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Factors influencing infection of mechanical wounds by *Fusarium circinatum* on Monterey pines (*Pinus radiata*)

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Pitch canker, caused by the fungus *Fusarium circinatum*, is a disease affecting many pine tree species. In California, *Pinus radiata* (Monterey pine) is the principal pine host affected by pitch canker. This investigation into factors affecting infection frequency by *F. circinatum* of *P. radiata* examined the influence of: (i) wound size; (ii) relative humidity; (iii) time of inoculation; (iv) inoculum density; and (v) wound age. Wounded branches sustained significantly more infections when large-diameter (1·6 mm) rather than small-diameter (0·5 mm) wounds were made. Infection frequencies tended to be higher at 100% RH than at ambient humidity, although these differences were not statistically significant. Infection frequencies were significantly higher on branches inoculated after 17·00 h than on branches inoculated before noon. Infection frequencies were significantly higher for wounded branches spray-inoculated with 5×10^7 rather than 1×10^7 spores mL^{-1}. Infection frequencies of pruning wounds declined as wounds aged.

Keywords: forest tree disease, pitch canker

Introduction

Pitch canker, caused by the fungus *Fusarium circinatum*, is a disease of pines found in many locations throughout the world (Gordon *et al*., 2001). In South Africa, pitch canker is a disease primarily of seedlings (Viljoen *et al*., 1995). In the south-eastern USA, pitch canker affects trees of all ages, with infections occurring principally where mechanical wounds are caused by storm damage or silvicultural practices (Dwinell *et al*., 1985). In California infections are associated with insect activity, especially that of bark beetles (Coleoptera: Scolytidae) (Fox *et al*., 1990; Hoover *et al*., 1996; Dallara, 1997; Gordon *et al*., 2001).

Previous work by Wikler *et al*. (2003) demonstrated a higher incidence and severity of pitch canker in native forests of Monterey pine (*Pinus radiata*) near the coast than further inland. The reasons for the differential development of pitch canker are not known, but could include climatic limitations on the infection process, differences in the availability of inoculum, and greater activity near the coast than further inland of insects acting as vectors, wounding agents, or both (Hoover *et al*., 1996). Little is known about the extent to which these factors may limit the frequency of infection by the pitch canker pathogen under field conditions in California. This study was undertaken to evaluate the effects on infection frequency of (i) wound size; (ii) relative humidity; (iii) time of inoculation; (iv) inoculum density; and (v) wound age.

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General methods

Inoculum

Spore suspensions were prepared from a known pathogenic isolate of *F. circinatum*, GL17 (= Fsp17, Gordon Laboratory, UC Davis, California), which was originally isolated from an infected *P. radiata* in California. Inoculum was obtained by growing GL17 on potato dextrose agar (PDA) in Petri dishes for at least 14 days at room temperature (23 ± 2°C) (Gordon *et al*., 1998). Unless otherwise stated, spore concentrations were adjusted to 1×10^7 spores mL^{-1}. Spore suspensions were prepared by flooding agar cultures with 10 mL 0·5% KCl, and lightly scraping the mycelium with a sterile bent glass rod. The suspension was passed through double layers of sterile cheesecloth and the spore concentration was estimated microscopically using a haemocytometer. The desired concentration was achieved by dilution with sterile 0·5% KCl. For each new inoculation, spore viability was assayed using an additional dilution to obtain *c*. 1500 spores mL^{-1} (target dose = 75 spores 50 μL^{-1}), 50 μL of which was spread onto each of five PDA dishes (a total of 95 Petri dishes for these experiments), followed by incubation for

3 days at room temperature (20°C). Germinated spores were counted and the average viability of spores used in these experiments (±SE) was 86·9 ± 1·6% of the target dose.

Inoculation and rating procedures

All experiments were conducted on trees in native stands at Pebble Beach, Monterey County, CA, or in a glasshouse at UC Davis. Inoculations were generally conducted between 10·00 and 14·00 h for these experiments (except in the time-of-day experiments). Inoculum was applied to branches (to runoff) using a hand-held spray bottle, and allowed to dry (2–4 h). After inoculation, except where indicated, branches were wounded to the depth of the xylem at 10 locations along their length, with wounding sites spaced *c.* 2 cm apart. The standard wounding implements were 1·6 mm in diameter and had been used for mechanical inoculations in previous work (Gordon *et al.*, 1998). Wounding implements were sterilized between branches by heating with a flame from a disposable butane lighter.

