CHITOSAN INDUCES RESISTANCE TO PITCH CANKER IN PINUS RADIATA

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(Received for publication 1 March 2004; revision 17 May 2004)

ABSTRACT

Foliar application of chitosan on Pinus radiata D.Don seedlings resulted in the induction of resistance to wound-inoculation with Fusarium circinatum Nirenberg & O’Donnell, the causal agent of pitch canker. Induced resistance was expressed as the suppression of symptom development on treated seedlings compared to the controls. Chitosan treatment protected seedlings against wound inoculation with ca100 spores per plant, and reduced disease incidence by 60% and deadtop development by 50%, compared to water-treated controls. However, chitosan efficacy was inoculum-dose dependent and no significant disease control was observed when treated seedlings were challenged with 500 or 8500 spores. At lower inoculum levels (10 spores/seedling) chitosan-treated seedlings remained more resistant to inoculation for at least 6 weeks after application. Furthermore, chitosan-induced resistance was shown to be systemic on 4-year-old P. radiata trees when chitosan application and inoculation were spatially separated. Due to the quarantine status of F. circinatum in New Zealand, inoculations were performed using Sphaeropsis sapinea (Fr.) Dyko & B.Sutton for this aspect of the study. Localised chitosan application induced an 86% reduction in average lesion length on adjacent untreated branches compared to their water-treated counterparts. These results demonstrate that chitosan can induce systemic disease resistance in P. radiata and there is potential for induced resistance in forest nursery disease management.

Keywords: induced resistance; chitosan; pitch canker; Pinus radiata; Fusarium circinatum; Sphaeropsis sapinea.

INTRODUCTION

Pitch canker disease of pines, caused by Fusarium circinatum, has proved to be extremely destructive to natural stands and amenity plantings of Pinus radiata in California (Correll et al. 1991; Storer et al. 1994, 2002), and has been devastating in pine-growing nurseries in a number of countries (Viljoen et al. 1994; Wingfield et al. 2002). It is potentially an extremely serious disease for plantation forestry in New Zealand (Dick
1998). Even in nurseries, conventional disease control strategies have not been totally effective against pitch canker (M.J. Wingfield pers. comm.). Whilst selection for resistance may be viable in the long term (Gordon et al. 1998; Storer et al. 1999), it can provide no protection for trees currently growing in plantations or nurseries. Alternative strategies to reduce disease incidence and spread in the event of an incursion of *F. circinatum* into New Zealand are therefore being sought.

There is increasing interest in the practical application of elicitors for crop protection (Lyon & Newton 1999; Tally et al. 1999). Elicitors function by stimulating plant defences, thereby accelerating and intensifying the resistance response to subsequent infections (Hammerschmidt 1999). Various elicitors, including salicylic acid, jasmonic acid, as well as several carbohydrates, lipids, proteins, and glycoproteins (Lyon et al. 1995; Hahn 1996), have been identified. Furthermore, topical application of elicitors has been reported to induce resistance against fungal, bacterial, and viral pathogens in several economically important crops (Kuc 1987; Tuzun & Kloeper 1995; Oostendorp et al. 2001).

Oligosaccharides from fungal cell walls — such as glucan, chitin, and chitosan — are naturally released at the plant/pathogen interface during the infection process, and are amongst the most potent elicitors known (Cote & Hahn 1994). Crude fungal wall extracts have been shown to elicit defence-related mechanisms in suspension cultures of *Pinus banksiana* Lambert (Campbell & Ellis 1992a, b) and *P. radiata* (Hotter 1997). Similarly, chitosan elicited phenylpropanoid and lignin biosynthesis in *Pinus elliottii* Engelmann (Lesney 1989; Mason & Davis 1997) and also stimulated monoterpene synthesis at wound sites in *Pinus contorta* Douglas (Miller et al. 1986; Croteau et al. 1987; Lieutier & Berryman 1988). These important defensive responses in conifers are involved with countering pathogen attack and sealing wounds. Chitosan has also been shown to be directly inhibitory to the growth of forestry root-rot pathogens, including *Fusarium oxysporum* Schlectendahl and *F. acuminatum* Ellis & Everhart (Laflamme et al. 1999) and has been investigated as a possible antifungal treatment for wood (Kumagai et al. 1990).

