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ORIGINAL ARTICLE

Inducible pine rosin defense mediates interactions between an invasive insect–fungal complex and newly acquired sympatric fungal associates

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Abstract

Mutualism between insects and fungi drives insect evolutionary diversification and niche expansion; for invasive insects, however, mechanisms by which they maintain mutualistic relationships with beneficial fungi have not been clearly explored. Here, we report that an invasive herbivorous insect, the red turpentine beetle (RTB), with its co-invasive mutualistic fungus, *Leptographium procerum*, has newly acquired a set of sympatric fungi during invasion, which could potentially outcompete the RTB mutualistic fungus. Host pine *Pinus tabuliformis* exhibited more rosin-based responses to the sympatric fungi than to RTB mutualistic fungus and, in return, the rapidly induced rosin suppressed the sympatric fungi more significantly than *L. procerum*. In addition, from direct fungal pairing competitions, we found that the antagonistic effects of sympatric fungi on *L. procerum* were drastically reduced under induced rosin defense. Our results together with previous findings imply that pine oleoresin defense (turpentine and rosin) might have been exploited by the invasive mutualistic fungus *L. procerum*, which helps to explain its invasion success and, by extension, its mutualistic partner RTB in China.

Key words: bark beetle–ophiostomatoid fungi–conifer interactions, diterpene resin acid, invasive species, mutualism, pine defense

INTRODUCTION

The mutualistic interaction between insects and fungi is one of the oldest and most successful relationships in animal–microbe symbiosis (Graham 1967; Mueller *et al*. 2005; Gibson & Hunter 2010). Fungal–insect mutualisms drive insects' rapid adaptation and worldwide-range distribution, by virtue of which insect vectors cultivate fungal cultures for nutrition (Mueller *et al*. 1998; Ayres *et al*. 2000), benefit from fungal ectosym-

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bionts for protection against pathogen infections (Konrad *et al*. 2015), or display expanded niches (host plant resources use) and lineage diversification (Joy 2013). Owing to the importance of mutualistic fungi, multiple strategies have evolved for insect vectors to keep the symbiotic interactions safe from competitive microbial "weeds" and parasites, including the behavior of tending to their fungal mutualists (Bass & Cherrett 1994) or application of antibiotics secreted from "auxiliary fungi" in the genus *Penicillium* (Wang *et al*. 2015). In categories of herbivorous insect vectors that attack or live in healthy plants, impacts of opportunistic competitive microbes on their fungal mutualists and mechanisms by which mutualists enhance their fitness have rarely been considered in the context of chemical mediations from the plant partner. Although complicated, such research is indispensable to elucidate the constancy of insect–fungal mutualism.

For aggressive bark beetles that attack healthy trees, their colonization successes in large part depend on behavioral and physiological regulations from symbiotic ophiostomatoid fungi (Paine *et al*. 1997; Hofstetter *et al*. 2006), among which some establish relatively stable mutualistic relationships with host vectors as primary symbionts, often transmitted between trees through beetle exoskeleton or even specialized body structure "mycangium" (Six & Klepzig 2004). The invasive bark beetle, the red turpentine beetle (RTB), *Dendroctonus valens* LeConte (Coleoptera: Scolytidae), was introduced from North America to China in the 1980s and has killed over 10 million *Pinus tabuliformis* since 1999 (Yan *et al*. 2005). The fungus *Leptographium procerum*, which is the most consistently associated with RTB in invaded regions (M. Lu *et al*. 2009), has been evidenced to have been introduced into China with RTB (Lu *et al*. 2011). This invasive fungus appears to form a mutualistic relationship with RTB vector due to its induction of attractant 3-carene for adult RTB recruiting to host pine *P. tabuliformis* and involvement in retention of nutrition for RTB larvae (Lu *et al*. 2010; Zhou *et al*. 2015, unpublished data). This insect–fungal mutualistic complex has also picked up an assemblage of fungal species found in association at relatively lower frequency, the majority of which might be newly acquired during the invasion process and have not been reported in association with RTB in North America, such as *Leptographium sinoprocerum*, *Leptographium truncatum* and *Hyalorhinocladiella pinicola* (M. Lu *et al*. 2009; Q. Lu *et al*. 2009; Sun *et al*. 2013; Taerum *et al*. 2013; Table 1). For maintaining its constant symbiosis with RTB vector, mechanisms whereby the invasive mutualist *L. procerum* enhances its fitness against competition from these sympatric fungi should be investigated, given the close relatedness in phylogeny between *L. procerum* and these sympatric fungi suggesting that intensive competition could occur in the same niches.

Bark beetle associated ophiostomatoid fungi are pathogenic to conifers; although these fungi do not play direct roles in tree killing, they initiate variable degrees of induced defense responses (Lieutier *et al*. 2009; Six & Wingfield 2011). Conifer oleoresin defense consists of two fractions, turpentine (monoterpene and sesquiterpene) and rosin (diterpene resin acid) (Phillips & Croteau 1999). The volatile turpentine fraction has been well studied and reported as being exploited as beetle chemical cues (Wood 1982; McLeod *et al*. 2005) or discouraging beetle and fungal attacks (Raffa & Smalley 1995); however, the higher molecular weight diterpene resin acids are relatively less explored and are suggested to mainly inhibit fungal associates with no obvious effects on bark beetle (Kopper *et al*. 2005). Of the oleoresin in Chinese pine, *P. tabuliformis*, rosin (within which abietic, neoabietic, palustric, levopimaric, and dehydroabietic acids altogether are grouped as abietane and account for 73% of all rosin) and turpentine make up 53.9% and 46.1% of oleoresin content, respectively (Song *et al*. 1993). The non-volatile diterpene resin acids can be retained in oleoresin around the sites of injury after volatile turpentine evaporates (Gijzen *et al*. 1993), suggesting their longer-term influences on infecting organisms. Therefore, induced rosin defense of *P. tabuliformis* should be essential for the colonization success of the invasive mutualist *L. procerum*, and its competitiveness with other sympatric fungi.

