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Rates of pitch canker induced seedling mortality among *Pinus radiata* families varying in levels of genetic resistance to *Gibberella circinata* (anamorph *Fusarium circinatum*)

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Abstract

Pitch canker, caused by *Gibberella circinata* Nirenberg & O'Donnell, is a problem for pines in both native and planted stands. The aerial phase of the disease results in shoot and canopy dieback, whereas soil or seedborne inoculum can cause damping off of emerging seedlings. Based on the extent of lesion development on inoculated shoots, families of *Pinus radiata* have been shown to differ significantly in resistance to pitch canker. This study was undertaken to determine if these same families also differ in mortality caused by *G. circinata* at the seedling emergence stage. For this purpose, seeds treated with a suspension of *G. circinata* spores were planted in a greenhouse and rated for pitch canker induced mortality. Variation between families, in mortality of emerging seedlings, was significant but the observed variation was not significantly correlated with measures of resistance based on stem inoculation tests. This suggests that mechanisms limiting the development of stem lesions do not confer measurable resistance to the seedling phase of the disease and therefore that early exposure to the pathogen may compromise selection for resistance to pitch canker in stands of *Pinus radiata*.

Keywords: Monterey pine; Radiata pine; Disease resistance; Fungal pathogen; Forest pathology

1. Introduction

Diseases caused by plant pathogenic fungi are a natural feature of forest ecosystems. However, in the absence of human intervention, native stands often show little evidence of damage from indigenous pathogens. This is presumably due, at least in part, to long-term selection for compatible interactions between host and parasite. Thus, highly aggressive pathogens are compromised by diminished competitiveness of their preferred host, and relatively resistant host genotypes are likely to leave more progeny than their more susceptible kin. This type of balance is lacking where non-native pathogens are introduced, and hence the great damage associated with exotic pathogens such as *Cryphonectria parasitca* and *Cronartium ribicola*, the causal agents of chestnut blight (Anagnostakis, 1987) and white pine blister rust (van Mantgem, 2004), respectively. A more recent example is pitch canker, caused by *Gibberella circinata* Nirenberg & O'Donnell (anamorph *Fusarium circinatum*), which was discovered in California in 1986, where it caused extensive mortality of Monterey pines (*Pinus radiata*) (McCain et al., 1987).

The initial phases of an epidemic caused by an exotic pathogen may reveal a nearly complete lack of resistance in the host population. Where genetic resistance does occur, it is likely to be manifested at a low frequency so a mitigating effect on disease development will depend on an increase in frequency of resistant genotypes through natural selection. Such directional selection requires that individuals in a regenerating population are challenged by a pathogen after they have reached a developmental stage where resistance can be expressed. If plants are killed by a disease while they are too young for the physiological mechanisms responsible for resistance to be operative, the influence of natural selection on the overall susceptibility of the population will be diminished, if not negated entirely.

Resistance to pitch canker has been identified in *Pinus radiata* (Monterey pine or radiata pine) (Gordon et al., 1998; Storer et al., 1999), which is the principal host to pitch canker in

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California (Gordon et al., 2001). Resistant *P. radiata* phenotypes are characterized by a reduced rate of lesion development, relative to more susceptible individuals. A slower rate of pathogen growth increases the likelihood that an infection will be fully contained before a girdling lesion develops. In the absence of girdling, an infected branch will not die so there will be no symptoms of the disease. Thus, relatively resistant trees sustain less dieback than more susceptible trees. However, the pitch canker pathogen can also infect seedlings, which may exhibit both pre- and post-emergence damping off (Storer et al., 1998). The seedling phase of the disease is of importance to pine nurseries in the U.S. (Huang and Kuhlman, 1990; Gordon et al., 2001), Chile (Wingfield et al., 2002), South Africa (Viljoen and Wingfield, 1994), and Spain (Landeras et al., 2005). It has not been established whether or not any form of genetic resistance to pitch canker is manifest in seedlings. For this reason, the present study was undertaken to determine if families differing in susceptibility based on an assay of 1.5-year-old trees (Matheson et al., 2006) showed any differential mortality at the seedling emergence stage.