Branches were rated for infection frequency 8–12 weeks after initiation of the experiment (Table 1). Infection frequency was calculated by dividing the number of infected wounds by the total number of wounds for each branch. Wounds were considered infected when tissue around the wound site was resin-soaked and discoloured. In most cases such lesions were obvious; however, if they were not, the presence of the pathogen was confirmed by isolation (as in the case of some control treatments). For this purpose, tissue was surface-decontaminated with 95% ethanol for 10 s, placed in 1·0% sodium hypochlorite for 2 min, then incubated on *Fusarium*-selective medium (Gordon *et al.*, 1998).

Field plot design

Trees were chosen from plots located within 2·8 km of the coast and at least 0·4 km from each other. Only trees with at least 10 branches of diameter 1·27 cm, that had not been used in other experiments, were chosen. Most plots contained 10 trees, although one experiment contained only five trees (in each of five plots) due to limited available trees that fulfilled the criteria.

Table 1 Dates of each trial for experiments on factors influencing wound infection of *Pinus radiata* by *Fusarium circinatum*

Experiment	Trial	Date
Wound size	1	18 January 2002
	2	12 March 2002
Field RH	1	10 July 2002
	2	27 August 2002
Field RH × time of inoculation	1	28 October 2002
	2	4 November 2002
Glasshouse RH × wound size	1	1 November 2002
	2	12 January 2003
Inoculum density	1	29 July 2002
	2	28 August 2002
Wound age	1	7 January 2003
	2	14 January 2003

Statistics

Data were analysed using SAS PROC MIXED (SAS Institute, Cary, NC, USA) unless the distribution of the residuals was not normal (determined *post hoc*), in which case data were analysed using nonparametric analyses. Degrees of freedom for *F*-tests of fixed effects were estimated using the Kenward–Rogers approximation. The likelihood-ratio χ^2 test (which tests the hypothesis that variation due to the random effect is >0) was used to test the random effects. Specific analyses and effects are listed under each experiment. Because the results were recorded as frequency data, the minimum and maximum limits were 0 and 100%, respectively. Bias-corrected, accelerated (BCa) bootstrap confidence intervals (CIs) were estimated using 5000 replications (Efron & Tibshirani, 1993) for all experiments except for experiment 6, in which exact CIs were calculated from the binomial distribution (Sokal & Rohlf, 2003). The 95% CIs are often asymmetrical because they reflect the distribution of the data.

Environmental data measurements

Data loggers were deployed to measure the RH and temperature in experiments in which RH was a factor. Mean RH and mean temperature for each RH experiment are recorded in Table 2.

Experiments

Experiment 1. Effect of wound size on infection

Inoculum was applied to branches as described above and wounds were created at 10 points per branch. Wounds were made using either a 1·6- or 0·5-mm drill bit to the depth of the xylem. The 0·5-mm drill bit approximates the diameter of the mandibles of twig beetles (*Pityophthorus* spp.) (0·48 ± 0·01 mm, $N = 25$ insects measured), an insect that has been demonstrated to be capable of creating wounds sufficient for infection under controlled conditions (Sakamoto, 2004). These two wounding regimes were also applied to noninoculated (negative control) branches. Each treatment was represented on one branch on each of 10 trees in the same stand, and replicated spatially in three plots, for a total of 30 treated trees. The experiment was conducted twice (Table 1). Branches were rated and infection frequency calculated 12 weeks after initiation in each case. Because residuals of data were not normally distributed, data were analysed with a nonparametric Mann–Whitney *U*-test (Sokal & Rohlf, 2003) on inoculated, wounded treatments. Confidence intervals (BCa 95%) were calculated as described above. Homogeneity of variance was confirmed using Levene's test ($F = 0{\cdot}03$, 1 df, $P = 0{\cdot}86$), and there was no significant trial effect ($\chi^2 = 0{\cdot}53$, 1 df, $P = 0{\cdot}46$); therefore data were pooled across trials.