Based on the above considerations, we set out experimental studies to investigate whether host pine *P. tabuliformis* differentially responds to the RTB invasive mutualist *L. procerum* and the newly acquired sympatric fungi, whether *L. procerum* and its sympatric fungi have differences in tolerance of the induced defensive component, and whether antagonisms from sympatric fungi on *L. procerum* are dramatically attenuated under elevated chemical defense.

MATERIALS AND METHODS

Pine seedlings, fungal isolates and inoculations

For the present study, 4–5-year-old *P. tabuliformis* seedlings (diameter: mean ± SE = 10.03 ± 0.13 mm;

measured at 2 cm above the soil line) were grown in plastic pots (diameter: 12 cm) in open-air conditions. Seedlings were transferred to a glasshouse (air temperature: 25 °C; relative humidity: 60%; 12 h photoperiod) for at least 1 month before the inoculation experiments. Fungal species used in this study were originally isolated from body surfaces and galleries of *D. valens* in its invaded regions in China (Table 1); cultures were collected in the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa and the Belgian Coordinated Collections of Microorganisms, Belgium (M. Lu *et al.* 2009; Q. Lu *et al.* 2009).

To avoid excessive mechanical damage, we did not apply multi-point inoculations on seedlings but rather chose to use single-point inoculation that was conducted by making a wound on each *P. tabuliformis* seedling with a sterile 5-mm-diameter cork borer on the main stem at 2 cm above the soil line (Lu *et al.* 2010). A 5-mm-diameter plug was taken from the margin of one actively growing fungal species cultured on 2% MEA (malt extract: 7 g; agar: 7 g; distilled water: 350 mL), placed into the hole to contact the cambium layer and wrapped by laboratory Parafilm (Pechiney Plastic Packaging, USA) to prevent contamination. We set seedlings inoculated with 2% MEA alone (mechanical wound) as controls. At the end of the inoculation experiments, fungal isolates were re-isolated from the inoculation areas to confirm that there had been no cross-contamination (Eckhardt *et al*. 2004).

Inoculation of main fungal associates of Chinese red turpentine beetle and identification of inducible rosin components in *Pinus tabuliformis* seedlings, after 12 days (Experiment 1)

We evaluated the inducibility of pine diterpene resin acids by fungal species that are mainly associated with RTB in China. Three seedlings per treatment were inoc-

Table 1 Fungal species associated with *Dendroctonus valens* in China and the representative isolates used in the experiments

Fungal species[†]	Type[‡]	Position where isolated[§]	Frequency (%)[§]	Isolate number[¶]
Leptographium procerum	North American/ Chinese RTB-associated; Invasive and mutualistic	Adult body surface and adult/larval gallery of RTB in Shanxi, Henan, and Shaanxi Provinces	47.4	CMW25626
Leptographium sinoprocerum		Adult/larval gallery of RTB in Shanxi and Hebei Provinces	10.5	MUCL46352
Leptographium truncatum		Adult/larval gallery of RTB in Shanxi Province	6.2	CMW25684
Leptographium pini-densiflorae	Chinese RTB-associated; Newly acquired in invaded regions	Adult body surface/gallery and larval gallery of RTB in Shanxi and Shaanxi Provinces	6.2	CMW25600
Ophiostoma minus (European variety)		Adult body surface and larval gallery of RTB in Shanxi Province	2.9	CMW26254
Ophiostoma rectangulosporium-like		Adult gallery of RTB in Shanxi Province	1.0	CMW26258
Hyalorhinocladiella pinicola		Adult body surface of RTB in Shanxi Province	0.7	CMW25613

[†]These fungal species are main associates of *D. valens* in China, which account for approximately 75% of all isolations. [‡]Strong population genetics evidence from Lu *et al*. 2011 showed that *L. procerum* populations associated with Chinese red turpentine beetle (RTB) originate from those with North American RTB. According to studies by M. Lu *et al*. (2009, as well as Table 2 in this reference), Q. Lu *et al*. (2009), Wang *et al*. (2013) and Taerum *et al*. (2013), these 6 fungal species mentioned here have not been observed in association with North American RTB during decades of researches, but have been found in association with RTB in China. "Newly acquired" means RTB has a new association with a fungus after its invasion into China. [§]Position and frequency of these fungal species referred to comprehensive studies by M. Lu *et al*. (2009), Q. Lu *et al*. (2009) and Wang *et al*. (2013), in which 133, 71 and 102 isolates were obtained, respectively. Our calculation of frequency for fungi was based on the overall 306 isolates. [¶]CMW, Cultures of Mike Wingfield, the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; MUCL, a part of the Belgian Coordinated Collections of Microorganisms (BCCM), Belgium.

ulated using the methods described above and after 12 days, trees were uprooted and phloem tissues around the inoculation points (5-mm below and above) were finely excised using a sterile razor blade and immediately frozen in liquid nitrogen. Tissues were homogenized into powder under cold temperature and diterpene resin acids were extracted in 0.5 mL HPLC-grade methanol (J & K Chemical, Beijing, China) twice for 14 h. For each sample, the overall 1 mL extract was filtered through a 0.45-μm PTFE syringe filter, then concentrated to 0.4 mL under ultrapure nitrogen gas, and stored at −20 °C prior to HPLC.

