Complex interactions among host pines and fungi vectored by an invasive bark beetle

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Summary

- Recent studies have investigated the relationships between pairs or groups of exotic species to illustrate invasive mechanisms, but most have focused on interactions at a single trophic level.
- Here, we conducted pathogenicity tests, analyses of host volatiles and fungal growth tests to elucidate an intricate network of interactions between the host tree, the invasive red turpentine beetle and its fungal associates.
- Seedlings inoculated with two strains of *Leptographium procerum* isolated from *Dendroctonus valens* in China had significantly longer lesions and higher mortality rates than seedlings inoculated with other fungal isolates. These two strains of *L. procerum* were significantly more tolerant of 3-carene than all other fungi isolated there, and the infection of Chinese pine (*Pinus tabuliformis*) seedlings by these two strains enhanced the production and release of 3-carene, the main attractant for *D. valens*, by the seedlings.
- Our results raise the possibility that interactions among the fungal associates of *D. valens* and their pine hosts in China may confer advantages to these strains of *L. procerum* and, by extension, to the beetles themselves. These interactions may therefore enhance invasion by the beetle-fungal complex.

Introduction

Recent studies have shown that the interactions of hosts, pathogens and vectors can manipulate vector insect behavior to enhance dispersal of the pathogens (Lacroix *et al.*, 2005; Mayer *et al.*, 2008), resulting in a positive feedback mechanism between the insect vector and its symbiotic pathogen. Most of the past studies on interactions between bark beetles, fungi and hosts have focused on the development and maintenance of symbioses, mutualistic relationships or host defenses (Paine *et al.*, 1997; Lieutier, 2004; Six & Klepzig, 2004; Klepzig *et al.*, 2009). However, such complex interactions have rarely been explored as a mechanism to enhance invasive success, which is the hypothesis presented here.

Approximately 25 yr ago, the red turpentine beetle, *Dendroctonus valens*, was accidentally introduced into China, where it attacks several pines, particularly Chinese pine (*Pinus tabuliformis*) (Yan *et al.*, 2005). Since its introduction into China, *D. valens* has become an aggressive, tree-

killing species there (Yan *et al.*, 2005), whereas in North America, it is widely considered to be a secondary pest (Smith, 1961). Differences in host attraction were ruled out as an explanation for this phenomenon (Sun *et al.*, 2004; Erbilgin *et al.*, 2007), and so we embarked upon a comparative study of *D. valens* fungal associates in its native and introduced regions in order to elucidate the possible role of symbiotic fungi in this behavioral shift. The symbiotic relationship between *D. valens* and its phoretic *Leptographium* spp. fungi, especially the two *Leptographium procerum* strains most commonly isolated in China, is probably mutualistic, because the fungi benefit from these symbioses by gaining transport to new host trees, and beetles benefit by using the fungi to help overcome tree defenses (Lieutier, 2004). In addition, laboratory studies have shown that *D. valens* larvae that were fed the two Chinese isolates of *L. procerum* gained significantly more weight than larvae fed other isolates (B. Wang, M. Lu & J. H. Sun, unpublished). Both the fungus and the beetle therefore appear to gain in fitness from the association. When we began this study, little was known about the possible acquisition of new ophiostomatoid (sap-staining) fungal associates by *D. valens* since its introduction into China, and we hypothesized that new Chinese fungal associates might be more virulent, more competitive or otherwise better adapted to kill host trees than the complement of sap-staining fungi normally associated with *D. valens* in North America. Past studies conducted in North America have focused on intact native beetle-fungal associations rather than on invasive beetle-fungal complexes (Paine *et al.*, 1997; Six & Klepzig, 2004), but new beetle-fungal-host associations arising from invasions could provide a Pandora's box of possible outcomes with potentially severe consequences for native forest ecosystems.

Several species of pathogenic fungi were isolated from *D. valens* in China (the introduced area) during the course of this study (Table 1), including the introduced *L. procerum*, as well as several new fungal associates (*L. sino-procerum*, *Hyalorhinocladiella pinicola*, *L. pini-densiflorae* and *Ophiostoma minus*; Lu, 2008; Lu *et al.*, 2009), whereas *L. terebrantis* and *L. procerum* are most often associated with this bark beetle in North America (the native area) (Wingfield, 1983; Klepzig *et al.*, 1995; Six *et al.*, 2003). Amongst the *D. valens*-associated fungi in China, only *L. procerum* appears to have been introduced from North America as an invasive beetle-fungal complex (Lu, 2008), although, oddly, *L. procerum* has actually not been reported from the northwestern United States, the putative source of the introduction to China. Here, we conducted pathogenicity tests, analyses of host volatiles and fungal growth tests to investigate complex interactions between the Chinese host tree (*P. tabuliformis*), the invasive red turpentine beetle *D. valens* and its fungal associates, especially *L. procerum*, that might explain its invasiveness in China.

