Bark beetle-mediated fungal infections of susceptible trees induce resistance to subsequent infections in a dose dependent manner

Nadir Erbilgin[1], Thomas R. Gordon*, David L. Wood and Andrew J. Storer†

Division of Organisms and Environment, University of California, 137 Mulford Hall, Berkeley, CA 94720, U.S.A.,*Department of Plant Pathology, University of California, 354 Hutchison Hall, Davis, CA 95616, U.S.A. and †School of Forest Resources and Environmental Science, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931-1295, U.S.A.

Abstract

1 Experiments were conducted to determine whether propagule loads on the twig beetles *Pityophthorus setosus* and *Pityophthorus carmeli* (Coleoptera: Scolytidae) influence the pathogen infection of the host tree in the Monterey pine-*Fusarium circinatum* system.

2 On an average, *F. circinatum* was isolated from 2.6% and 3.3% of trapped *P. setosus* and *P. carmeli*, respectively, although the isolation percentages varied over the season, being highest in the spring and lowest in late summer and fall for both species. Mean pathogen load was 13.4 and 22.6 propagules per beetle, on *P. setosus* and *P. carmeli*, respectively, and decreased from May to November for both species. The pathogen was also isolated from approximately 55% of both beetle species that emerged from infected branches. Mean propagule load on emerged *P. setosus* and *P. carmeli* was 39 and 66.5, respectively.

3 On the basis of these data, beetle species were treated with one of three propagule loads (low, medium, high) and caged onto live branches to determine whether they could transmit the pathogen. At all propagule loads, both species transmitted the pathogen, and transmission percentage and lesion length, a measure of tree susceptibility, were positively correlated with propagule load.

4 To investigate further whether the previous transmission by beetles could affect response of the same trees to subsequent infection with *F. circinatum*, different branches were inoculated on the same trees used in the transmission study, and lesion lengths were measured. Lesion lengths were lower on trees that had been previously exposed to beetles treated with high or medium propagule loads than on trees that had previously been exposed to beetles treated with low propagule loads. This suggests that the initial infection by beetles carrying high or medium propagule loads induced resistance to subsequent infections of the host, whereas infections caused by beetles with low propagule loads did not.

Keywords Bark beetles (Coleoptera: Scolytidae), fungal transmission, *Fusarium circinatum*, isolation percentage, propagule load, systemic induced resistance.

Introduction

Coniferous trees provide an environment for a multitude of organisms, including invertebrates, vertebrates, fungi and

Correspondence: Nadir Erbilgin. Tel: +1 780 492 8693; fax: +1 780 492 1767; e-mail: erbilgin@ualberta.ca

[1]Present address: Department of Renewable Resources, 230A Earth Sciences Building, University of Alberta, Edmonton, Alberta, T6G 2E3, Canada

bacteria, during their long lifespans (Raven *et al.*, 1998). Although trees are a source of food for many of these organisms, trophic relationships are generally balanced and not conspicuously detrimental to trees in native forest stands. For example, twig beetles (Coleoptera: Scolytidae) in the genus *Pityophthorus* in North America preferentially colonize branch tips that are recently killed, broken, dying or diseased (Bright, 1981). Because they exploit tissue that is no longer productive, twig beetles generally have little or no negative

impact on their host trees. However, such relationships can be altered by the introduction of exotic microbes, as happened recently in California, where the pitch canker pathogen was first recognized in 1986 (McCain *et al.*, 1987).

Pitch canker, caused by *Fusarium circinatum* Nirenberg & O'Donnell (=*Fusarium subglutinans* (Wollenweb & Reinking) Nelson, Toussoun & Marasas f. sp. *pini*), was originally described in the southeastern U.S. States in 1946 (Hepting & Roth, 1946) and is now a problem for pines in many parts of the world (Gordon *et al.*, 2001). In California, pitch canker has been reported from 19 coastal counties from Mendocino Co. (north of San Francisco) to San Diego Co. near the Mexican border (Gordon *et al.*, 2001). Although many pine species are affected, Monterey pine (*Pinus radiata*) has suffered the greatest damage from this disease (Gordon *et al.*, 2001). The pitch canker pathogen requires a wound in order to infect, and it appears that twig beetles are among the most important wounding agents in California (Gordon *et al.*, 2001). Adults emerging from diseased branches often carry spores of *F. circinatum* (McNee *et al.*, 2002) and, in the process of seeking a suitable substrate for colonization, they may create and transmit the fungus to wounds on healthy branches of both pitch canker-infected and adjacent uninfected trees, even though the insects may not colonize the trees on which they establish infection courts (Storer *et al.*, 2004; Erbilgin *et al.*, 2005; Sakamoto *et al.*, 2007).

The establishment of the pathogen on small branches leads to death of shoot tips as a result of lesions that girdle the branch. The disease intensifies through multiple independent infections, which can also affect large branches and the main stem, leading to extensive canopy dieback and death of the entire tree (Gordon *et al.*, 2001).

Studies of pitch canker in both native and planted stands of Monterey pine have found that most individuals are susceptible to the infection (Storer *et al.*, 2002; Wikler *et al.*, 2003) but that some trees manifest resistance (Storer *et al.*, 1999). It has also been shown that some trees sustaining relatively severe damage from *F. circinatum* may eventually recover (Gordon *et al.*, 2001), in part through the operation of systemic induced resistance (SIR). Evidence for the involvement of SIR derives from correlative studies conducted both in the laboratory (Bonello *et al.*, 2001) and in the field (Gordon, 2006).