The abietane-type resin acids were separated by a reversed-phase HPLC (Agilent 1260 series, CA, USA) equipped with a photo-diode array detector. An Agilent Zorbax XDB column in tandem with a HC-C18 column was carried out for chromatographic separation. The injection volume was set at 20 μL. The mobile phase, multi-wavelength detection and data processing followed the rapid analysis methods described by Kersten *et al*. (2006), with minor modifications. Briefly, an isocratic ternary solvent system (85%, 5% and 10%: methanol, 5% acetic acid, water, respectively) was run at a flow rate of 1 mL/min; four wavelengths (240, 268, 293 and 300 nm) could optimize quantification of underivatized dehydroabietic, levopimaric, palustric, neoabietic and abietic acids; peak identification of these phytochemicals were conducted on Agilent ChemStation through comparison of retention times and co-injection tests with each of the following standards: dehydroabietic acid (99+%, Orchid cellmark), levopimaric acid (95+%, Orchid cellmark), palustric acid (90–95%, Orchid cellmark), neoabietic acid (98+%, Fluka), and abietic acid (85%, Acros); peak areas were quantified by standard calibration curves of standards and resin acid concentrations were determined on a dry weight of phloem tissue after extraction.

Characterization of rosin components inductions by *L. procerum* and 3 newly acquired sympatric fungi across time (Experiment 2)

We assessed the induction dynamics of diterpene resin acids in 4–5-year-old *P. tabuliformis* seedlings across time by three newly acquired sympatric fungal species (*H. pinicola*, *L. truncatum* and *L. sinoprocerum*) and the invasive mutualist *L. procerum*. Seedlings received single-point inoculation by each of the 4 fungal species following the steps mentioned above. At 3, 6, 9, 12, 18 and 24 days after inoculation, seedlings were uprooted and necrotic tissues around inoculation points were sampled and quickly transferred into liquid nitrogen (5 seedlings per treatment for 18 and 24 days, and 10 seedlings per treatment for each of other time points) and extracted diterpene resin acids were identified and analyzed as in Experiment 1.

Evaluation of growth rates of fungal associates under elevated concentrations of abietic acid, the main component of *P. tabuliformis* rosin (Experiment 3)

Abietic acid is a characteristic component of oleoresin and also one of the most abundant constitutive diterpenes (approximately 20% of all rosin) in mature *P. tabuliformis* trees (Song *et al*. 1993); of the abietanes induced by fungi in *P. tabuliformis* seedlings, abietic acid is abundantly produced and maintained at a level more than other compounds in phloem (see results); and abietic acid seems more easily synthesized and accessible than other chemicals for bioassays. Based on these reasons, we determined the effects of abietic acid on the growth of 4 fungi (*H. pinicola*, *L. truncatum*, *L. sinoprocerum* and the invasive mutualistic fungus *L. procerum*) using the methods of Kopper *et al*. (2005), with minor modifications. Briefly, we amended 2% MEA with abietic acid by dissolving different quantities of this chemical in ethyl acetate and added this into the molten media to yield the appropriate percentages by dry mass (milligrams of abietic acid per gram of dry MEA media). Five levels of abietic acid (0.01, 0.1, 1, 10 and 100 mg/g) were applied (abietic acid was filter-sterilized before being added into media). Ethyl acetate alone was used as control. Ethyl acetate as solvent did not affect fungal growth rates (*L. procerum*: $t = 0.69$, df = 7, $P = 0.51$; *H. pinicola*: $t = -1.66$, df = 8, $P = 0.14$; *L. truncatum*: $t = 2.10$, df = 8, $P = 0.08$; *L. sinoprocerum*: $t = 1.16$, df = 7, $P = 0.28$). A 5-mm-diameter plug of MEA colonized by actively growing fungal mycelia was added to the centre of 90-mm diameter plates of MEA, so that the mycelial side was in contact with media surface. Plates were incubated at 25 °C in the dark and linear growths were recorded daily until fungal mycelia in control plates reached the edges. Reduced growth rate was measured as: (average growth rate of fungus in control − growth rate of fungus in treatment)/average growth rate of fungus in control. Each treatment contained 4 to 5 replicates.

Determination of antagonistic effects of three newly acquired sympatric fungi on *Leptographium procerum* with and without abietic acid (Experiment 4)

We quantified the antagonistic effects of the three sympatric fungi (*H. pinicola*, *L. truncatum*, and *L. sinoprocerum*) on *L. procerum* in the presence or absence of abietic acid, using the dual culture experimental design (Campanile *et al*. 2007). Each Petri dish (90 mm in diameter) containing 20 mL autoclaved 2% MEA was inoculated with 2 5-mm-diameter plugs at a distance of 50 mm after solidification: one was from *L. procerum* and the other was from 1 of the 3 fungi. MEAs were amended with ethyl acetate (solvent) or abietic acid at the 100-mg/g level following the methods in Experiment 3. Petri dishes inoculated with *L. procerum* and sterile 2% MEA plug were set as control. Each combination was repeated 5 times. The area of *L. procerum* was measured at the time when the mycelia of this fungus reached the edges in control plates with solvent alone (at 15 days after inoculation). An antagonism index (AI) of each of the 3 fungi on *L. procerum* was calculated following this formula: $AI = (C_{area} - T_{area})/C_{area} \times 100\%$. C_{area} is the average area of *L. procerum* in control Petri dishes and T_{area} is the area of *L. procerum* in the presence of another fungal species. Area was quantified through taking a photograph of the plate followed by outlining fungal edge using Image J software (National Institutes of Health, Maryland, USA).

Statistical analysis

For Experiment 1, we used principal components analysis to describe the composition of diterpene resin acids in *P. tabuliformis* seedlings infected with various fungal species. Then, we used one-way ANOVA to determine differences in PC1 values and the concentration of individual diterpene resin acid induced by fungal associates, and employed the Duncan test for pairwise comparisons among treatments. For Experiment 2, we applied a 2-way ANOVA with a full factorial model (time, treatment, and time-treatment interaction) for each of the 5 compounds in phloem (mg/g phloem dry mass); as new seedlings were used at each time point, all the responses were assumed to be independent of each other. For Experiment 3, we used 1-way ANOVA to analyze differences in reduced growth rates between treatments for each of the 4 fungi. For all ANOVA analyses, normality of residues and homogeneity of variances were tested. When ANOVA consumptions of homogeneity were not met, data were Box–Cox transformed, and in Experiments 2 and 3, were followed by Bonferroni pairwise comparisons. For Experiment 4, the antagonism index of each of the 3 fungi on *L. procerum* in MEA amended with solvent alone and 100 mg/g abietic acid was compared by independent-sample *t*-test. All analyses were performed using SPSS Statistics 20 (IBM, Armonk, NY, USA).

