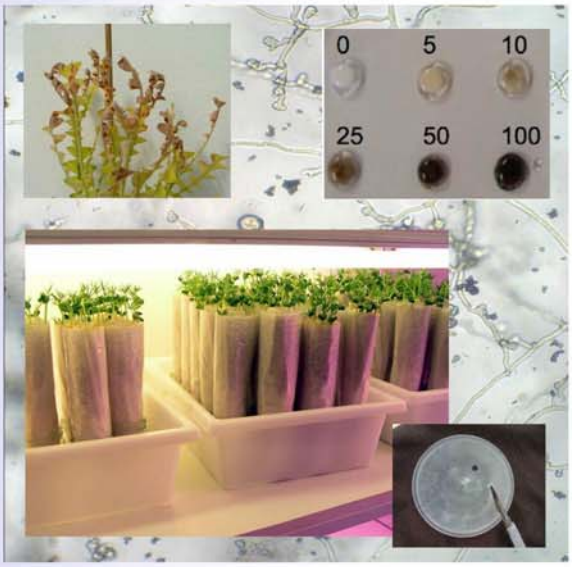
May 2008

Research into natural and induced resistance in Australian native vegetation of *Phytophthora cinnamomi* and innovative methods to contain and/or eradicate within localised incursions in areas of high biodiversity in Australia



Enhancing the efficacy of phosphite with the addition/ supplementation of other chemicals such as those known to be involved in resistance

Tender Number 19/2005
Sub project 19.2.2

Murdoch LINK

Australian Government
Department of the Environment and Heritage

CPSM
Centre for *Phytophthora* Science and Management

Research into natural and induced resistance in Australian native vegetation of *Phytophthora cinnamomi* and innovative methods to contain and/or eradicate within localised incursions in areas of high biodiversity in Australia.

Tender Number: 19/2005DEH Sub Project 19.2.2 30 May 2008

Project Period and Period Covered by this Report
25 May 2005 – 30 May 2008

Project Manager Details
Associate Professor Giles Hardy
Forest Pathology
Rm 3.037 Biological Sciences Building
Murdoch University
Murdoch WA 6150
Ph: 08 0360 6272
Email: G.Hardy@murdoch.edu.au

Report compiled by:
Dr Kay Howard
Email: K.Howard@murdoch.edu.au

Sub-Project Manager Details
Emeritus Professor Jen McComb
Rm 3.017 Biological Sciences Building
Murdoch University
Ph: 08 0360 2336
E-mail: J.McComb@murdoch.edu.au

Other Sub-Project Staff
Dr Emer O'Gara (CPSM) - Sub-Project Co-ordinator (2005-2007)
Dr Kay Howard (CPSM) - Sub-Project Co-ordinator (2008)
Dr Philip O'Brien (Murdoch University) – Supervisor and Project Advisor
Associate Professor Bernie Dell (CPSM) – Project Advisor
Bryan Shearer (DEC) – Project Advisor
Colin Crane (DEC) – Project Advisor
Gregory Freebury (DEC) – Project Advisor
Associate Professor Mike Calver (Murdoch University) – Statistician
Murdoch University
PhD Students: Patricia Stasikowski, Nathan Jardine, Leila Eshraghi
Graduate Research Assistant: Julie Ellery
Research Officers: Joshua Gilovitz (2005-2007), Peter Scott (2008)
Internal Study Contract Student: Jayden O'Brien

How to cite this report:
McComb JA, O'Brien P, Calver M, Staskowski P, Jardine N, Eshraghi L, Ellery J, Gilovitz J, Scott P, O'Brien J, O'Gara E, Howard K, Dell B, Hardy GEStJ (2008) Research into natural and induced resistance in Australian native vegetation of *Phytophthora cinnamomi* and innovative methods to contain and/or eradicate within localised incursions in areas of high biodiversity in Australia. Enhancing the efficacy of phosphite with the addition/supplementation of other chemicals such as those known to be involved in resistance. Prepared by the Centre for Phytophthora Science and Management for the Australian Government Department of the Environment, Water, Heritage and the Arts.

Contents

EXECUTIVE SUMMARY

Chemically–induced resistance to *Phytophthora cinnamomi*

To test the efficacy of treatments against a pathogen it is necessary to have a standard pathosystem. We assessed the suitability of intact *Arabidopsis* plants from 20 ecotypes, root inoculated with 29 *P. cinnamomi* isolates, as *Arabidopsis* offers so many advantages for physiological and molecular work and allows fast throughput of trials. It was shown that intact *Arabidopsis* plants, grown hydroponically and root inoculated with *P. cinnamomi* zoospores are not a suitable pathosystem, as although root mass is reduced, shoots are qualitatively and quantitatively unaffected and plants remain healthy. An ideal pathosystem is one where susceptible *Arabidopsis* ecotypes die and resistant *Arabidopsis* ecotypes survive.

Detached leaves of 4-week old *Arabidopsis* (ecotype Landsberg erecta) plants inoculated with zoospores or mycelium of *P. cinnamomi* provided a good model system for some of the analysis of the effects of phosphite as infection could be assessed through lesion size and abundance of callose papillae. Leaves treated with phosphite showed reduced lesion size and increased numbers of callose papillae. Using qPCR an increase in the level of expression of the defense gene PRI was quantified. Although not an ideal pathosystem, *Arabidopsis* can be used to examine very early defense responses (in the first few days following inoculation) after treatment with phosphite.

A rapid assay was developed to compare the effect of phosphite and metabolic inhibitors on pathogenicity of *P. cinnamomi.* Filter paper discs overgrown with *P. cinnamomi* were treated with 20 µL drops of phosphite or inhibitors, then tested for pathogenicity (ability to colonise lupin roots), or growth on NARPH plates. It was shown that c-AMP is likely to be involved in the reduction by phosphite of *P. cinnamomi* pathogenicity. The technique provides a means of screening compounds that might enhance phosphite efficacy, and to explain the mode of action of phosphite.

Quantification of phosphite uptake and movement in the plant is hampered by the lack of a quick, cheap method of measuring accurately the concentration of phosphite in different plant tissues. Costs of the existing HPLC method (~$30 per sample) prevent such studies, and analyses in the region of $1 - $5 per sample are required. We examined two methods (a silver nitrate assay, and a phosphite dehydrogenase assay) as potential methods of accurately and cost effectively measuring phosphite in plant tissues. Both methods proved promising and represent exciting advances in phosphite analysis. Some additional fine tuning is required to ensure that the methods are reliable across a range of plant species from different families. A cheap, accurate and robust analytical method will allow many important questions about phosphite uptake and movement to be investigated.

Improving the efficiency of phosphite

For experiments which required responses from root infection by *P. cinnamomi*, a model system using lupins (*Lupinus angustifolius*) was developed. Lupin seedlings 4 - 6 days old are infected using *P. cinnamomi* on 5 mm diam. Miracloth discs. Plants are sprayed with the desired test solutions the following day, and lesion lengths assessed on day 13 - 14 after spraying. In this model system phosphite (0.5%) significantly reduced lesion lengths but the addition of the penetrant Pulse at 0.1 or 0.25% did not enhance the control exhibited by phosphite alone.

However, it was shown that Pulse significantly increased the uptake of phosphite in *Eucalyptus staeri* and *Banksia baxteri*, and all further experiments included 0.1% Pulse in the phosphite sprays. In this experiment it was also shown that phosphite uptake in both species reached a maximum after approximately 15 minutes. The speed at which phosphite is taken up has never been demonstrated before.

Three plant defense activators were used in combination with phosphite to determine whether they enhanced the effect of phosphite in the lupin model plant system and in the field using *B. baxteri* underbark inoculated *with P. cinnamomi.* They were also tested using native species in the glasshouse which allowed, in addition, assessment of whether the plant defense activators increased the longevity of the effect of the phosphite.

Benzothiadiazole (BTH), DL-ß-amino-n-butyric acid (BABA) and Methyl jasmonate (MeJ) did not increase the efficacy of phosphite in the lupin trials and in some cases appeared to increase lesion lengths. They had no effect on the development of lesions in *Banksia* in the field, although variability amongst these plants indicated that larger replicate numbers were needed.

The glasshouse trial assessed stem colonisation by *P. cinnamomi* after underbark inoculation of 13-month old *Banksia baxteri, Eucalyptus staeri* and *Lambertia inermis*. Plants were sprayed with phosphite (0.5%) in combination with the defense activators (each at two concentrations), and infected at 1, 6 and 11 weeks after spraying. There was no evidence that the addition of defense activators increased the effectiveness of phosphite over this time-period.

The ability of the defense activators BTH and MeJ were tested for their ability to increase the responsiveness of 11-month old *B. grandis* seedlings sprayed with phosphite at 0, 0.1, 0.25 and 0.5 g/L phosphite. At 9 weeks after inoculation there was no evidence that the addition of plant defense activators enhanced the ability of the lower phosphite concentrations (0.1 and 0.25%) to contain the pathogen.

Some plants when sprayed with phosphite do not show any resistance to *P. cinnamomi*. One such plant selected from the above experiments was a *Lambertia inermis* genotype. Cut shoots of this plant were sprayed with phosphite together with BTH or MeJ (at two concentrations),and then the cut end of the shoot was exposed to *P. cinnamomi* mycelium in an *in vitro* assay. BTH (0.15 mM) reduced lesion length compared to phosphite alone, but the protection was not as strong as seen in genotypes that respond to phosphite.

In summary, no single model plant system can fill all experimental requirements. *Arabidopsis* cut leaves are appropriate for some analyses while lupins provide a convenient method for quick screening trials. The results of trials using herbaceous model plants must be used with caution and results confirmed by further tests on the woody native plants in the glasshouse and eventually the field.

While the penetrant Pulse can be recommended for inclusion in phosphite sprays, there is little evidence of benefit from inclusion of the defense activators BTH, MeJ or BABA, except possibly for species that have genotypes that do not respond to phosphite.

RECOMMENDATIONS

Scientific:

1. To understand phosphite uptake and movement within plants further research should include the optimisation of the two simple methods for determining the phosphite content in plant tissue developed in this study: (1) an enzymatic method based on the conversion of phosphite to phosphate; and (2) a method of chemically staining plant tissues for the presence of phosphite. The enzymatic method has a number of parameters that need optimising, including the reduction of the level of background staining that occurs in some plant species. For both detection methods we need to: test a wider range of species and tissue types and validate results by comparing them with those obtained using the HPLC method of detection.
2. The experiments utilising detached leaves show phosphite induces expression of defense genes in *Arabidopsis*. Future research should focus on identifying which of the defense pathways are important for resistance to *P. cinnamomi*. This will provide more specific information on what type of supplements to use to enhance the efficacy of phosphite.

3. Our experiments provided no evidence that the plant defense agents BABA, BTH or MeJ could replace phosphite or enhance its

effects on *P. cinnamomi*. Further experiments should test the ability of these compounds to prolong the longevity of the protection afforded by phosphite, beyond the 11 weeks tested here.

4 In the current study we scanned the literature for other possible defense activators other than the ones we used. To our knowledge there are not yet other compounds derived from different chemicals to the ones we used. However, it is important that other defense activators are screened alone, and in combination with phosphite as and when they become available.

5 A detailed study of the plants that do not respond to phosphite is required. When a cheap method of analysing the phosphite content of plant tissues is finalised, a detailed comparison should be made between the phosphite uptake, transport and metabolism in genotypes known to be responsive or non–responsive to phosphite, and of the effect of adding defense activators to the phosphite sprays.

6 The cut shoot method is recommended as a potential technique for screening rapidly and consistently under controlled environmental conditions, a wide range of native plant species for their responses to phosphite and plant defense activators when challenged with *P. cinnamomi.* This will be particularly valuable for species difficult to propagate for glasshouse trials and situations where it would be unwise to introduce the pathogen into the field for trials.

Management:

1 Pulse significantly increased the uptake of phosphite in *Eucalyptus staeri* and *Banksia baxteri*. It is recommended that 0.1% Pulse is used as the penetrant when spraying phosphite.