Table 1. *Pinus tabuliformis* seedling health and lesion length associated with inoculations of Chinese-invasive, American and Chinese *Dendroctonus valens*-associated fungi after 2 months.

Inoculum	Collection locality (native, invasive[1])	Isolate number	% Seedlings			Lesion length (cm) ± SD
			Healthy	Dying	Dead	
Leptographium procerum	China (invasive)	CMW 25569	25	65	10	2.32 ± 0.79 a
L. procerum	USA (native)	CMW 10217	95	5	0	0.57 ± 0.13 c
L. procerum	China (invasive)	CMW 25614	15	55	30	2.36 ± 0.68 a
L. terebrantis	USA (native)	CMW 1764	95	5	0	0.76 ± 0.17 b
Hyalorhinocladiella pinicola	China (native)	CMW 25613	100	0	0	0.46 ± 0.02 d
L. pini-densiflorae	China (native)	CMW 25600	100	0	0	0.44 ± 0.04 d
Ophiostoma minus	China (native)	CMW 26254	100	0	0	0.44 ± 0.03 d
Control			100	0	0	0.41 ± 0.01 e

Twenty 2-yr-old seedlings were inoculated in each treatment. Letters indicate significant differences across treatments ($P < 0.05$).
[1]Native or invasive with respect to the collection locality.

Materials and Methods

Strains, seedlings and inoculations

Three isolates of *Leptographium procerum* (Kendr.) Wingf. (one isolated from *Dendroctonus valens* LeConte (Coleoptera: Scolytinae) in the USA and two isolated from *D. valens* in China) and one isolate each of *L. terebrantis*, *H. pinicola*, *L. pini-densiflorae* and *O. minus* were used in *Pinus tabuliformis* Carrière seedling inoculations (Table 1). Hereafter, we refer to the two strains of *L. procerum* originating from the USA but isolated in China as 'Chinese-invasive' strains, whereas the strain isolated from *D. valens* in the USA and *L. terebrantis* from the USA are referred to as American strains, and the native Chinese fungi are referred to as Chinese strains/species. A separate population genetics study (M. Lu, M. Wingfield & J. H. Sun, unpublished) provided substantial evidence that the two Chinese-invasive *L. procerum* strains originated in North America, because all alleles were shared by North American *L. procerum* and no allele was unique to the Chinese populations. One Chinese-invasive *L. procerum* (CMW25614) was collected from *D. valens* specimens in the Yaopin Forest Station (35°46′N, 109°16′E; average elevation, 1000 m), Shaanxi Province. The other Chinese fungal associates, including the other Chinese-invasive strain of *L. procerum* (CMW25569), were collected from *D. valens* specimens in Tunlanchuan Forest Station (37°48′N, 111°44′E; average elevation, 1400 m), Shanxi Province. The American *L. procerum* and *L. terebrantis* were collected from *D. valens* in Idaho and Vermont (Lu, 2008). All isolates were maintained on malt extract agar (MEA) in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. We chose not to perform multiple inoculations on seedlings in order to avoid excessive mechanical damage to the seedling stems. Although some reports have indicated that low-density inoculations are poorly correlated with virulence (Krokene & Solheim, 1999), other studies have shown good correlation between responses from mass-inoculated mature trees and lesion length in wound-inoculated seedlings, monoterpene production by inoculated trees and/or fungal growth on malt agar (Krokene & Solheim, 1998; Eckhardt *et al*., 2004; Lieutier *et al*., 2004).

Two-year-old *P. tabuliformis* seedlings (stem diameter, 6-8 mm) were planted in plastic pots (diameter, 12 cm) and established for 4 wk in a glasshouse at c. 25°C. For inoculations (one inoculation per seedling), wounds were made on the bases of seedlings using a cork borer (diameter, 4 mm) to remove the bark and expose the cambium. Plugs of mycelium were taken from 7-d-old fungal cultures grown on MEA using the same sized cork borer and were placed into the wounds with the mycelial surface facing the cambium. Plugs of MEA alone (without fungi) were applied to trees in the same manner to serve as controls. To prevent desiccation and contamination, inoculated wounds were sealed with laboratory film (Parafilm M; Pechiney Plastic Packaging, Chicago, IL, USA).

Experiment 1

We tested the pathogenicity of the Chinese, Chinese-invasive and American *D. valens*-associated fungi to *P. tabuliformis* seedlings by inoculating 20 seedlings per treatment using the methods described above. In the phloem, fusiform necrotic lesions formed above and below the inoculation points as a reaction to the inoculum. Virulence was evaluated by measuring the length of these necrotic lesions in the phloem. After 2 months, seedlings were recorded as living, dying (chlorotic) or dead; all seedlings were uprooted and the lesion length resulting from inoculation was measured. Re-isolation of the fungus was attempted on 2% MEA from the inoculation area. After 7 d, the plates were examined for the presence of the respective fungi (Eckhardt *et al*., 2004) to confirm that there had been no cross-contamination.