RESULTS

Three Chinese red turpentine beetle fungal associates induce high amounts of diterpene resin acids in *Pinus tabuliformis* seedlings after 12 days

Among these main fungal associates of RTB in China (Table 1), principal component analysis showed that compositions of diterpene resin acids (abietic, neoabietic, palustric, levopimaric and dehydroabietic acids) in phloem infected by three newly acquired fungal associates (*H. pinicola*, *L. truncatum* and *L. sinoprocerum*) differed greatly from those with other treatments (Fig. 1a); this was further confirmed by significant differences in the first principal component (PC1) values among treatments, with the 3 fungi having higher PC1 values than all others (Fig. 1b; 1-way ANOVA: $F_{7,16} = 8.04$, $P = 0.0003$).

For individual compounds, abietic acid and palustric acid were significantly induced in phloem by the 3 fungi, compared to mechanical wound (Fig. 1c,d; 1-way ANOVAs: $F_{7,16} = 10.52$, $P < 0.0001$; $F_{7,16} = 6.25$, $P = 0.0012$; respectively); neoabietic acid, levopimaric acid and dehydroabietic acid were shown to have significant inductions only by *L. truncatum* and *H. pinicola* after 12 days of inoculations (Fig. 1e–g; 1-way ANOVAs: $F_{7,16} = 6.88$, $P = 0.0007$; $F_{7,16} = 9.39$, $P = 0.0001$; $F_{7,16} = 14.23$, $P < 0.0001$, respectively).

The 3 newly acquired fungal associates exhibit higher inductions of diterpene resin acids than *L. procerum* across time

There were significant differences in inductions of the 5 diterpene resin acids in host pine phloem among *H. pinicola*, *L. truncatum*, *L. sinoprocerum* and *L. procerum* across time (Fig. 2a–e; 2-way ANOVAs: dehydroabietic acid: treatment, $F_{3,176} = 67.07$, $P < 0.0001$, time, $F_{5,176} = 146.45$, $P < 0.0001$, treatment × time, $F_{15,176} = 5.28$, $P < 0.0001$; abietic acid: treatment, $F_{3,176} = 38.36$,