Sub Project 19.2.2
Chemically–induced resistance to *Phytophthora cinnamomi* and improving efficiency of phosphite

GENERAL INTRODUCTION

Phytophthora is a serious threat to biodiversity in Australia, and imposes major costs to farming, horticulture, forestry, mining and natural resource management. The scale of plant death and landscape degradation caused by *Phytophthora cinnamomi* is so large that it has been identified as one of 13 Key Threatening Processes to Australia's biodiversity (Environment Australia 2001). Currently, the only way to deal with the diseases caused by *P. cinnamomi* is through disease management practices. Phosphite is the only practical management tool for *Phytophthora* available to farmers and environmental managers. The appearance of resistance to phosphite in the pathogen population would be a devastating blow to our ability to manage the disease, and previous work from our laboratory has indicated that this is a real possibility (Dobrowolski *et al*. 2008).

Our previous research has demonstrated phosphite to be highly effective in reducing the impact of *P. cinnamomi* on susceptible species from a range of native plant communities in WA (Shearer *et al.* 2004a, Tynan *et al.* 2001) and Victoria (Ali and Guest 1998). Phosphite does not kill the pathogen but slows its growth making it possible for plants to wall-off infection. Phosphite treated plants may still produce zoospores (Wilkinson *et al.* 2001), however, phosphite reduces the rate at which disease fronts move through a plant community for between six months to more than five years (Shearer and Fairman 1997a, b). While the fungicide successfully protects normally susceptible plant species against *P. cinnamomi,* the protective properties induced by phosphite, and its persistence *in planta,* vary between species (Barrett 2001, Barrett *et al.* 2003, 2004), thus influencing the management of the phosphite regime for a given vegetation community.

There are concerns about the build up of resistance to phosphite and also between species variability in the efficacy of phosphite (i.e. robustness and duration of the protective effect). This means that on a specific site protection of the vegetation is constrained or compromised by the 'weakest link'. The interval between applications of phosphite, and application rates are dependent on species that respond poorly and/or are susceptible to phosphite phytotoxicity.

A number of studies have shown that phosphite induces expression of the plant defense responses that serve to protect the plant against colonisation by pathogens. Phosphite induced pathways include: a) the hypersensitive response which results in programmed death of the infected cell (Perrone *et al.* 2000); b) enzymes of the phenylpropanoid pathway leading to accumulation of antimicrobial phytoalexins (Groves 2002, Guest and Bompeix 1984, Jackson *et al.* 2000); and c) the systemic acquired resistance pathway that protects against subsequent challenge by the same or unrelated pathogens (Chuang *et al.* 2003, Molina *et al.* 1998).

Although defense pathway induction may be at least partly responsible for the

protective effect of phosphite it fails to explain all of the observations. Jackson *et al.* (2000) showed that the protective effect in *Eucalyptus marginata* did not coincide with induction of defense enzymes, and Roetschi *et al.* (2001) showed that protection of *Arabidopsis thaliana* from *Phytophthora* was independent of known defense pathways. Pilbeam *et al.* (2000) reported that the *in planta* concentration of phosphite and the percent recovery of *P. cinnamomi* from the roots of treated plants were directly related to the concentration of phosphite applied to the plant. This suggests a direct action of phosphite on the pathogen in the plant, an argument that is further supported by the observation that the persistence and accumulation of phosphite in the roots of treated plants varies from one species to another (Hardy *et al.* 2001). It seems logical therefore that processes that affect the transport and distribution of phosphite within the plant would determine the extent and duration of the protective effect of phosphite. Delays in transport to the roots may lead to increased loss from the plant through leaf fall or sequestration in fruit or seeds such as occurs in avocado (van der Merwe *et al.* 1994), ultimately reducing the amount effectively transported to the roots. Studies of phosphite concentrations in plant tissues are hampered by the high costs of the only available method of analysis and in this project we will develop new, cheaper techniques for phosphite analysis.

In this project we will investigate the mechanisms by which both the plant and the pathogen respond to phosphite. One aspect will be to identify the plant genes that are transcriptionally altered (repressed or induced) by treatment with phosphite and for this work we will test the suitability of the model plant system *A. thaliana.* The main advantages of this species are its fast growth, the availability of mutants, genome wide arrays and the sequence of its entire genome.

AIMS

The three aims of this section are to:

- elucidate the indirect and direct mechanisms of action of phosphite by determining which plant genes are affected by phosphite and which biochemical pathways in the pathogen are affected by phosphite;
- develop cheap but reliable methods for qualitative and/or quantitatitve analysis of phosphite in plant tissues to enable study of the phosphite uptake, transport and storage in plant tissues; and
- achieve additive or multiplier effects in the strength and/or duration of host responses to *Phytophthora* by increasing the efficiency of uptake of phosphite through use of previously untested surfactants, and by using phosphite in combination with other compounds that elicit plant defense responses.

Sub Project 19.2.2.1
Assessment of root inoculation of intact *Arabidopsis thaliana* plants as a pathosystem for *Phytophthora cinnamomi*

INTRODUCTION

Phytophthora cinnamomi is a devastating root-rot pathogen of a number of economically important crops and native plant species worldwide. In the South West Botanical Province of Western Australia, *P. cinnamomi* is a serious threat to the conservation of native plant communities, causing root rot disease in over 2300 of the region's 5710 described species (Shearer *et al*. 2004b). Despite this alarmingly wide host range, identifying a plant that both exhibits genetic variation in its susceptibility to *P. cinnamomi* and is amenable to analysis of this genetic variation has not been straightforward. Most research into the interactions between plants and *P. cinnamomi* has focussed on tree and shrub species (Jackson *et al*. 2000, Daniel *et al*. 2005, Williams *et al*. 2003) not well-suited to genetic analysis due to their large size, long intergenerational times and uncharacterised genomes.

In contrast, *A. thaliana* is small in size, has a rapid generation time of 6 weeks, a compact and fully-sequenced genome and a wide range of commercially available ecotypes and mutants (Meyerowitz and Somerville 1994). These features have enabled the identification of *A. thaliana* defense pathways (reviewed by Glazebrook 2001) activated in response to bacterial (Lu *et al*. 2001), fungal (Van Hemelrijck *et al*. 2006) and oomycete (Holub *et al.* 1994) pathogen challenge. Several pathosystems between *A. thaliana* and oomycete pathogens have been recently developed, most notably with the downy mildew pathogen *Peronospora parasitica* (Bittner-Eddy 2003), but also with *Phytophthora palmivora* (Daniel and Guest 2006), *Phytophthora porri* (Roetschi *et al.* 2001) and *P. cinnamomi* (Robinson and Cahill 2003).

The *A. thaliana – P. cinnamomi* pathosystem described by Robinson and Cahill (2003) ranked 20 *A. thaliana* ecotypes from highly susceptible to moderately resistant to *P. cinnamomi* after treating roots and excised leaves of two, four and six week old seedlings with zoospore suspensions. In roots, chlamydospores, sporangia and callose papillae production were measured and in excised leaves, chlamydospores, hyphae, dead cell clusters and callose papillae production were assessed. On the basis of these interactions ecotypes Ler and Cvi-0 were rated as *P. cinnamomi* 'susceptible'.

Preliminary trials in our glasshouse and *in vitro*, using isolates from the Murdoch Culture Collection, provided little evidence of *P. cinnamomi* pathogenicity towards *A. thaliana* ecotype Ler. This raised the possibility that we were using an inappropriate *P. cinnamomi* isolate and that a more effective *P. cinnamomi - A. thaliana* pathosystem might be developed by screening a range of *P. cinnamomi* isolates against a variety of *A. thaliana* ecotypes.

Sub Project 19.2.2.1
Assessment of root inoculation of intact *Arabidopsis thaliana* plants as a pathosystem for *Phytophthora cinnamomi*

AIM

To develop an *A. thaliana* - *P. cinnamomi* pathosystem in which susceptible and resistant plants can be easily distinguished by visual disease symptoms.

METHODS

Experimental Design

In the first trial, 20 *A. thaliana* ecotypes were exposed to an inoculum containing mixed zoospores of 29 *P. cinnamomi* isolates. Six of the most susceptible *A. thaliana* ecotypes which were selected from this trial to use in a smaller-scale repeat of the experiment, were exposed to an inoculum containing mixed zoospores of 10 *P. cinnamomi* isolates (Trial 2).

Plant growth conditions

Trial 1

Seeds of 20 randomly selected *A. thaliana* ecotypes were purchased from Lehle Seeds (Round Rock, Texas). These ecotypes (abbreviations in brackets) were: Aua/Rhön (Aa-0), Bensheim (Ben), C24, Cape Verde Islands (Cvi-0), Columbia-0 (Col-0), Columbia-3 (Col-3), Columbia-PRL (Col-PRL), Dijon-G (Dij), Estland (Est), Greenville (Gre-0), Kendalville (Kin-0), Landsberg erecta (Ler), Mühlen (Mh-0), Niederzenz (Nie), Nossen (No-0), RLD, RLD1, S96, Turk Lake (Tur) and Wassilewskija (Ws-2).

Plants were grown hydroponically to facilitate zoospore inoculation and to enable observation and recovery of the roots. Rockwool plugs of approximately 3 cm^3 were inserted into frames above light-proofed plastic tubs (Figure 1). Each tub received 500 mL of commercial hydroponics nutrient solution (Ag-Grow®, Aquaponics, Canning Vale WA) made to half strength (as directed by the manufacturer) in deionised water with pH adjusted to 6.5. This solution was replaced weekly. Seeds were sown in excess onto rockwool plugs and stratified at 4ºC for 2 days before being placed in a growth chamber at 23°C with a 16 h photoperiod. Ten to 14 days after germination, germinated seedlings were thinned to 8 plants per plug and grown for 4 weeks in the hydroponics solution before inoculation.

Trial 2

The *A. thaliana* ecotypes Aa-0, Ben, Col-0, Dij, Gre-0 and Nie were used in the second trial. Growth conditions were similar to the first trial except that the Ag-Grow® nutrient solution was diluted to quarter strength and the photoperiod was reduced to 12 h. Plants were grown individually in rockwool plugs with approximate dimensions of 3 × 1.5 × 1.5 cm. The plugs were inserted into 2.5 cm high × 1 cm diameter tubes (the cut-off tops of 5 mL pipette tips) and fitted into holes in the lids of light-proofed hydroponic tubs so that the lower part of each rockwool plug was submerged in the nutrient solution (Figure 1). Six tubs

were set up with inoculated plants and 6 tubs had non-inoculated controls, with two plants of each ecotype in each tub.

P. cinnamomi *isolates*
Twenty-nine *P. cinnamomi* isolates from the Murdoch Culture Collection were used in the experiment (Table 1). Isolates were chosen to cover a range in virulence and growth rate (Wilkinson *et al.* 2001). All isolates were passaged through apples to ensure virulence had not been lost prior to experiments (Ribeiro 1978).

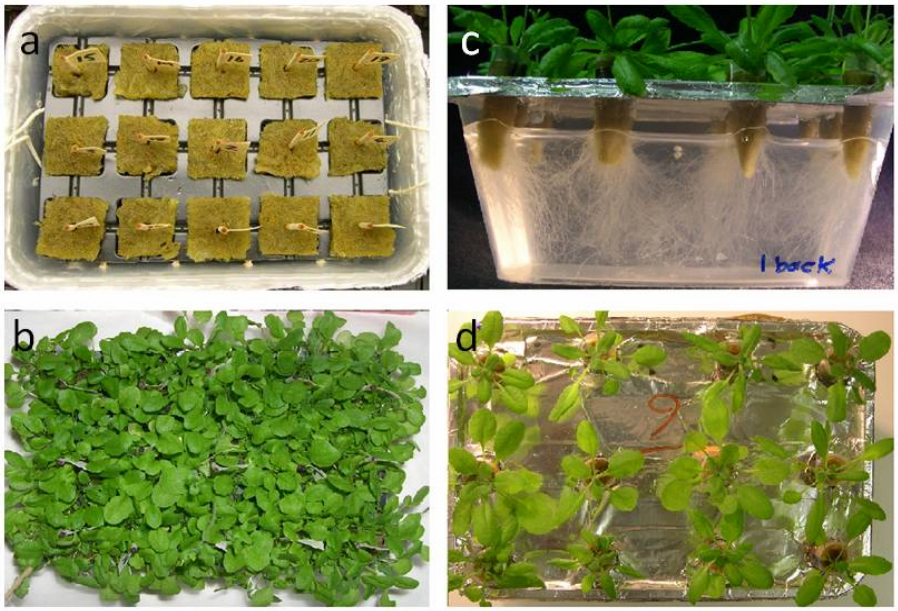


Figure 1 The hydroponics systems used to grow *Arabidopsis thaliana.* **a**) system used in Trial 1, showing rockwool plugs before stratification. **b**) same container after 4 weeks (8 plants per rock wool plug). **c** and **d**) hydroponics system used in Trial 2 after 4 weeks. The outer foil layer has been removed to allow roots to be viewed. Tubs were 17.5 cm across at their widest point.