Our current understanding of the role of plant induced defenses in conifer-pathogen interactions is quite limited (Brignolas *et al.*, 1995; Bonello *et al.*, 2001; Krokene *et al.*, 2003). SIR was first demonstrated to occur in Monterey pine in response to *F. circinatum* (Bonello *et al.*, 2001). SIR has also been documented in Austrian pine (*Pinus nigra*) inoculated with the necrogenic canker pathogen *Sphaeropsis sapinea*, and its less aggressive sister species *Diplodia scrobiculata* (Blodgett & Bonello, 2003). In subsequent studies, Blodgett *et al.* (2007) demonstrated that molecular signals move both acropetally and basipetally in the tree to elicit the SIR response.

Experimental documentation of SIR is generally based on studies using direct inoculations with the pathogen, whereas natural infections are thought to occur primarily through wounds created by insects (Gordon *et al.*, 2001). The occurrence of SIR under field conditions implies that this response can occur when infection courts are created by insects,

although this has not been confirmed experimentally. Much remains to be learned about SIR, and the *F. circinatum*-Monterey pine system provides good opportunities to address this issue. The main objective of the present study was to determine whether an insect-mediated infection elicited a response to a subsequent infection that was consistent with SIR. To make our tests as realistic as possible, we first determined naturally-occurring propagule loads on twig beetles by assaying *Pityophthorus* spp. that were trapped or emerged from host branches. On the basis of this information, the two most prevalent *Pityophthorus* spp. were treated with pathogen spore suspensions to obtain appropriate propagule loads. These insects were used to demonstrate transmission of the disease to healthy branches and, thereafter, we tested for SIR by challenging previously infected trees with the pitch canker pathogen to determine whether their response was influenced by prior exposure to the pathogen.

Materials and methods

Trapping dispersing beetles

Flight intercept traps (Advanced Pheromone Technologies, Marylhurst, Oregon) baited with attractive compounds [(*E*)-(+)-pityol and a combination of (*E*)-(−)-conophthorin and (*E*)-(+)-pityol] (Dallara *et al.*, 2000)) for *P. setosus* and *P. carmeli,* respectively, were set up to monitor beetle seasonal flight phenology in native Monterey pine forests at Año-Nuevo State Reserve (37°06′N, 122°17′W) (San Mateo Co.), Cambria (35°34′N, 121°05′W) (San Luis Obispo Co.) and the Monterey Peninsula (36°36′N, 121°54′W) (Monterey Co.) from April to November, 2005. On the basis of the number of study plots selected per location, various numbers of traps (range 6–12) were placed with 50–100 m spacing at each location. Crumpled paper towels were placed into the trap cups to reduce contact among beetles. Traps were emptied every 2 weeks.

We quantified isolation percentage and the number of propagules carried by individual beetles that emerged from pitch canker-infected and twig beetle-colonized Monterey pine branches on the Monterey Peninsula in May, July, September and November 2005. We returned branches to the laboratory where they were placed into emergence tubes, which had been disinfected by treatment with 0.5% sodium hypochlorite. Beetles were collected daily from glass vials attached to emergence tubes, as described by McNee *et al.* (2002), which minimized contact between emerged beetles. All trapped and emerged *Pityophthorus* spp. were identified to species and sexed. Beetles were stored at 4 °C before they were used for fungal isolation.

Fungal isolations from beetles

The natural propagule load was determined for individual beetles that were trapped in the field or reared from infected branches in the laboratory. First, sterilized water (0.5 mL) and tergitol (0.0l mL) (Sigma-Aldrich Chemical Co, St Louis,

Missouri) were mixed in a microcentrifuge tube containing a single adult beetle. The tube was then placed on a vortex mixer (Thermolyne/Sybron model M16715; Sybron International, Milwaukee, Wisconsin) for 60 s to dislodge fungal propagules present on the exoskeleton of individual beetles. The resulting suspension was then serially diluted in three separate 0.5-mL aliquots. Each of three dilutions was spread over the surface of a separate plate containing *Fusarium*-selective medium (Aegerter & Gordon, 2006), after which the plates were incubated in a plastic container at room temperature (23–27 °C) in the dark for 3–5 days. Colonies were identified as *F. circinatum* based on colony morphology and, where necessary, the structure of the microconidiophore, as described by Gordon *et al.* (1996). *Fusarium circinatum* colony-forming units on each plate were enumerated and used to estimate the percentage of beetles carrying pathogen propagules (isolation percentage) and the number of propagules carried by individual beetles. If the standard error of the mean propagule load was greater than 10% of the mean for any beetle species, additional individuals were washed until the standard error was less than 10% of the mean. Because we were interested in quantifying the seasonal variability in fungal isolation from beetles trapped in different landscapes of Monterey pine forests along the coast of central California, beetles trapped every 2 weeks from each location were pooled together to represent all trap data from that particular location. A minimum of 100 specimens for each beetle species was randomly selected from this pool of beetles to represent each location for each month.

Field assessment of fungal transmission by beetles

Based on results obtained using the methods described above, seasonal propagule loads of *F. circinatum* were estimated for each beetle species for each location. Beetles were then treated with propagule suspensions to obtain individuals carrying a natural load, and three and 30 times the natural load to determine whether fungal transmission was influenced by the infestation level. To determine the dose required to achieve these levels, suspensions were prepared, as described by Gordon *et al.* (1998), in a log series of propagule densities, in the range 10^3 to 10^7 propagules/mL of water. Beetles were immersed in these suspensions, allowed to dry on filter paper and subsequently assayed to estimate propagule loads. The relationship between the concentration of propagules in the suspension and the number of propagules carried by each insect was established. From this relationship, we determined the propagule density required to treat beetles to obtain the desired propagule loads. Live beetles used in these experiments were collected from the Monterey Peninsula and stored at 4 °C for up to 24 h before use. Because all of the beetles used in our experiments were obtained from nature, they would have had some unknown level of naturally occurring pathogen propagules. We have assumed that beetles used in validating the infestation procedure (described above) were representative of those used in our experiments and, therefore, that the final infestation levels obtained were an aggregate of both naturally occurring and artificially reared propagules.