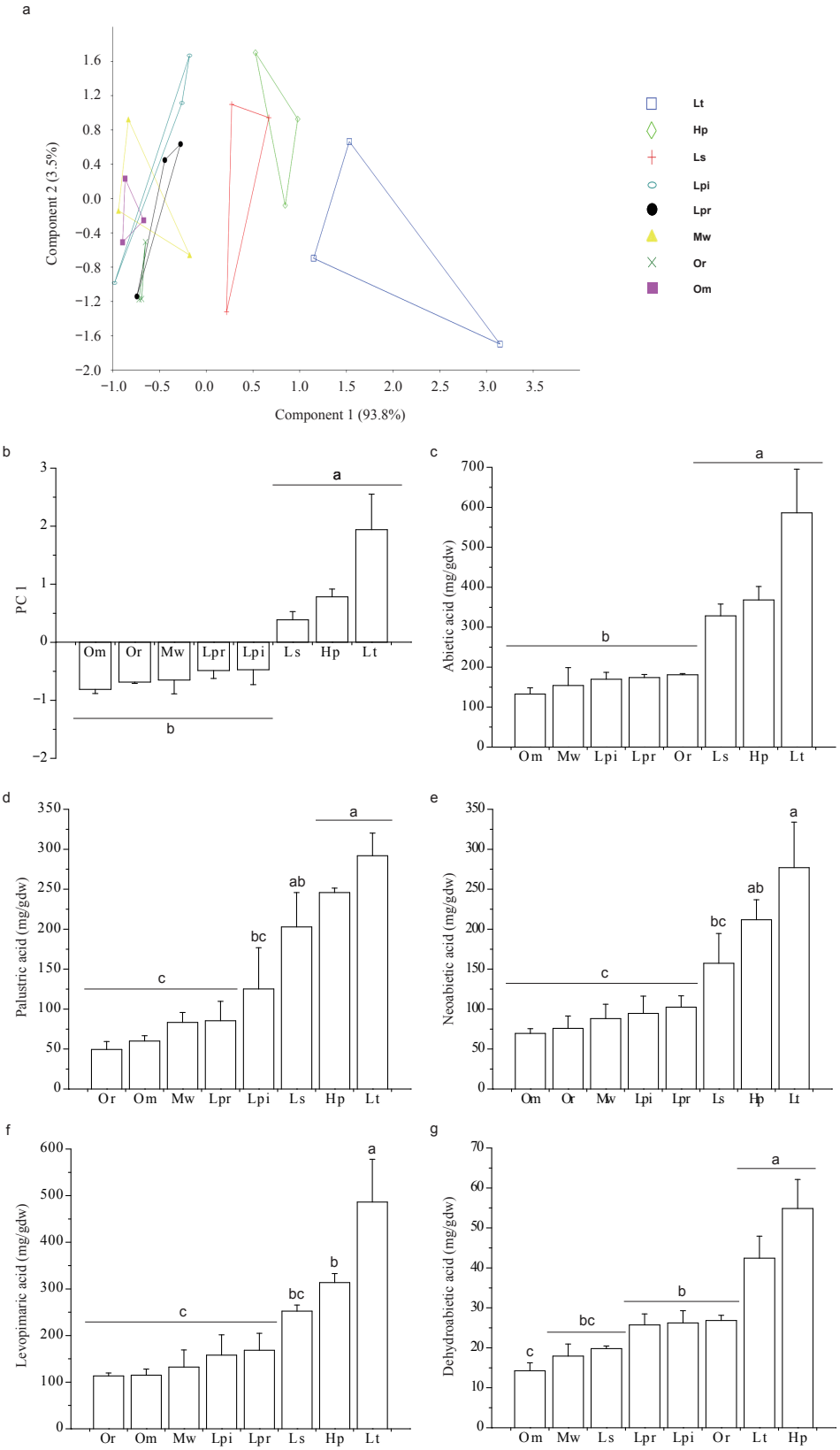

Figure 1 Rosin composition (a), the first principal component (b), and concentrations (Mean + SEM) of abietic acid (c), palustric acid (d), neoabietic acid (e), levopimaric acid (f) and dehydroabietic acid (g) in phloem of 4–5-year-old *Pinus tabuliformis* seedlings induced by mechanical wound and *Dendroctonus valens* fungal associates. Different letters on bars indicate significant differences between treatments ($P < 0.05$). Mw, mechanical wound; abbreviations of fungal names are based on full names shown in Table 1.

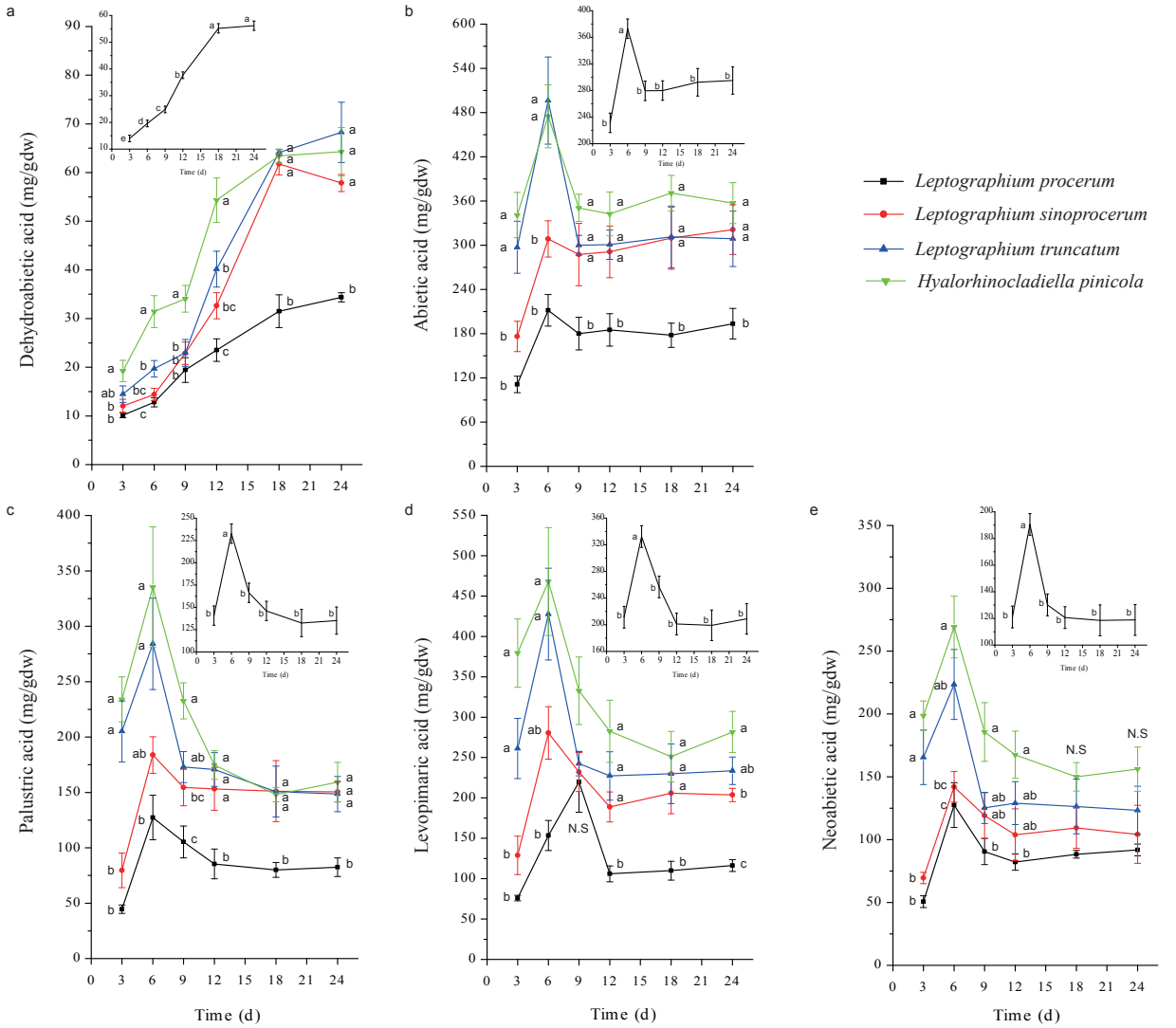

Figure 2 Induction dynamics of concentrations (mean ± SEM) of dehydroabietic acid (a), abietic acid (b), palustric acid (c), levopimaric acid (d), and neoabietic acid (e) by the invasive mutualist *Leptographium procerum* and 3 newly acquired sympatric fungi, *L. sinoprocerum*, *L. truncatum* and *Hyalorhinocladiella pinicola*, in phloem of 4–5-year-old *Pinus tabuliformis* seedlings. Different letters on curves indicate significant differences between treatments within each time point ($P < 0.05$); N.S, not significant. Inset: Changes in average concentrations (± SEM) of diterpene resin acids of fungal treatments across time points; different letters on curves indicate significant differences between time points ($P < 0.05$).

