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Systemic induced resistance in Monterey pine

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Summary

The pathogenic fungus *Fusarium circinatum* causes pitch canker of pines. This study shows that Monterey pine (*Pinus radiata*), one of the most economically important pine species in the world and the main host in California, responds to infection by *Fusarium circinatum* in a manner consistent with systemic induced resistance. Repeated mechanical inoculations of the same trees in the field produced progressively smaller lesions over a period of 2 years, with mean lesion length decreasing significantly from 2.89 ± 0.42 cm to 1.04 ± 0.17 cm. In the greenhouse, predisposing inoculations with the pathogen induced a significant lesion length reduction, from 5.5 ± 0.21 cm in control trees to 4.46 ± 0.36 cm in predisposed trees over a period of 6 weeks. Under constant environmental conditions in a growth chamber, predisposing inoculations also induced a significant reduction in lesion size, from 3.01 ± 0.15 cm to 2.55 ± 0.18 cm over a period of 4 weeks. This is the first unequivocal report of systemic induced resistance in a conifer.

1 Introduction

Plants rely on both constitutive and induced defensive mechanisms for protection against invasive microorganisms. One induced defensive mechanism is systemic acquired resistance (SAR), a phenomenon in which plants inoculated with pathogenic organisms exhibit enhanced resistance to subsequent challenges with the same or different pathogens (STICHER et al. 1997). According to this descriptive definition, this phenomenon has been demonstrated in many angiosperm plants, including major crop species and their associated bacteria, viruses, fungi, and nematodes (OGALLO and MCCLURE 1996; STICHER et al. 1997). Recently, SAR has become synonymous with salicylic acid-mediated induced resistance, in which pathogenesis related (PR) proteins are the end products of the host response transduction pathways (VAN LOON et al. 1998). This differentiation of SAR from other types of induced resistance (e.g. ISR – VAN LOON et al. 1998) is based on a few angiosperm model systems and has been the source of some confusion (see editors' note on terminology in AGRAWAL et al. 1999). Consequently, throughout this paper, infection by a pathogen resulting in greater resistance to subsequent challenge by that pathogen will be referred to as systemic induced resistance (SIR), which implies nothing about the underlying mechanism.

In this paper we show that *Pinus radiata* D. Don (Monterey pine, or radiata pine) responds to infection by the plant pathogenic fungus, *Fusarium circinatum* Nirenberg and O'Donnell (ex *F. subglutinans* (Wollenw. and Reinking) Nelson f.sp. *pini* Correll et al.), the cause of pitch canker, in a manner consistent with SIR.

Pitch canker, a destructive disease of *Pinus* spp., is a chronic problem in plantations in the south-eastern US, and is currently causing extensive mortality in Monterey pine, its

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principal host in the western US (STORER et al. 1997). The disease is of great concern for its potential to interfere with commercial production of Monterey pine in the southern hemisphere, where this species is grown on 4 million hectares (BALOCCHI et al. 1999). Although most trees are susceptible, the native populations of Monterey pine show a wide range of variation in their response to the pathogen (STORER et al. 1999). This variation can be quantified by measuring the length of the lesion that develops following mechanical inoculation with the fungal pathogen (GORDON et al. 1998). In addition to demonstrating the existence of this natural variation in resistance, two field studies (GORDON et al. 1998; STORER et al. 1999) indicated that repeated, widely spaced inoculations of the same trees lead to progressively smaller lesions, suggesting a systemically inducible mechanism of resistance in this pathosystem. In light of this, we re-analysed the data from GORDON et al. (1998) to more directly assess the impact of repeated inoculations on the susceptibility of Monterey pine to subsequent infections by *F. circinatum*. That study was not designed to test for the occurrence of SIR, so it did not include control inoculations on each date of trees not previously inoculated. Consequently, possible effects resulting from differences in environmental factors between dates could not be separated from effects of prior inoculations. For this reason, we proceeded to test the hypothesis that SIR was operative in Monterey pine through additional experimentation using genetically identical trees under controlled conditions.

