

**INDUCED RESISTANCE IN *PINUS RADIATA*
SEEDLINGS TO PINE PITCH CANKER**

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EXECUTIVE SUMMARY

A study was conducted to assess the potential for using elicitors to protect *Pinus radiata* seedlings against infection with *Fusarium circinatum*, the causal agent of pine pitch canker. Elicitor treatments, applied either six weeks or one week before inoculation, were compared with a commercial fungicide applied 1 week before inoculation. Treated plants were wounded then inoculated with either 10 spores or 100 spores at the wound site in order to mimic low and high disease pressure conditions. Elicitor treatment and fungicide treatment reduced the onset of disease development, as indicated by a reduction in incidence and lesion length. At low inoculum levels all of the treatments significantly reduced disease incidence and severity. At high inoculum levels there were no significant differences between treatments during the early stages of symptom development. However, by 50 days post inoculation it was apparent that there was a significant reduction in plant mortality on seedlings that had been treated with elicitor at 1 week before inoculation. These results confirm our previous findings that elicitor treatment can reduce the severity of infections caused by *F. circinatum*. There are indications that the plant response may be overcome by high inoculum pressure, particularly when the duration between treatment and inoculation extends to six weeks. Repeated elicitor applications may be advisable under high disease pressure conditions.

Recommendations

- Further testing of the efficacy of elicitors against pitch canker should be carried out overseas in locations where the fungus occurs naturally and there are fewer constraints to experimental design.
- Elicitors should be considered for their potential to suppress disease in *P. radiata* seedling nurseries. An assessment of the combined use of a fungicide and an elicitor treatment for disease control is particularly recommended. Such trials could be conducted in a nursery(ies) in New Zealand against the range of pine seedling diseases and concurrently against insect pests.

INDUCED RESISTANCE IN *PINUS RADIATA* SEEDLINGS TO PINE PITCH CANKER

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INTRODUCTION

Pitch canker disease of pines, caused by *Fusarium circinatum*, has proven extremely destructive to wild and amenity *Pinus radiata* stands in California, and is a potentially devastating disease to *P. radiata* plantation forestry in New Zealand. Conventional disease control strategies have proven ineffective against pitch canker and whilst selection for resistance appears viable in the long term (Gordon *et al.* 1998; Storer *et al.* 1999, 2001, 2002) it can provide no protection for trees currently growing in plantations or nurseries. Alternative strategies that might reduce disease incidence and spread in the event of an incursion of *F. circinatum* have therefore been sought and studied.

Scientists at HortResearch, Ruakura, have investigated the use of “elicitors” to control and manage fungal diseases in a number of plant hosts subject to serious disease, including *P. radiata*. Elicitors are compounds that function by accelerating and intensifying the plant’s natural resistance response to infection. Plant disease resistance typically relies upon the activation of various physical and chemical defences, including callose and lignin deposition, phytoalexin synthesis, the production of proteinase inhibitors and the formation of lytic enzymes such as glucanases and chitinases (Lyon *et al.* 1995). Disease occurs when the response is too slow rather than due to the absence of any particular defence mechanism. The topical use of elicitors to accelerate or intensify the resistance response, resulting in an enhanced disease resistance, has been demonstrated for several host/pathogen interactions (eg Kuc 1987; Bonnet *et al.* 1996; Reglinski *et al.* 1994, 1997, 1998). This has sometimes been called ‘induced resistance’ and it is proposed that elicitor treatment ‘sensitizes’ the plant to subsequent infection. Induced resistance against fungal, bacterial and viral pathogens has been demonstrated in several important crops worldwide, including cereals, legumes, solanaceous plants, trees and small fruits (Tuczun and Kloepper 1995).

Elicitors may have no direct anti-microbial activity or they may have a double mode of action, acting directly on a pathogen as well as eliciting host responses. In conifers elicitors have been shown to induce resistance mechanisms in suspension cultures of *P. elliotii* (Lesney 1989), *P. banksiana* (Campbell and Ellis 1992) and *P. radiata* (Hotter 1997), and at wounds in *P. contorta* (Miller *et al.*, 1986). Reglinski *et al.* (1998) demonstrated that treatment with 5-chlorosalicylic acid induced phenylalanine ammonia lyase (PAL) activity in *P. radiata* seedlings and protected the plants from subsequent infection by *Sphaeropsis sapinea* (syn. *Diplodia pinea*). This compound had antifungal activity in *in vitro* tests but this did not fully account for the prevention of disease development observed. Furthermore, plants were protected against fresh inoculation challenge for up to 32 days after application of the elicitor.