There are very few reports demonstrating the use of elicitors to protect conifers against subsequent pathogenic infection. Foliar application of 5-chlorosalicylic acid (5CSA) caused a reduction in susceptibility of *P. radiata* seedlings to infection with *Sphaeropsis sapinea* (Reglinski et al. 1998). Similarly, exposure of Norway spruce (*Picea abies* (L.) Karsten) seedlings to methyl jasmonate for 3 days resulted in a reduction in the incidence of damping-off after inoculation with *Pythium ultimum* Trow (Kozlowski et al. 1999). In both examples the reduction in disease incidence was attributed to the induction of host defence mechanisms.

We investigated the potential for using chitosan to induce resistance in *P. radiata* seedlings against fungal infection. The two specific objectives were to determine the effectiveness of chitosan in protecting *P. radiata* seedlings against *F. circinatum*, and to establish whether the induced response was local or systemic.

**METHODS**

**Trial Location and Plant Material**

Experiments were conducted in the Forest Research Quarantine Facility or a shade house at Forest Research, Rotorua, New Zealand. *Pinus radiata* seed obtained from the
Forest Research nursery was stratified in water for 24 hours before being sown. For Experiment One, seedlings were grown in pots (170 × 170 mm) with 9–12 plants per pot. For Experiment Two, seedlings were grown in HIKO V93 trays (40 plant cells/tray). In each experiment the plants were reared in a glasshouse and transferred to the Quarantine Facility before inoculation. The trees used for Experiment Three were 4-year-old *P. radiata* shoot cuttings with a physiological age of approximately 7 years. These trees were too large for the Quarantine Facility and so, for biosecurity reasons, inoculations were made using *S. sapinea*.

### Chitosan Preparation

Chitosan (crab shell, minimum 85% deacetylated, Sigma Chemical Co., St Louis, Mo. USA) was ground to a powder and then 10 g were solubilised by stirring for 4 h in 500 ml of 1 M acetic acid. Insoluble material was removed by filtration through three layers of cheesecloth. The filtrate was then precipitated by neutralisation with 2 M NaOH and the chitosan recovered by centrifugation at 10 000 rpm for 20 minutes. The chitosan pellets were thoroughly washed with milliQ water to remove salts and then freeze dried. The freeze-dried powder was dissolved in 0.025 M acetic acid and the pH adjusted to 6 with 1 M NaOH to obtain a 1 mg/ml stock solution. The stock was diluted in water to obtain a chitosan concentration of 0.1 mg/ml and a pH of 5.5 for all experiments.

### Inoculum Preparation

*Fusarium circinatum* (NZFS 308B ex *P. radiata* Santa Cruz, California) was maintained on 3% w/v malt extract agar (MEA). Fresh cultures, incubated for 5 days at 25°C in the dark, were used for inoculum preparation. Spores were harvested by pouring 2 ml of sterile distilled water on to the culture, agitating the surface of the mycelium with a sterile spatula, and then collecting the resultant suspension. The concentration of spores was estimated using a haemocytometer and adjusted to the desired concentration (Tables 1 and 2) with sterile deionised water. Spore viability was routinely tested by carrying out a serial dilution, pipetting a standard amount from each dilution to 3% (w/v) MEA petri plates (three replicates per dilution), and counting the resultant colonies. *Sphaeropsis sapinea* (NZFS 897) was cultured on 3% w/v MEA. Mycelial plugs taken from 1-week-old *S. sapinea* cultures were used as inoculum.