2 Materials and methods

2.1 General inoculation and disease assessment techniques

For all the following experiments, trees were inoculated with a known pathogenic strain of *F. circinatum* using the procedures described by GORDON et al. (1998). Briefly, small wounds deep enough to reach the sapwood were made on branches of young trees using a drill bit (1.6 mm diameter), and 5 μl of an aqueous spore suspension were deposited therein. Spores were collected from 7- to 10-day-old PDA cultures of *F. circinatum* by flooding the plates with sterile water and spore concentrations were adjusted to 50–250 spores per inoculation (depending on the experiment) using a haemocytometer. After incubation, the inoculated branches were excised from the trees and disease susceptibility estimated by measuring the size of the sapwood lesion after removal of the outer bark.

2.2 Review of field data (GORDON et al. 1998)

The data were obtained from trees of known susceptibility, which were planted out on public land in Santa Cruz County, California, in 1993, at which time they were approximately 2 years old. Thirty trees were inoculated between 1995 and 1997, on branches selected at random; all trees were inoculated at least once, but 10 of them were inoculated on four different occasions (July 1995, Nov. 1995, May 1996 and March 1997) with either 50 or 250 spores per inoculation. These trees were used in the data review. Disease susceptibility was estimated 40–70 days post-inoculation. The significance of any trend in lesion size development over time was tested using analysis of variance (ANOVA) incorporating a linear contrast.

2.3 Greenhouse experiment

The test trees were 4-year-old cuttings (ramets) derived from mother plants (genets) selected from a native stand of regenerating Monterey pine in the Monterey peninsula,

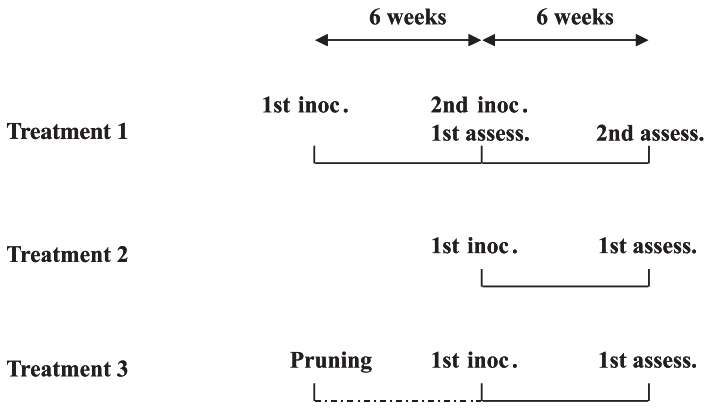

Fig. 1. Design of greenhouse experiment. One ramet from each of eight genets of *Pinus radiata* was assigned to each of the three treatments. Differences across treatments (i.e. between trees) were tested at the end of the experiment, based on lesion lengths following either the second inoculation in treatment 1, or the first inoculation in treatments 2 and 3. (See text for details.)

California, and used in previous experiments (STORER et al. 1999). The mother plants used in this study displayed a range of susceptibilities to the pathogen, but none were considered resistant. Eight genotypes, represented by three clones each, were used. Within each genotype, each clone was assigned to one of three treatments, as illustrated in Fig. 1. All inoculations were performed on three branches per tree, using 250 spores per inoculation. Branches were selected at random within the crown, with no preference with respect to height. As a control, each tree also received one water mock inoculation at the same time as the pathogenic inoculations. Treatments 1 and 2 (Fig. 1) tested for an effect of prior inoculation on lesion development resulting from a second set of inoculations. Because lesion length was always assessed from cut branches, treatment 3 was a control to test the effect of prior branch removal on the final lesion length. Comparisons across treatments (i.e. between trees) were based on lesion lengths measured 12 weeks after the first inoculation in treatment 1. Differences between treatments were tested using ANOVA, with orthogonal contrasts to compare treatments.

2.4 Growth chamber experiment

This experiment was conducted in a growth chamber with constant temperature, humidity and photoperiod regimes (temperature: 25°C day, 18°C night; relative humidity: 75% constantly; 12 h photoperiod – PAR intensity at tree top: 570 mol m^{-2} s^{-1}).

Trees from five of the same genotypes used in the greenhouse experiment were transferred to a growth chamber 2 weeks before the start of the experiment for acclimation. Each genotype was represented by one to three clones, for a total of 10 plants. The trees were inoculated in the same way as in the greenhouse experiment on 4 November 1998, and again after 4 weeks on 2 December 1998. Thus, 30 branches were inoculated with the pathogen, and 10 were mock inoculated with water, on each date. Differences in mean lesion length between the two dates (i.e. within trees) were tested using ANOVA.