Experiment 2

We inoculated seedlings with the Chinese, Chinese-invasive and American *D. valens*-associated fungi, and then uprooted a subset of the seedlings at 5-d intervals, excised the necrotic lesions, measured them and quantified the monoterpenes extracted from them. Three to seven seedlings per

treatment were sampled at 3, 8, 13, 18, 23, 28 and 33 d; the number per sample varied because of seedling mortality during the sampling period. The monoterpene contents were determined using the methods described previously (Raffa & Berryman, 1982). Briefly, phloem samples were finely chopped with a razor blade and monoterpenes were extracted in 10 ml hexane for 24 h. We added 0.1% p-cymene (99%) purchased from Pherotech International Inc. (Delta, BC, Canada) to the hexane solution as an internal standard. This monoterpene is not present in *P. tabuliformis* phloem and is easily separated from the naturally present monoterpenes. The extract was separated from phloem by vacuum filtration, and dried over calcium chloride for 1 h. Separations were performed on a gas chromatograph-mass spectrometer (GC-MS) (Hewlett Packard 6890N GC model coupled with 5973 MSD) equipped with a DB-WAX column (60 m length × 0.25 mm i.d. × 0.25 m film) (J&W Scientific, Folsom, CA, USA). The GC oven temperature program was set at 50°C for 2 min, increased to 220°C at 5°C min^{-1}, increased to 230°C at 4°C min^{-1} and then set at 230°C for 5 min. The on-column injector temperature was 220°C and helium was the carrier gas (flow rate, 1 ml min^{-1}). The MS electron impact source was operated in scan mode (30-300 (atomic mass units (amu)) with the MS source temperature at 230°C and the MS Quad at 150°C. The identifications of chromatogram peaks were based on comparisons with retention times and mass spectra of known standards and those in the NIST02 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA).

The total quantities of monoterpenes in each lesion were determined by comparison with the known quantities of p-cymene and corrected for differences in relative response factors, as described by Raffa & Steffeck (1988). Monoterpene concentrations were also determined on a dry weight of phloem basis by oven drying (following extraction) and weighing each phloem lesion sample.

Experiment 3

We quantified the headspace monoterpenes released over time from seedlings inoculated with the Chinese, Chinese-invasive and American *D. valens*-associated fungi. Three to seven seedlings per treatment were sampled at 3, 8, 13, 18, 23, 28 and 33 d (sample number varied because of seedling mortality). An effluvial headspace sampling method, modified from that of Andersson (2003), was used to collect volatiles from seedlings with different treatments. Each potted plant was enclosed in a 42 × 45 cm^2 plastic oven bag (Reynolds, Richmond, VA, USA) sealed with self-sealing strips at the opening around each stem, at a height of 1-2 cm above soil level. Compressed air (Beijing Gas Main Plant, Beijing, China) was purified and humidified through three 500 ml glass jars filled with molecular sieve (0.5 nm; Beijing Chemical Company, Beijing, China), freshly activated charcoal (Beijing Chemical Company) and distilled water, respectively. The filtered and moisturized air was pushed into the bag at the rate of 200 ml min^{-1}, and then drawn from the bag via an in-line collector (a glass tube with an internal diameter of 3 mm) containing 100 mg of Super Q chromatographic substrate (80-100 mesh, Alltech Association Inc., Deerfield, IL, USA) at a rate of 200 ml min^{-1} by a membrane pump (Beijing Institute of Labour Instruments, Beijing, China) for 24 h. This positive/negative pressure system ensured that no ambient air was pulled into the bag. Volatile compounds were extracted from the Super-Q using 1 ml high-performance liquid chromatography-grade hexane with 0.1% p-cymene as internal standard. Extracts were stored at -20°C until chemical analyses. Immediately after headspace sampling, oven-dried plants were weighed. Collected volatiles were analyzed and identified as in Expt 2.

Experiment 4

We determined the effects of individual volatile compounds on the growth of each test fungus using a modification of the methods of Hofstetter *et al.* (2005). We placed a 0.5 cm disk of MEA colonized with actively growing hyphae of each of the seven test isolates (Table 1) at the centers of 90 mm Petri dishes containing 2% MEA. We assayed eight volatile compounds commonly found in healthy and *D. valens*-attacked *P. tabuliformis*, including (+)-α-pinene, camphene, (−)-β-pinene,

myrcene, (−)-limonene, (+)-3-carene, β-phellandrene and terpinolene (purities of 85%, 86%, 80%, 84%, 87%, 86%, 85% and 88%, respectively) purchased from Pherotech International Inc. (Delta, BC, Canada). Each compound (1.0 ml) was absorbed onto sterile filter paper (diameter, 55 mm) and glued inside the lid of the Petri dish with the actively growing fungus. We incubated each plate upside down at 25°C in darkness. Each treatment and a control (filter paper alone) were replicated five times for each fungus. We traced the outer edge of fungus growth on the outside of the dish every 2 d using a map tracer. The colony diameter was then measured in four directions (0°, 90°, 180° and 270°) and the average of these measurements was taken to give a growth value for each sampling interval. We ended the assay when the fungus reached the edges of the Petri dishes.

