

Temporal variation in contamination of pine engraver beetles with *Fusarium circinatum* in native Monterey pine forests in California

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The relative importance of beetle species associated with *Fusarium circinatum*-infected Monterey pines was investigated in three Monterey pine forests along the coast of central California, USA from April to November in 2004 and 2005. *Fusarium circinatum* was frequently isolated from *Ips mexicanus* and *I. plastographus*. The mean percentage isolation based upon numbers of *I. mexicanus* and *I. plastographus* carrying propagules of *F. circinatum* was 17.7 and 10.9% in 2004 and 16.7 and 17.3% in 2005, respectively. The mean percentage isolation was high in the spring and early summer and low in late summer and autumn in all three locations for both species. Isolation was higher from beetles emerging from harvested *F. circinatum*-infected pine-stems than for trapped beetles, 42.4% for *I. mexicanus* and 45.9% for *I. plastographus*. The mean (\pm SE) propagule load of trapped *I. mexicanus* was 269.5 (\pm 14.1) in 2004 and 281.7 (\pm 35.7) in 2005 and was 216.1 (\pm 28.9) in 2004 and 251.9 (\pm 28.4) in 2005 for *I. plastographus*. Mean propagule loads decreased from May to November in all locations for both species. Propagule loads of beetles emerged from infected stems were lower than that of trapped beetles, with means of 89.4 (\pm 23.2) and 93.0 (\pm 23.2) for *I. mexicanus* and *I. plastographus*, respectively. Thus beetles must acquire fungal propagules from more than one infected host. These results also suggest that higher contamination rates and propagule loads in spring and early summer may indicate a higher risk of pitch canker transmission, relative to late summer or autumn.

Keywords: bark beetles, disease transmission, isolation rate, pitch canker, propagule load

Introduction

The focus of the current study was pine pitch canker, caused by *Fusarium circinatum* (= *Fusarium subglutinans* f. sp. *pini*), and two associated bark beetle species that occur in three native Monterey pine (*Pinus radiata*) forests along the central coast of California, USA.

Pitch canker was discovered in 1946 in the southeastern United States, where it was described as a disease affecting plantation-grown pines (Hepting & Roth, 1946). In 1986, pitch canker was identified as the cause of extensive damage to planted Monterey pines in coastal California (McCain *et al.*, 1987). The pathogen is considered a recent introduction into California (Wikler & Gordon,

2000). Typical symptoms of the infection include death of shoot tips, which results from girdling lesions on young branches. The infection intensifies through multiple independent infections, which can also affect large branches, leading to extensive canopy dieback. Infections on the main stem may lead to the death of an entire tree (Gordon *et al.*, 2001). Pitch canker is now found in 19 coastal counties in California from Mendocino in the north to San Diego in the south (Gordon *et al.*, 2001).

Previous work has shown that many bark beetle species (Coleoptera: Scolytidae), including several species of twig beetles (*Pityophthorus* spp.), one cone beetle (*Conophthorus radiatae*), and the pine engraver beetles (*Ips* spp.) are associated with pitch canker-infected Monterey pines in nature. In this system, the relative importance of the pine engraver beetles as vectors and/or wounding agents has not been fully assessed, unlike the other beetle species (Storer *et al.*, 2004; Erbilgin *et al.*, 2005; Sakamoto *et al.*, 2007). In this study, the pine engraver beetles *I. mexicanus* and *I. plastographus* were emphasized because they are the only beetle species that could potentially colonize

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(Wood, 1982) and transmit the fungus to large branches and stems (Fox *et al.*, 1990, 1991). Fungal infections on such tree parts could be initiated by beetle-borne inoculum, in which case the beetle is truly a vector, and/or by inoculum already present on the branch surface, with the beetle serving as a wounding agent.