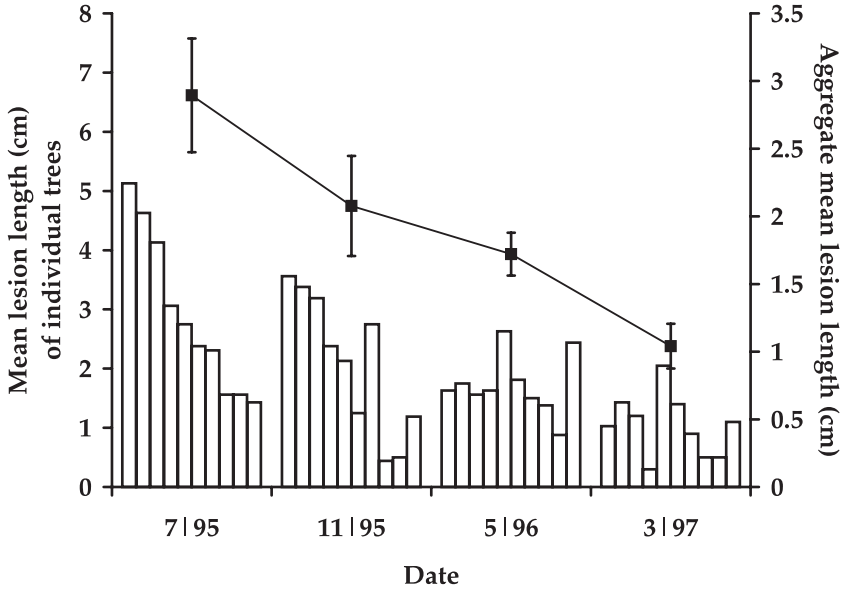

Fig. 2. Lesion lengths from field-inoculated Monterey pines. Mean lesion lengths on 10 individual trees (bars), and aggregate mean lesion lengths of all trees on each date (line). Error bars on individual trees were omitted for clarity. Bars on line are standard errors. Ordering of trees within dates was determined by lesion lengths on the first date. The left and right *Y* axes were assigned different scales to separate the bar chart from the line chart, respectively

3 Results

3.1 Review of field data

For the first inoculation, the aggregate mean lesion length from the 10 trees was 2.89 ± 0.42 cm. The mean lesion length following the fourth and final inoculation was 1.04 ± 0.17 cm, a 68% decrease, relative to the first inoculation. ANOVA showed a significant effect of date of inoculation on lesion length ($F = 6.58$; d.f. 3, 36; $p = 0.001$) and lesion length declined consistently over the four dates (linear contrast: $p < 0.001$) (Fig. 2).

3.2 Greenhouse experiment

The water inoculations never produced any lesions, and were therefore excluded from the analysis. Treatment was a significant factor in lesion development at the end of the experiment ($F = 3.35$; d.f. 2, 63; $p = 0.042$). The mean lesion length from the induced trees in treatment 1 was 4.46 ± 0.36 cm, which was 18.8% smaller than the average lesion from treatments 2 and 3 combined (5.53 ± 0.21 cm) (Fig. 3). Orthogonal contrasts between treatments revealed that treatment 1 was significantly different from treatments 2 and 3 combined ($p = 0.013$), but that treatments 2 and 3 were statistically indistinguishable ($p = 0.704$).

3.3 Growth chamber experiment

Mean lesion length on the second assessment date was 2.55 ± 0.18 cm, which was 15.5% smaller than at the first assessment 4 weeks earlier (3.01 ± 0.15 cm) (Fig. 4). This difference was statistically significant ($F = 4.16$; d.f. 1, 51; $p = 0.047$).