### Seedling Treatment and Wound Inoculation

#### Experiment One

Six-month-old *P. radiata* seedlings were sprayed to run-off with either chitosan (0.1 mg/ml) or sterile water. Pulse® (a.i. 1.02 g/litre polyether modified polysiloxane, Monsanto, Wellington, New Zealand) at 0.05% (v/v) was included as a wetting agent/penetrant in each treatment. Treatments were applied using a pressurised sprayer with eight replicate pots containing 9–12 seedlings per pot for each treatment. Chitosan was applied on two occasions, 14 days and 7 days before inoculation. Water was applied 7 days before inoculation. When dry, the seedlings were transferred to the Quarantine Facility where the ambient temperature was 19–20°C with 16:8 h light:dark. Seedlings were inoculated by removing one needle fascicle, 2–5 cm from the apex of each seedling, and placing a 5-µl
droplet of *F. circinatum* spore suspension containing $2 \times 10^4$, $1 \times 10^5$, or $1.7 \times 10^6$ spores/ml on the wound site. This equated to approximately 100, 500, or 8500 spores per inoculation respectively. Plants were examined twice weekly for symptom development. The first indication of infection was a purplish oily lesion at the inoculation point. Infection was scored on each occasion in the following categories: (a) lesion length, (b) dead top — wilting and death of the crown, and (c) mortality — death of the seedling. Isolations were carried out from representative plants exhibiting various levels of dieback. Plants were harvested, surface sterilised in 10% hypochlorite, rinsed in sterile water, and then cut into segments and plated on to potato dextrose agar (PDA). Petri dishes were incubated at 22°C and examined after 7 days for fungal colonies.

**Experiment Two**

Three-month-old *P. radiata* seedlings were sprayed to run-off with either chitosan (0.1 mg/ml), thiabendazole at 30 g a.i./litre, or sterile water. Pulse at 0.05% was added to each treatment. There were 27 replicate plants per treatment. Chitosan was applied at either 6 weeks or 1 week prior to inoculation. Water and thiabendazole were each applied 1 week before inoculation. Seedlings were inoculated with *F. circinatum* suspensions containing $2 \times 10^3$ spores/ml (*ca* 10 spores per inoculation site) using the same techniques as described in Experiment One. Symptom development was recorded at regular intervals up to 50 days post-inoculation. Isolations from inoculated plants were carried out as described above.

**Experiment Three**

Experiment Three was carried out on 4-year-old *P. radiata* cuttings (physiological age of approximately 7 years) in the shade house. Chitosan (0.1 mg/ml) in 0.05% Pulse was applied with a hand sprayer to one single branch per tree on two occasions, 8 days prior to inoculation and again 1 day before inoculation. Control plants were similarly treated with 0.05% Pulse in water. There were 25 replicates per treatment. Inoculations were carried out on adjacent untreated branches of each tree by cutting a shallow wound (12–15 x 5–8 mm x 1 mm deep) on the upper side of the branches and then placing a 5-mm-diameter plug of *S. sapinea* mycelium on to the fresh wound. The plug was wrapped with moist cotton wool and held in place by a strip of plastic film. The inoculated branches were harvested 4 weeks later and the lengths of visible lesions were measured.

**Statistical Analysis**

Treatment effect on disease incidence and lesion size were determined by analysis of variance (ANOVA), with each seedling treated as an individual replicate. Differences between treatment means were separated by the Least Significant Difference (LSD) at $p>0.05$, $p>0.01$, and $p>0.001$. All statistical analyses were carried out using GENSTAT 5.

**RESULTS**

There was a significant reduction ($p<0.05$) in disease incidence on *P. radiata* seedlings that were treated with chitosan before wound inoculation with 100 *F. circinatum* spores per wound site (Table 1). At 21 days post inoculation, only 25% of chitosan-treated plants had developed disease symptoms compared to 63% of the water-treated control plants.
Furthermore, disease symptoms were less severe in treated plants than control plants, with dead tops occurring in 21% of chitosan-treated seedlings compared to 42% of water-treated seedlings. No significant treatment effect was observed on seedlings that were inoculated with 500 or 8500 spores per wound site.