Table 2 Ranges of temperature and relative humidity for experiments 2–4, assessing factors affecting infection frequency by *Fusarium circinatum* of *Pinus radiata*

Experiment	Trial	Treatment	Temperature		RH	
			Low (°C)	High (°C)	Low (%)	High (%)
2. Field RH	1	Ambient	7·9	20·8	79	100
		Humidification	7·9	19·0	100	100
	2	Ambient	13·3	23·5	73·8	98
		Humidification	10·5	19·2	100	100
3. Field RH × time of inoculation	1	Day[a]-Amb	6·6	19·8	56·8	95·9
		Day[a]-Humid	6·6	20·8	100	100
		Eve[b]-Amb	6·5	15·2	75	95·9
		Eve[b]-Humid	6·5	15·2	100	100
	2	Day[c]-Amb	5·7	22·1	66·8	100
		Day[c]-Humid	5·7	22·1	100	100
		Eve[d]-Amb	5·7	16·4	72·3	100
		Eve[d]-Humid	5·7	16·4	100	100
4. Glasshouse RH × wound size	1	Ambient	16·8	28·3	72	97
		Humidification	16·9	28·3	100	100
	2	Ambient	16·8	26·7	49·4	82·9
		Humidification	16·6	26·5	100	100

[a]10·00 h 28 October to 07·00 h 29 October 2002.
[b]17·00 h 28 October to 09·00 h 29 October 2002.
[c]11·30 h 4 November to 8·30 h 5 November 2002.
[d]18·00 h 4 November to 10·00 h 5 November 2002.