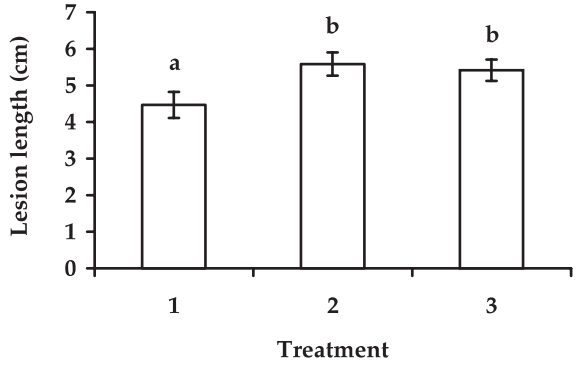

Fig. 3. Lesion lengths from the greenhouse experiment. Trees with a history of prior infection (treatment 1) developed smaller lesions ($p < 0.05$) than trees with no history of prior infection (treatment 2) or trees that were only pruned before the challenge infection (treatment 3). Bars are standard errors. For a full description of the treatments see text and Figure 1

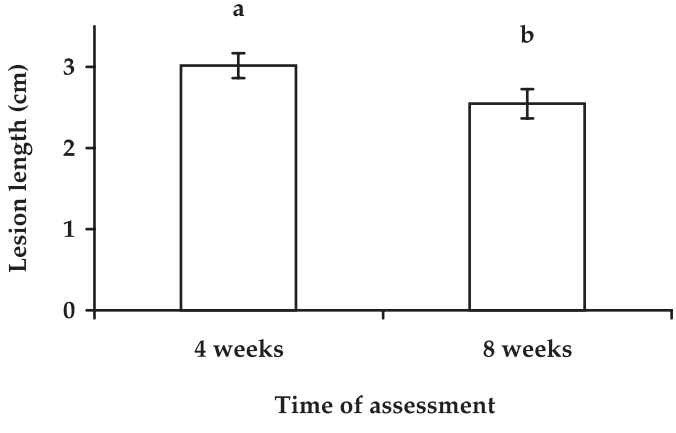

Fig. 4. Lesion lengths from the growth chamber experiment. Trees with a history of prior infection developed smaller lesions in response to subsequent inoculations ($p < 0.05$). Bars are standard errors

4 Discussion

The results of the field experiment reviewed here indicate that lesion size decreased as a result of multiple inoculations over time. A similar trend was also evident in previous work conducted on 40 trees in native stands of Monterey pine on the Monterey peninsula (Monterey County, California). In those trees, lesion length decreased over the course of three inoculations in less than a year (STORER et al. 1999). Thus, results from two independent field studies are strongly suggestive of a systemic effect of prior inoculations on Monterey pine's susceptibility to pitch canker.

The results of these experiments strongly support the occurrence of SIR in Monterey pine, the first time this phenomenon has been demonstrated in a conifer. The pitch canker pathosystem is well suited for studying SIR because disease development can be quantified precisely using mechanical inoculations, which have been proven to be highly predictive of the response of different host genotypes to the disease in the field (GORDON et al. 1998).

Other studies provide evidence suggestive of induced resistance in tree species, particularly in angiosperms. HELTON and BRAUN (1971) demonstrated SIR in *Prunus domestica* infected with the canker fungus, *Cytospora cincta*. A number of investigations explored the possibility of inducing resistance in elm trees threatened by Dutch elm disease, caused by *Ophiostoma ulmi sensu lato*, with variable results in terms of inducing agents (bacteria and fungi), range of effects, and applicability to disease management (JENG et al. 1983; MYERS and STROBEL 1983; SCHEFFER 1983; SHI and BRASIER 1986; SCHEFFER 1989; SCHEFFER 1990; SUTHERLAND et al. 1995).

In conifers, recent examples of localized induced resistance have been reported in Norway spruce against bark beetle-associated pathogenic fungi (CHRISTIANSEN et al. 1999; KROKENE et al. 1999). However, these authors did not observe systemic induced resistance in their pathosystems. Localized induced protection was also demonstrated in roots of Douglas-fir seedlings, where prior infection with an ectomycorrhizal fungus, *Laccaria laccata*, afforded protection against subsequent lethal root rot caused by *Fusarium oxysporum*, apparently by inducing accumulation of phenolics in the roots (SYLVIA and SINCLAIR 1983). REGLINSKI et al. (1998) reported induction of Monterey pine resistance to the fungal pathogen *Sphaeropsis sapinea*, which causes shoot blight, crown wilt, canker and sap stain on a number of conifer species. In this case, resistance was induced chemically by spraying the trees to run-off with salicylic acid derivatives, which are known chemical inducers of plant resistance (STICHER et al. 1997). Thus, the effect was not necessarily systemic, as the whole tree crown was treated. A case of induction of systemic protection against fusiform rust (caused by *Cronartium quercuum* f.sp. *fusiforme*) in loblolly pine (*Pinus taeda*) seedlings was reported by ENEBAK and CAREY (1998), but this involved the use of non-invading plant growth-promoting rhizobacteria as inducing agents, a case of induced systemic resistance (ISR) (VAN LOON et al. 1998). Finally, an *in vitro* investigation showed that resistance of Scots pine seedlings to the foliar pathogen *Botrytis cinerea* was induced systemically by infection of the root system with the mycorrhizal fungus *Hebeloma crustuliniforme* (P. BONELLO and R. PEARCE, unpublished).