Table 1 *Phytophthora cinnamomi* isolates used in combined infection of *Arabidopsis thaliana* ecotypes. All isolates are from the Murdoch University culture collection.
* = isolates used in the second experiment.

Isolate	Isolate
A15 (MP135)*	MP91
A2394 (MP132)	MP94-03
A26 (MP134)	MP94-13
DCE210 (MP27)	MP94-17
MP100	MP94-18
MP102*	MP94-20*
MP103	MP94-30*
MP114	MP94-37
MP125*	MP94-48*
MP128*	MP97*
MP32	MP97-12
MP37	MP98
MP62*	MP99
MP80*	MPD039
MP86	

Production of mycelia and zoospores

Zoospores were generated using a modified method of Dolan and Coffey (1986) using non-sterile soil extract. Pure cultures of *P. cinnamomi* were grown on V8 agar (Hardham *et al.* 1991) in the dark at 25°C for 4 - 5 days until they reached approximately 6 cm in diameter. Agar soil water extract (100 g of potting mix in 1 L of deionised water incubated for 5 h at room temperature and filtered through Whatman No. 1 filter paper) was added to the washed agar plugs. Plates were then incubated for a further 2 days under cool fluorescent light at 27°C. Cultures were checked under the microscope for the presence of sporangia then placed at 4°C for 30 min to synchronise release of zoospores. Plates were kept at 25°C until zoospores were released. Zoospore concentrations varied between 1×10^3 and 1×10^5 zoospores/mL, depending on the isolate.

Inoculation procedure

Trial 1

20 mL of zoospore suspension and 5 mycelial plugs from each isolate were added directly to 1 L hydroponic tubs containing 4-week-old *A. thaliana* seedlings. Roots of the seedlings were immersed in a mixture of zoospores and mycelial plugs from 29 *P. cinnamomi* isolates at 23°C in the dark for 24 h. Non-inoculated control plants were subjected to identical treatment, except that they were incubated in filtered soil-water extract rather than the zoospore suspension mix. After 24 h the plants were removed from the inoculum, rinsed in deionised water and transferred to clean tubs containing fresh nutrient solution. Plants were then grown for a further 3 weeks before harvest.

Trial 2

An identical inoculation procedure was used in Trial 2 except that the inoculum contained ten *P. cinnamomi* isolates instead of twenty-nine (Table 1).

Lupin seedling controls

Lupin (*L. angustifolius*) seedlings were used to confirm the zoospore virulence of each of the 29 *P. cinnamomi* isolates. Three-day-old lupin seedlings were inoculated with 100 µL of zoospore suspension from each *P. cinnamomi* isolate as it was added to the inoculum mixture. Controls were inoculated with soil water extract. Seedlings were incubated on filter paper, in Petri dishes in the dark, at 25°C for 3 days and then checked for lesions (dark discolouration of root tissue). Root sections 1 cm in length were transferred to a *Phytophthora* selective medium NARPH (Hüberli *et al.* 2001). *P. cinnamomi* growth was scored after 2 days. Three-day-old lupin seedlings were also placed alongside *Arabidopsis* seedlings in the infection vessel in the presence of the combined zoospore suspension (two lupin seedlings per tub). After 24 h, the lupin seedlings were removed, blotted dry, plated out on NARPH and incubated as described above.

Harvesting of inoculated plants

Trial 1

Three weeks after inoculation, rockwool plugs were removed from the hydroponic tubs. Plants were photographed and the maximum root length (extent of protrusion from the base of the rockwool) for each plug was measured to the nearest mm. The above ground portions of the 5 largest plants from each rockwool plug were removed and their dry weight determined after drying at 70°C for 10 days. Root sections 1 cm in length were taken in duplicate from two randomly selected plants of each ecotype, blotted dry, immersed for 1 min in 1% sodium hypochlorite, blotted, immersed for 1 min in 70% ethanol, blotted and then rinsed twice in sterile deionised water. Root sections were then plated onto a *Phytophthora* selective medium (NARPH), and incubated in the dark for 2 days at 25°C, and the presence of mycelia was recorded.

Trial 2
Plants were also harvested 3 weeks after inoculation and 7 weeks after germination. For each ecotype, the root sections of 2 plants of inoculated and control treatments were excised, surface sterilised and plated onto NARPH as described in Trial 1. Above ground plant parts and rockwool plugs (in which roots were embedded) were dried at 70^{o}C for 1 week and weighed. Root weight was calculated as the increase in dry mass of each rockwool plug before planting and after the harvest.

Statistical analyses
The statistical significance of differences between the means of zoospore-treated and control plants of each ecotype were assessed with independent samples t-tests. Data were first analysed with Levene's test for equality of variances. When Levene's test indicated variances were heterogeneous, an independent samples *t*-test with unequal variances assumed was used. If Levene's test did not indicate heterogeneous variance between control and treatment data, an independent samples *t*-test with equal variances assumed was used. The two-tailed limit of 0.05 was used as the criterion for significance in all statistical analyses, all of which were conducted using SPSS 15.0 software.

RESULTS
Trial 1
Shoots
Three weeks after *A. thaliana* seedlings were inoculated with the mixed zoospore suspension, there were no plant deaths and the foliage of both inoculated and non-inoculated plants appeared healthy (Figure 2). Measurements of shoot dry weight, however, indicated the zoospore treatment reduced shoot growth of some ecotypes (Figure 3a). When data for all ecotypes were bulked, average shoot dry weight was significantly ($p = 0.017$) decreased from 17.3 mg (± 0.35 mg, $n = 60$) in non-inoculated control plants to 15.9 mg (± 0.89 mg, $n = 240$) in zoospore treated plants. Amongst individual ecotypes, shoot dry weight of zoospore treated Gre-0 ($p = 0.006$) and Ben ($p = 0.015$) plants was significantly less than that of non-inoculated controls (Figure 3a).

Roots
The effect of zoospore inoculation was more evident in the roots than in the foliage. Roots of treated plants were significantly shorter than those of non-inoculated controls in all 20 ecotypes (Figure 3b).

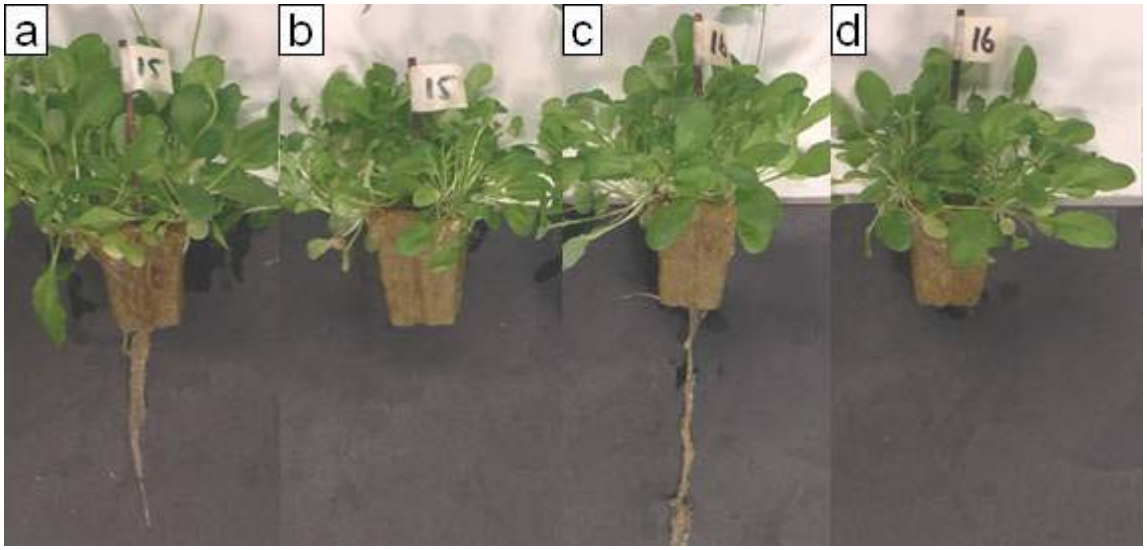


Figure 2 The effect of *Phytophthora cinnamomi* zoospore inoculation on foliage and roots of *Arabidopsis thaliana*. **a**) ecotype Bensheim, control, **b**) ecotype Bensheim, inoculated, **c**) ecotype Kendalville, control, and **d**) ecotype Kendalville, inoculated. Rockwool plugs are 3 cm in height and each contain 8 seedlings. Roots were inoculated with a mixed zoospore suspension of 29 *P. cinnamomi* isolates 4 weeks after germination, and photographed after a further 3 weeks of growth.

Lupin controls

Lesions developed in the roots of lupin seedlings inoculated with zoospore suspensions from individual isolates, indicating that all zoospore suspensions were viable and virulence had not been lost during storage in culture. No lesions developed in lupin seedlings sham inoculated with soil water extract. Lesions also developed in lupin seedlings incubated alongside *A. thaliana* seedlings in the mixed zoospore suspension, but did not develop in seedlings incubated alongside *A. thaliana* controls immersed in soil water extract rather than the mixed zoospore suspension.

P. cinnamomi *recovery from inoculated roots*

P. cinnamomi was recovered from 100% of *A. thaliana* root sections plated onto the *Phytophthora* selective medium (2 per ecotype). No *Phytophthora* was isolated from the root sections of non-inoculated plants. In the lupin controls, *P. cinnamomi* was not reisolated from zoospore-treated seedlings despite the formation of necrotic lesions. *P. cinnamomi* was not recovered in any lupin seedlings sham inoculated with soil water extract.