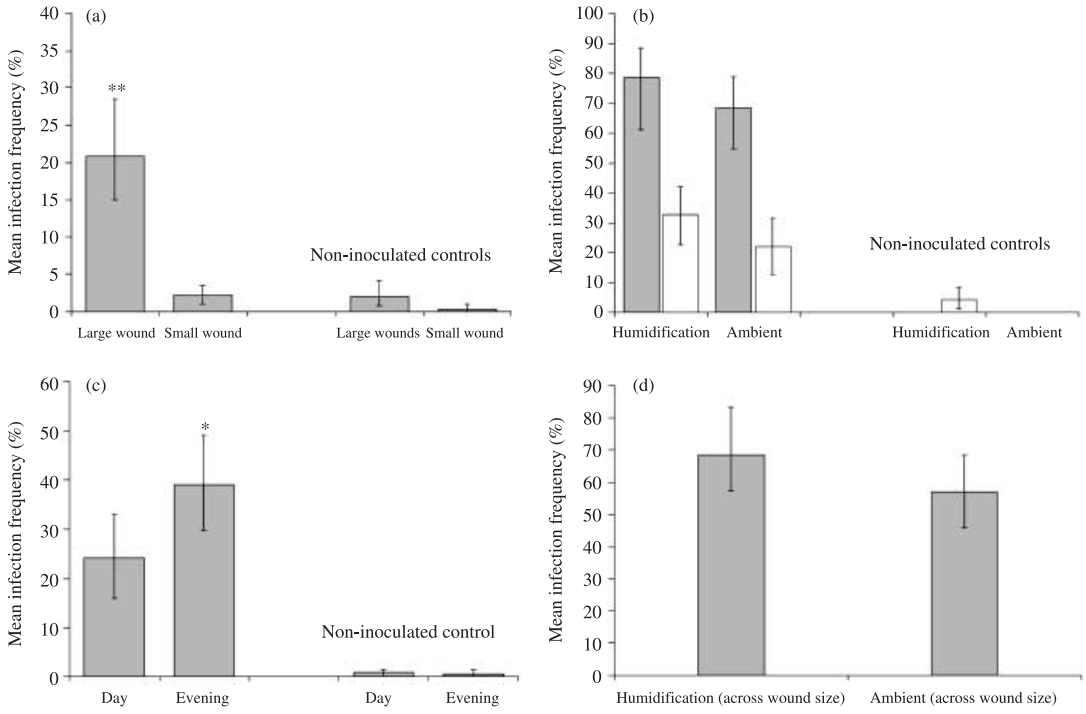

Figure 1 Experiments 1–4. Mean infection frequencies of branches of *Pinus radiata* sprayed with a *Fusarium circinatum* suspension of 1×10^7 spores mL^{-1} and (a) wounded with either a 1·6- or 0·5-mm drill bit under field conditions; (b) wounded with a 1·6-mm drill bit and subjected to humidification treatment or exposed to ambient field conditions; (c) wounded with a 1·6-mm drill bit and inoculated in either the day or evening under field conditions; or (d) wounded with either a 1·6- or 0·5-mm drill bit and subjected to humidification treatment or ambient glasshouse conditions. For (b) and (d) filled bars represent means for trial 1, open bars represent means for trial 2. Error bars represent 95% CI calculated using BCa bootstrapping procedure. **, $P < 0{\cdot}01$; *, $P < 0{\cdot}05$.

Large wounds had significantly higher infection frequencies than small wounds (Mann–Whitney $U = 5{\cdot}26$, 1 df, $P < 0{\cdot}01$) (Fig. 1a). Noninoculated control branches had low mean infection rates (Fig. 1a).

Experiment 2. Field relative humidity

Branches were sprayed with inoculum and wounded to the xylem with a 1·6-mm drill bit. Half the branches were sealed overnight (*c.* 16 h, from 17·00 to 9·00 h the following

Table 3 PROC MIXED random effects for experiments 2, 3 and 5, assessing factors affecting infection frequency by *Fusarium circinatum* of *Pinus radiata*

Experiment	Variable	df	χ^2	*P*
2. Field RH	Trial	1	41·5	<0·001***
	Tree(trial)	1	21·3	<0·001***
3. Field RH × time of inoculation	Trial	1	0	0·99 NS
	Tree(trial × time of inoculation)	1	13·4	<0·001***
5. Inoculum density	Trial	1	8·81	0·003**
	Tree(plot × trial)	1	13·15	<0·001***
	Plot(trial)	8	9·75	0·283 NS
	Trial × inoculum density	9	6·81	0·66 NS

***, $P < 0·001$; **, $P < 0·01$; *, $P < 0·05$; NS, not significant.

day) in plastic bags, onto the inner surface of which water had been sprayed. Bagged branches are hereafter referred to as the humidification treatment (Table 2). Each treatment was applied to one branch on each of 20 trees. The experiment was conducted twice (Table 1). Branches were rated and infection frequency was calculated 8 weeks after initiation. Data were analysed using SAS PROC MIXED. Humidification was treated as the fixed effect, while trial and tree(trial) were treated as random effects (Table 3). Confidence intervals (BCa 95%) were calculated as described above.

Humidified treatment branches were not significantly different from nonhumidified treatment branches ($F_{1,59}$ = 3·32, P = 0·073), although means for humidified treatments were higher than for nonhumidified (Fig. 1b). All noninoculated control branches remained uninfected, except for humidified, noninoculated branches in trial 2 (Fig. 1b).

Experiment 3. Field relative humidity × time of inoculation

Field experiments were conducted to test the interaction between humidification and time of inoculation (time of day) on infection. Branches were inoculated or not (negative controls), and wounded with a 1·6-mm drill bit. Half the branches were enclosed in plastic bags as described above. On one set of 10 trees, branches were inoculated during the day and covered for 21 h (Table 2). Branches on the second set of 10 trees were inoculated in the evening and covered with bags for 16 h (Table 2). The experiment was conducted twice (Table 1). Branches were rated and infection frequency calculated 10 weeks after initiation. Data were analysed using SAS PROC MIXED. Time of day and humidification were treated as fixed effects, while trial and tree(trial × time of day) were treated as random effects (Table 3). Confidence intervals (BCa 95%) were calculated as described above.

Humidification was not significant ($F_{1,77}$ = 0·67, P = 0·42) and treatments were homogeneous by Levene's test (F = 0·01, 1 df, P = 0·93), so data were pooled across humidification treatments in a second analysis looking at the effect of time of day. Branches inoculated during the evening treatment had significantly higher infection rates than those inoculated during the day ($F_{1,77}$ = 4·97, P = 0·028) (Fig. 1c). Noninoculated control branches had low mean infection rates (Fig. 1c).