After application of a propagule suspension, live beetles were used in an experiment to assess the efficacy of transmission of *F. circinatum* to branches, using established procedures (Hoover *et al.*, 1996). The experiment included eight treatments: (i) *P. setosus* with low propagule load (seasonal mean average = 13 propagules/beetle); (ii) *P. setosus* with medium propagule load (3 × seasonal mean average = 39 propagules/beetle); (iii) *P. setosus* with high propagule load (30 × seasonal mean average = 390 propagules/beetle); (iv) *P. carmeli* with low propagule load (seasonal mean average = 22 propagules/beetle); (v) *P. carmeli* with medium propagule load (3 × seasonal mean average = 66 propagules/beetle); (vi) *P. carmeli* with high propagule load (30 × seasonal mean average = 660 propagules/beetle); (vii) direct inoculation with *F. circinatum* (propagule load = 250); and (viii) mechanical wounding only. The experiment was initiated on 140 randomly selected Monterey pine trees on the Monterey Peninsula in January 2006.

All trees were initially asymptomatic, and in the range of 10.0–12.0 m tall, with a mean diameter of 20 cm. One hundred and twenty trees were randomly assigned to one of the first six treatments (n = 20/treatment), whereas 20 trees were randomly assigned to the remaining treatments (n = 10/treatment). On each tree, three branches above breast height were randomly selected and utilized for treatment applications. In treatments 1–6, one beetle was caged on each of three branches on each tree. Pieces of drinking straws, 1 cm in length, were used to confine the beetles to branches. One end of each piece was cut to fit to the branch surface, and then it was vertically placed over a beetle on the branch, and secured with tape. For direct inoculations with *F. circinatum* in treatment 7, a mechanical wound was made on each branch using a drill bit (diameter 1.6 mm) to create a wound deep enough to reach the sapwood and 5 μL of a fungal propagule suspension was deposited into the wound (Gordon *et al.*, 1998). Earlier studies showed that the extent of lesion development resulting from such inoculations provided a quantitative measure of tree susceptibility to pitch canker (Gordon *et al.*, 1998; Bonello *et al.*, 2001). In treatment 8, each branch was wounded but not inoculated.

In May 2006, 4 months after the beetle caging experiment was established, we cut all three branches from each tree and brought them to the laboratory where we isolated the fungus from branches to determine the transmission percentage of *F. circinatum*, and measured lesion lengths to the nearest millimeter on each branch. Three branches on mechanically wounded trees were also cut to evaluate whether wounding and cutting had any effect on the lesion development in the subsequent experiment. Branch diameter at the point of inoculation was measured to determine whether size had any effect on differences in transmission percentage and lesion development.

Assessment of systemic induced resistance in the field

If the initial inoculation triggered SIR, subsequent infections with the same pathogen should result in shorter lesions on trees subjected to that treatment, relative to the control trees

(and trees subjected to treatments not resulting in SIR) (Gordon *et al.*, 2001; Bonello *et al.*, 2006; Gordon, 2006). To test for this possibility, at the time of branch removal in the original experiment in May 2006, three new branches in the same whorl on each of the 140 test trees were mechanically inoculated with *F. circinatum*. Another ten asymptomatic trees that were not previously subjected to any experimental treatment were also inoculated with *F. circinatum*, as described above, to serve as controls. Four months after these direct inoculations with the pathogen (September 2006), all three branches from 150 trees were cut and returned to the laboratory. Transmission percentage and lesion length on each branch was recorded. The diameter of each branch at the point of inoculation was measured to determine whether branch size had any effect on transmission percentage and lesion development.

Statistical analysis

Mean isolation percentage (per location and per month) for beetles was estimated, assuming an over-dispersed Binomial distributed response (Isolation: Yes or No), using a Logit model from the family of Generalized Linear Models (GLM) (McCulloch & Searle, 2001) regressed on location, collection date and sex variables. The functional shape (linear) of the date effect was determined visually from the partial residuals from a fitted Generalized Additive Model (GAM) (Venables & Ripley, 1997) for a Logit response using the R-GAM (R-Statistics, 2006) routine. The parameters were estimated by likelihood-based techniques with the GLIMMIX procedure available in SAS software (SAS Institute, Inc., 2003).

Mean numbers of propagules from trapped beetles per site and per month were estimated with a Poisson regression model from the family of the Mixed GLM for over-dispersed Poisson distributed responses (counts) to address potential over-dispersion arising from the repeated measurements. The counts per trap were not independent because the same site was measured during the eight occasions. The counts were regressed on a third degree polynomial on date, with sex as a fixed effect and site as a random effect. The functional shape (third degree polynomial) of the date effect was determined visually from the partial residuals from a fitted GAM or a Poisson response using the R-GAM routine. The model's parameters were estimated with SAS GLIMMIX procedure, and the Wald type test statistics used for comparisons among sites, times and sex for an experiment-wise error rate of 0.05.

The estimation of the mean number of propagules on each emerged beetle was calculated using the logit model for an over-dispersed Binomial distributed response regressed on date and sex. The functional shape (linear) of the date effect was determined visually from the partial residuals from a GAM for the Logit response using the R-GAM routine. The mean number of propagules was estimated using a Poisson regression for over-dispersed Poisson distributed counts regressed on date and sex.