The studies reported here aimed to (i) determine the relative abundance of dispersing pine engraver beetles responding to synthetic lures in Monterey pine forests in three coastal locations from April to November, (ii) determine the percentage of dispersing beetles of each species carrying propagules of *F. circinatum*, and (iii) estimate the number of viable *F. circinatum* propagules on individual beetles as a measure of the infective potential of each beetle species during the same time period.

Materials and methods

Trapping dispersing beetles

Flight intercept traps (Advanced Pheromone Technologies) baited with the attractive lures (ultra high release of α -pinene and ethanol, Phero Tech Inc.) for bark beetles were set up to monitor beetle activity in Monterey pine forests at the three locations, Año-Nuevo State Reserve (San Mateo Co.), Cambria (San Luis Obispo Co.), and the Monterey Peninsula (Monterey Co.), from April to November in 2004 and May, July, September and November in 2005 (Fig. 1). Monterey pine was the dominant tree species in all three forests with an occasional second canopy layer of coast live oak (*Quercus agrifolia*). Forests

in all locations had a similar understory composition. The most commonly occurring shrub species were coyote brush (*Baccharis pilularis*), poison-oak (*Toxicodendron diversilobum*), California huckleberry (*Vaccinium ovatum*), bush monkey-flower (*Mimulus aurantiacus*) and California blackberry (*Rubus ursinus*). There was also a regular herbaceous layer consisting of *Sanicula* spp., *Galium* spp. and a variety of grasses.

At each location, several traps were placed at various intervals ranging from 100 m to 1 km between traps depending on the size of each location. Traps were emptied every two weeks. Samples of *F. circinatum*-infected Monterey pine trees on the Monterey Peninsula were also cut in May and brought to the laboratory where they were placed into emergence containers. All trapped and emerged beetles were identified to species level and sexed. All beetles were kept in a refrigerated unit (4°C) for up to one month before processing, as described below.

Fungal isolations from beetles

Sterilized water (0.5 mL) and tergitol (0.01 mL) (Sigma-Aldrich Chemical Co.) were mixed in a microcentrifuge tube containing a single adult beetle (trapped in the field or emerged from infected stems in the laboratory). The tube was subjected to sonication (60 s) using a tip sonicator (Ultrasonic Homogenizer, Cole-Parmer) 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 plated onto a separate plate containing



Figure 1 The three study locations along the coast of central California, USA. Native Monterey pine forests only occur in three disjunct locations along the coast of central California, Año-Nuevo State Reserve (San Mateo Co.), Cambria (San Luis Obispo Co.) and the Monterey Peninsula (Monterey Co.).

Fusarium-selective medium (FSM) (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. The number of propagules carried by individual beetles was determined by counting the number of *F. circinatum* colonies on each plate, relative to the dilution factor. Identification of the fungus was based on its distinctive colony morphology on FSM and in some cases structure of the microconidiophores (Gordon *et al.*, 1996). If the standard error of the mean spore load was greater than 10% of the mean for any beetle species, further individuals were washed until the standard error was less than 10% of the mean. Beetles trapped every two weeks from each location were pooled together, and a minimum of 100 specimens for each beetle species was randomly selected from this pool of beetles to represent each location for each month. Viable *F. circinatum* propagule loads were determined for these beetles and all individual beetles emerged from *F. circinatum*-infected stems as described above. These investigations enabled the determination of percentage of beetles carrying propagules of *F. circinatum* and the mean propagule load on individual beetles.

Data analyses

Mean percentage isolation of *F. circinatum* propagules (per location and per month) for beetles trapped or emerged from infected stems 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 SAS (SAS Institute, Inc., 2003) GLIMMIX procedure.

Mean number of propagules (per beetle per month and per location) was 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 were regressed on a third degree polynomial on date and sex as fixed effects and location 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 the SAS GLIMMIX procedure, and the Wald-type test statistics used for comparisons among locations, dates and sex for an experiment-wise error rate of 0.05.