In Experiment Two, chitosan treatment slowed down the onset and severity of disease whether applied 1 week or 6 weeks before inoculation (Table 2). Average lesion length was significantly smaller (p<0.05) on chitosan-treated seedlings than on untreated controls for the duration of the experiment. By 32 days post-inoculation, 82% of water-treated controls had developed dead tops compared to only 44% of the seedlings treated with chitosan 6 weeks earlier (p<0.05). Furthermore, by this phase of the trial 11% of water-treated controls had died compared to 0% mortality amongst the fungicide- and chitosan-treated seedlings. By 50 days post-inoculation, the mortality level had risen to 78% for water-treated controls compared with only 29–37% for chitosan-treated seedlings (p<0.01). There was 52% mortality amongst fungicide-treated seedlings but this was not significantly different to the control. In the *F. circinatum* studies the pathogen was isolated from plants expressing symptoms and confirmed as the causal agent; no other pathogenic fungi were isolated.

Localised chitosan application induced systemic resistance to wound inoculation with *S. sapinea* in 4-year-old *P. radiata* cuttings (Table 3). Average lesion lengths on branches that were untreated, but were adjacent to chitosan-treated branches on the same tree, were significantly (p<0.001) smaller (19.6 mm) than lesions on respective water-treated controls (49.6 mm). Isolations from infected plant material confirmed *S. sapinea* as the causal agent.

**DISCUSSION**

There is increasing interest in the practical implementation of induced resistance for disease management (Lyon & Newton 1999; Oostendorp *et al.* 2001; Tuzun & Kloepper 1995). In this study we demonstrated that foliar application of chitosan can induce resistance in *P. radiata*, and that the induced resistance response is systemic. Chitosan is a potent elicitor of defence mechanisms in conifers (Miller *et al.* 1986; Lieutier & Berryman...
<table>
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<tr>
<th></th>
<th>Incidence (%)</th>
<th>Average lesion length (mm)</th>
<th>Dead top (%)</th>
<th>Mortality (%)</th>
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<tr>
<td></td>
<td>15 days 32 days 50 days</td>
<td>15 days 32 days 50 days</td>
<td>15 days 32 days 50 days</td>
<td>15 days 32 days 50 days</td>
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<tr>
<td>Water</td>
<td>81 96 97</td>
<td>10 35 36</td>
<td>7 82 0</td>
<td>0 11 78</td>
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<td>Fungicide</td>
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<td>7* 28* 16*</td>
<td>15 66 0</td>
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<td>Chitosan 6 weeks pre-inoculation</td>
<td>52* 85 85</td>
<td>6* 25* 23*</td>
<td>15 44* 0</td>
<td>0 0 30*</td>
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<tr>
<td>Chitosan 1 week pre-inoculation</td>
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<td>6* 21* 27*</td>
<td>18 74 0</td>
<td>0 0 37*</td>
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<tr>
<td>LSD</td>
<td>26 17 16</td>
<td>1.3 8 8</td>
<td>19 26 0</td>
<td>0 10 27</td>
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* Significantly (p<0.05) better than untreated control.
Reglinski et al. — Chitosan and pitch canker

1988; Lesney 1989; Mason & Davis 1997) but, to our knowledge, it has not previously been shown to elicit systemic resistance to pathogens in coniferous species.

Induced resistance is generally characterised by a reduction in the size and incidence of lesions that develop on elicitor-treated plants after inoculation (Hammerschmidt 1999). Foliar application of chitosan reduced disease incidence on *P. radiata* seedlings inoculated with ca100 *F. circinatum* spores/wound, but did not elicit effective resistance in seedlings challenged with the higher inoculum levels (500 and 8500 spores/wound). This inoculum dose dependence may indicate the limitations of the induced host response. However, it is estimated that some twig beetle vectors (*Pityophthorus* spp.) carry less than 10 spores per individual (Gordon *et al.* 1998), and so this result may not necessarily reflect the potential for induced resistance in natural conditions. *Fusarium circinatum* had a devastating effect on container-grown pine seedlings in South Africa (Viljoen *et al.* 1994), and it was reported (M.J.Wingfield pers. comm.) that fungal mycelia spread rapidly across the soil to infect tender young plants. Chitosan efficacy in these circumstances, where the relevance of inoculum concentration is not known, remains to be determined.