Mean transmission percentage from three branches (eight treatment levels for the beetle-mediated infection experiment and nine treatment levels for the challenge inoculation) was estimated for an over-dispersed Binomial distributed response (Transmission: Yes or No) using a logit model for repeated measures (three branched from the same tree). The estimation was quantified with SAS GENMOD procedure. The test of treatment pairwise comparisons was performed using Bonferroni's approach for an experiment-wise error rate of 0.05. For challenge inoculations, the fungal infection of at least one branch was estimated using the logit model regressed on treatment and branch mean diameter using SAS GENMOD procedure. The functional shape (linear) of the mean diameter effect was determined visually from the partial residuals from a GAM for the logit response using the R-GAM routine.

The mean lesion length difference between the challenge inoculation and the beetle-mediated infection trials was estimated for 8 of the 9 treatments levels assuming an analysis of variance model for continuous response using the SAS MIXED procedure, and the treatment pairwise comparisons were tested using the Bonferroni's approach for an experiment-wise error rate of 0.05.

To further test for a linear relationship between lesion lengths and SIR induction, we coupled treatments 1 and 4; 2 and 5; and 3, 6 and 7, and also regressed lesion lengths induced by the challenge inoculations on lesion lengths induced by beetles.

Results

Trapping dispersing beetles

We trapped 9562 twig beetles, representing five species of *Pityophthorus* in three locations. The two most abundant species, *P. setosus* and *P. carmeli*, accounted for more than 95% of the total catch and were the focus of subsequent studies. The remaining 5% of twig beetles trapped included *Pityophthorus nitidulus*, *Pityophthorus tuberculatus* and *Pityophthorus californicus*.

Fungal isolations from beetles

The mean isolation percentage from trapped *P. setosus* and *P. carmeli* was not statistically different and was 2.6% and 3.3%, respectively ($F_{1,4790} = 1.1$, $P = 0.3$). The mean isolation percentage for beetle species varied significantly through time, but not with location or sex (Table 1). For *P. setosus*, the mean isolation percentage peak occurred in April/May in all three locations with a second peak in September/October for Cambria and Año-Nuevo State Reserve (Fig. 1A). For *P. carmeli*, the mean isolation percentage steadily declined from 6.3% to 1.3% in April to November for all three locations (Fig. 1B).

The mean propagule loads on trapped *P. setosus* and *P. carmeli* varied between species and were 13.4 ± 6.5 and 22.6 ± 12.8 per beetle, respectively ($F_{1,132} = 8.9$, $P = 0.03$). The mean propagule load showed significant variation with time but not with location or sex (Table 1). The mean propagule load per *P. setosus* beetle declined from 17.9 in April to 7.3 in November (Fig. 2A). For *P. carmeli*, the mean propagule

Table 1 Statistical summary of isolation percentage and propagule loads on individual twig beetles (*Pityophthorus setosus* and *Pityophthorus carmeli*) trapped in native Monterey pine forests infested with *Fusarium circinatum* on the central coast of California, U.S.A. in 2005

	Date (month)		Location		Sex	
	F	P	F	P	F	P
Isolation percentage[a]						
Trapped						
Pityophthorus setosus	4.9	< 0.001	1.3	0.7	2.2	0.1
Pityophthorus carmeli	16.1	< 0.001	0.1	0.9	0.3	0.6
Emerged[b]						
Pityophthorus setosus	0.9	0.5	NA	NA	1.8	0.2
Pityophthorus carmeli	0.4	0.8	NA	NA	0.7	0.5
Propagule loads of *Fusarium circinatum*[c]						
Trapped						
Pityophthorus setosus	21.8	< 0.001	2.8	0.07	1.9	0.18
Pityophthorus carmeli	200.8	< 0.001	2.6	0.08	2.2	0.15
Emerged						
Pityophthorus setosus	0.2	0.9	NA	NA	2.5	0.1
Pityophthorus carmeli	0.2	0.9	NA	NA	3.2	0.08

[a]Isolation percentage refers to the proportion of beetles carrying propagules of *F. circinatum*.
[b]Beetles were only emerged from infected branches collected from Monterey pines on the Monterey Peninsula, Monterey County.
[c]Propagule loads refer to the mean number of viable *F. circinatum* propagules on individual beetles.NA, not available.

load per beetle was 28.5 in April, and declined steadily to 8.2 in November (Fig. 2B).

Pityophthorus setosus and *P. carmeli* also represented approximately 95% of beetles that emerged from *F. circinatum*-infected Monterey pine branches. The mean isolation percentage did not differ between *P. setosus* and *P. carmeli*, and was approximately 55% for both species, or approximately 15-fold higher than the same species caught in traps ($F_{1,382} = 0.8$, $P = 0.4$). There were no time or sex effects on the isolation percentage for either species (Fig. 3A, Table 1). Mean isolation percentage was different for the two beetle species ($F_{1,207} = 6.8$, $P = 0.01$). There were no significant effects of time or sex on mean propagule loads on either beetle species (Fig. 3B, Table 1). The mean propagule load per beetle that emerged from infected branches was approximately three-fold higher (39.0 ± 8.9 for *P. setosus*; 66.5 ± 9.5 for *P. carmeli*) than that of trapped beetles.