The potential for such methods to enhance resistance of *P. radiata* seedlings to *F. circinatum* was tested in studies carried out in the Quarantine Facility at the New Zealand Forest Research Institute (Reglinski and Dick 2001). Seedlings were challenged with a range of either 100, 500 or 8500 *F. circinatum* spores per inoculation. Elicitor-treated seedlings that were inoculated with 100 spores/wound exhibited reduced disease incidence and severity. Over 70% of the treated seedlings showed no disease symptoms and, where infection was apparent, the average lesion length was reduced compared to the controls. There was no significant treatment effect at inoculum levels greater than 500 spores/wound suggesting that the induced resistance response may have been overwhelmed. In previous studies on induced resistance to *S. sapinea* in

P. radiata it was shown that the induced response could be overcome by high inoculum levels (unpublished data).

It is considered unlikely that the highest level of inoculum tested in these experiments (8500 spores per wound site) would be experienced under natural conditions in the field. Most infections of *P. radiata* in California are vectored by insects, primarily by twig, cone and bark beetles. Spore loads carried by some of the twig beetle vectors (*Pityophthorus* spp.) have been estimated to be as low as <10 spores per individual (Gordon *et al.* 1998).

The preliminary results obtained were encouraging and indicate that elicitors can be used to induce resistance of *P. radiata* to the pitch canker fungus. However, the data also show that there are limitations to the efficacy of the elicitor treatment and that the induced response may be overwhelmed by extremely high inoculum levels. The aim of this further study was two-fold:

1. to verify and extend the results previously attained by challenging elicitor-treated seedlings with a lower spore concentration (closer to that experienced in the field).
2. investigate the onset and duration of the induced resistance response (i.e. vary the time between elicitor treatment and challenge inoculation).

MATERIALS AND METHODS

Location of trial. Experiments were carried out in the Quarantine Facility at Forest Research, Rotorua.

Plant material: The susceptibility to pitch canker of much of New Zealand's current breeding stock of *P. radiata* has been amply demonstrated through the extensive use of New Zealand seed for plantings throughout California (Dick 1998). Seed from a standard seedlot (GF 14) used throughout New Zealand were sown in HIKO V93 trays (40 plant cells/ tray) (Fig 1). The plants were reared in a glasshouse and transferred to the Quarantine Facility the day before inoculation. Plants were 3 months old when inoculated and of variable size, ranging from 35 to 120mm in height.

Preparation of inoculum: Three percent malt extract agar (MEA) plates were each seeded with ½ ml of spore suspension prepared from an isolate of *F. circinatum* (NZFS 308B ex *P. radiata* Santa Cruz, California). Plates were incubated for 5 days at 25°C in the dark. Spore suspensions were prepared by pouring 2ml of sterile distilled water onto each petri dish containing the fungus, 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 with sterile distilled water. Each concentration was checked using the haemocytometer, and spore viability tested by carrying out a serial dilution, pipetting a standard amount from each dilution to 3% MEA petri plates (3 replicates per dilution) and counting the resultant colonies. Two inoculum concentrations were selected; 2×10^3 and 2×10^2 spores/ml. equating to 100 and 10 spores per inoculum site.

Inoculation method: Two inoculation methods were used. It had been planned to inoculate all plants on the stem through a small wound attained by the removal of a fascicle. However many of the smaller plants carried only the primary leaves and not begun to form fascicles. Consequently these plants were inoculated at the apex; two needles were removed from the terminal rosette and a 5 µl droplet of spore suspension placed on the position. For the larger plants one needle fascicle was removed from the upper stem of each seedling approximately 15-20 mm from the apex and a 5 µl droplet of spore suspension placed on the wound site. The $2 \times$

10^3 and 2×10^2 spores/ml equated to 100 and 10 spores applied per inoculation site. A small mark was made on the adjacent needle with a marker pen so the inoculum point could be located. All the plants were inoculated. Immediately prior to inoculating the plants, 10 replicates of the 5 μ l inoculum droplet were dispensed, for each spore concentration, on to MEA petri dishes from the auto pipette.