The estimation of the mean number of propagules of *F. circinatum* from individual beetles emerging from harvested infected trees was again done using the logit model for an over-dispersed binomial distributed

Table 1 Statistical summary of percentage isolation and propagule loads on individual pine engraver beetles (*Ips mexicanus* and *I. plastrographus*) trapped in native Monterey pine forests infected with *Fusarium circinatum* on the central coast of California, USA in 2004 and 2005.

	Date (Month)		Location		Sex	
	F	P	F	P	F	P
Percentage isolation ^a						
2004						
<i>I. mexicanus</i>	10.2	<0.0001	7.3	0.0007	0.01	0.93
<i>I. plastrographus</i>	3.7	0.0007	3.3	0.04	1.2	0.28
2005						
<i>I. mexicanus</i>	3.8	0.01	0.4	0.69	1.2	0.27
<i>I. plastrographus</i>	7.0	0.0001	0.09	0.92	0.5	0.46
Propagule loads of <i>F. circinatum</i> ^b						
2004						
<i>I. mexicanus</i>	216.2	<0.0001	1.2	0.29	0.2	0.65
<i>I. plastrographus</i>	217.7	<0.0001	1.8	0.19	1.7	0.2
2005						
<i>I. mexicanus</i>	175.4	<0.0001	2.8	0.07	25.7	<0.0001
<i>I. plastrographus</i>	38.5	<0.0001	1.5	0.23	15.3	<0.0001

^aPercentage isolation of beetles carrying propagules of *F. circinatum* in a given beetle sample size.

^bPropagule loads refer to the mean number of viable *F. circinatum* propagules on individual beetles.

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. Mean numbers of propagules from contaminated beetles was estimated using a Poisson regression for over-dispersed Poisson distributed counts regressed on date and sex.

Results

More than ten thousand beetle specimens associated with Monterey pine trees were caught in the three native Monterey pine forests along the coast of central California. The most abundant bark beetle species in all three Monterey pine forests were *I. mexicanus* and *I. plastrographus*, which accounted for more than 97% of the total catch in both 2004 and 2005.

The overall mean isolation of *F. circinatum* from *I. mexicanus* (17.7%) was higher than from *I. plastrographus* (10.9%) in 2004 ($F_{1,4537} = 5.88$, $P = 0.01$). For either species, effects on isolation percentage were significant for location and date (month), but not for sex in 2004 (Table 1). Among locations, both Cambria (21.3% for *I. mexicanus*; 11.4% for *I. plastrographus*) and Año-Nuevo State Reserve (19.0% for *I. mexicanus*; 10.9% for *I. plastrographus*) were significantly higher than the Monterey Peninsula (16.1% for *I. mexicanus*; 9.3% for *I. plastrographus*) (Fig. 2).

For *I. mexicanus*, the highest percentage isolation occurred in May–June in all three locations with a second peak in September–October for Cambria, and October–November

