of *Ips pini* and *D. ponderosae* (Wallin & Raffa, 2000; Reid & Purcell, 2011), and also in previous studies (Xu *et al.*, 2014). Xu *et al.* (2014) documented that the gallery lengths of adult female *D. valens* were negatively correlated with the α-pinene concentrations, besides which, our results showed that high levels of α-pinene concentrations significantly suppressed the feeding and drilling behaviors of both sexes of *D. valens* compared to control in 6 h (Fig. 1). High monoterpene concentrations present not only a behavioral challenge but a physiological and metabolic challenge for bark beetles. Previous studies suggested that bark beetles need more energy reserves, lipid stores and detoxification enzymes, like P450 monooxygenases to metabolize the monoterpene α-pinene (Sturgeon & Robertson, 1985; Wallin & Raffa, 2000; Lípez *et al.*, 2011; Reid & Purcell, 2011). Considering the negative effects of α-pinene to bark beetle, the tolerance or detoxification of α-pinene is vital for *D. valens* to overcome host defense and then successfully colonize on host pine.

The findings of this study also suggested that α-pinene not only have negative effects on bark beetles, but also influence the growth of *D. valens*-associated microorganisms. In our previous study, we found that the three bacterial species, including *Serratia* sp., *Pseudomonas* sp. and *R. aquatilis* account for more than 69% of bacterial isolates both in *D. valens* gut and frass (Xu *et al.*, 2015); also, the three yeasts *Ca. piceae*, *Cy. americana* and *Ca. oregonensis* account for more than 70% of yeast isolates in *D. valens* surroundings (Lou *et al.*, 2014). Further, the influences of α-pinene to these dominant microorganisms are varied depending on the species. *Cy.*

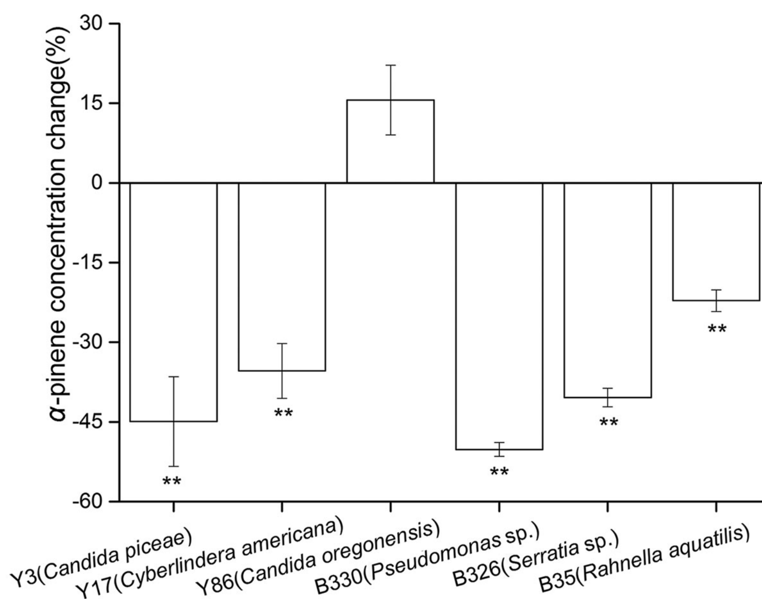

Fig. 3 Percent changes in α-pinene concentrations applied to media relative to controls in presence of three yeast strains and three bacterial strains, including Y3 (*Candida piceae*), Y17 (*Cyberlindera americana*), Y86 (*Candida oregonensis*), B326 (*Serratia* sp.), B330 (*Pseudomonas* sp.) and B35 (*Rahnella aquatilis*) associated with *Dendroctonus valens*. The data were analyzed using independent *t*-test (*Ca. piceae*, $t = -6.74$, $P < 0.01$; *Cy. americana*, $t = -5.77$, $P < 0.01$; *Ca. oregonensis*, $t = 1.98$, $P = 0.08$; *Serratia* sp. $t = -15.83$, $P < 0.001$, *Pseudomonas* sp. $t = -22.24$, $P < 0.001$, *R. aquatilis*, $t = -8.03$, $P < 0.001$). Asterisks indicate a statistically significant difference ($P < 0.01$) between media with strains and equal amounts of inactivated strains.

Americana, *Ca. oregonensis*, *R. aquatilis* and *Pseudomonas* sp. were significantly suppressed by α-pinene at three concentrations compared to controls, while α-pinene promoted the growth of *Ca. piceae* and *Serratia* sp. (Fig. 2). Plant defense chemicals have been shown to possibly influence herbivore gut microbiota, which in turn influence host utilization (Mason *et al.*, 2015). Future studies should focus on how associated microorganisms of *D. valens* are influenced dynamically by host defensive monoterpenes.

Our results demonstrated that yeasts and bacteria frequently associated with *D. valens* degraded conifer defense chemical α-pinene *in vitro*. *Pseudomonas* spp. and *R. aquatilis* associated with *D. ponderosae* are capable of α-pinene degradation (Mason *et al.*, 2014). *Pseudomonas* sp., *Serratia* sp. and *Candida piceae*, the most frequently isolated microorganisms in *D. valens* surroundings (Lou *et al.*, 2014; Xu *et al.*, 2015), could reduce more than 40% of α-pinene-amended media within 24 h compared to controls. The metabolization of α-pinene by yeasts and bacteria has also been reported in the utility of terpene for commercial bioprocessing (Wright *et al.*, 1986; Yoo *et al.*, 2001; Rottava *et al.*, 2010). The α-pinene degradation by microorganisms may benefit microbes sensitive to α-pinene, such as *Ca. oregonensis* and *R. aquatilis* (Fig. 2), both of which are capable of verbenone pheromone production (Xu *et al.*, unpublished data; Xu *et al.*, 2015) and the bacteria is involved in nitrogen fixation for bark beetles (Morales-Jimenez *et al.*, 2009).

The degradation of α-pinene by microorganisms may have an adaptive benefit to *D. valens* as the monoterpene is a toxic and highly lipophilic compound that must be transformed before excretion (Lípez *et al.*, 2011). Yeasts and bacteria capable of α-pinene degradation account for more than 50% and 70% of the isolates from *D. valens*, respectively (Lou *et al.*, 2014; Xu *et al.*, 2015), further suggesting that associated microorganisms might assist *D. valens* in host monoterpene detoxification. Since all degradation experiments were conducted *in vitro*, here, we did not supply enough direct evidence to assess the importance of the microbial contribution to overcome host pine defense *in vivo*. Experiments on the removal of microorganisms from bark beetles by antibiotic treatments or axenically reared bark beetles may help to ascertain the tolerances and detoxification capabilities of bark beetles that are relatively free of microbial symbionts, and also prove the contribution of microorganisms to tolerances and detoxification capabilities.

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Disclosure

The authors declare that they have no conflict of interest.

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