Field assessment of fungal transmission by beetles

Regardless of propagule load, both *P. setosus* and *P. carmeli* successfully transmitted *F. circinatum* to Monterey pines when confined on branches, with no significant differences in transmission percentage between these two beetle species ($F_{1,111} = 1.6$, $P = 0.1$; Table 2). However, transmission percentage and lesion lengths varied based on the propagule load on beetles. For beetles with the highest propagule loads (660 for *P. carmeli* and 390 for *P. setosus*), transmission percentages were not significantly different from that obtained using direct inoculations with *F. circinatum*, but they were significantly greater than those caused by beetles with low or medium propagule loads (22 and 66 for *P. carmeli*, 13 and 39 for *P. setosus*, respectively). Mechanical wounding alone created 3–4 mm scars in the sapwood of the branch but not the discoloration typical of *F. circinatum*-induced lesions, and data from this treatment were thus excluded from the percentage transmission analysis, but included in the lesion length comparisons. Both beetles treated with high propagule load and direct inoculations with *F. circinatum* created longer lesions than beetles with medium or low propagule loads. The mean diameter of branches used did not vary significantly among treatments ($\chi = 0.4$, d.f. = 1, $P = 0.5$) and the branch diameter × treatment interaction was not significant ($\chi = 1.6$, d.f. = 8, $P = 0.9$).

Assessment of systemic induced resistance in the field

The direct inoculation of branches with *F. circinatum* yielded a similar transmission percentage among previously treated or untreated trees, in the range 92–98%; there was, however, a significant treatment effect on lesion lengths resulting from the challenge inoculations (Table 2). Mean lesion lengths were 21.4 ± 2.2 mm and 19.2 ± 2.0 mm for trees that were previously either mechanically wounded or untreated, respectively. By contrast, mean lesion lengths for trees that were previously subjected to direct or indirect (via beetles) inoculation treatments were significantly altered relative to trees in other treatment groups (Fig. 4) (time × treatment interaction, $F_{15,131} = 37.3$, $P < 0.001$). Mean lesion lengths on branches previously treated with beetles with high or medium propagule loads or subjected to direct inoculation with *F. circinatum* were lower than that induced by beetles with low propagule loads. Mean lesion lengths on branches that were previously treated with beetles with low propagule loads increased relative to the first lesions and were not significantly different from those on mechanically wounded or untreated branches (Fig. 4).
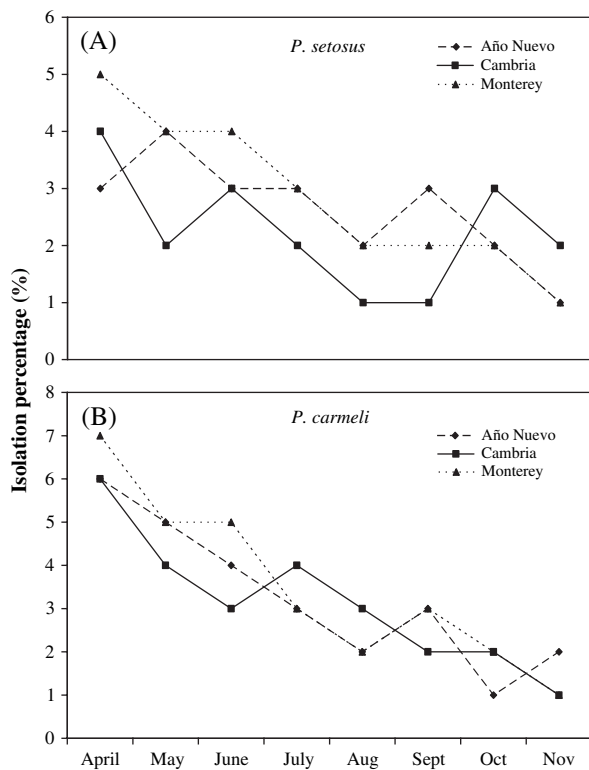
Figure 1 Mean isolation percentage of *Fusarium circinatum* on trapped *Pityophthorus setosus* and *Pityophthorus carmeli* in three Monterey pine forests along the coast of central California, U.S.A. (A, B) From *P. setosus* and *P. carmeli*, respectively, from April to November, 2005. For each species, we randomly selected 100 specimens at each location/month (total 2400 specimens per species).

Regression analyses between lesion lengths revealed a significant inverse relationship between lesion length and SIR induction (for treatments 1 and 4, $lesion_2 = -0.55$ $lesion_1 + 3.65$, $r^2 = 0.71$, $F_{1,39} = 35.9$, $P < 0.001$; for treatments 2 and 5, $lesion_2 = -0.53$ $lesion_1 + 3.8$, $r^2 = 0.53$, $F_{1,39} = 5.2$, $P = 0.028$); for treatments 3, 6 and 7, $lesion_2 = -0.53$ $lesion_1 + 3.8$, $r^2 = 0.63$, $F_{2,49} = 12.3$, $P < 0.0001$).

Discussion

The present study reveals four key findings. First, induction of systemic resistance in Monterey pines to pitch canker was dependent on propagule load on beetles, and not the wounding created by beetles on branches. Second, there appeared to be a threshold lesion length (in the beetle-vectored inoculations) below which subsequent challenge reveals no evidence of SIR. Third, *P. setosus* and *P. carmeli* were equally competent as agents of fungal transmission but propagule load had a significant effect on both transmission percentage and lesion lengths for both species. Fourth, propagule loads varied with beetle species and season, and there were no differences in propagule load vectored by males and females of each beetle species, or among beetles from different sites.
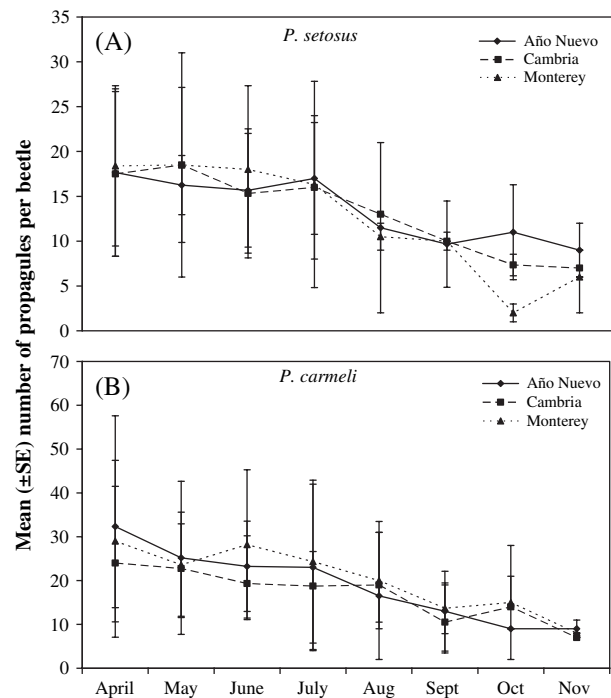