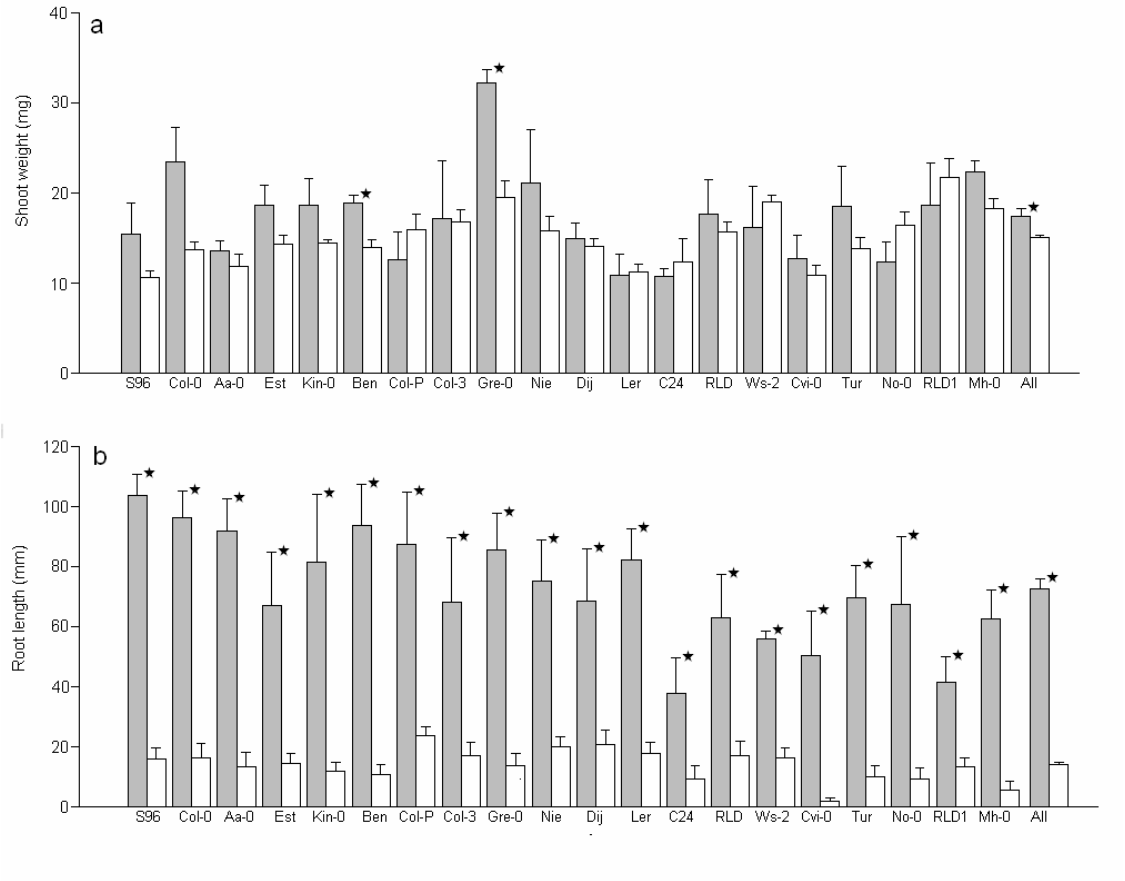


Figure 3 Effect of a mixed *Phytophthora cinnamomi* zoospore inoculation on **a**) shoot dry weight and **b**) root length across 20 *Arabidopsis thaliana* ecotypes. 'All' represents combined data of all ecotypes. Zoospore treated plants (□) and untreated controls (❑). Stars (★**)** indicate a significant ($p < 0.05$) difference between inoculated and control means. Vertical bars represent one standard error of the mean. For each ecotype inoculated $n = 12$ and control $n = 3$.

Trial 2

Shoots

Six relatively 'sensitive' ecotypes to *P. cinnamomi* infection (as determined by a reduction in foliar mass and root length) were selected from the first trial for use in a smaller-scale repeat of the experiment. These ecotypes were Aa-0, Ben, Col-0, Dij, Gre-0 and Nie. In the second trial, seedlings were grown in individual plugs to enable the root dry weight of individual plants to be calculated (Figure 1a). Under the less crowded conditions, the plants grew larger (mean shoot dry weight 201.0 mg) than those in the first trial (mean shoot dry weight 20.7 mg). Trends were otherwise similar to the first experiment. Treating seedlings with the inoculum containing zoospores of 10

P. cinnamomi isolates (rather than 30 as in the first trial) did not significantly alter the shoot dry weight of any of the ecotypes tested individually, but when data of all ecotypes were bulked, zoospore treatment led to a significant ($p < 0.05$) reduction in shoot dry weight (Figure 5a).

Roots
Zoospore treatment again led to a reduction in root length, although this reduction was less pronounced than in the first trial. *A. thaliana* roots that were treated with zoospores appeared dark and thinner in comparison to non-inoculated roots (Figure 4). Root length was significantly ($p < 0.05$) reduced in response to inoculation in ecotypes Aa-0 and Col-0, and when root length data of all 6 ecotypes were bulked together (Figure 5b). Zoospore treatment also reduced root weight. This reduction was not statistically significant ($p > 0.05$) when ecotypes were analysed individually, but was significant ($p < 0.05$) when the dry weight of inoculated roots of all ecotypes was compared to non-inoculated roots (Figure 5c).

Lupin controls
As in the first trial, lesions developed in all lupin seedlings treated with zoospores and not in lupins sham inoculated with soil water extract, indicating that zoospores used were viable and virulent at the time of inoculation.

P. cinnamomi *recovery from inoculated roots*
P. cinnamomi was re-isolated from all the roots sampled for inoculated plants of *Arabidopsis* and lupin, but not from the control plants of either species.

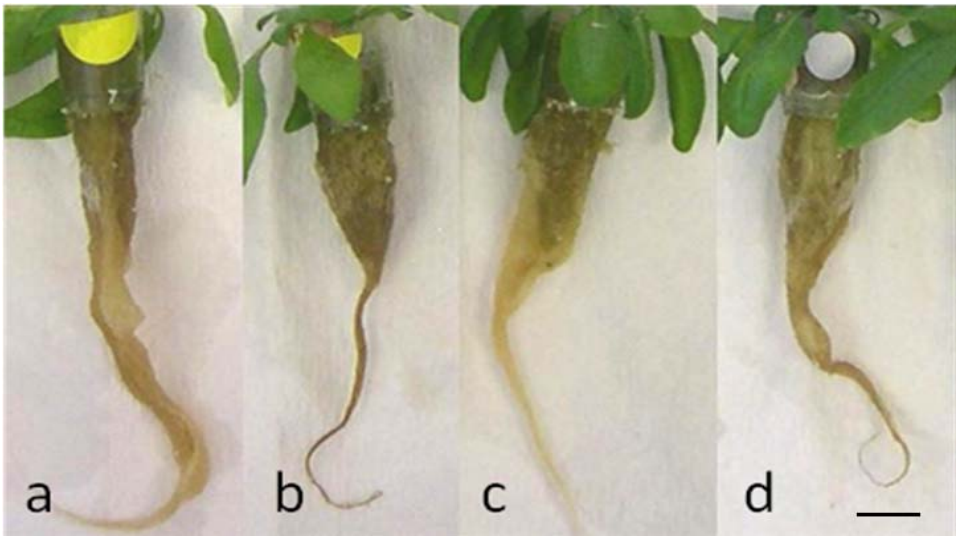


Figure 4 Effect of *Phytophthora cinnamomi* zoospore inoculation on the roots of *Arabidopsis thaliana* showing darker & thinner roots of inoculated ecotypes compared with control ecotypes. **a**) ecotype Greenville, control, **b**) ecotype Greenville, inoculated, **c**) ecotype Niederzenz, control, and **d**) ecotype Niederzenz, inoculated. Scale bar = 1 cm.

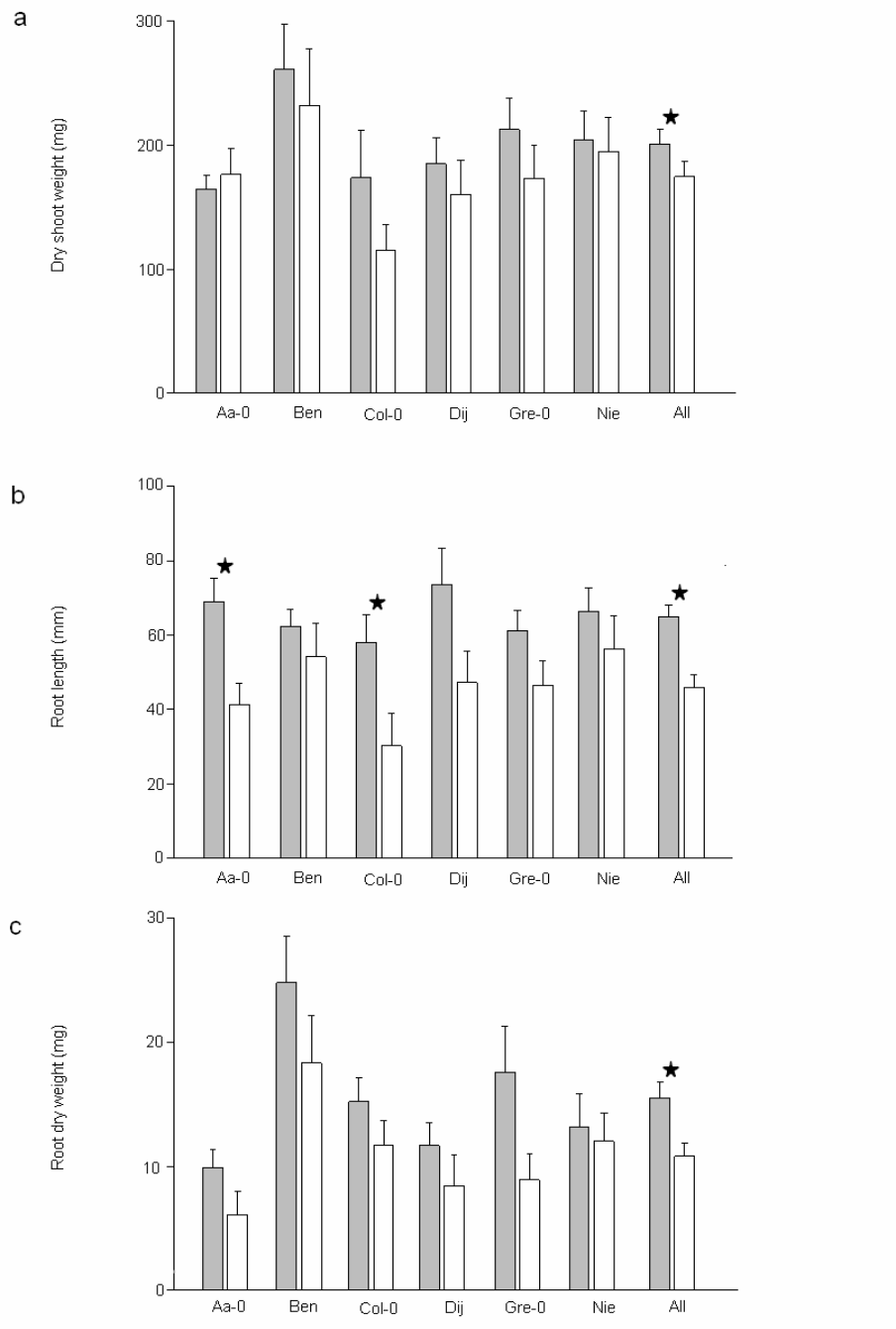


Figure 5 Effect of zoospore inoculation with a mixture of 10 *Phytophthora cinnamomi* isolates on **a**) dry shoot weight, *n* = 10; **b**) root length, *n* = 12; and **c**) root dry weight, *n* = 10, in 6 *Arabidopsis thaliana* ecotypes. The roots of seedlings were inoculated with a mixed *P. cinnamomi* zoospore suspension 4 weeks after germination and harvested after a further 3 weeks of growth. 'All' represents combined data of all ecotypes. Stars (★**)** indicate a significant ($p < 0.05$) difference in the means of zoospore treated (□) and non-treated plants (■). Vertical bars represent one standard error of the mean.

DISCUSSION

Twenty *A. thaliana* ecotypes grown hydroponically and inoculated with a mixed suspension of 29 *P. cinnamomi* isolates showed no disease symptoms in the foliage after 3 weeks. Plants treated with zoospores did show a significant reduction in root length and *P. cinnamomi* was re-isolated from all inoculated plants sampled. It is possible that growth of the pathogen in infected roots is quickly walled off and the growth of lateral roots initiated. However, as the upper portion of the root was encased in a rockwool plug, it was not possible to determine whether this was the case. Regardless of whether roots regenerated in this manner, it is clear that the pathogen did not spread beyond the roots, and that sufficient functioning root tissue survived to support shoot growth.

Ecotypes Gre-0 and Col-0 were found to be among the most sensitive to *P. cinnamomi* inoculation based on a reduction in plant mass and root length. These findings differ from those of Robinson and Cahill (2003) who used *P. cinnamomi* isolate DU026 to inoculate roots and detached leaves of the same 20 *A. thaliana* ecotypes used in the present study. For root inoculations Robinson and Cahill (2003) transferred two and four week old seedlings into 1 mL of zoospore suspension and measured chlamydospore and sporangia production after 96 h. The number of callose papillae in roots of 4-week old seedlings was also determined 12, 24 and 48 h post inoculation. For leaf inoculations, zoospore droplets were placed on detached leaves excised from 2 and 4 week old plants. Pathogen colonisation of leaf tissue (hyphae, sporangia and chlamydospore production) and defense response (callose, papillae and cell cluster production) was monitored post inoculation. Cvi-0 and Ler were identified as the most susceptible ecotypes to *P. cinnamomi*, as they supported the most growth of the pathogen on leaves and roots, and produced less defense responses. Under our experimental conditions these ecotypes were not markedly affected. This discrepancy could be due to the different *P. cinnamomi* isolates used or conditions under which the plants were inoculated. Alternatively, the parameters used by Robinson and Cahill (2003) to determine susceptibility to *P. cinnamomi* may not correlate to macroscopic indicators of disease such as changes in root length or shoot biomass.