$P < 0.0001$, time, $F_{5,176} = 9.85$, $P < 0.0001$, treatment × time, $F_{15,176} = 1.55$, $P = 0.0937$; palustric acid: treatment, $F_{3,176} = 29.63$, $P < 0.0001$, time, $F_{5,176} = 11.14$, $P < 0.0001$, treatment × time, $F_{15,176} = 2.14$, $P = 0.0102$; levopimaric acid: treatment, $F_{3,176} = 32.22$, $P < 0.0001$, time, $F_{5,176} = 9.49$, $P < 0.0001$, treatment × time, $F_{15,176} = 1.93$, $P = 0.0233$; neoabietic acid: treatment, $F_{3,176} = 33.05$, $P < 0.0001$, time, $F_{5,176} = 11.32$, $P < 0.0001$, treatment × time, $F_{15,176} = 1.53$, $P = 0.0991$).

Fungal infections gradually elevated the inductions of dehydroabietic acid until 18 days after inoculation to reach a plateau (Fig. 2a inset); *H. pinicola* induced higher concentrations of dehydroabietic acid than *L. procerum* from three days and all three newly acquired fun-

gi exhibited higher inductions after 18 days compared to *L. procerum* (Fig. 2a). Whereas for the other four compounds the trends were substantially different, with fungal inductions starting at 3 days and rapidly achieving peak induction at 6 days, then descending at 9 days and then maintaining at the steady level (Fig. 2b–e inset); all 4 compounds were more highly induced by *H. pinicola* and *L. truncatum* at 3 and 6 days after inoculation, while, in comparison to by *L. procerum*, after 12 days only neoabietic acid was not induced at higher levels by the 3 newly acquired fungi (Fig. 2b–e).

Invasive mutualistic *L. procerum* is more tolerant of abietic acid than the 3 newly acquired fungal associates

As an important component of diterpene resin acids, abietic acid significantly reduced the fungal growth rates of *H. pinicola*, *L. truncatum* and *L. sinoprocerum*, as well as the invasive mutualistic fungus *L. procerum* (Fig. 3a–d; Brown–Forsythe one-way ANOVA, $F_{4,14.47} = 604.94$, $P < 0.0001$; one-way ANOVA, $F_{4,18} = 508.57$, $P < 0.0001$; 1-way ANOVA, $F_{4,20} = 364.95$, $P < 0.0001$; Brown–Forsythe 1-way ANOVA, $F_{4,6.72} = 428.86$, $P < 0.0001$; respectively). There were great variations in their tolerances of abietic acid: beginning at 1 mg/gdw of abietic acid, the reduced growth rates of *H. pinicola* and *L. truncatum* became higher than those at 0.01 mg/gdw of abietic acid, while *L. sinoprocerum* and *L. procerum* had significantly higher reduced growth rates until 10 mg/gdw; at 100 mg/gdw, which was the magnitude of induced concentration in seedling phloem, the extent of the reduced growth rate for *L. procerum* was around 60%, smaller than that for *H. pinicola*, *L. truncatum* and *L. sinoprocerum*, which were near 97%, 100% and 77%, respectively (Fig. 3a–d).

Invasive mutualistic *L. procerum* suffers from weaker suppression by newly acquired fungal associates in the presence of abietic acid

The competitive suppression by each of *H. pinicola*, *L. truncatum* and *L. sinoprocerum* on *L. procerum* was compared in dual cultures with and without abietic acid. Compared to those in media with solvent alone, antagonistic effects of the three newly acquired fungal associates on *L. procerum* were significantly reduced in the presence of abietic acid (Fig. 4), indicating that *L. procerum* could enhance its fitness when host pine (*P. tabuliformis*) produces high levels of diterpene resin acids in response to newly acquired sympatric fungi (Fig. 5).

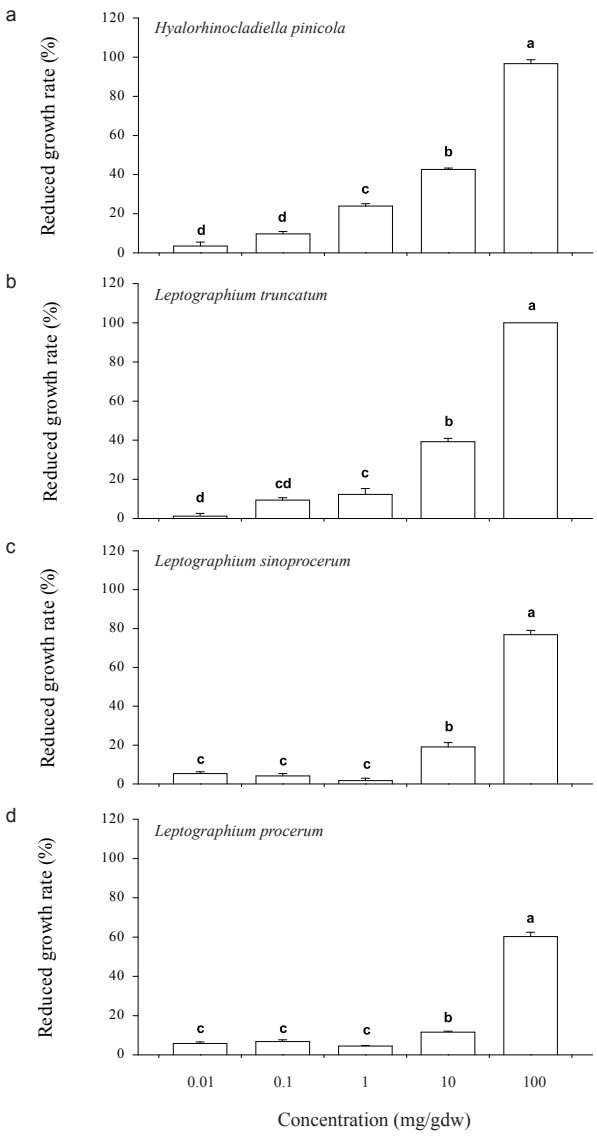

Figure 3 Reduced growth rates (%; mean + SEM) of *Hyalorhinocladiella pinicola* (a), *Leptographium truncatum* (b), *L. sinoprocerum* (c) and *L. procerum* (d) under elevated concentrations of abietic acid. Different letters on bars indicate significant differences between treatments ($P < 0.05$).