Figure 2 Mean $\pm$ SE number of propagules of *Fusarium circinatum* on trapped *Pityophthorus setosus* and *Pityophthorus carmeli* in three native Monterey pine forests along the coast of central California, U.S.A. (A) and (B): on *P. setosus* and *P. carmeli*, respectively, from April to November, 2005. For each species, we randomly selected 100 specimens at each location/month (total 2400 specimens per species).

For beetles reared from infected host branches, the only significant difference occurred between the two beetle species, with *P. carmeli* having higher propagule loads. Whether beetles were trapped or reared, isolation percentage and propagule loads were higher during spring and early summer than during late summer or fall. The mean isolation percentage was 15-fold higher and the propagule load was three-fold higher from beetles that emerged from infected host branches compared with trapped beetles.

In the present study, a large portion of *Pityophthorus* species had low propagule loads (≤ 20), a small percentage of the beetles (2–3%) had propagule loads in the range 50–150, and a few beetles that emerged from infected host tissues had more than 1000 propagules of *F. circinatum*. Furthermore, a single branch (50–60 cm long) can yield up to 30–60 *Pityophthorus* spp. individuals, including the two most abundant species identified in the present study (McNee *et al.*, 2002; Erbilgin *et al.*, 2005). Thus, various propagule loads utilized to contaminate beetles in the present study are reasonable and fall within the actual range carried by beetles capable of attacking Monterey pine branches in nature.

The successful establishment of pitch canker-infections by *Pityophthorus* species is consistent with previously published results (Storer *et al.*, 2004; Erbilgin *et al.*, 2005). These earlier studies have shown that infections can result when beetles are confined on the surface of a branch (Storer *et al.*, 2004) and when they are attracted to trees by pheromone baits with
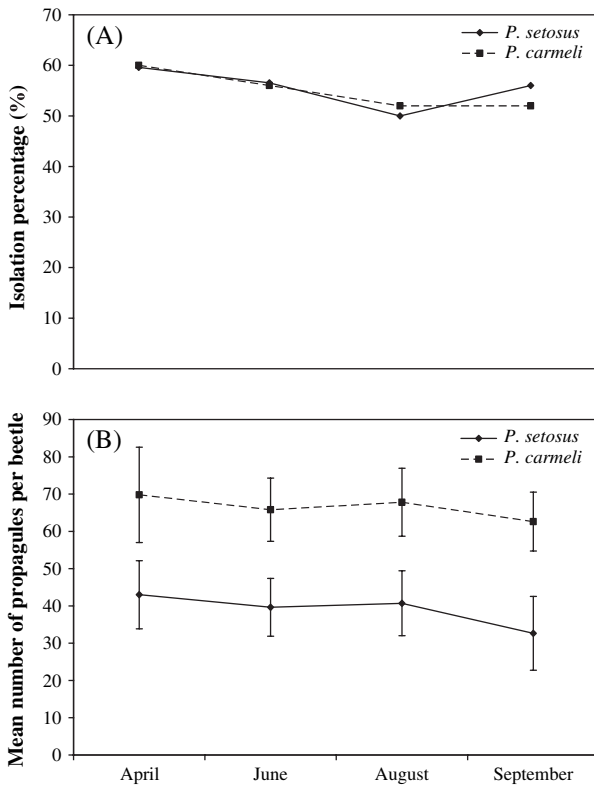
Figure 3 Mean isolation percentage (A) and mean ± SE propagule loads (B) on *Pityophthorus setosus* and *Pityophthorus carmeli* emerged from *Fusarium circinatum*-infected branches of Monterey pines in native forests on the Monterey Peninsula, Monterey, California, U.S.A., in April, June, August and September 2005. For each species, we randomly selected at least 50 specimens per month.

minimal (Sakamoto *et al.*, 2007) or no restriction on their range of movement (Storer *et al.*, 2004; Erbilgin *et al.*, 2005). The present study extends these results by demonstrating that

the infective potential of *F. circinatum*-contaminated beetles may be influenced by propagule loads of *F. circinatum* (Gordon *et al.*, 2001). Moreover, susceptibility to subsequent infection by the same pathogen depended on propagule load on beetle species, and not on the wounding created by beetles on branches. Given the stochastic nature of dynamics in the insect component of host–pathogen interaction, insects could play a significant role in mediating the outcome of coupled host–pathogen interactions at least in the pitch canker–Monterey pine system. Such interactions between insects and pathogen may influence the ecological and co-evolutionary dynamics of host plant–pathogen interactions.

The results obtained in the present study suggest that the occurrence of induced plant resistance against the fungal pathogen depends on the size of the dose transmitted by insects. When the initial number of propagules transmitted to the infection court was low, trees sustained infections by *F. circinatum* (as indicated by short lesions), and further infection by the same fungus demonstrated positive growth. This initial transmission was apparently not sufficient to induce systemic resistance. By contrast, when the initial transmission rate was high, trees initially sustained longer lesions and did manifest SIR for subsequent inoculations.