Statistical analyses

For the first experiment, we analyzed the lesion length of fungi among treatments for each of the strains using one-way ANOVA, and employed the Bonferroni approach for pair-wise comparisons among treatments. For Expts 2 and 3, we applied a two-way ANOVA with treatment, time (in days) and treatment-time interaction as fixed effects to each of the following responses: the lesion length (cm), eight compounds (α-pinene, camphene, β-pinene, myrcene, 3-carene, limonene, β-phellandrene and terpinolene) from phloem (ng g^{-1} phloem within lesion) and headspace (ng g^{-1} dry seedling h^{-1}). As new seedlings were used for measurement at each occasion, we assumed all the responses to be independent of each other. For Expt 4, we analyzed the mean linear growth of fungi among treatments for each of the strains using one-way ANOVA for each sampling date. The Bonferroni approach was used to test pair-wise comparisons among treatments for 3, 18 and 33 d periods for an experiment-wise error rate equal to 0.05. For all ANOVA analyses, we tested the normal distribution (normality diagnostics) and homogeneity (Levene's test) of the variances of the responses for each treatment. We used SAS 9.2 (SAS Institute, Inc, Cary, NC, USA) and SPSS 11.5 (SPSS Inc., Chicago, IL, USA) for the statistical procedures.

Results

In Expts 1, 2 and 3, all seedlings from which re-isolations were attempted yielded only the inoculated fungus. None of the test fungi were isolated from discolored tissue associated with control wounds.

Tests comparing the pathogenicity of Chinese, Chinese-invasive and American *D. valens*-associated fungi on host seedlings (Expt 1) showed that 2 months following inoculation, 15 of 20 seedlings inoculated with one Chinese-invasive *L. procerum* (CMW 25569) and 17 of 20 seedlings inoculated with the other Chinese-invasive *L. procerum* (CMW 25614) were either dead or dying (Table 1). However, only one of 20 seedlings was dead or dying among those inoculated with the American *L. procerum*. The lesions caused by the Chinese-invasive *L. procerum* were also significantly longer than those caused by American *L. procerum* (Table 1). As for other isolates, only one of 20 seedlings inoculated with American *L. terebrantis* was dying. All seedlings inoculated with Chinese *H. pinicola*, *L. pini-desiflorae* and *O. minus* were healthy, and no control seedlings died. Among all inoculated isolates, the two Chinese-invasive *L. procerum* strains caused the longest lesions in host seedlings. In the first phase of Expt 2 (lesion lengths), the two Chinese-invasive *L. procerum* strains caused significantly longer lesions than any other strains or species 8, 13, 18, 23, 28 and 33 d after inoculation (Fig. 1).
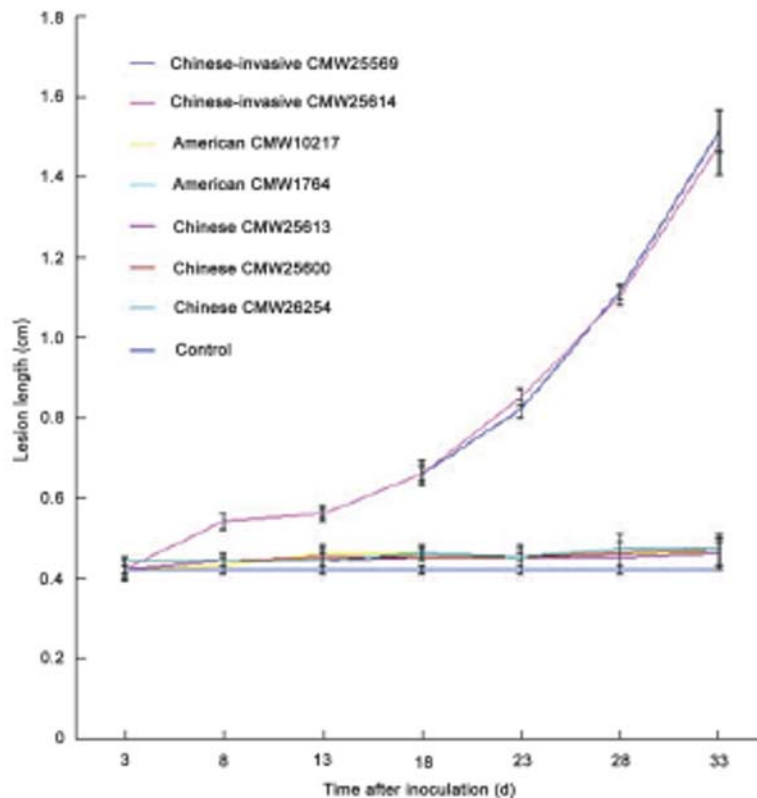
Figure 1. Lesion lengths on *Pinus tabuliformis* seedlings (± SD) after inoculation with Chinese-invasive, American and Chinese fungi associated with *Dendroctonus valens* after 3, 8, 13, 18, 23, 28 and 33 d. Three to seven 2-yr-old seedlings were inoculated in each treatment. Type III tests of fixed effects results: isolate, $F_{7,305} = 20\,314.4$, $P < 0.0001$; time, $F_{6,305} = 8859.45$, $P < 0.0001$; isolate $\times$ time, $F_{42,305} = 3235.94$, $P < 0.0001$.