Ambient temperature in the containment room ranged from 15 - 22°C with 16h /8h light/dark cycle. In order to ensure that there was no risk attached to testing *F. circinatum* all containment criteria set by ERMA New Zealand, as outlined in their decision to application no. NOC00003 (21 December 2000), and in the Ministry of Agriculture and Forestry approved quality manual for the operation of the Forest Research containment facility were adhered to.

Elicitor: The elicitor formulation used in this study contained a heterogeneous mixture of oligosaccharins and also the wetter/penetrant DuWett at a final concentration of 0.075%. The elicitor was applied at either 6 weeks or 1 week prior to inoculation. Plants were sprayed to runoff and left to dry.

Treatments: In addition to the elicitor/time treatments, and two water control treatments, a fungicide (thiabendazole at 30 g a.i./litre + 0.05 % Pulse Penetrant (Monsanto) to aid spread and uptake) that has been used to mitigate *F. circinatum* infections in nurseries in the United States was applied 1 week before inoculation to two treatments. This represented another control on the elicitor treatments. The various combinations of elicitor application period, inoculum concentration and the water and fungicide controls resulted in 8 treatments as follows:

1. Water control (100 spores per wound)
2. Water control (10 spores per wound)
3. Fungicide control(100 spores per wound)
4. Fungicide control(10 spores per wound)
5. Elicitor (time interval 1 (6 weeks prior to inoculation) & 100 spores per wound)
6. Elicitor (time interval 1 (6 weeks prior to inoculation) & 10 spores per wound)
7. Elicitor (time interval 2 (1 week prior to inoculation) & 100 spores per wound)
8. Elicitor (time interval 2 (1 week prior to inoculation) & 10 spores per wound)

Assessments: Plants were examined twice weekly for symptom development. The number of plants in each treatment exhibiting signs of infection was recorded 15, 32 and 50 days after inoculation. At each assessment the length of visible lesion below the inoculation point was measured. For those plants inoculated at the apex the entire length of dead stem was measured. Mortality was recorded as it occurred.

Reisolations: Isolations were carried out from representative plants after 50 days. The entire stem of plants exhibiting various levels of dieback, including some visually healthy plants, were harvested, surface sterilised in 10% hypochlorite, rinsed in sterile water, and then cut into segments and plated onto potato dextrose agar (PDA). Petri dishes were incubated at 22°C and examined after 7 days for fungal colonies.

Disposal of material: At completion of the trial all material including plants, pots, soil, Petri dishes etc was double bagged and transferred immediately to the incinerator within the New Zealand Forest Research Quarantine Facility. Any non-burnable instruments were soaked overnight in bleach and then autoclaved.

RESULTS

Seven days after inoculation small lesions were visible at the inoculation point on some plants only (Fig 2). The number of plants with visible infection increased with each assessment up to 32 days (Figs 3 and 4). No further incidence was recorded but many of the infected plants continued to deteriorate and die. A final evaluation was made 50 days after inoculation. Some plants that exhibited only a very small lesion and one or two dead needles at 7 days did not deteriorate further (Fig 5).

Disease incidence and severity on *P. radiata* seedlings 15 days after inoculation are given in Table 1. At the low inoculum loading the incidence was lower for the fungicide and for the 6-week pre-inoculation elicitor treatment, but not the 1-week pre-inoculation elicitor treatment, compared to the untreated control. Furthermore, lesions were significantly smaller on treated seedlings, for all treatments, than on untreated controls. At the high inoculum loading the incidence of infection was lower in all treatments than for the untreated control, though this was not significant. None of the treatments caused a significant reduction in lesion length when challenged with the high inoculum loading.