The ability of *P. cinnamomi* to produce sporangia and chlamydospores in a plant species may not be a reliable indicator of susceptibility if measured in isolation. *P. cinnamomi* has previously shown to be capable of producing chlamydospores and sporangia on the roots of *P. cinnamomi* resistant species such as *Corymbia calophylla* and *Gahnia radula* (Cahill *et al*. 1989). They investigated a number of plant species ranging from highly susceptible to *P. cinnamomi* to highly resistant. The pathogen penetrated and formed small lesions on all species tested, regardless of the susceptibility of the species in the field. The difference between resistant and susceptible species was their ability to contain the spread of lesions. Resistant species were able to contain

lesions by the deposition of phenolic material and through morphological changes in cellular organisation at the site of infection. Lesions were not contained in susceptible species but progressed through the roots, eventually leading to wilting of shoots and plant death. Root growth ceased within 24 h after inoculation of both susceptible and resistant plants, however, in resistant species root growth continued within 48 h, usually from lateral branching roots (Cahill *et al*. 1989). The response of *A. thaliana* to *P. cinnamomi* inoculation was consistent with a resistant interaction as described by Cahill *et al*. (1989). While *P. cinnamomi* did infect root tissue and slow their growth, lesions were contained and symptoms did not spread to the above ground plant parts.

The effect of zoospore inoculation on root length was less marked in the second trial than in the first. There are several possible explanations for this discrepancy. In the second trial, zoospores of 10 rather than 29 *P. cinnamomi* isolates were used to inoculate the plants (Table 1); these isolates were selected due to their reliability for zoospore production rather than on pathogenicity towards *Arabidopsis*, and it is therefore possible that isolates with the greatest pathogenicity towards *Arabidopsis* were omitted from the second trial. The difference may also be due to a reduced inoculum load or altered environmental conditions. In the second trial, plants were grown individually in rockwool plugs, rather than in groups of 8. The reduced competition resulted in larger plants which may have been more capable of mounting defense against *P. cinnamomi* infection. The strength of nutrient solution was also reduced by half, and this reduction in nutrient concentration may have stimulated root growth prior to inoculation.

Our results lead us to question whether *A. thaliana* is susceptible to *P. cinnamomi*. Our failure to kill plants growing in soil in the glasshouse by inoculation with zoospores, and the healthy growth of hydroponically grown plants after heavy inoculation with zoospores from a wide range of isolates leads us to conclude that this is not a suitable pathosystem from which to develop novel treatments for controlling *P. cinnamomi*. In susceptible host plants, *P. cinnamomi* and other *Phytophthora* species can be highly virulent, causing symptoms such as lesion development, reductions in shoot and root weights and plant deaths (Conn *et al.* 1991, Shearer *et al.* 2006). The absence of any plant deaths 3 weeks after zoospore inoculation indicates that *A. thaliana* should not be considered highly susceptible to any of the 29 *P. cinnamomi* isolates tested in this experiment. We conclude that the usefulness of *A. thaliana* as a model *P. cinnamomi* host is limited due to the plant's resistance towards this normally aggressive pathogen.

Sub Project 19.2.2.2
Use of detached leaves of *Arabidopsis thaliana* to assess phosphite induced expression of defence genes

INTRODUCTION

In previous experiments (Section 19.2.2.1), we showed that hydroponically grown *Arabidopsis* was not a suitable pathosystem for infection with *P. cinnamomi*. However, Roetschi *et al*. (2001) demonstrated infection of *A. thaliana* by *Phytophthora brassica*, and Robinson and Cahill (2003) described infection of detached leaves by *P. cinnamomi*, and reported differences in the susceptibility of different ecotypes to *P. cinnamomi.* We therefore further explored the potential of *A. thaliana* as a pathosystem for *P. cinnamomi* by analysing the infection by *P. cinnamomi* of detached leaves.

Several methods of measuring levels of expression of defense were used including lesion size and callose papillae formation. Callose formation is a measure of the resistance of a plant as deposition of callose at infection sites helps to prevent colonisation by *P. cinnamomi*.

In addition, quantitative real time polymerase chain reaction (qRT-PCR) technology (SYBR Green®, Applied Biosystems) was used to assess gene expression. Using qRT-PCR we could screen for expression of the different defense genes by measuring RNA. This meant that instead of the requirement to conduct different enzyme assays for each gene we used the same technique for different genes. If primer sets designed for *A. thaliana* also prove to be effective in native species then q-RT PCR can be applied to material harvested and stored from selected species and treatment combinations from glasshouse experiments.

For measuring levels of gene expression it is important to have housekeeping genes whose expression is not affected by the treatment so results can be corrected for variation in the amounts of RNA. We have identified three genes; Ubiquitin, Glyceraldehyde-3-phosphate dehydrogenase and Tubulin whose expression is not affected by phosphite and can therefore be used as housekeeping genes.

In the longer term, comparison of the reactions of control and phosphite-treated plants of the model plant *Arabidopsis* when infected with *P. cinnamomi* may help explain the basis of the results from treatments with phosphite, and other plant defense activators in glasshouse and field experiments.

AIMS

The two aims of this experiment were to:

- determine whether phosphite could protect cut leaves of *A. thaliana* from infection with *P. cinnamomi;* and
- use this pathosystem to determine which defense genes were induced when *Arabidopsis* was treated with phosphite.

Sub Project 19.2.2.2
Use of detached leaves of *Arabidopsis thaliana* to assess phosphite induced expression of defence genes

METHODS

Plant material

A. thaliana seeds of ecotype *Landsberg erecta* (Ler) were purchased from (Lehle Seeds). Seeds were sown on the surface of seed raising mix (Debco, Australia) in 5 cm free-draining plastic pots pre-moistened for 30 min with distilled water. After sowing, the seeds were stratified for 3 days at 4^{o}C in the dark (Ticconi *et al.* 2001) before transferring to a growth chamber with a 10/14 h day/night photoperiod at 20 ± 2^{o}C (Robinson and Cahill 2003). Pots were watered every second day to container capacity and half strength Hoagland's nutrient solution (Hoagland and Arnon 1938) was applied every 4 days. Phosphite was applied by spraying plants to runoff with 20 mM phosphite.

Inoculum preparation

The *P. cinnamomi* isolates (MP125, DP55 and MP94-48) were obtained from the Murdoch University Culture Collection. These isolates were maintained on 20% V8-juice agar (Miller 1955) at 25ºC in the dark and subcultured every 7 days.

For zoospore production, a method described by (Byrt and Grant 1979) was used with some modifications. The isolates were grown on the surface of 10% V8-juice agar medium by placing a single mycelial plug into the centre of a 9-cm Petri dish plate and incubating at 25^{o}C for 4 days in the dark. Mycelial plugs (3 mm^2) from the actively growing edge of the colony were placed onto fresh 10% V8 agar plates containing sterile Miracloth (Calbiochem, USA) discs (30 mm diameter) and incubated in the dark at 25^{o}C for 3 days. The *P. cinnamomi* colonised Miracloth discs were aseptically transferred to a sterile 250 mL Erlenmeyer flask containing 100 mL of 5% clarified V8 juice broth and incubated overnight on an orbital shaker (90 rpm) at 25^{o}C under the light. The V8 broth was then discarded and the discs were washed four times at 15 min intervals with sterile distilled water. The discs were then incubated in 100 mL of mineral salts solution overnight at 25^{o}C under light on an orbital shaker (90 rpm). Finally, the discs were removed from the flask and covered with 20 mL sterile distilled water and incubated at 20^{o}C until sporangia were formed. The discs were cold shocked at 4^{o}C for 30 min to stimulate synchronous release of zoospores. The zoospore density was determined using a haemocytometer and was adjusted to a concentration of 1 x 10^5 zoospores/mL using sterile distilled water.

Inoculation of plants with *P. cinnamomi*

Leaves of 4-week old *Arabidopsis* plants were washed with sterile distilled water one day after application of phosphite. The leaves were excised and placed with the abaxial surface facing upwards into the well of cell culture plates on pre-moistened 2.5 cm filter paper. Each leaf was inoculated with 3 µL of 1 x 10^5 zoospores/mL along the midvein. Plates were sealed and incubated in the dark at 25^{o}C for 12 h, then transferred to a growth chamber with a 10/14 h day/night photoperiod at 20 ± 1^{o}C to allow lesions to develop.

Lesion size

Disease symptoms were evident 10 days after inoculation. Lesions caused by *P. cinnamomi* were photographed using a digital camera attached to a dissecting microscope and the area of the lesion was calculated using the software program ASSESS (APS, Minnesota).

Aniline blue staining for callose formation

To detect callose within the leaf cells, a staining method modified from Keogh *et al*. (1980) was used. A minimum of 10 leaves per treatment were examined. Leaves were collected at 3, 6, 12, 24, 48 and 72 h post inoculation. Prior to staining, the leaves were cleared in 97% ethanol overnight at room temperature (RT), and rinsed in 50% ethanol followed by a rinse in dH_2O. The leaves were stained using a 0.01% (w/v) solution of aniline blue buffered in 150 mM K_2HPO_4 (pH 9.5) at RT for 30 min. Leaf tissues were mounted on glass slides in 70% glycerol and examined under blue light epifluorescence. Callose was detected by the occurrence of light blue to white fluorescence within cells and along cell walls. The number of callose papillae formed were counted in the 10x field view (Thatcher *et al.* 2005). Callose was quantified using the software program ASSESS to count the callose depositions.

Measurement of Gene Expression by qRT-PCR

Preparation of plants and extraction of RNA

Arabidopsis seeds were surface sterilized and sown on 1% (w/v) MS (Murashige and Skoog's medium) agar medium. Seeds were stratified for 2 days at 4^{o}C in the dark to induce germination then placed in a growth chamber at 22 – 23^{o}C with a 14h photoperiod (Ticconi *et al.* 2001). After 2 weeks incubation, a phosphite solution was applied to the medium to give a final concentration of 15mM phosphite. After 24 h the leaves were collected and snap-frozen in liquid nitrogen.

RNA extracted from the frozen leaf tissue using the QIAGEN RNeasy plant mini kit (QIAGEN). Genomic DNA was removed by digestion with an RNAase free DNAase (Invitrogen). Gene expression was measured using the SYBR® GreenER™ Two-Step qRT-PCR Kit (Invitrogen) as described by the manufacturer.

Primer design

Primers for the housekeeping genes, EIFa and PPR and the PR1 gene were designed by alignment of sequences retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). From the aligned sequences the primers were designed using Primer Express 1.5 software (Applied Biosystems). Preliminary experiments have shown that expression of the housekeeping genes is not affected by phosphite treatment. Primer details are given in the following Table 2.

Table 2 Details of primers used for measuring transcription of genes in *A. thaliana* by qRT-PCR.