Pitch canker may now serve as a good model system for studying SIR in trees. The importance of this system follows from the value of conifers as dominant species in many northern temperate forests and their role as a key renewable resource. Monterey pine, in particular, is one of the most economically important conifer species in the world, with nearly 4 million hectares planted world-wide (BALOCCHI et al. 1999).

Furthermore, because some of the biosynthetic pathways involved in plant defence in conifers may be different from those in model annual crop plants (e.g. lignin synthesis – STRACK 1997), where SIR has mostly been studied, characterization of the biochemical basis for SIR in pines may provide new insights into the generality of this phenomenon.

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Résumé

Résistance systémique induite chez le Pinus radiata

Fusarium circinatum est l'agent causal du 'pitch canker' des pins. Le *Pinus radiata* est l'un des pins les plus importants économiquement dans le monde, et le principal hôte de la maladie en Californie. Cette étude montre que *P. radiata* réagit régulièrement à l'infection de *Fusarium circinatum* d'une façon qui peut être de la résistance systémique induite. Des inoculations mécaniques répétées sur de mêmes arbres en nature ont produit progressivement des lésions dont la taille diminuait au cours d'une période de deux ans; la longueur des lésions diminuait significativement de 2.89 ± 0.42 cm à 1.04 ± 0.17 cm. En

serre, des inoculations de pré-conditionnement avec le parasite ont entraîné une réduction significative de la longueur des lésions, de 5.5 ± 0.21 cm chez les témoins à 4.46 ± 0.36 cm chez les arbres pré-conditionnés, au cours d'une période de six semaines. En conditions environnementales constantes en chambre climatique, les inoculations de pré-conditionnement ont aussi induit une réduction significative de la taille des lésions, de 3.01 ± 0.15 cm à 2.55 ± 0.18 cm, en une période de 4 semaines. Ceci est la première mention non équivoque d'une résistance systémique induite chez un conifère.

Zusammenfassung

Induzierte systemische Resistenz in Monterey-Kiefer

Der pathogene Pilz *Fusarium circinatum* verursacht einen Krebs an Kiefern (pitch canker). Die vorliegende Untersuchung zeigt, dass die Monterey-Kiefer (*Pinus radiata*), eine der ökonomisch wichtigsten Kiefernarten der Welt und Hauptwirt in Kalifornien, auf Infektionen durch *Fusarium circinatum* mit induzierter systemischer Resistenz reagiert. Wiederholte mechanische Inokulationen der gleichen Bäume im Feld führten zu zunehmend kleineren Läsionen über einen Beobachtungszeitraum von zwei Jahren. Die Länge der Läsionen nahm von 2.89 ± 0.42 cm auf 1.04 ± 0.17 cm signifikant ab. Im Gewächshaus bewirkten prädisponierende Inokulationen mit dem Pathogen eine signifikante Reduktion der Läsionslänge von 5.5 ± 0.21 cm in den Kontrollen auf 4.46 ± 0.36 cm in den prädisponierten Pflanzen über einen Beobachtungszeitraum von sechs Wochen. Prädisponierende Inokulationen bewirkten auch unter konstanten Umweltbedingungen in einer Klimakammer eine signifikante Abnahme der Läsionsgrösse von 3.01 ± 0.15 cm auf 2.55 ± 0.18 über eine Periode von vier Wochen. Es handelt sich hier um den ersten eindeutigen Nachweis von induzierter systemischer Resistenz bei einer Konifere.

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