Treatment	Low inoculum level (10 spores/wound)		High inoculum level (100 spores/wound)	
	Incidence (%)	Average lesion length (mm)	Incidence (%)	Average lesion length (mm)
Untreated	81	10	78	5
Fungicide	48 [#]	7 [#]	56	8 ^{##}
Elicitor (6 weeks pre-inoculation)	52 [#]	6 [#]	59	7 ^{##}
Elicitor (1 week pre-inoculation)	63	6 [#]	59	5
lsd	26	1.3	26	1.9

Table 1: Disease incidence and severity on *P. radiata* seedlings 15 days after inoculation with *F. circinatum*. Numbers in blue[#] are significantly (P<0.05) better than untreated controls and numbers in red^{##} are significantly poorer than untreated controls.

After 32 days (Table 2) the incidence of infection in elicitor treated seedlings did not significantly differ from the untreated controls regardless of inoculum loading. A slightly lower incidence was recorded in the fungicide treatment at the lower inoculum load. Lesion length, for both inoculum loadings, was smallest in the 1-week pre-inoculation treatment and was significantly smaller than in fungicide and untreated control seedlings. However at the higher spore loading both the fungicide and the 6-week treatment had an average lesion length longer than that of the control.

Treatment	Low inoculum level (10 spores/wound)		High inoculum level (100 spores/wound)	
	Incidence (%)	Average lesion length (mm)	Incidence (%)	Average lesion length (mm)
Untreated	96	35	85	23
Fungicide	78	28	82	27
Elicitor (6 weeks pre-inoculation)	85	25**	74	26
Elicitor (1 week pre-inoculation)	89	21**	78	16**
Isd	17	1.2	21	1.9

Table 2: Disease incidence and severity on *P. radiata* seedlings 32 days after inoculation with *F. circinatum*. Numbers in blue are significantly ($P < 0.05$) better than untreated controls and numbers in red are significantly poorer than untreated controls. ** indicates significantly better than untreated control and fungicide.

By 50 days many of the infected plants had died and the beneficial effect of elicitor treatment in enhancing plant resistance was visible (Figs 6, 7). At low inoculum loadings, elicitor application at 6 weeks or 1 week pre-inoculation, significantly reduced percentage mortality compared with untreated controls. At high inoculum levels the only effective treatment was the elicitor applied at 1 week pre-inoculation and this was significantly better than both the untreated control and the fungicide treatment. The fungicide treatment also significantly reduced mortality at the low spore loading.

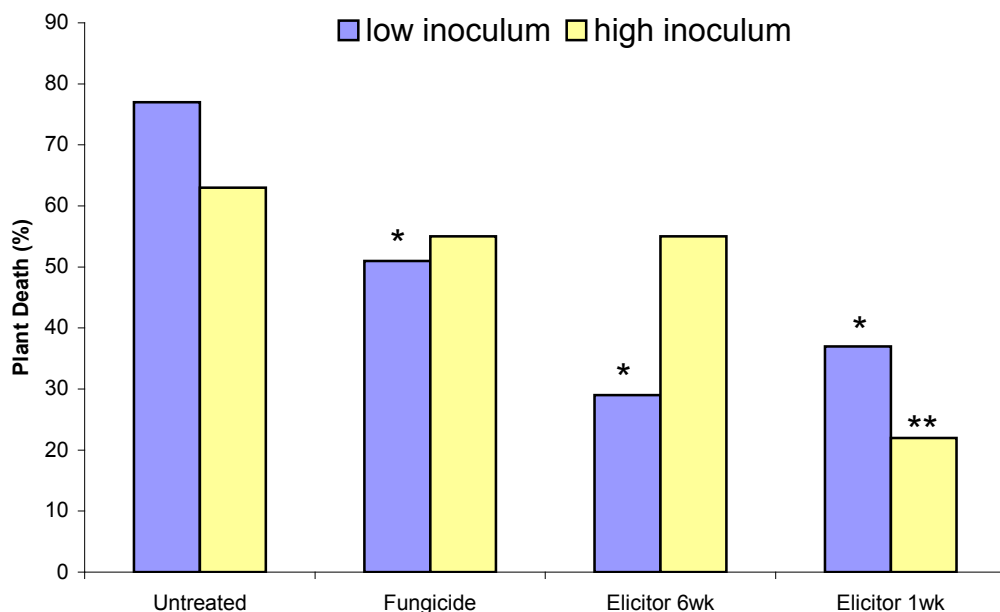


Figure 7: Incidence of plant death (%) 50 days after inoculation with *F. circinatum*. * = significantly different to untreated control ($P < 0.05$), ** = significantly different to untreated control and fungicide ($P < 0.05$). No association could be found within any treatment between the incidence or severity of infection and plant height (data not given).