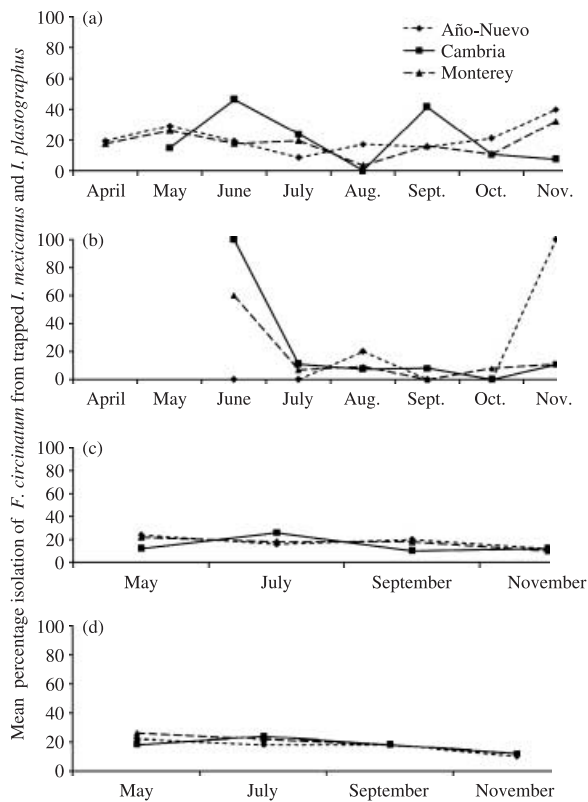


Figure 2 Mean percentage isolation of *Fusarium circinatum* from both trapped *Ips mexicanus* and *I. plastographus* in three native Monterey pine forests along the coast of central California, USA. (a) and (b): from *I. mexicanus* and *I. plastographus*, respectively, from April to November, 2004; (c) and (d): from *I. mexicanus* and *I. plastographus*, respectively, in May, July, September and November, 2005.

for both Año-Nuevo State Reserve and the Monterey Peninsula (Fig. 2a). Since the lures did not attract sufficient numbers of *I. plastographus*, the within-year isolation rate data for *I. plastographus* was incomplete, but it appears that it also had two peaks in all three locations (Fig. 2b).

In 2005, the overall isolation of the fungus was statistically similar between beetle species (16.7% for *I. mexicanus* and 17.3% for *I. plastographus*) ($F_{1,1190} = 2.33$, $P = 0.13$). Isolation varied with date (month), but not with location (*I. mexicanus*: 18.3% in Año-Nuevo State Reserve, 17.3% in the Monterey Peninsula, and 15.1% in Cambria; *I. plastographus*: 18.3% in Cambria, 17.2% in the Monterey Peninsula, and 17.1% in Año-Nuevo State Reserve) or sex for either species (Table 1; Fig. 2c,d). Although the temporal pattern of oscillation in 2005 was not as clear as in 2004 (because insect samples were collected and analyzed for every two months rather than monthly), overall percentage isolation was high in late spring or early summer and low in late summer or autumn for both species in all three locations.

Large numbers of both species of beetles emerged from *F. circinatum*-infected stems in the laboratory. The overall

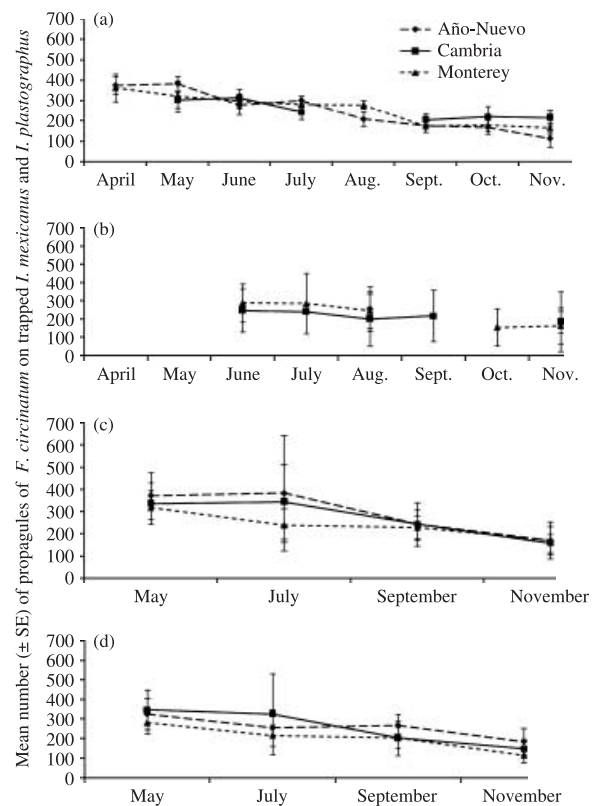


Figure 3 Mean number (\pm SE) of propagules of *Fusarium circinatum* on trapped *Ips mexicanus* and *I. plastographus* in three native Monterey pine forests along the coast of central California, USA. (a) and (b): on *I. mexicanus* and *I. plastographus*, respectively, from April to November, 2004; (c) and (d): on *I. mexicanus* and *I. plastographus*, respectively, in May, July, September and November, 2005.

percentage contamination of both species was higher than those trapped: 42.4% for *I. mexicanus* and 45.9% for *I. plastographus*. There was no sex effect on isolation rate for either species (*I. mexicanus*: $F_{1,177} = 1.49$, $P = 0.22$; *I. plastographus*: $F_{1,177} = 0.3$, $P = 0.59$).