DISCUSSION

The interactions between bark beetle-associated fungi were shown to be significantly mediated by conifer defensive chemicals, in which monoterpenes have been greatly explored (Bridges 1987; Delorme & Lieutier 1990). In most situations, mycangial fungi that are ben-

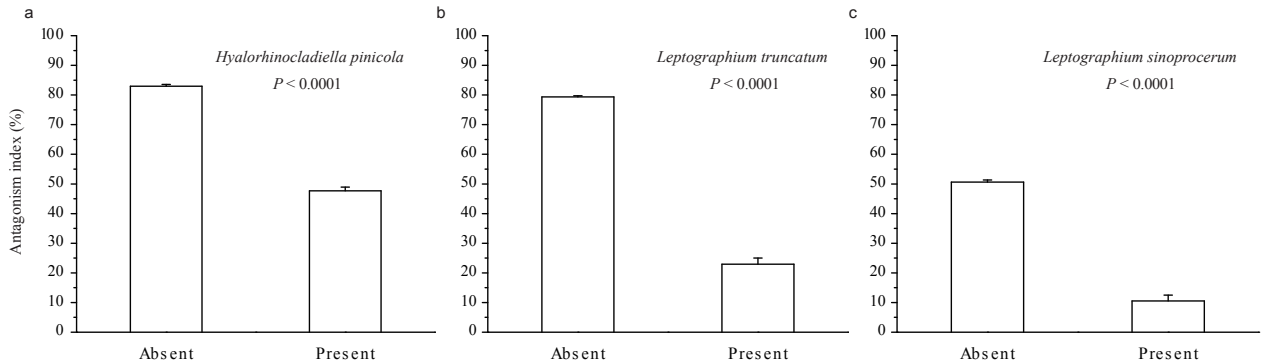

Figure 4 Antagonism indices (%; mean + SEM) of *Hyalorhinocladiella pinicola* (a), *Leptographium truncatum* (b) and *L. sinoprocerum* (c) on the invasive mutualist *L. procerum* under solvent control (absent) or 100 mg/g abietic acid (present).