Genes	Forward and reverse primers (5→3')	Amplicon size (bp)	Temperature (°C)
EF-1a	TGAGCACGCTCTTCTTGCTTTCA GGTGGTGGCATCCATCTTGTTACA	76	78.7
PPR	GAGTTGCGGGTTTGTTGGAG CAAGACAGCATTTCCAGATAGCAT	61	75.0
PR1	AGCCTATGCTCGGAGCTACG ACCCCAGGCTAAGTTTTCCC	91	82.0

Q-PCR

Four samples per treatment were randomly chosen and analysed to prevent bias in the results. PCR reactions were set up with the electric Eppendorf Pippetor (Quantum Scientific Pty Ltd) to minimize pippetting errors. The real Time PCR reactions were conducted using iQ SYBR Green Supermix Kit (Bio-Rad). Each sample was analysed in triplicate in a total 25 µL reaction volume of; 6 µL of cDNA, 12.5 µL 2 x mix buffer (containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/mL iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM flourescein and stabilizers), 4.5 µL Ultra Pure water (Fisher Biotech, Australia) and the required amount of forward and reverse primers. Reactions were run on an icycler® thermocycler (Applied Biosystems) using the following cycling conditions: 95°C for 5 min and 40 cycles at 95°C for 15 s and 60°C for 1 min followed by 1 cycle at 95°C for 1 min, 1 cycle at 55°C for 1 min for data collection and subsequent real-time analysis to be enabled. For each experiment, a non-template reaction was included as a negative control. The specificity of the PCR reactions was confirmed by melting curves analysis of the products as well as by size verification of the amplicons in a conventional agarose gel.

The threshold cycle (Ct) values were determined at the same fluorescence threshold line for each gene. The Ct value for each sample was obtained by calculating the arithmetic mean of the triplicate values, when the standard deviation was lower than 0.2. Ct values were transformed into raw quantity values (Q) according to the following equation, Q = E (Min Ct - Sample Ct) (geNorm user manual, http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf), where "E" is the efficiency of the real-time PCR for each gene (housekeeping genes and PR genes) and "Min Ct" is the minimum Ct value for the samples analysed. E values were calculated for each gene from the given slope after running serial dilutions of cDNA and the following formula E = [10(-1/ slope)].

RESULTS

Infection of leaves of *A. thaliana* by *P. cinnamomi* zoospores resulted in the appearance of lesions on the leaves (Figure 6) that were much more extensive on untreated leaves compared to leaves from plants treated with 20 mM phosphite. When lesion size is expressed as percent affected area the difference between the plants is very significant (Figure 7). The results show that phosphite does induce resistance in *A. thaliana*.

Callose formation was measured in phosphite treated and untreated plants over a period of 72 h post treatment. Tissue sections stained for callose deposition 12 h post-inoculation are shown in Figure 8. Callose deposition was more extensive in sections from the phosphite treated plants. Changes in callose deposition over a 72 h period post treatment are shown in Figure 9. After a lag period of 12 h, callose formation increased in both the treated and untreated plants (Figure 9). At 24 and 48 h higher levels of callose were observed in the treated plants. By 72 h the levels of callose were the same in plants treated or not treated with phosphite.

Figure 6 Lesion size in *Phytophthora cinnamomi* infected *Arabidopsis thaliana* leaves treated with phosphite (20 mM) (**a**, **b**) and untreated (**c**, **d**).

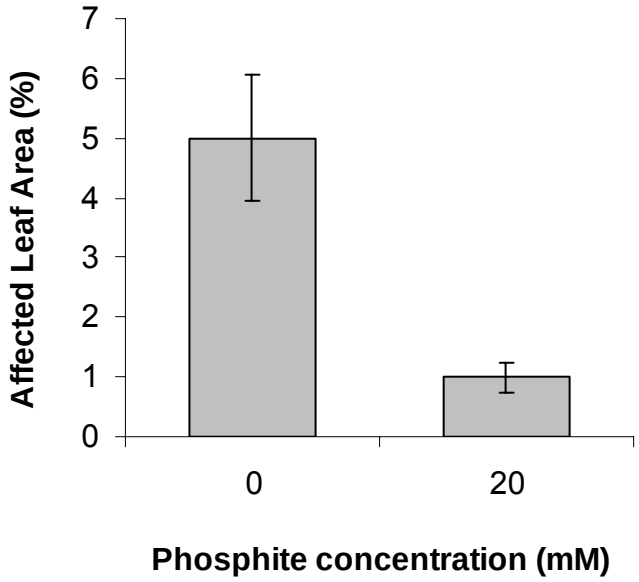


Figure 7

Comparison of lesion size in phosphite treated and untreated *Arabidopsis thaliana* plants. 25 leaves were assessed for each treatment. Vertical bars represent two standard errors of the mean.

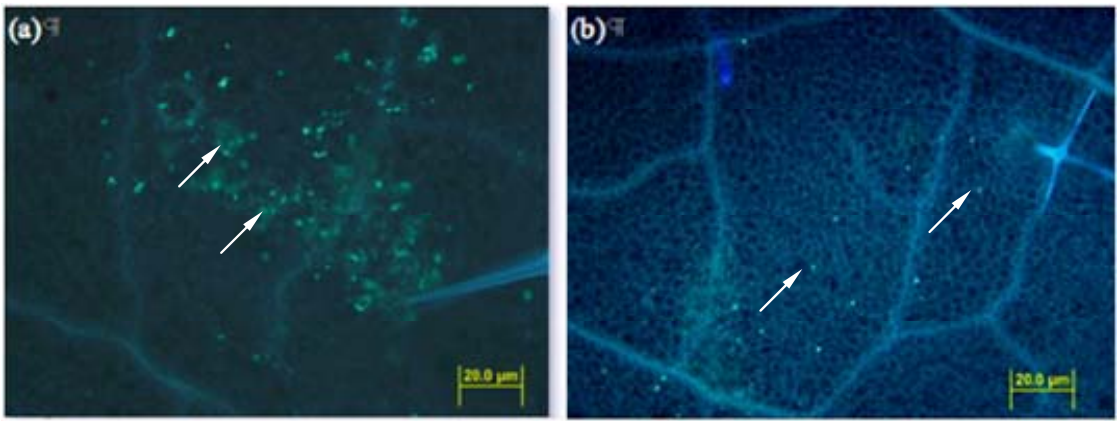


Figure 8 Callose deposition in leaves of *Arabidopsis thaliana* ecotype *Landsberg erecta* (Ler) following inoculation with *Phytophthora cinnamomi.* **a**) Aniline-blue-induced fluorescence image of a phosphite treated 4-week old leaf 48 h post inoculation. Light blue areas are callose deposits at sites of penetration. **b**) Same preparation as in **a**) but showing blue-light-induced fluorescence of callose in a non-phosphite treated 4-week old leaf 48 h post inoculation. Arrows indicate sites of callose deposition.

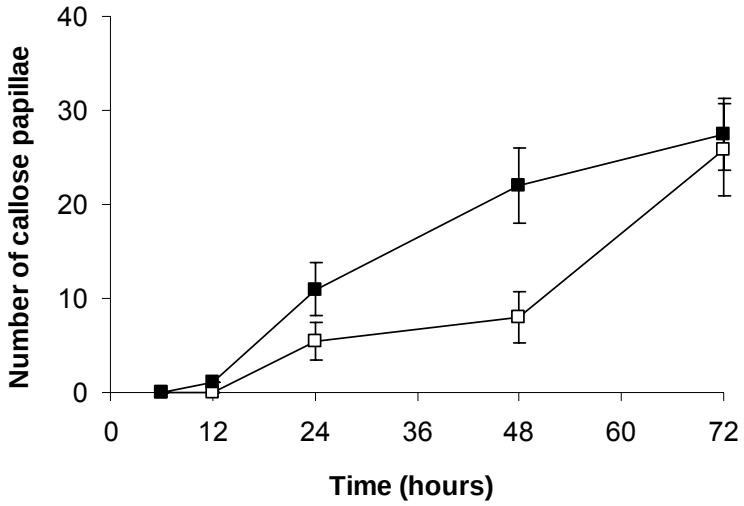


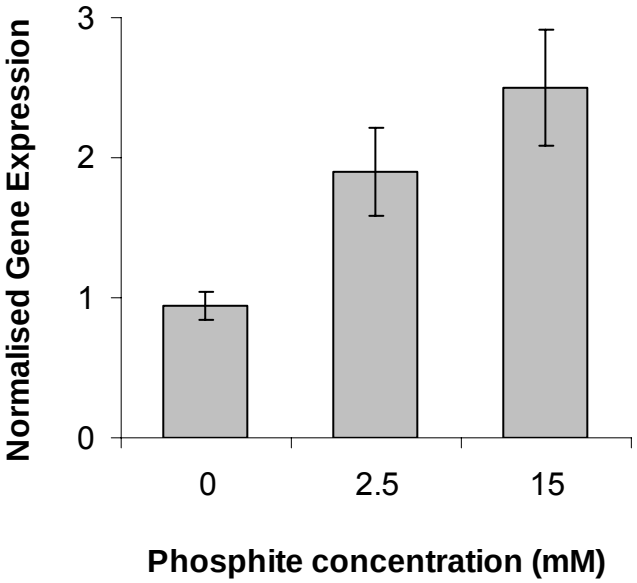
Figure 9

Callose formation in untreated and phosphite treated plants of *Arabidopsis thaliana*. (□) no phosphite, (■) 20 mM phosphite. Vertical bars represent two standard errors of the mean.

The effect of phosphite on defense gene expression was measured in *A. thaliana* grown in MS liquid medium. Phosphite was added to the medium, and 24 h later the leaves were harvested for RNA extraction and analysis of gene expression by qRT-PCR. Expression of the PR1 gene was normalised to expression of the housekeeping genes to allow for variation in the amounts of RNA. The data shows that expression of the PR1 gene was increased by exposure of the plants to 2.5mM phosphite (Figure 10). Further increasing the level of phosphite to 15 mM phosphite resulted in an even higher level of expression of the PR1 gene.

Figure 10

Effect of phosphite on transcription of the PR1 gene of *A. thaliana*. Expression of the PR1 gene was normalised to that of the housekeeping genes (EIFa, PPR). Vertical bars represent two standard errors of the mean.



DISCUSSION

The results show that for a short period of time after inoculation *A. thaliana* ecotype Ler is susceptible to infection by *P. cinnamomi* and application of phosphite makes the plants more resistant. This was indicated by lesion size and callose formation. Phosphite application also induced the expression of host defense genes.

Callose is the deposition of lignin at the site where the mycelium enters the host cell. It is essentially a layer of lignin laid down around the hyphal tips as they attempt to enter a cell and has the effect of containing the mycelium and preventing its further spread through the plant. Callose production is stimulated by H_2O_2 released in response to pathogen infection although a lag phase was evident in both the treated and untreated plants; there was greater deposition in the treated plants at 24 and 48 h post treatment. However, by 72 h post-inoculation callose deposition was similar between phosphite treated and non-treated plants.

Expression of the PR1 gene is induced by phosphite treatment of the plants. PR-1 is required for salicylic acid signalling pathway leading to activation of Systemic Acquired Resistance (SAR) (Thatcher *et al.* 2005). The PR-1 induced response is initiated locally at first and then systemically throughout the plant, resulting in long lasting systemic resistance to a broad range of bacteria, fungi and viruses. Prior induction of the system such as would occur with the application of phosphite would have the advantage of inducing a higher level of resistance in the plant at the time of infection. This would enhance the plants ability to ward off the infection.

Although intact plants of *Arabidopsis* were unsuitable for experimentation with *P. cinnamomi*, we have shown that detached leaves during the first 3 days after infection with zoospores, show significant differences in response to *P. cinnamomi* depending on whether or not they have been sprayed with phosphite. Defense responses appear to be switched on earlier in the phosphite-treated leaves resulting in smaller lesions, more callose papillae, and higher expression of the PR1 gene. Therefore, the cut leaf system appears to have potential for further molecular based studies on determining the mechanisms of resistance induced by phosphite application.

Sub Project 19.2.2.3
The effects of phosphite on *Phytophthora cinnamomi* and of inhibitors of pathogenicity

INTRODUCTION

One way of determining which metabolic processes or signal transduction pathways are important in the effect of phosphite on the pathogenicity of *P. cinnamomi* is to treat the pathogen with a series of signal transduction inhibitors. Inhibitors which reduce pathogenicity can then be further tested to see if they are involved in the inhibition of pathogenicity by phosphite and thus elucidate the pathways of phosphite action. Such inhibitors may lower the concentration of phosphite necessary to inhibit growth, or enhance the efficacy of phosphite.

AIMS

The aims of this experiment were to:

- develop a rapid test for assessing the effects of phosphite on plant infection; and
- determine which inhibitors of metabolic processes or signal transduction pathways reduce pathogenicity of *P. cinnamomi*.