Fusarium circinatum was reisolated from plant parts with visible lesions or tissue death. The fungus was not reisolated from the basal parts of plants exhibiting top death only.

Fusarium colonies grew from all of the droplets dispensed from the 100-spore solution but from only 9 of the 10-spore solution droplets. This indicated a high percentage variability in the inoculum loading at this concentration. Some plants may have received considerably more spores than the anticipated 10/inoculum site and some will have received <10 or perhaps none.

DISCUSSION

The purpose of this study was to further investigate the potential for using biological control methods to protect *P. radiata* seedlings from infection with the causal agent of pitch canker, *F. circinatum*. Results obtained in the studies reported by Reglinski and Dick (2001) were extremely encouraging and indicated that elicitors could be used to induce resistance of *P. radiata* to the pitch canker fungus. However, the data also showed limitations to the efficacy of the elicitor treatment and that the induced response may be overwhelmed by extremely high inoculum levels. In a series of inoculation experiments carried out in California on both clonally propagated *P. radiata* (Gordon *et al.* 1998) and in naturally regenerated stands (Storer *et al.* 1999) considerable variation in resistance to *F. circinatum* was encountered. Length of lesion produced by the inoculated plants was used as the measure of host response and although some seasonal effects were encountered the differences were primarily the result of individual host interaction with the pathogen.

Storer *et al.* (1999), applied inoculation doses of 25, 125, 250, 500 and 1000 spores in 5µl droplets, and found that lesions on inoculated *P. radiata* branches were significantly longer with higher spore loads. However, Gordon *et al.* (1998) inoculated 4-year-old *P. radiata* with doses of 250 and 500 spores in 5µl droplets and did not find a significant difference in response. More recently, Bonello *et al.* (2001) successfully inoculated 2-year old trees and 4-year old cuttings using 50-250 spores per inoculation.

In the earlier series of tests in New Zealand (Reglinski and Dick 2001) two elicitors and a biocontrol agent were tested separately and in combination and compared with a standard fungicide treatment. Many of the treated seedlings developed significantly shorter lesions (25-35% reduction) than their counterparts that were treated with water. Several inoculum levels were applied and the response varied with the inoculum dose. At the lowest inoculum level, the elicitor 1 treatment significantly reduced the severity of *F. circinatum* infection on *P. radiata* seedlings with over 70% of the plants showing no symptoms. Furthermore, the average lesion length on elicitor treated plants that were infected was smaller than on the control plants. Thus, elicitor treatment reduced both the incidence and the severity of *F. circinatum* infection. These results were particularly encouraging when one considers that the seedlings were inoculated with 100 spores per wound site whilst spore loads carried by some of the twig beetle vectors (*Pityophthorus* spp.) have been estimated to be as low as <10 spores per individual (Gordon *et al.* 1998).

The results obtained in this current study however did not demonstrate a high level of consistency with regard to the plant response to different inoculum levels and the elicited resistance response. It is not known why there was such a poor relationship between inoculum level and disease development and this complicates interpretation of the data. However, despite these problems it is encouraging that elicitor treatment, when applied 1 week before inoculation, reduced average lesion length and percent mortality at both high and low inoculum levels.

Indeed by 50 days post-inoculation these elicitor-treated plants had significantly lower mortality than both fungicide and untreated control plants. The 6-week pre-inoculation elicitor treatment appeared to be less effective than the 1-week-pre-inoculation treatment. Whilst this may be indicative of the duration of the induced response it cannot be ruled out that this is also, in part, due to the ‘elicitor-competence’ of the more mature seedlings. Studies in other plant species have shown that some inducible defences that are developmentally regulated are only expressed as the plant matures. It is not known if similar age-related processes contribute to the differential induced resistance response of *P. radiata* seedlings in this study.