In host volatile analyses (second phase of Expts 2 and 3), the common terpenes produced from phloem inoculated with all of the *D. valens*-associated fungi were α-pinene, camphene, β-pinene, myrcene, 3-carene, limonene, β-phellandrene and terpinolene, whereas 3-carene and terpinolene were not detected from controls. There were no differences in the concentrations of α-pinene, camphene, β-pinene, myrcene, limonene and β-phellandrene among all fungal treatments (Expt 2: α-pinene isolate, $F_{6,170} = 4.23$, $P = 0.21$; camphene isolate, $F_{6,174} = 5.11$, $P = 0.17$; β-pinene isolate, $F_{6,178} = 3.11$, $P = 0.40$; myrcene isolate, $F_{6,175} = 2.10$, $P = 0.56$; limonene isolate, $F_{6,178} = 3.48$, $P = 0.32$; β-phellandrene isolate, $F_{6,180} = 3.56$, $P = 0.39$; Expt 3: α-pinene isolate, $F_{6,201} = 5.78$, $P = 0.31$; camphene isolate, $F_{6,212} = 6.72$, $P = 0.23$; β-pinene isolate, $F_{6,207} = 2.99$, $P = 0.67$; myrcene isolate, $F_{6,211} = 4.15$, $P = 0.34$; limonene isolate, $F_{6,212} = 5.48$, $P = 0.29$; β-phellandrene isolate, $F_{6,209} = 3.72$, $P = 0.31$). Neither were there differences in the concentrations of 3-carene and terpinolene after only 3 d (Figs 2a,b, 3a,b). However, there were significant differences in 3-carene and terpinolene concentrations among all fungal treatments 8, 13, 18, 23, 28 and 33 d after inoculation (Figs 2a,b, 3a,b), with the two Chinese-invasive *L. procerum* isolates inducing higher concentrations of 3-carene and terpinolene than all other isolates.
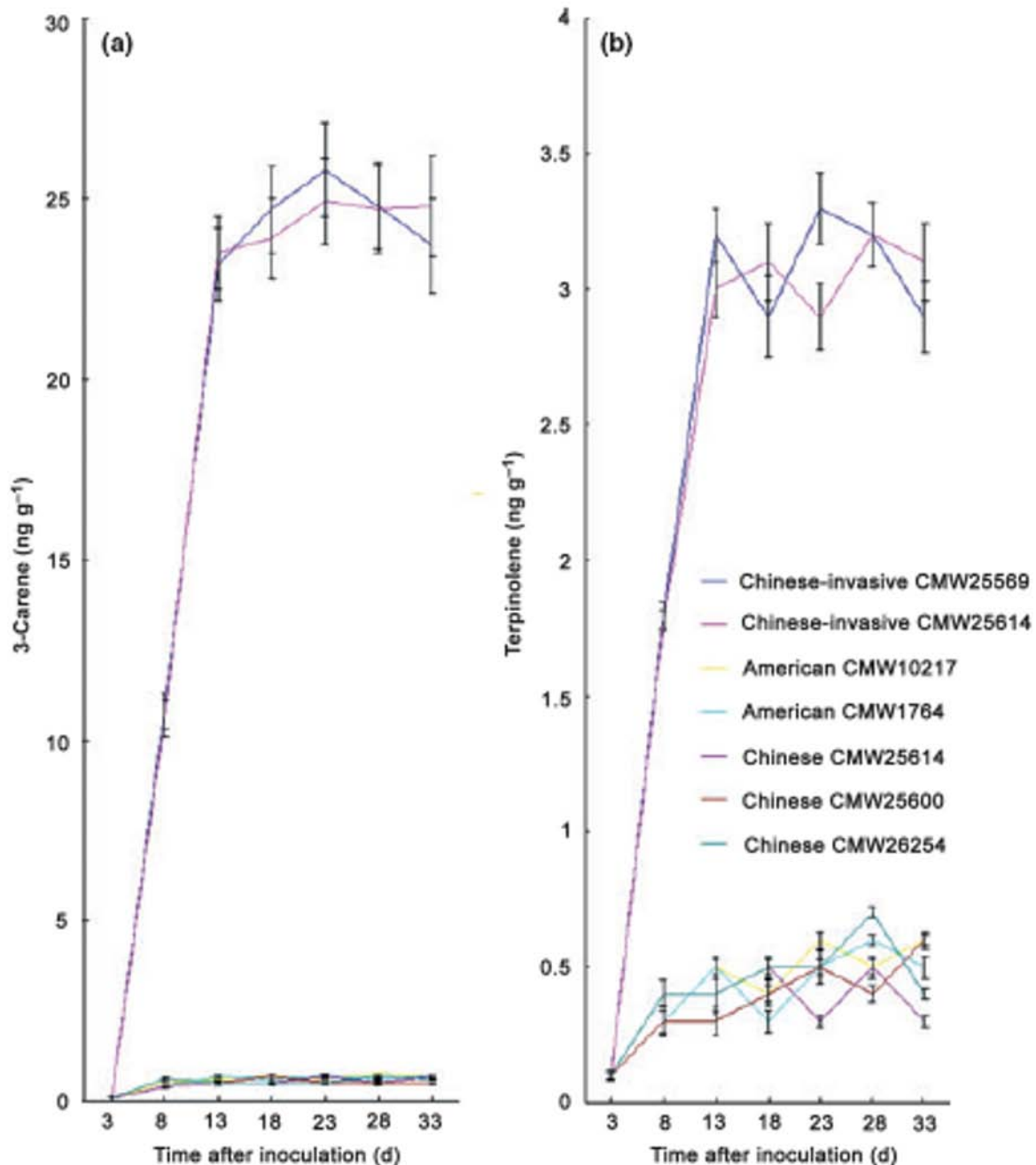