The mean (\pm SE) propagule load was statistically higher for trapped *I. mexicanus* (269.5 ± 14.1) than for trapped *I. plastographus* (216.1 ± 28.9) in 2004 ($F_{1,774} = 4.67$, $P = 0.03$). There was a significant date (month) effect, but no location or sex effects on the mean number of propagules on either beetle species (Table 1). The mean propagule load decreased from April to November in all three locations for both species (Fig. 3a,b). Among locations, the mean number of propagules on *I. mexicanus* was $274.1 (\pm 23.7)$ in the Monterey Peninsula, $271.3 (\pm 20.6)$ in Año-Nuevo State Reserve, and $257.1 (\pm 17.5)$ in Cambria, and were $240.5 (\pm 47.4)$ in Cambria, $208.7 (\pm 98.2)$ in Año-Nuevo State Reserve, and $201.4 (\pm 40.9)$ in the Monterey Peninsula for *I. plastographus* (Fig. 3a,b). Males of *I. mexicanus* and *I. plastographus* had means of $271.9 (\pm 19.5)$ and $220.3 (\pm 37.2)$ propagules per individual, and females had means of $266.9 (\pm 22.4)$ and $208.3 (\pm 47.7)$ propagules, respectively.

In 2005, there was no significant difference on the overall mean propagule load between *I. mexicanus* (281.7 ± 35.7) and *I. plastographus* (251.9 ± 28.4) ($F_{1,197} = 1.93$, $P = 0.17$). There were significant date and sex, but no location effects on the mean number of propagules (Table 1). Similar to the temporal pattern of propagule loads observed in 2004, mean propagule loads in all three locations declined from May to November for both species in 2005 (Fig. 3c,d), although fewer samples were collected and analyzed. Among locations, mean propagule loads of *I. mexicanus* and *I. plastographus* were $305.2 (\pm 59.9)$ and $270.4 (\pm 39.4)$ in Año-Nuevo State Reserve, $288.1 (\pm 76.4)$ and $269.1 (\pm 70.3)$ in Cambria and $251.2 (\pm 36.3)$ and $219.7 (\pm 32.1)$ in the Monterey Peninsula, respectively. Males of *I. mexicanus* and *I. plastographus* had mean propagule loads of $327.4 (\pm 55.1)$ and $302.8 (\pm 50.1)$, and females had means of $221.2 (\pm 38.1)$ and $203.9 (\pm 27.4)$ per individual beetles, respectively.

Propagule loads of beetles emerged from infected stems were lower than that of trapped beetles. The means were $89.4 (\pm 23.2)$ and $93.0 (\pm 23.2)$ for *I. mexicanus* and *I. plastographus*, respectively. The mean propagule load for males (*I. mexicanus*: 85.2 ± 39.1 ; *I. plastographus*: 125.1 ± 42.7) was not different from that of females (*I. mexicanus*: 92.5 ± 28.1 ; *I. plastographus*: 63.7 ± 21.1) (*I. mexicanus*: $F_{1,73} = 2.4$, $P = 0.13$; *I. plastographus*: $F_{1,82} = 2.9$, $P = 0.09$).