Further examination of data for individual trees revealed a significant inverse relationship between lesion length after the first infection and SIR. This indicates that tree resistance to subsequent infection was dependent on the size of the initial dose. Trees challenged with a high level of inoculum are primed to respond to subsequent infections more quickly, thus limiting lesion development, whereas trees exposed to lower inoculum levels may not be capable of such a response. This dichotomy may help to explain the differential occurrence of SIR among trees that initially appear to be comparable in susceptibility. Some such trees are eventually killed by pitch canker, whereas others recover (Storer *et al.*, 2002; Gordon, 2006), and this may reflect chance quantitative differences in their initial exposure to the pathogen. Our findings pertain to

Table 2 Mean transmission percentage of *F. circinatum* and lesion lengths on Monterey pine branches containing beetles treated with different propagule loads of *F. circinatum* or directly inoculated with *F. circinatum*

Treatments	No propagules/beetle	Beetle-mediated infection		Challenge inoculation	
		% Transmission[a]	Lesion length (mm)[a]	% Transmission[a]	Lesion length (mm)[a]
Pityophthorus carmeli	High (660)	90.0[a]	14.7 ± 1.3[a]	98.3	6.4 ± 0.2[b]
Pityophthorus setosus	High (390)	88.3[a]	14.5 ± 1.1[a]	98.3	5.3 ± 0.3[b]
Pityophthorus carmeli	Med (66)	53.3[b]	9.9 ± 0.7[b]	96.7	5.7 ± 0.3[b]
Pityophthorus setosus	Med (39)	52.6[b]	7.9 ± 0.6[b]	98.3	4.3 ± 0.2[b]
Pityophthorus carmeli	Low (22)	20.0[c]	5.3 ± 0.5[c]	98.3	17.7 ± 1.8[a]
Pityophthorus setosus	Low (13)	13.3[c]	4.1 ± 0.4[c]	98.3	18.7 ± 1.4[a]
Inoculation with fungus	250	100.0[a]	17.4 ± 0.7[a]	96.6	5.9 ± 0.4[b]
Mechanical wounding[b]	N/A		3.2 ± 2.0	93.3	21.4 ± 2.2[a]
Untreated control[c]				92.6	19.2 ± 2.0[a]
F		42.2	62.8	1.8	23.4
P		< 0.0001	< 0.0001	0.09	< 0.0001

[a]Means in the same column followed by different superscript letters are significantly different (Bonferroni adjustments: $\alpha = 0.05/7 = 0.007$ for the beetle-mediated infection and $\alpha = 0.05/9 = 0.005$ for the challenge inoculations).
[b]Mechanical wounds were not included in transmission percentage estimates. Mechanical wounds alone created only 3–4 mm scars without the discoloration typical of *F. circinatum*-induced lesions.
[c]Untreated control trees were only included in the challenge inoculations.
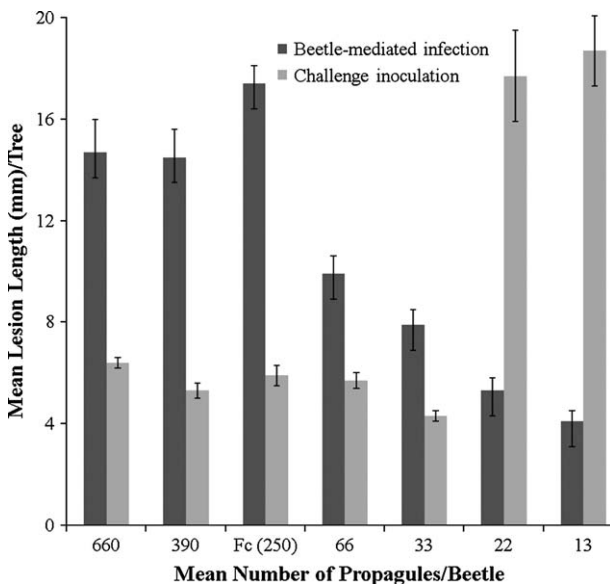
Figure 4 Mean lesion lengths on Monterey pine branches exposed to beetles treated with various propagule loads of *Fusarium circinatum* or directly inoculated with *F. circinatum* (Fc). The treatments corresponding to each propagule load are shown in Table 2. The mean lesion length difference between the challenge inoculation and the beetle-mediated infection was significant at each given point (analysis of variance model for continuous response using the SAS MIXED procedure, and the treatment pairwise comparisons were tested using the Bonferroni's approach for an experiment-wise error rate = 0.05). A reduction in the mean lesion lengths between the challenge inoculation and the beetle-mediated infection indicates systemic induced resistance on trees.

a plant response to an exotic pathogen, and it remains to be determined whether they are equally applicable to interactions in co-evolved host–pathogen systems.

Variation in percentage contamination and propagule load on beetle species suggests that insects may influence the dynamics of disease development. They can promote the spread of the pathogen and thereby produce more dead or dying trees in which to breed, although they may also limit possibilities for development where initial infection triggers SIR. For example, beetles with a low percentage contamination and low inoculum loads are expected to cause a lower rate of infection, whereas higher inoculum loads increase the likelihood of infections becoming established. However, where insects mediate infections that lead to extensive lesion development, they may facilitate the operation of a feedback mechanism against *F. circinatum* by enhancing host resistance to subsequent infections (Bonello *et al.*, 2001).

Acknowledgements

References

Aegerter, B.J. & Gordon, T.R. (2006) Rates of pitch canker induced seedling mortality among *Pinus radiata* families varying in levels of genetic resistance to *Gibberella circinata* (anamorph *Fusarium circinatum*). *Forest Ecology & Management*, **235**, 4–17.