2. Materials and methods

2.1. Radiata pine families

Seed for our study was received from a radiata pine tree improvement program in New Zealand. Each family corresponds to seed collected from a single open-pollinated tree (treated as half-sibs). Each seed lot was divided, with a portion being grown out for use in a stem inoculation assay conducted at 1.5 years of age (Matheson et al., 2006), and another portion being used in assays conducted at the seedling emergence stage, as described below.

2.2. Artificial infestation of seed

Seeds from 118 families were immersed in 1% sodium hypochlorite for 2 min. They were then soaked overnight in sterile de-ionized water, vortexed 10 s in a suspension of 10^3 spores of *G. circinata* per ml of water and air-dried. The spore suspension was produced as described by Schmale and Gordon (2003) from a single virulent isolate of *G. circinata* originally recovered from an infected *P. radiata* tree on the Monterey Peninsula, California. This isolate, designated GL17, is available on request from the second author. Seeds were sown individually in a sterilized potting mix, which was a blend of 40% coarse sand, 20% sphagnum peat, 20% redwood compost and 20% pumice rock, amended with 1.78 kg each of dolomite, oyster shell and single super phosphate per M^3 of mix in 3.8 cm (diameter) planting cells. Non-infested seeds of each family were treated with sodium hypochlorite and soaked in water as described above, and sown in the same manner as infested seed to serve as negative controls. Water was supplied as needed by overhead misting, and emerging seedlings were monitored visually for symptoms of disease. Seedlings that appeared diseased were removed, surface sterilized by brief immersion in 70% ethanol followed by 30–90 s in 1% sodium hypochlorite (shorter treatments for smaller seedlings) and cultured on a selective medium (FSM). This medium was a modification of the one used by Correll et al. (1991) and was composed of 15.0 g peptone, 20.0 g agar, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g PCNB and 10 ml of a streptomycin sulfate stock solution (30 mg/ml) in 1.0 l of de-ionized water. Colonies growing from cultured seedlings were identified as *G. circinata* based on colony morphology on FSM and when necessary, morphology on carnation leaf agar as described by Gordon et al. (1996). Eight weeks after sowing, all remaining seedlings in the infested treatment were harvested and cultured as described above, to determine if they were infected with *G. circinata*.

Each experiment included 10 seeds from each family that were infested, and another 10 seeds that were not (control group); one seed per planting cell in each case. Estimates of rates of pre-emergent damping off in the infested seed treatment were made by comparing emergence rates of infested and control seed. For the analysis (described below), the number of seedlings killed was estimated as the number of diseased seedlings from which *G. circinata* was cultured plus the number estimated to have died prior to emergence. Families with low germination rates of control seed were excluded from the analysis. The experiment was conducted four times over a 1-year period, with each experiment being treated as a replicate block. Greenhouse temperatures ranged from: 16 to 39 °C, 15 to 28 °C, 17 to 31 °C; and 21 to 38 °C during replications 1–4, respectively.

2.3. Sowing of seed under naturally-infested needles

Based on rates of mortality observed in the experiments described above, the 30 *P. radiata* families with the lowest and highest mortality rates were selected for further evaluation. Within each group, seed lots were bulked (15 low and 15 high) for use in the experiments described below.

Pine needles were collected in proximity to pitch canker-infected trees in a native *P. radiata* stand on the Monterey Peninsula. Needles were either recently fallen from an infected tree or were dead or senescent but still attached below a resinous canker. Fallen needles were also collected in a non-infested stand of *P. radiata* for use in a control treatment, as described below. Needles were assayed by washing in sterile de-ionized water and plating of the washate on FSM. Colonies of *G. circinata* were counted after 5–7 days incubation at room temperature. Inoculum levels in the repeated experiments ranged from 975 to 4197 colony-forming units (CFUs) per gram of needles (measured on a fresh weight basis). In the planting tray, seeds were underlain by a 1 cm layer of needles mixed with a steam-sterilized field soil (Yolo sandy loam) and overlain by a 3 cm layer of needles. As a control, separate planting trays were set-up using needles collected in a non-infested stand. These needles were determined to carry only very low levels of pathogen inoculum (<15 CFUs per gram). Trays were irrigated by short periods of misting every 2–4 h depending on conditions. The experiments were terminated 6–7 weeks after planting. Seedling mortality rates reported are numbers of seedlings killed by *G. circinata* (confirmed by

recovery of the pathogen from seedlings cultured on FSM) out of the total number of seedlings emerged. Each experiment included four replicate trays of 40–60 seeds per treatment, and the experiment was conducted four times. Greenhouse temperatures ranged from: 22 to 37 °C, 21 to 40 °C, 22 to 32 °C; and 16 to 30 °C during replications 1–4, respectively.