METHODS

Plants and growth conditions

Lupinus angustifolius (narrow leaf lupin) seeds (Department of Agriculture, Wongan Hills Research Station) were germinated in the dark at 25^{o}C, in deionised water. Re-sealable plastic bags (40 x 25 cm) were split open until flat, a layer of bench coat (Bench Roll, Kimberly-Clark, Australia), two paper towels and a piece of Whatman No. 1 filter paper were laid on one side of an open bag and soaked with deionised water. Eight to 10 seeds were placed 2 cm from the top of the bag, and the bag loosely rolled up. The roll was placed into a container filled with deionised water, and put under lights (150 µE) on a 16 h day cycle at 25°C. Water was added to the container as needed. In some instances an extra layer of aluminium foil was added prior to rolling to exclude light from the roots.

Phytophthora cinnamomi isolates

P. cinnamomi isolates MP125 or MP94-48 (Murdoch Culture Collection) were used throughout these experiments. Isolates were chosen because they varied in virulence, growth rate and their sensitivity to phosphite (Wilkinson *et al*. 2001). The pathogen was passaged through apples to ensure pathogenicity prior to all experiments (Ribeiro 1978).

Colonisation of discs

Unless otherwise stated the isolate was grown on V8 medium, which consisted of 10% cleared V8 juice (Campbell's Soups Australia P/L) and 2% Difco bacteriological agar (Bacto Laboratories P/L, Liverpool, NSW). Approximately 20 sterilised 0.5 mm discs of GF/B filter paper (Whatman) were placed aseptically onto a 13 mm Petri dish containing V8 medium that had been point inoculated in 3 places with recently passaged isolates of *P. cinnamomi*. Plates

were incubated in the dark at 25ºC for 5 days to allow the mycelium to colonise the discs.

Treatment of *P. cinnamomi* colonised discs with phosphite
For infusion with inhibitors, the filter paper discs colonised with *P. cinnamomi* were transferred to fresh agar plates and 20 µL of a solution of the inhibitor added to the disc. The discs were then placed onto the roots of lupin seedlings to assay pathogenicity, or onto the *Phytophthora* selective medium NARPH (Hüberli *et al*. 2001) to assay growth of *P. cinnamomi*. The NARPH plates were incubated in the dark at 25ºC for 24 h.

Testing for the presence of phosphite
Prior to inoculation of plants, the phosphite treated *P. cinnamomi* colonised discs were tested for the presence of phosphite using a silver nitrate stain (Section 19.2.2.4).

Inoculation of plants
Colonised and treated discs were placed on to the root tip of 4-day-old *L. angustifolius* seedlings. Bags were incubated and assessed as described under "assessment of disease symptoms and infection by *P. cinnamomi*".

Reisolation of *P. cinnamomi*
At the end of each experiment the colonised discs that had been used to inoculate the plants were plated aseptically onto NARPH medium. All plant roots were also plated out on to NARPH to ensure disease symptoms were due to the presence of *P. cinnamomi* and not a contaminant.

Assessment of disease symptoms and infection by *P. cinnamomi*
Measurements of total visible lesion length and root growth below the point of inoculation were taken on alternate days. At the end of the experiment, at day 10 post germination, the height of the plant was measured and the root was plated out on to NARPH and incubated in the dark for 4 days at 25ºC, to recover the pathogen.

Treatment of *P. cinnamomi* colonised discs with inhibitors
P. cinnamomi colonised discs were placed aseptically onto fresh agar plates or onto sterile, empty Petri dishes. Solutions containing various concentrations of the inhibitors (Table 3) were added to the discs before using them to infect the lupin roots. Pathogen viability after treatment was checked by placing treated discs on to NARPH medium and incubating in the dark at 25ºC for 24 h.

Table 3 Inhibitors tested for their ability to enhance the efficacy of phosphite for controlling *Phytophthora cinnamomi* pathogenicity

Inhibitor[1]	Metabolic processes/enzymes inhibited
Phenyl Arsine Oxide	Protein tyrosine phosphatases
Okadaic Acid	Protein phosphatases
di-butryl-cAMP	Mitigates inhibition of adenylate cyclase, enhances signal transduction

[1]Inhibitors were obtained from Sigma-Aldrich, Australia

RESULTS

Phosphite treatment of *P. cinnamomi*

Treatment of colonised discs with phosphite showed that the ability of the pathogen to infect lupin seedlings and produce disease symptoms was completely inhibited in 2 isolates of *P. cinnamomi* at phosphite concentrations of 3 mM and above (Table 4 - data for one isolate shown). In all phosphite treatments subsequent pathogen growth on V8 agar was not significantly affected.

Phenyl arsine oxide (PAO) treatment of *P. cinnamomi*

Inhibition of growth and pathogenicity in *P. cinnamomi* isolate MP94-48 was not evident at PAO concentrations below 30 mM (Table 4). Complete inhibition of growth and pathogenicity was only observed at concentrations of 100 mM.

Okadaic acid treatment of *P. cinnamomi*

Okadaic acid marginally inhibited growth of *P. cinnamomi* on agar at 10 mM. Greater inhibition was observed at higher concentrations, although complete inhibition was not observed even at concentrations up to 1000 mM (Table 4). A marginal effect on pathogenicity was observed only at the highest concentration tested (1000 mM).

di-butryl-cAMP treatment of phosphite treated *P. cinnamomi*

Di-butryl-cAMP inhibited pathogenicity at the 3mM concentration with a significant reduction at 10 mM (Table 4). At these concentrations growth of the pathogen was not affected.

Table 4 The effect of phosphite, Phenyl Arsine Oxide (PAO), Okadaic Acid and di-butryl-cAMP on growth and pathogenicity of *Phytophthora cinnamomi* isolate MP94-48.

Treatment		Inhibitor concentration (mM)							
		0	**1**	**3**	**10**	**30**	**100**	**500**	**1000**
Phosphite	Growth[1]	+++	+++	+++	+++	++	++	nt	nt
	Pathogenicity[2]	+++	+-	-	-	-	-	nt	nt
PAO	Growth[1]	+++	+++	+++	++	++	-	nt	nt
	Pathogenicity[2]	+++	+++	+++	+++	=-	-	nt	nt
Okadaic Acid	Growth[1]	+++	+++	nt	++	nt	++	+	+
	Pathogenicity[2]	+++	+++	nt	+++	nt	+++	+++	++
di-butryl-cAMP	Growth[1]	+++	+++	+++	+++				
	Pathogenicity[2]	+++	+++	++	+				

[1]Growth refers to the growth of *P. cinnamomi* on V8 agar.
[2]Pathogenicity was measured on lupin seedlings.
+ shows the relative amount of growth or pathogenicity with +++ representing most.
- denotes no growth. nt indicates not tested.

DISCUSSION

We have developed a rapid test to assess the effects of phosphite on plant infection. Filter paper discs covered with *P. cinnamomi* mycelium, placed in contact with the root of germinating lupin seeds lead to infection and lesion development. Infusing the disc with phosphite reduces pathogenicity at concentrations that do not affect mycelial growth when this is tested on V8 agar. The system has enabled fast evaluation of the effects of various inhibitors to determine which of those mimic the effect of phosphite.

Phosphite was the most effective compound at inhibiting the pathogenicity of *P. cinnamomi* as pathogenicity was reduced at 1mM concentration. Neither okadaic acid nor PAO inhibited pathogenicity to any appreciable extent when applied separately, suggesting that protein phosphatases are not important in the inhibitory effect of phosphite. However, treatment of mycelium with di-butryl-cAMP had a significant effect on pathogenicity at 10mM concentration, with no reduction in growth. di-butryl-cAMP is thus likely to be involved in phosphite action on pathogenicity. In future experiments we will continue to refine the experimental system and screen further compounds to explore their effects on pathogenicity, elucidate the pathway of phosphite action, and the possibility that they may enhance the effects of phosphite.

Sub Project 19.2.2.4
Development of methods of assaying *in planta* phosphite to allow assessment of phosphite uptake and distribution throughout the plant

INTRODUCTION

The presence of phosphite in plant material is usually determined by extraction and quantification by HPLC (Roos *et al*. 1999). This is an accurate but time consuming and expensive method. The techniques also require equipment and chemicals that are not readily available. In addition, the techniques are low throughput and expensive (> $30/sample). For these reasons phosphite analysis has posed a major limitation on the scope of this project. Considerable benefit would be derived from being able to use a simple assay/stain to detect phosphite visually in plant material. This will provide us with the ability to a) do phosphite budgets in all plant tissues across a range of plant species with time after phosphite application, and b) determine the time required for phosphite to translocate to the roots, and determine in which tissues it is eventually stored.

We used two approaches to develop simple, accurate and inexpensive assays for determining phosphite in plant tissues:

- Method 1: a chemical staining method that can be used in field situations; and
- Method 2: a more accurate enzyme assay.

Method 1: Chemical staining method for phosphite detection

An assay was developed by Dr D Clarke (Chemistry Department) and P Stasikowski (PhD student) of Murdoch University. Silver nitrate (1 M) and Nitric acid (1 M) are mixed in varying proportions and applied to solutions or to tissues known to contain phosphite. After a variable amount of time (1 min to 2 h depending on material and concentration of phosphite) the phosphite reduces the silver in the silver nitrate to elemental silver which precipitates.

The overall equation for the reaction is:

$$2AgNO_{3\,(aq)} + H_3PO_3 \Rightarrow 2Ag_{\,(s)} + H_3PO_4 + 2H^+_{\,(aq)} + 2NO_3$$

Initially, the precipitate is colloidal and brown (known as a Tyndall effect). This Tyndall effect is caused by the reflection of light by very small particles in suspension and is an indication of the sensitivity of the assay. However, this sensitivity may be offset by cross reactivity with other molecules in organic material. As the reaction proceeds the precipitate may become charcoal grey and insoluble, depending on the concentration of phosphite present.

Preliminary trials with the stain on standard aqueous solutions of phosphite, and phosphite treated plant material showed that considerable background darkening made it difficult to pick up phosphite in all but very high concentrations (in excess of 30 mM). Variation in the time taken for the precipitate to develop was also noted. Silver salts are generally sensitive to light and although at high phosphite concentrations the chemical reduction of

silver nitrate is fairly rapid, at low concentrations the reaction is slow. The assay therefore needs to be carried out in the dark to prevent the development of background.

Method 2: Spectrophotometric method for phosphite measurement *in planta*

The spectrophotometric method was developed based on the enzyme phosphite dehydrogenase produced by the soil bacterium *Pseudomonas stutzeri*. The enzyme converts phosphite to phosphate and at the same time converts NAD to $NADH_2$ (Relyea and van der Donk 2005). The $NADH_2$ can be used to reduce a tetrazolium salt to a coloured compound so that it can be easily observed. The amount of coloured compound produced is proportional to the amount of phosphite in the original sample.

AIM

The aim of these experiments was to develop simple, rapid and cost effective alternative methods to the existing HPLC method for the determination of phosphite concentrations in plant tissues.

METHODS

Method 1: Chemical staining method for phosphite detection

Development of the assay

The assay was tested at different temperatures to determine the optimum at which distinct colour separation occurs.

Phosphite (0, 5, 10, 25, 50 and 100 mM) was added to small absorbent discs which were placed into Petri dishes and chilled at 4^{o}C for 15 min. 5 µL of (20:1 $AgNO_3/HNO_3$) was added to each of the discs. The dishes were then incubated at 20, 25, 30 and 35^{o}C. Colour formation on the disks was monitored and recorded at 5, 10, 15 and 20 min using a colour intensity scale that was established at the beginning of the experiment. There were three replicate dishes for each temperature.

Development of reference colours for standard concentrations of phosphite

Glass fibre filters were infused with different concentrations of phosphite (0 - 100mM) and then treated with Ag^{++}.