Blodgett, J.T. & Bonello, P. (2003) The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. *Forest Pathology*, **33**, 15–19.

Blodgett, J.T., Eyles, A., Bonello, P. (2007) Organ-dependent induction of systemic resistance and systemic susceptibility in *Pinus nigra* inoculated with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. *Tree Physiology*, **27**, 511–517.

Bonello, P., Gordon, T.R. & Storer, A.J. (2001) Systemic induced resistance in Monterey pine. *Forest Pathology*, **31**, 99–106.

Bonello, P., Gordon, T.R., Herms, D.A., Wood, D.L. & Erbilgin, N. (2006) Nature and ecological implications of pathogen-induced systemic resistance in conifers: a novel hypothesis. *Physiological & Molecular Plant Pathology*, **68**, 95–104.

Bright, D.E. (1981) Taxonomic monograph of the genus *Pityophthorus* Eichhoff in North and Central America (Coleoptera: Scolytidae). *Memoirs of the Entomological Society of Canada*, **118**, 378.

Brignolas, F., Lacroix, B., Lieutier, F. *et al.* (1995) Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with Ophiostoma polonicum, a bark beetle-associated fungus. *Plant Physiology*, **109**, 821–827.

Dallara, P.L., Seybold, S.J., Meyer, H., Tolasch, T., Francke, W. & Wood, D.L. (2000) Semiochemicals from three species of *Pityophthorus* (Coleoptera: Scolytidae): identification and field response. *Canadian Entomologist*, **132**, 889–906.

Erbilgin, N., Storer, A.J., Wood, D.L. & Gordon, T.R. (2005) Colonization of cut branches of five coniferous hosts of the pitch canker fungus by *Pityophthorus* spp. (Coleoptera: Scolytidae) in central, coastal California. *Canadian Entomologist*, **137**, 337–349.

Gordon, T.R. (2006) Pitch canker disease of pines. *Phytopathology*, **96**, 657–659.

Gordon, T.R., Storer, A.J. & Okamoto, D. (1996) Population structure of the pitch canker pathogen, *Fusarium subglutinans* f sp *pini*, in California. *Mycological Research*, **100**, 850–854.

Gordon, T.R., Wikler, K.R., Clark, S.L., Okamoto, D., Storer, A.J. & Bonello, P. (1998) Resistance to pitch canker disease, caused by *Fusarium subglutinans* f.sp. *pini*, in Monterey pine (*Pinus radiata*). *Plant Pathology*, **47**, 706–711.

Gordon, T.R., Storer, A.J. & Wood, D.L. (2001) The pitch canker epidemic in California. *Plant Disease*, **85**, 1128–1139.

Hepting, G.H. & Roth, E.R. (1946) Pitch canker, a new disease of southern pines. *Journal of Forestry*, **44**, 742–744.

Hoover, K., Wood, D.L., Storer, A.J., Fox, J.W. & Bros, W.E. (1996) Transmission of the pitch canker fungus, *Fusarium subglutinans* f. sp. *pini* to Monterey pine, *Pinus radiata*, by cone- and twig-infesting beetles. *Canadian Entomologist*, **128**, 981–994.

Krokene, P., Solheim, H., Krekling, T. & Christiansen, E. (2003) Inducible anatomical defense responses in Norway spruce stems and their possible role in induced resistance. *Tree Physiology*, **23**, 191–197.

McCain, A.H., Koehler, C.S. & Tjosvold, S.A. (1987) Pitch canker threatens California pines. *California Agriculture*, **41**, 22–23.

McCulloch, C.E. & Searle, S.R. (2001) *Generalized, Linear, and Mixed Models*. John Wiley and Sons, Inc., New York, New York.

McNee, W.R., Wood, D.L., Storer, A.J. & Gordon, T.R. (2002) Incidence of the pitch canker pathogen and associated insects in intact and chipped Monterey pine branches. *Canadian Entomologist*, **134**, 47–58.

R-Statistics 2006. *The R Foundation for Statistical Computing. R Version 2.4.1. A Language and Environment*. URL http://www.r-project.org

Raven, P.H., Evert, R.E. & Eichhorn, S.E. (1998) *Biology of Plants*, 6th edn. W.H. Freeman/Worth, New York, New York.

Sakamoto, J.M., Gordon, T.R., Storer, A.J. & Wood, D.L. (2007) The role of *Pityophthorus* spp. as vectors of pitch canker affecting Monterey pine, *Pinus radiata*. *Canadian Entomologist*, **139**, 864–871.

SAS Institute, Inc. 2003. *SAS Procedures Guide, Version 9, Release 9.1*. SAS Institute, Inc., Cary, North Carolina.

Storer, A.J., Bonello, P., Gordon, T.R. & Wood, D.L. (1999) Evidence of resistance to the pitch canker pathogen (*Fusarium circinatum*) in native stands of Monterey pine (*Pinus radiata*). *Forest Science*, **45**, 500–505.

Storer, A.J., Wood, D.L. & Gordon, T.R. (2002) The epidemiology of pitch canker of Monterey pine in California. *Forest Science*, **48**, 694–700.

Storer, A.J., Wood, D.L. & Gordon, T.R. (2004) Twig beetles, *Pityophthorus* spp. (Coleoptera: Scolytidae), as vectors of the pitch canker pathogen in California. *Canadian Entomologist*, **136**, 685–693.

Venables, W.N. & Ripley, B.D. (1997) *Modern Applied Statistics With S-PLUS*. Springer, New York, New York.

Wikler, K., Storer, A.J., Newman, W., Gordon, T.R. & Wood, D.L. (2003) The dynamics of an introduced pathogen in a native Monterey pine (*Pinus radiata*) forest. *Forest Ecology & Management*, **179**, 209–221.