2.4. Statistical analysis

Seedling mortality data were evaluated by mixed model analysis of variance using the PROC MIXED procedure of SAS (Version 9.1). To resolve issues of non-homogeneity of variance for lesion length between families, families were divided into two groups based on their variance. The analysis was then conducted separately on each group. Prior to the analysis, mortality data from inoculated seed were square root transformed, while mortality data for seedlings emerging through infested needles were arcsine square root transformed.

Analysis of lesion length data from stem inoculation tests was conducted as described by Matheson et al. (2006). It was assumed that families were half-sibs and that heritabilities could be estimated as follows:

$$h^2 = \frac{4V_f}{V_f} + V_p + V_e$$

in which V_f represents the family variance component, V_p represents the variance due to a 'plot' of five pots of the same seed lot in each replicate and V_e represents the residual variance.

The relationship between lesion length and seedling mortality was evaluated by correlation analysis using the PROC CORR procedure in SAS. This analysis included the mean values for 103 families that had sufficient data from each type of resistance assay.

3. Results

When exposed to *G. circinata* at the emergence stage (i.e., inoculum applied to seed), variation in seedling mortality between families of *P. radiata* was significant ($P = 0.039$), with family mean mortality rates ranging from 3.5 to 52%. Likewise, these same families showed considerable variation in lesion length when subjected to the stem inoculation test. Mean lesion lengths for the families ranged from 10.3 to 38.7 mm and heritability was estimated at 0.490 ± 0.077, indicating strong genetic control of the observed variation (Matheson et al., 2006). However, there was not a significant correlation (Pearson correlation coefficient = −0.00814, $P = 0.935$) between family means for seedling mortality and means for lesion lengths in the stem inoculation test (Fig. 1). Even when the 30 families with the most extreme seedling mortality rates (15 lowest and 15 highest) were considered, a *t*-test indicated the two groups did not differ significantly based on the stem inoculation test ($P = 0.799$).

Within the control group (non-infested seed), over all four replications, a total of 30 seedlings died. Isolations were attempted from all 30 seedlings, and *G. circinata* was recovered from only two. We attribute this very low rate of infection to chance contamination. The germination rate for non-infested seed was variable and ranged from 53 to 100% for the 118 families tested. Using this as an estimate of the percent germination of inoculated seed, we tested for a correlation with disease-induced mortality. Based on this test, germination rate was not predictive of seedling mortality (Pearson correlation coefficient = −0.11960, $P = 0.2289$).

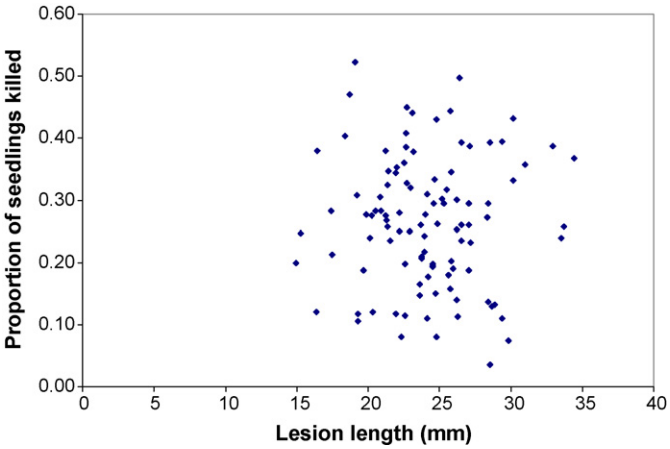

Fig. 1. Relationship between two measurements of susceptibility to pitch canker among families of *P. radiata*: seedling mortality vs. lesion length on 1.5-year-old saplings. Each point represents a single family, values plotted are family means adjusted for other variables in the model.