Experiment 4. Glasshouse relative humidity × wound size

The combined effects of RH and wound size on infection were examined on seven 3- to 4-year-old *P. radiata* trees (*c.* 1 m high, main stem *c.* 2 cm diameter) in 9-L pots in a glasshouse. Inoculum was applied to all branches and, after drying, branches were wounded with either 0·5- or 1·6-mm drill bits. Half the branches from each wounding regime were humidified as described above (Table 2). Bags were removed after 12 h. The experiment was conducted twice (Table 1). Branches were rated for infection frequency 5–6 weeks after inoculation.

Because residuals for the data were not normal, a two-way nonparametric ANOVA [Scheirer–Ray–Hare (SRH) extension of the Kruskal–Wallis test (Sokal & Rohlf, 2003)] was used to test the significance of the effects on infection frequency of humidification, wound size, and their interaction. BCa confidence intervals were calculated as described above.

No significant effects were detected for wound size (trial 1: SRH χ^2 = 3·5, 1 df, P = 0·062; trial 2: SRH χ^2 = 0·084, 1 df, P = 0·77); humidification (trial 1: SRH χ^2 = 1·9, 1 df, P = 0·17; trial 2: SRH χ^2 = 2·0, 1 df, P = 0·15); or their interaction (trial 1: SRH χ^2 = 0·68, 1 df, P = 0·41; trial 2: SRH χ^2 = 0·0052, 1 df, P = 0·94). Across wound sizes, mean infection frequencies were higher for humidified, inoculated branches than for nonhumidified, inoculated branches, but the difference was not significant (Fig. 1d).

Experiment 5. Effect of inoculum density on infection frequency

To determine the effect on infection frequency of inoculum density, branches were sprayed with an aqueous suspension of (i) 1×10^7 or (ii) 5×10^7 spores mL^{-1} and allowed to dry fully; or (iii) left unsprayed as negative controls. Branches were wounded as described above with a 1·6-mm drill bit. Each treatment was represented on one branch on each of 25 trees, separated spatially into five plots of five trees each. The experiment was conducted twice (Table 1). Branches were rated, and infection frequency was calculated 9–10 weeks after inoculation. Data were analysed using SAS MIXED and BCa 95% confidence intervals were calculated. Inoculum was treated as a fixed effect, while trial, plot(trial), and tree(plot × trial) were treated as random effects (Table 3).

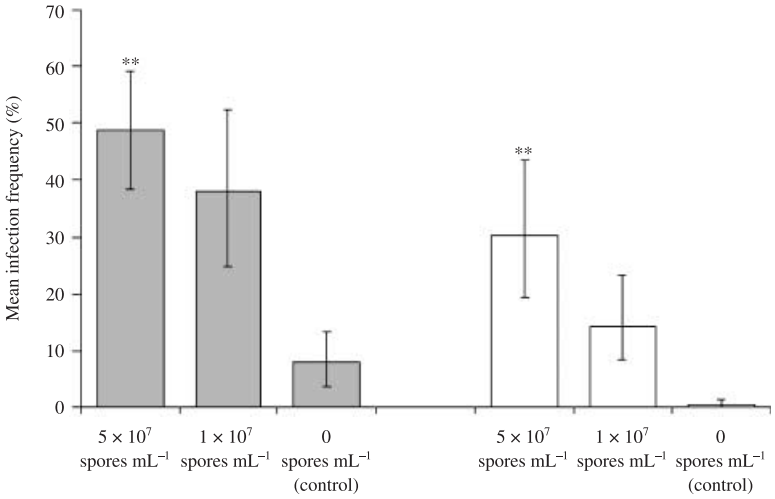

Figure 2 Mean infection frequencies of branches of *Pinus radiata* sprayed with a suspension of *Fusarium circinatum* either 5×10^7 or 1×10^7 spores mL^{-1}, and wounded with a 1·6-mm drill bit. Error bars represent 95% CI calculated using BCa bootstrapping procedure. Filled bars represent means for trial 1, open bars represent means for trial 2. **, $P < 0{\cdot}01$.