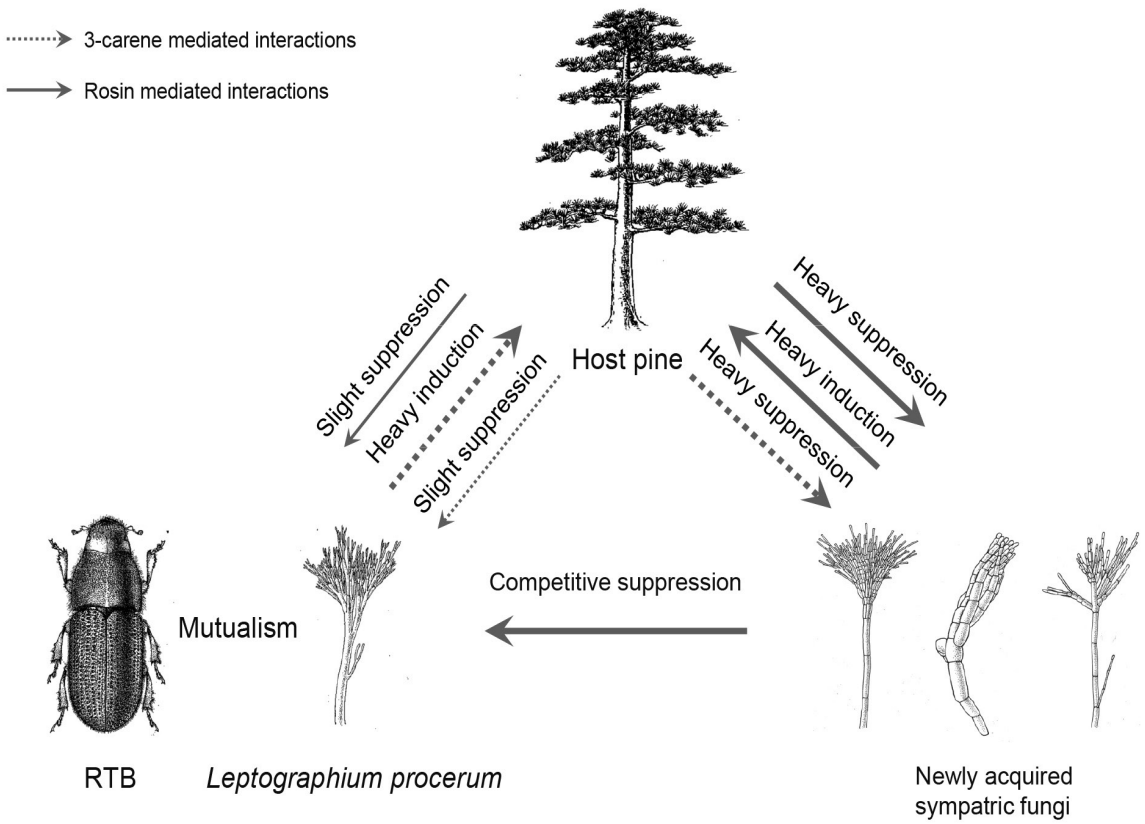

Figure 5 Schematic presentation of *Pinus tabuliformis* oleoresin defense in mediation of interactions between invasive mutualist *Leptographium procerum* and newly acquired sympatric fungi by red turpentine beetle (RTB) during invasion. The invasive bark beetle, RTB, gains benefits from co-introduced mutualistic fungus *L. procerum*; however, this fungus is competitively suppressed by newly acquired sympatric fungi. *L. procerum* not the sympatric fungi induces host pine to produce 3-carene (turpentine) that heavily suppresses sympatric fungi while slightly influences itself (Lu *et al.* 2010) (see dotted lines); the other component of oleoresin-rosin (also diterpene resin acid) of host pine, contrarily, is induced by sympatric fungi not by *L. procerum*, which also more heavily suppresses sympatric fungi than *L. procerum*, leading to dramatic reduction in competitive suppression of sympatric fungi on *L. procerum* (see solid lines). The combined strategies of utilizing host pine oleoresin defense by *L. procerum* may enhance its own fitness in invaded regions, providing a basis for its mutualism with co-introduced vector RTB.

eficial to their vector hosts are more adaptable to conifer monoterpenes than opportunistic ones and, thus, it has been regarded as an important mechanism for explaining the sustainable symbiotic relationship between bark beetles and mycangial fungi (Hofstetter *et al*. 2005). Beetle colonization of coniferous hosts goes through several stages, including aggregation attacking, reproduction, and offspring development until emergence; however, considering the nature of conifer defensive volatiles, these types of chemicals were mainly described to have effects on initial stages of beetle colonization, such as aggregation and initial attacks (Paine *et al*. 1997). This means that roles of monoterpenes in mediating interactions of symbiotic fungi might only be part of the whole story. In fact, by means of constitutive and inducible production (Christiansen *et al*. 1999; Zhao *et al*. 2010), conifer oleoresins also contain diverse non-volatile defensive chemicals that could potentially confer persistent selection forces on the fitness of symbiotic fungi throughout the course of beetle colonization. As an invasive beetle–fungus complex, the fitness of *L. procerum* directly links to the invasiveness of *D. valens* in *P. tabuliformis* forests. It would be comprehensive and interesting to assess mechanisms by which *L. procerum* enhances its fitness to mitigate competitions from sympatric fungi in the context of whole pine oleoresin defense, including both volatile (turpentine) and non-volatile (rosin) chemicals.

During the invasion process of *D. valens–L. procerum* complex, a set of ophiostomatoid fungi have been picked up by the invasive complex in China. Among those found in galleries or body surfaces of Chinese RTB (Chinese-RTB-associated fungi), it was the three newly acquired sympatric species (*H. pinicola*, *L. truncatum* and *L. sinoprocerum*), not the invasive *L. procerum* itself, that induced high amounts of diterpene resin acids in host pines; however, the *L. procerum* showed stronger tolerance of these non-volatile chemicals than the inducers, which means that this fungus may benefit from the elevated chemical stress provided by sympatric fungi. The dual culture results further implied that the antagonistic effects of sympatric fungi on the invasive fungus *L. procerum* were significantly weakened when diterpene resin acids were induced in host pine phloem. The higher induction/lower tolerance of diterpene resin acids by the three sympatric fungi and lower induction/higher tolerance by *L. procerum* may partly result from the slower evolutionary recognition of invasive fungus *L. procerum* by *P. tabuliformis* rosin defense. Host pine rosin defense could evolve more rapidly to recognize newly acquired sympatric fungi and respond effectively, possibly due to longer co-adaptation time between them, while *L. procerum* could, therefore, escape from inducible rosin defense. Our previous study demonstrated that at the initial stage of beetle colonization, *L. procerum* induces host pine to produce high amounts of 3-carene, a monoterpene not only recruiting beetle attacks but also suppressing other beetle-associated fungi (Lu *et al*. 2010). The higher induction of 3-carene from host pine by *L. procerum* might attribute to its rapid evolution (Lu *et al*. 2011) in putative effectors whereby host pine could more easily recognize it and express monoterpene-related elicitors. The higher tolerance of 3-carene and diterpene resin acids may also derive from faster evolution of *L. procerum* than other species or this fungus may have innately acquired this ability before its introduction into China. The enhanced fitness of the invasive fungus *L. procerum* appears to be attained by totally taking advantage of the host pine's induced oleoresin (turpentine and rosin) defense, contributing to its release from competitive suppression by sympatric fungi (Fig. 5).

From the induction dynamics curves of diterpene resin acids across time, we found that the five abietanes that accounted for considerable percentages in induced pine oleoresin were rapidly increased in the 3 days following fungal infection; more importantly, for abietic acid, neoabietic acid, palustric acid and levopimaric acid, there were obvious peaks in their productions at 6 days after fungal inoculations; these phenomena indicate that pine non-volatile rosin chemicals may even exert early-period selection forces, to determine colonization success at the initial stages after fungal and beetle landing. Future studies should focus on their bioactivities on both beetles and fungi, although studies on diterpene resin acids have lagged behind research on other pine chemicals such as monoterpenes and phenolics, mostly due to rather recent breakthroughs in rapid detection technology and the costly biosynthesis of pure chemicals for bioassays.

In the most recent 3 to 4 years, increasing examples have demonstrated that insect invasion successes are boosted by mutualistic microbes (Himler *et al*. 2011; Vilcinskas *et al*. 2013; Zhao *et al*. 2014). Symbionts confer enhanced fitness or extended phenotypes to invasive insect hosts and, to some extent, the fitness of these microbes determines the persistence of insect invasions. Therefore, elucidating interactions between mutualists of invasive insects and newly associated sympatric microbes can provide novel insights into the fates of sym-

biotic invasions in introduced regions. Future risk assessments for symbiont-driven insect invasions should not simply pay attention to characteristics of the "player" symbionts, but should also consider potential positive or negative network interactions from other sympatric microbial species, acquired by insect vectors during their invasion processes.

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