Figure 2. (a) 3-Carene and (b) terpinolene concentrations (± SD) from *Pinus tabuliformis* seedling phloem associated with inoculations of Chinese-invasive, American and Chinese *Dendroctonus valens*-associated fungi after 3, 8, 13, 18, 23, 28 and 33 d. Three to seven 2-yr-old seedlings were inoculated in each treatment. Type III tests of fixed effects results for 3-carene (a): isolate, $F_{6,172} = 160\ 335$, $P < 0.0001$; time, $F_{6,172} = 219\ 444$, $P < 0.0001$; isolate × time, $F_{36,172} = 35\ 741.1$, $P < 0.0001$. Type III tests of fixed effects results for terpinolene (b): isolate, $F_{6,210} = 60\ 674.2$, $P < 0.0001$; time, $F_{6,210} = 213\ 387$, $P < 0.0001$; isolate × time, $F_{36,210} = 28\ 149.4$, $P < 0.0001$.
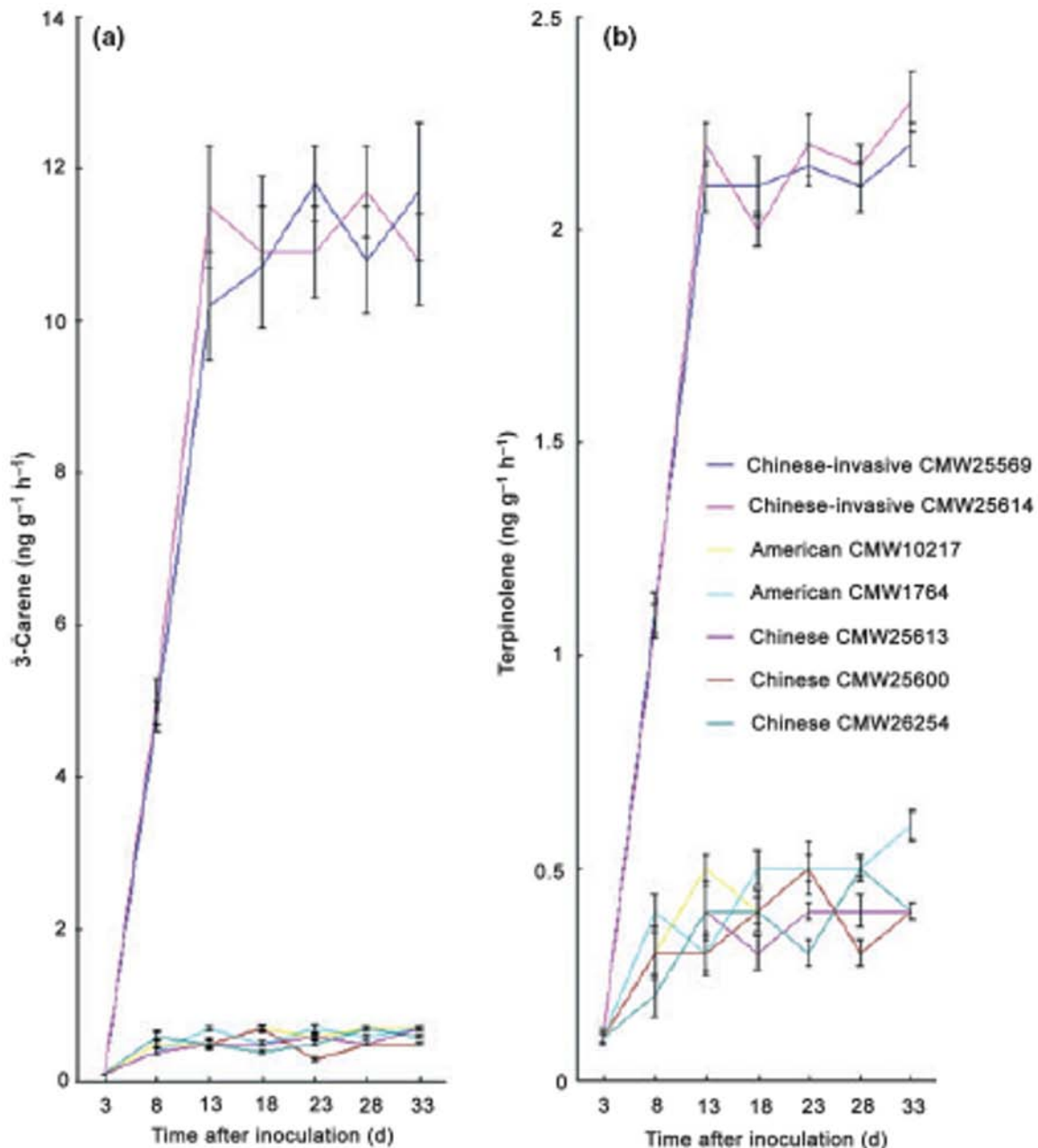
Figure 3. (a) 3-Carene and (b) terpinolene headspace concentrations (± SD) from *Pinus tabuliformis* seedlings associated with inoculations of Chinese-invasive, American and Chinese *Dendroctonus valens*-associated fungi after 3, 8, 13, 18, 23, 28 and 33 d. Three to seven 2-yr-old seedlings were inoculated in each treatment. Type III tests of fixed effects results for 3-carene (a): isolate, $F_{6,213} = 91\ 062.3$, $P < 0.0001$; time, $F_{6,213} = 1\ 681\ 834$, $P < 0.0001$; isolate × time, $F_{36,213} = 45\ 914.7$, $P < 0.0001$. Type III tests of fixed effects results for terpinolene (b): isolate, $F_{6,214} = 57\ 066.8$, $P < 0.0001$; time, $F_{6,214} = 73\ 383.7$, $P < 0.0001$; isolate × time, $F_{36,214} = 18\ 351.2$, $P < 0.0001$.