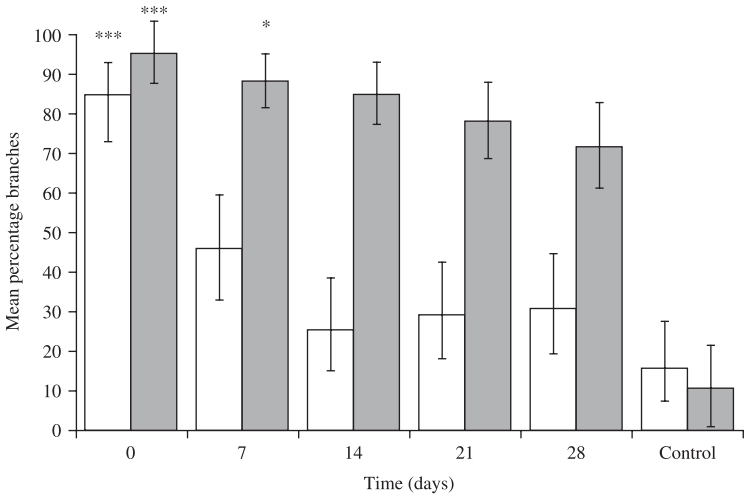

Figure 3 Mean percentage of branches with pitch canker symptoms (open bars) and mean percentage of branches with positive *Fusarium circinatum* recovery from branch terminals cut with surface-decontaminated pruning shears and sprayed to runoff with a suspension of 10^7 spores mL^{-1} at 0, 7, 14, 21 or 28 days after the initial cut ($N = 59$ per treatment) (filled bars). No inoculum was applied to control branches. Error bars represent exact CI calculated from the binomial distribution. ***, $P < 0{\cdot}001$; *, $P < 0{\cdot}05$.

There was a significant difference in infection frequencies between high and low inoculum densities ($F_{1,49} = 9{\cdot}30$, $P = 0{\cdot}0037$) (Fig. 2). Unsprayed control branches had low infection frequencies for both trials (Fig. 2).

Experiment 6. Effect of wound age on infection of pruned branches

To examine the effect of wound age on infection frequency under field conditions, branches with diameters between 1·3 and 2·5 cm were cut with pruning shears, *c.* 2·5 cm distal to the first branch node, and sprayed to runoff with a suspension of 1×10^7 spores mL^{-1} at 0, 7, 14, 21 or 28 days after the wound was made. Noninoculated branches served as negative controls. Each treatment was represented once on each of 29 trees in the first trial (one tree was lost to storm damage), and 30 trees in the second trial (Table 1). Branches were rated for symptoms and for pathogen presence 10–12 weeks after inoculation. A binary logistic regression (Sokal & Rohlf, 2003) was used to analyse the effect of wound age on infection frequency and recovery of the pathogen. Exact CIs (95%) were calculated from the binomial distribution.

There was a significant negative correlation between age of wound and infection frequency (maximum likelihood $\chi^2 = 77{\cdot}84$, 5 df, $P < 0{\cdot}001$) (Fig. 3). Odds ratios of infected to noninfected were highest for branches sprayed immediately after being cut (day 0) compared with the control. Despite the lower infection frequency of older wounds, the pathogen was still recovered from all wound age classes (Fig. 3).

Discussion

Wounds created by insect activity in *P. radiata* tissue have been demonstrated experimentally to be susceptible to infection by *F. circinatum* (Sakamoto, 2004). The wound size experiments described here compared the likelihood of infection through the standard inoculation wound size (1·6 mm diameter) with those that might be created by a tunnelling or feeding insect (0·5 mm diameter). Large

wounds were infected at a significantly higher frequency than small wounds in field experiments, but not in glasshouse experiments. This difference in infection frequency might be explained by a more rapid host response to small rather than large wounds. In addition, the greater susceptible surface area associated with larger wounds may result in more spores in proximity to the infection court. This suggests that, while small wounds simulating those made by twig beetles do become infected at low levels, simple feeding wounds may be insufficient for infection in the presence of a natural inoculum load.