Discussion

Fusarium circinatum was successfully isolated from both *I. mexicanus* and *I. plastographus*. Overall, the proportion of beetles carrying propagules of *F. circinatum* and the propagule loads on individual beetles were higher in the spring and early summer than in late summer and autumn. Beetles emerging from *F. circinatum*-infected stems were more contaminated, but had lower propagule loads than trapped beetles. The infective potential of *F. circinatum*-contaminated beetles may be influenced by both the numbers of insects contaminated with fungal inoculum and propagule loads (Gordon *et al.*, 2001). Thus beetles with a low contamination rate and a low propagule load are expected to cause a lower rate of infection than when both the contamination and propagule loads are higher, as demonstrated for *Pityophthorus* spp. (NE, unpublished data).

Several lines of evidence suggest that the pine engraver beetles play an important role in transmission of *F. circinatum* in native Monterey pine forests in California (Fox *et al.*, 1990, 1991). In the current study, about 45% of beetles emerged from infected hosts and about 15% of trapped *Ips* spp. were contaminated with propagules of *F. circinatum*. From April to November, the proportions of beetles contaminated were the highest during the May-June and September-October periods. However, the highest propagule load for both species occurred in May. This implies that there is temporal variability in the importance of each beetle species as a vector of *F. circinatum*.

Potentially, offspring beetles contaminated with propagules of *F. circinatum* disperse pathogen propagules, suggesting that they are important in transmission of *F. circinatum*. In the current study, a high percentage (ca. 45%) of *Ips* progeny was contaminated with the fungus when they were emerged from infected Monterey pine stems. Fox *et al.* (1990) also reported that when either one or both parents of *I. paraconfusus* were experimentally contaminated by *F. circinatum*, the fungus was successfully isolated from emerging progeny, immature beetles and beetle galleries. This indicates a vertical transmission of *F. circinatum* to a high percentage of beetle progeny from contaminated parents and/or contaminated host tissues under bark. In addition, higher propagule loads of trapped beetles relative to beetles emerged from infected hosts further suggests that beetles may also acquire additional propagules when they visit potential hosts during dispersal flights, although this needs to be verified.

Successful transmission of *F. circinatum* has been demonstrated for another *Ips* species, *I. paraconfusus* (Fox *et al.*, 1991). In the earlier study, *I. paraconfusus* experimentally contaminated with *F. circinatum* transmitted the fungus to both seedlings in the laboratory and mature pines in the field. The fungus was isolated from about 50% of the wounds created by beetles feeding on seedlings, and each seedling had at least one successful inoculation. This suggests that stem-colonizing bark beetles may be responsible for introducing the pathogen into the stem and large branches of Monterey pines in nature. This proposition is consistent with observations from long term monitoring plots where many individual trees sustained a few infections on branch terminals before any of them sustained stem infections (Storer *et al.*, 2002). This pattern also fits with the generalized role of beetles as vectors that wound trees and thereby inoculate healthy host tissues more or less randomly (Paine *et al.*, 1997).

Finally, some species of the pine engraver beetles are capable of attacking branches and boles of healthy pines (Schultz & Bedard, 1987; Fox *et al.*, 1990). For example, *I. mexicanus* was found tunnelling in living, healthy trees in the field (Fox *et al.*, 1990, 1991). This suggests that *Ips* spp. are capable of disseminating the fungus from infected host material to both healthy parts of an infected tree as well as to uninfected trees.

The results of this study were parallel to those reported by Ambourn *et al.* (2005) in the oak wilt disease-sap beetle system. Ambourn *et al.* (2005) reported that sap beetles associated with the disease posed the greatest risk to healthy oak stands when their contamination rates were higher in the spring. Similarly, in the pitch canker pathosystem, both beetle contamination rate and propagule load were higher in the spring and early summer, and therefore the risk of fungal transmission may be greatest during this period of the year. One practical aspect of this conclusion is that forestry operations, such as pruning or wounding of pines, should be conducted when the risk of fungal transmission is low in late summer

and early autumn, to reduce a risk of infection. Further studies are needed to confirm the within-year trends observed here and to investigate the effect of beetle propagule load on the frequency of infection of Monterey pine stems.

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