Fungal growth tests (Expt 4) showed that, of the secondary metabolites produced by trees, (+)-α-pinene, (−)-β-pinene and (+)-3-carene had the most striking impact on the rate of growth of fungi on MEA (Fig. 4). (+)-3-Carene significantly reduced the growth of all fungi, except for the two Chinese-invasive strains of *L. procerum*, and (+)-α-pinene and (−)-β-pinene enhanced the growth of Chinese-invasive L. procerum more than any other fungi.
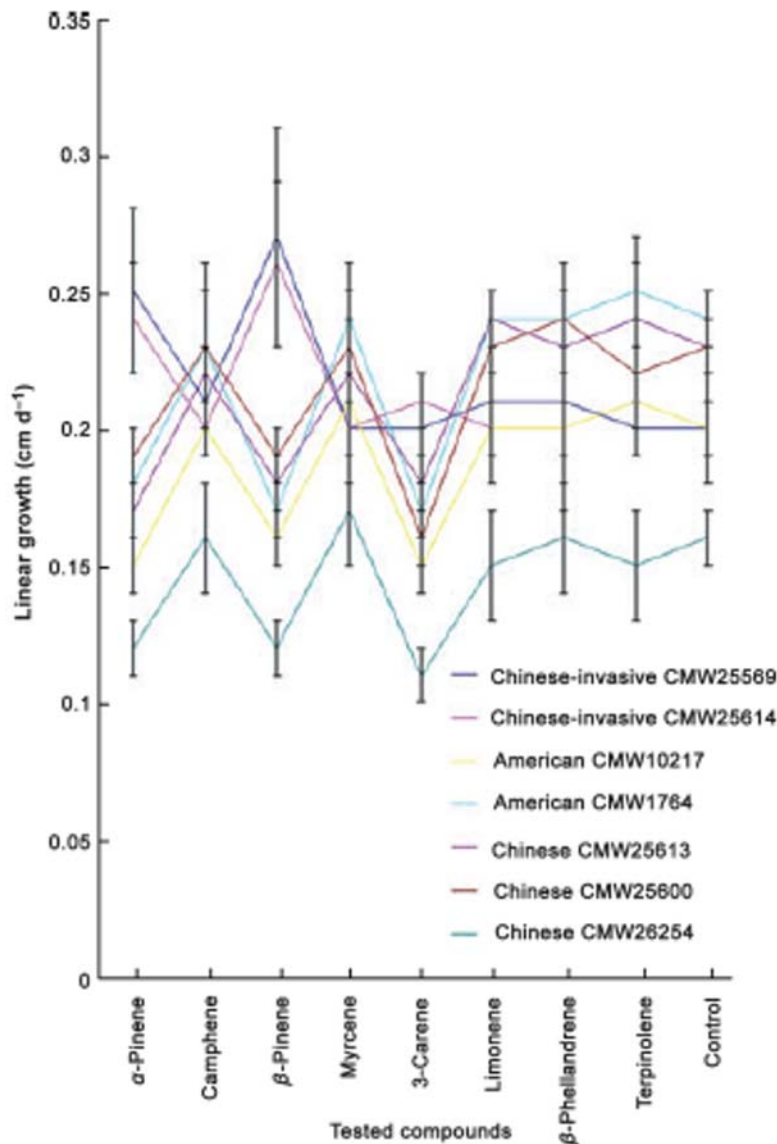
Figure 4. Mean linear growth rate (± SD) of each fungus on 2% malt extract agar in the absence (control) or presence of volatiles from a particular monoterpene. CMW25569, $F_{8,36} = 2.987$, $P = 0.021$; CMW10217, $F_{8,36} = 4.187$, $P = 0.003$; CMW25614, $F_{8,36} = 3.173$, $P = 0.019$; CMW1764, $F_{8,36} = 5.099$, $P < 0.001$; CMW25613, $F_{8,36} = 4.201$, $P = 0.003$; CMW25600, $F_{8,36} = 4.419$, $P = 0.002$; CMW26254, $F_{8,36} = 5.396$, $P < 0.001$.