When seed from these two groups (highest and lowest seedling mortality) were bulked and sown under naturally-infested needles, there was a significant difference in mortality between those planted under highly-infested needles and those planted under needles with a low infestation level (Table 1).

Table 1
Gibberella circinata-induced mortality rates of seedlings emerging through pine needles with high or low infestation levels

Needle infestation level[a]	Seedling mortality group[b]	Trial 1	Trial 2	Trial 3	Trial 4
		Mortality (%)[c]			
High	High mortality	29.7	23.8	43.6	91.7
	Low mortality	12.7	29.5	73.3	93.4
Low	High mortality	0	0.3	1.3	0.2
	Low mortality	0.5	0	0.3	2.6
		P-value[d]			
Statistical significance	Mortality group	ns	ns	ns	ns
	Needle infestation level	0.0007	<0.0001	<0.0001	<0.0001
	Interaction	ns	ns	0.04	ns

[a] High level: colony-forming units (CFUs) per gram of needles of 1650, 975, 2250 and 4200 in trials 1–4, respectively; low level: less than 15 CFUs per gram of needles in all trials.

[b] Mortality groups were identified based on experiments in which inoculum was applied to seed (see text for details). The high and low groups represent bulked seed lots from each of 15 families, with the highest and lowest mortality rates, respectively.

[c] Values are the means of four replications, adjusted for the other variables in the model and back-transformed after analysis.

[d] ns: not statistically significant at $\alpha = 0.05$.

However, mortality rates did not differ between the two groups of families (Table 1).

4. Discussion

Gibberella circinata is a cause of stem and branch cankers in many pine species, and is also important as a cause of seedling mortality (Viljoen and Wingfield, 1994). Previous work on *P. radiata* has confirmed infection of seedlings from contaminated seed collected in native stands of this species (Storer et al., 1998). The seedling phase of the disease is a problem for commercial nurseries because of lost productivity and possible quarantine restrictions on shipment of plant materials. The latter concern is enhanced by the fact that an infected seedling can remain symptomless (Storer et al., 1998) and thereby serve as a vehicle for long distance transport of the pathogen. This means of dissemination appears to have contributed to the establishment of pitch canker in California (Gordon et al., 1996).

In California, pitch canker is not only a problem for nursery grown pines, but also affects both urban and native forests of *P. radiata*. Although most trees are susceptible, varying degrees of resistance in *P. radiata* have been demonstrated (Storer et al., 1999). Susceptibility to pitch canker appears to be genetically determined and therefore it is possible that the frequency of resistance may increase over time through natural selection. However, relative resistance based on mean lesion lengths induced in 1.5-year-old trees was not predictive of mortality rates resulting from exposure to *G. circinata* during or immediately after their emergence from soil or through infested leaf litter. Thus, it appears the resistance that is operative in older trees, is not expressed in emerging seedlings. That differences in seedling mortality rates between families were significant may imply that some other mechanism of resistance is operative at this developmental stage. Alternatively, mortality rates might simply vary according to differences in vigor between seed lots, but the lack of a significant correlation between germination rate (as a proxy for vigor) and mortality argues against this hypothesis.

Resistance to pitch canker that has been characterized in *P. radiata* appears to be quantitative, with a nearly continuous range of variation in lesion lengths (Gordon et al., 1998). Trees sustaining relatively short lesions are less likely to suffer branch girdling and hence should have less canopy die-back than more susceptible trees. However, even trees that appear highly resistant by this measure typically allow some growth of the pathogen before it is contained. Consequently, even if mechanisms that restrict pathogen development function at the seedling stage, the amount of growth that can be tolerated is very much less in a hypocotyl of perhaps 2 mm in diameter than in a stem or branch, which has a diameter several times greater.

If, as our results suggest, resistance to *G. circinata* is not operative in *P. radiata* seedlings, the effect of natural selection on susceptibility of native stands to pitch canker will be diminished. However, unless inoculum pressure is very high, some seedlings may escape infection and survive to an age when resistance to *G. circinata* is expressed. In this event, heritability of disease resistance will be a critical determinant of the rate at which the frequency of resistant phenotypes increases over time.

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