Pine pitch canker disease can have devastating effects on very young seedlings. Viljoen *et al.* (1994) reported root rot and damping-off of *P. patula* in South Africa. Dick and Simpson (2003), in a report to the Forest Health Research Collaborative described 100% mortality of germinating *P. radiata* seedlings when seed was sown in inoculated potting mix. Although it was 3 months after sowing the seedlings used in this experiment were small (ranging from 35 -120 mm high) and, having been glasshouse reared, were very succulent. It is possible that the plants were so ‘tender’ and *P. radiata* is so susceptible to this fungus that even a small inoculum loading is difficult to combat.

Nurseries overseas where *F. circinatum* has had a devastating effect on pine seedlings have reared their plants largely in containers (Viljoen *et al.* 1994). Typically, container-rearing results in a more constantly humid environment than would be encountered with bare-root stock. The fungus can spread rapidly on the surface of the soil and young plants so that their succulent tissues are quickly overcome. This was also observed in the damping-off trial carried out in the Quarantine Facility at Forest Research for the Forest Health Research Collaborative (Dick and Simpson 2003). Under these circumstances there does not appear to be an “infection court” requirement such as is evident in older plants where the fungus is unable to invade uninjured tissue.

Reports from Chile where the fungus is now established in some nurseries (Wingfield *et al.* 2001; W. Brown, Forest Research pers. com.) are that the disease has caused substantial losses in cutting stool beds, and in container-reared cuttings that have been collected from those stool beds. It has not been evident in the bare-root seedling beds. Cutting stool plants, by their nature, have many injuries that will provide openings for infection. Infections in such circumstances are unlikely to be mediated by insect vectors. Instead the many wounds provided by the operation of cutting collection are probably invaded by spores dispersed by water-splash. Numbers of spores dispersed in this manner and reaching a wound are likely to be considerably higher than those carried by insects (estimated to be often <10).

CONCLUSION

The conditions under which the trials have been carried out in New Zealand are, of necessity due to the quarantine status of the fungus, very contrived. There are a number of external conditions (eg artificial lighting) that could be contributing to plant reactions that may be different to those that would occur in the field. Also the level of response variability in the material tested in New Zealand is unknown. To take account of the probable variability in the host population, a very large number of plants need to be challenged with the pathogen (not feasible in containment facilities). Such work should be carried out where large numbers can be accommodated in natural environments and where appropriate clonal material is available. Further testing of the efficacy of elicitors should therefore be carried out overseas in locations where the fungus occurs naturally and there are fewer constraints to experimental design.

There are limitations for any method of disease control and it is for this reason that integration of different control methods should be considered. From an ecological perspective the use of elicitors for disease control is arguably a more complex process than fungicide use since elicitors operate through the plant defences and do not affect the pathogen directly *per se*. Any factor that affects either the plant and/or the pathogen will influence the outcome of a plant/pathogen interaction. Evidence to date indicates that field efficacy of elicitors rarely attains the levels reported in controlled laboratory or glasshouse conditions. Even highly potent synthetic elicitors, such as acylbenzolar-s-methyl (ActiGard), show variable efficacy in the field when used as stand alone products. It is possibly for this reason that, new elicitor products such as Elexa[®] and Messenger[®] are recommended for use as part of an integrated pest management (IPM) program in rotation with conventional fungicides. In an integrated program fungicides would weaken the pathogen and so indirectly favour the plant whilst elicitors boost the plant's defences and thereby improve disease resistance. It may be interesting to consider the combined use of a fungicide and an elicitor treatment for disease control since their different modes of action will complement one other and favour healthy plants.

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Fig 1: *P. radiata* seedlings immediately before inoculation



Fig 2: Early symptom development in some plants



Fig 3: Seedling tops wilting at 34 days



Fig 4: Severe dieback at 50 days



Fig 5: Death of one needle at inoculation point (50 days)



Fig 6: Treatment 10 (low spore loading, elicitor treated 1-week before inoculation) at 53 days. Many plants with restricted top death