Having established the ability of chitosan to induce resistance in *P. radiata* seedlings, our next goal was to investigate the duration of the induced response. For this study ca 10 *F. circinatum* spores were applied per wound site in order to reflect more natural inoculum levels, as discussed above. Previous studies on *P. radiata* indicated that treatment with 5-chlorosalicylic acid induced resistance to *S. sapinea* inoculation for up to 32 days after application (Reglinski *et al.* 1998). In this investigation, chitosan treatment enhanced resistance in *P. radiata* seedlings to *F. circinatum* inoculation for at least 42 days. Induced resistance was expressed as a reduction in disease severity rather than a reduction in disease incidence. Indeed, by 50 days post-inoculation there was no difference in disease incidence between treatments. However, average lesion length and seedling mortality were significantly lower in chitosan-treated seedlings than in water-treated controls.

The next objective of this study was to determine whether the chitosan-induced resistance response in *P. radiata* was systemic. The quarantine status of *F. circinatum* in New Zealand dictated that all experiments involving this pathogen should be carried out in a containment facility. However, since the 4-year-old trees selected for systemic studies could not be relocated to this facility we elected to challenge the treated trees with *S. sapinea* which shares similarities with *F. circinatum* in that it can infect through wounds in shoots and stems, and dieback may follow. The finding that chitosan application induced systemic resistance to *S. sapinea* in 4-year-old *P. radiata* trees is arguably the most interesting and exciting result in our investigation. To our knowledge this is the first report of foliar elicitor application resulting in the systemic induction of disease resistance in

<table>
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<th>Treatment</th>
<th>Average lesion length (mm)</th>
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<tr>
<td>Water control</td>
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<tr>
<td>Chitosan</td>
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<td>LSD</td>
<td>7.5</td>
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* Significantly (p<0.001) better than untreated control
coniferous trees. Induction of resistance to *S. sapinea* has previously been reported in SCSA-treated *P. radiata* (Reglinski et al. 1998), and to *Pythium ultimum* in methyl jasmonate-treated Norway spruce (Kozlowski et al. 1999). However, in each of these studies small seedlings were used and the whole plant was exposed to the elicitor, so negating the opportunity to investigate a systemic response.

Our data indicate that induced resistance may have potential as a disease control method in *Pinus radiata* seedlings and young trees. Furthermore, the finding that chitosan treatment elevated resistance to both *F. circinatum* and *S. sapinea* suggests that the induced response is not pathogen specific and therefore may offer broad-spectrum control. This may be a particularly attractive option for forest nurseries where elicitor application could be easily integrated with existing management practices. Further investigations are required to determine more accurately the onset and duration of the chitosan-induced response in *P. radiata*.

From an ecological perspective, induced resistance is arguably a more complex disease control option than the use of fungicides. This is because elicitor efficacy is reliant upon a physiological and biochemical response by the plant, and therefore is affected by climatic and agronomic factors that influence general plant health. For example, light is known to affect plant defence signalling (Karpinski et al. 2003) and so the induced resistance response of seedlings under glass or under artificial light may be different from that of those in the field. Furthermore, glasshouse-reared plants are generally more tender and more susceptible to infection than their field-grown counterparts. This may also affect elicitor-competence and partly explain the failure of chitosan to elicit an effective resistance response against high inoculum levels in these seedlings. A final note of caution is warranted since plant resistance against pathogens and insects is regulated by several interconnecting, and sometimes antagonistic, pathways (Feys & Parker 2000; Thaler et al. 2002). Pathogen-induced resistance has been shown to compromise subsequent resistance to insects and vice versa (Bostock et al. 2001) and so elicitation of resistance to fungi may predispose *P. radiata* to insect attack. Further experiments are required to confirm such issues before proceeding with implementation of elicitors on a large scale.

**ACKNOWLEDGMENTS**

Thanks to members of the Forest Health Group at Forest Research for technical assistance during part of this work. Also to Martin Upsdell (AgResearch, Ruakura Research Centre) for statistical analysis of the data. The Forest Health Research Collaborative funded this work and the authors are appreciative of contributions from City Forests Limited.

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