Furthermore, noninoculated wounds of either 1·6 or 0·5 mm diameter are not infected by naturally occurring inoculum. The pitch canker pathogen is known to require wounds in order to establish infection (Dwinell *et al*., 1985; Gordon *et al*., 2001). However, Correll *et al*. (1991) demonstrated that mechanical wounds left exposed to naturally occurring inoculum do not provide good infection courts in coastal California. The current study on wound sizes is consistent with this finding: mechanical wounds became infected at a very low rate in the absence of applied inoculum. Even where inoculum was applied prior to wounding, infection frequencies were rather low, possibly due to a reduction in spore viability during the drying process.

There was no significant effect of increased RH on infection frequency in either glasshouse or field experiments. The wounds made in these experiments completely penetrated the bark, and this may have allowed the pathogen sufficient access to water within the branch, reducing the influence of atmospheric moisture on the likelihood of infection. Wounds induced naturally by insects or other means may be more superficial, rendering the pathogen more dependent on external sources of moisture. Experiments utilizing implements that induce shallower wounds might allow for a test of this hypothesis. It is also possible that the ambient levels of RH that occurred during both glasshouse and field experiments were above the threshold where an effect on infection frequency would be observed. Future studies testing lower levels of RH might better detect an effect of humidity on infection frequency.

Temperature may also play an important role in the epidemiology of pitch canker. McDonald (1994) demonstrated that pitch canker lesion lengths were positively correlated with temperatures between 14 and 26°C, and lesions did not develop on trees inoculated and maintained at 10°C. The temperatures in glasshouse experiments were between 16·8 and 28·3°C, well within the permissive range for infection, but temperatures during field experiments often fell below 10°C, which may explain the low infection frequencies observed. However, the first field RH trial had a higher infection frequency than the second, despite the fact that the range of temperatures for the first trial was lower than the second. Thus there does not appear to be a simple relationship between ambient temperatures and infection frequencies in these field experiments.

Although these experiments did not reveal a significant effect of RH on infection frequency, the timing of the humidification treatment appeared to be important. When the humidification interval was initiated earlier (before noon rather than early evening, after 17·00 h), infection frequency was significantly lower. It is possible that inoculum applied during the day suffered reduced viability from greater exposure to UV, as daytime inoculations occurred during full sun in both trials. A time-course study would be needed to determine how long *F. circinatum* remains viable after application to a branch under sunny field conditions.

A limiting effect of inoculum is suggested by a significantly higher infection frequency when the spore suspension applied to branches prior to wounding contained 5×10^7 rather than 1×10^7 spores mL^{-1}. Further work is needed to determine the threshold concentration below which infection will not occur consistently. Naturally occurring inoculum loads in infested and pitch canker-free areas are unknown. This is an important consideration because the availability of inoculum on branch surfaces will influence the likelihood of insect-induced wounds becoming infected.

The data presented here suggest that susceptibility of pruning wounds to infection by *F. circinatum* decreases significantly with wound age. Freshly cut and 7-day-old wounds were the most frequently infected. Previous studies by Kuhlman (1987) showed a similar trend in inoculated seedlings of loblolly pine (*Pinus taeda*) and slash pine (*Pinus elliottii* var. *elliottii*). The phenomenon of decreasing susceptibility postwounding has also been well documented in other pathosystems (Ramos *et al*., 1975; Teviotdale, 1991; Xu *et al*., 1998).

While susceptibility decreased with wound age, *F. circinatum* was recovered at a fairly high frequency, even from symptomless branches. Presumably the pathogen had invaded the tissue to a limited extent, as material from the cut tissue was surface-disinfested prior to assay, as described in the methods. Perhaps the lack of water in older cuts prevents successful invasion by the pathogen. Alternatively, the increasing viscosity of pitch as it ages may act as a physical barrier that does not kill the pathogen, but prevents entry.

The results of this study do not explain satisfactorily the observed spatial variation in the incidence and severity of pitch canker. Although it seems plausible that wounds would be more likely to become infected near the coast, where fog would often limit evaporative demand, the experiments did not demonstrate a significant effect of atmospheric moisture on infection frequency. Future studies investigating the role of environmental conditions in limiting infection are needed to address this issue.

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