Discussion

The Chinese-invasive strains of *L. procerum* induced significantly higher concentrations of 3-carene – in both phloem tissue and seedling headspace volatiles – than other fungi associated with *D. valens* in either the USA or China, and yet they were more tolerant of the monoterpene than all of the other fungal strains tested. (+)-α-Pinene and (−)-β-pinene, which are normally released from both healthy and *D. valens*-attacked hosts (Zhang, 2006), enhanced the growth of Chinese-invasive *L. procerum* more than any other fungi. These are the same two strains that caused significantly longer lesions and higher mortality in inoculated seedlings than in other isolates. Host seedlings responded more strongly to the Chinese-invasive *L. procerum* than to other fungal strains, presumably because the Chinese-invasive *L. procerum* were more virulent to host seedlings. Other studies have shown good correlation between responses from mass-inoculated mature trees and lesion length in wound-inoculated seedlings, monoterpene production by inoculated trees and/or fungal growth on malt agar (Krokene & Solheim, 1998; Eckhardt *et al.*, 2004; Lieutier *et al.*, 2004).

Therefore, we suggest that the Chinese-invasive strains may be better adapted than other strains or have a pre-existing tolerance to the host response by tolerating high concentrations of 3-carene, α-pinene and β-pinene.

Previous research has shown that 3-carene is more attractive to *D. valens* in the field and laboratory than any other host volatile (Sun *et al*., 2004; Zhang, 2006; Erbilgin *et al*., 2007), and the effect is positively related to the release rate of 3-carene at rates ranging from 110 to 210 mg d^{-1}, the highest rate tested (Sun *et al*., 2004). Although the results from our study were based on inoculations of seedlings rather than mature trees, it is possible that these two strains of *L. procerum* result in greater numbers of *D. valens* on infected host trees than do the other fungi. Further work based on field trapping or laboratory olfactometer tests should be performed to test this conjecture.

Other evidence has shown that *D. valens* larvae gain more weight by feeding on these *L. procerum* strains than on other fungi, suggesting that these two strains may contribute to beetle fitness (B. Wang, M. Lu & J. H. Sun, unpublished). The inferences we can draw are limited because this study was conducted with seedlings and in vitro fungal cultures rather than mature trees, but, taken together, they suggest a possible mutualistic relationship that could enhance invasion by and/or spread of a beetle-fungal complex. In a similar vein, Adams *et al*. (2009) have shown that both α-pinene and volatiles of some bacterial associates are capable of stimulating the growth of native American *L. procerum*, with complex interactions between host volatiles, bacteria and fungi that can affect beetle fitness. Although all of these cases describe native, coevolved systems, adaptations that enhance fitness in the native ecosystem may also confer advantages for symbiotic associations during the invasion of a new ecosystem.

In the past, fungal and bacterial symbionts have received relatively little attention from researchers because of the difficulties imposed by a fundamental lack of information about microbial ecology, biodiversity and biogeography (Desprez-Loustau *et al*., 2007). Recent technological advances have greatly stimulated research of this type, but more recent studies (Scott *et al*., 2008; Adams *et al*., 2009; Klepzig *et al*., 2009) and our results underscore the need for further investigations of this type. Future risk analyses for invasive species should not simply focus on the nominal invading species, but should also consider potential mutualistic and antagonistic relationships between the exotic species and its microbial symbionts (Klepzig *et al*., 2009), particularly in situations in which the phoretic insect has shown the capacity to acquire new microbial symbionts in the invaded region.

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