Detection of phosphite in planta

Three to four-week-old *L. angustifolius* plants grown in paper rolls (described previously) were sprayed to run-off with 0.5% phosphite (Agri-fos). Plants were harvested, separated into root and stem sections and stained, either as stem or root segments, or after crushing 50 mg of plant material and adding 50 µL of the silver nitrate/nitric acid (20:1) mixture. Tubes were incubated in the dark at

25ºC for 15 min. Stained stem and root segments were also sectioned whilst frozen and visualised under a light microscope to determine where phosphite was retained at a cellular level.

Method 2: Spectrophotometric method for phosphite measurement *in planta*

A comparison was made of the effectiveness of the crude extract of the enzyme phosphite dehydrogenase produced by the soil bacterium *Pseudomonas stutzeri* with that of a purified sample. The enzyme was purified to homogeneity as assayed by polyacrylamide gel electrophoresis (Figure 11). Preliminary tests showed that the crude extract performed better in phosphite measurement assays compared to the highly purified enzyme (data not shown) and the following experiments were conducted using crude extracts of the enzyme. The enzyme appears to be stable when stored at -20^{o}C.

A standard curve was constructed using different phosphite concentrations, but when plant extracts containing known concentrations of phosphite were added to the reaction the results were not linear. The effect of incubation of the extracts with 50 gm/mL of activated charcoal was tested to reduce interference.

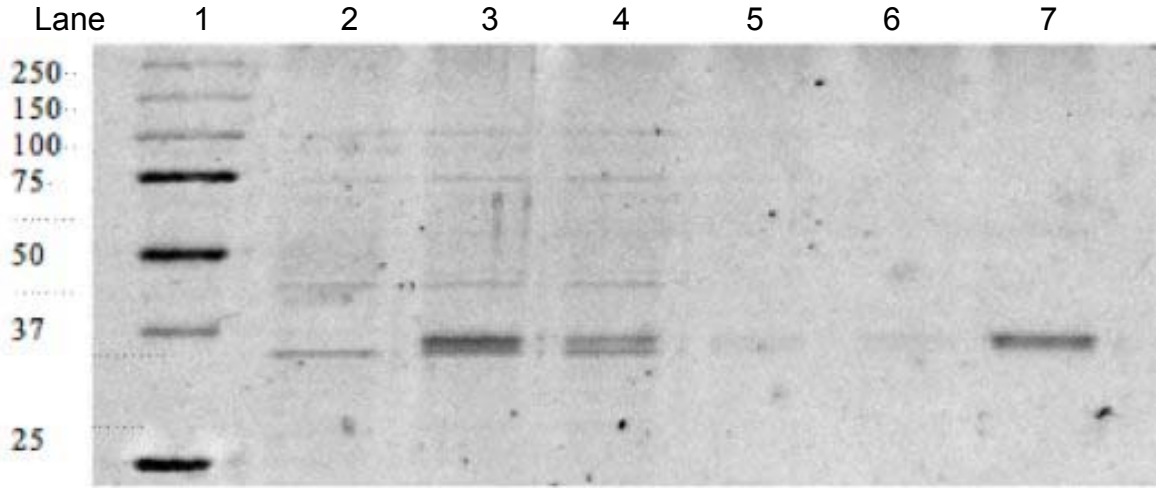


Figure 11 Over-expression and purification of phosphite dehydrogenase from *E. coli* BL21 (DE3) pET15b-*ptxD.*

Lane 1 - molecular weight markers (Precision Plus Protein Standards, Bio-Rad);
Lane 2 - lysate of an *E. coli* culture grown in LB (Luria-Bertani) broth in the absence of IPTG (Isopropyl-thio-galactoside);
Lane 3 - lysate of an identical culture after incubating for 3 h in the presence of 0.1 mM IPTG;
Lanes 4 to 6 - flow through solutions of consecutive wash steps using the MagneHis protein purification kit;
Lane 7 - purified phosphite dehydrogenase.

RESULTS

Method 1: Chemical staining method for phosphite detection

The rate of reaction increased with increasing temperature. The optimal temperature was 30°C, as at 35°C the reaction occurred too rapidly to detect differences between the higher concentrations of phosphite. The lower temperature was therefore used. At this temperature some phosphite detection was possible in 10 - 15 min.

Staining intensity on the glass fibres was directly related to the concentration of phosphite on the filter (Figure 12).

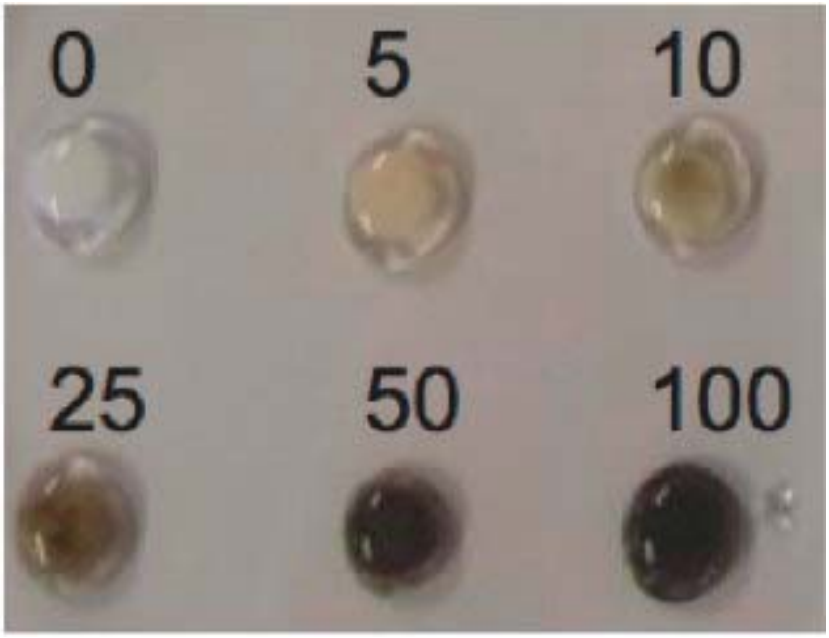
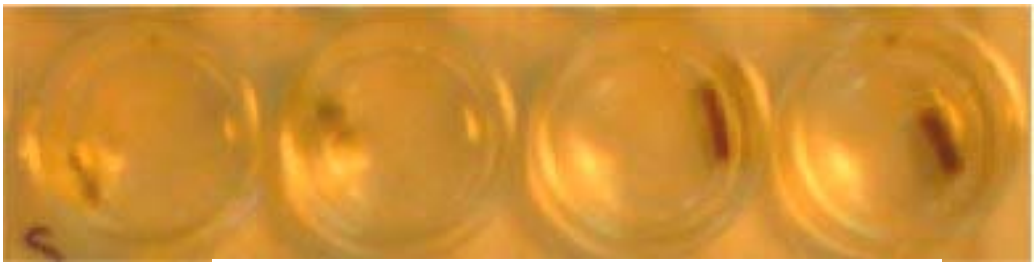


Figure 12 Silver staining intensity on filter discs impregnated with 0 – 100 mM concentration (mM) phosphite.

It was possible to detect phosphite in the plant tissues using segments of different tissues and staining for the presence of phosphite using the Ag^{++} reagent.

Phosphite was detected in the upper stem segments 4 h after treatment with 0.5% phosphite (Figure 13). It was found that keeping plant material very dry, almost semi-desiccated during the reaction enhanced the uptake of silver nitrate and reduced background staining. Desiccation was achieved by placing the whole plant or plant sections between absorbent paper overnight.

The alternative method of treatment of the plant samples by crushing and assaying the suspension was quicker and gave clearer results (Figure 14).



Control **Test**

Figure 13 Measurement of the translocation of phosphite in upper stem segments of *Lupinus angustifolius*. Sections were harvested 4 h after treatment with phosphite and stained for the presence of phosphite. The control sections are shown on the left.

Control **Test**

Figure 14 A comparison of phosphite levels in macerated tissue segments of *Lupinus angustifolius* untreated (control) and 6 h after spraying with 0.5% phosphite after 6 h.

Sectioned material showed a high level of background staining, and observation using the light microscope caused continuation of the reactions with Ag^{++}. Success of the stain using thin sections will depend on being able to fix the stain and stop the continued development of the silver precipitate.

Method 2: Spectrophotometric method for phosphite measurement *in planta*

The amount of coloured tetrazolium salt produced shows a linear response to the amount of phosphite in test solutions in the range (Figure 15). The

treatment of the plant extract with activated charcoal resulted in a linear measure of absorbance against phosphite concentration (Figure 16).

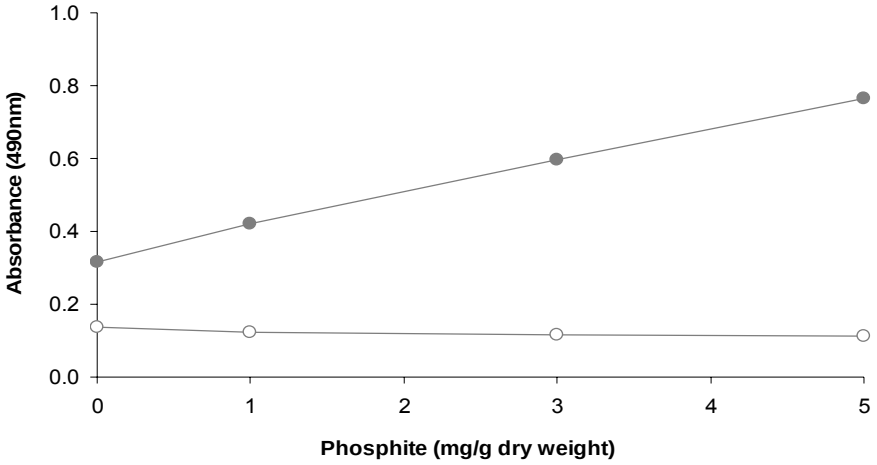


Figure 15 Phosphite standard curve using the phosphite dehydrogenase assay. Absorbance at 490 nm in response to increasing phosphite concentration with (—●—) and without (—○—) the addition of phosphite dehydrogenase enzyme. Data points represent means of triplicate measurements.

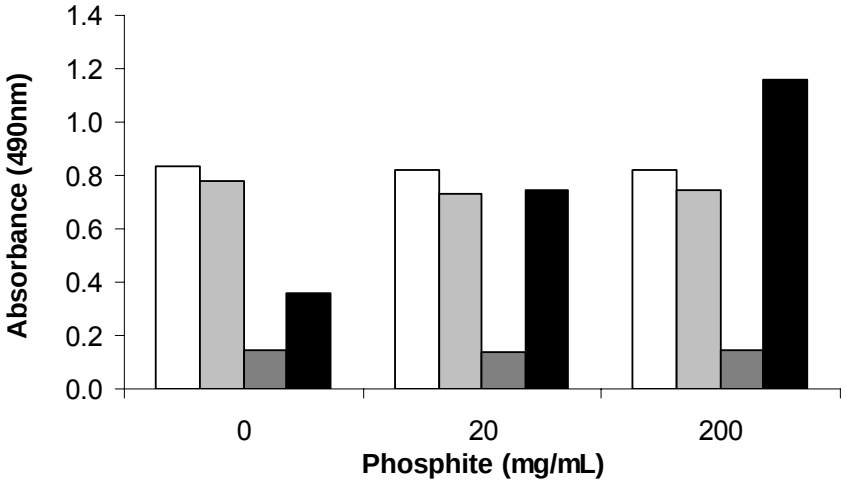


Figure 16 Effect of treating plant extracts with activated carbon on the enzymatic phosphite assay. Jarrah leaf extracts containing 0, 20 and 200 µg of phosphite/mL were either not treated (controls), or incubated overnight with 50 mg/mL of activated carbon. Samples were then added to the assay buffer with or without phosphite dehydrogenase enzyme. Control – enzyme (☐); control + enzyme (□); activated carbon – enzyme (■); and activated carbon + enzyme (■).

Sub Project 19.2.2.4
Development of methods of assaying *in planta* phosphite to allow assessment of phosphite uptake and distribution throughout the plant

DISCUSSION

Phosphite measurement in plant tissues over time since application is one of the major limitations on the scope of experiments designed to analyse the transport and accumulation of phosphite in different plant species. In this project we have designed two simple, cheap and reliable methods for measuring phosphite. The chemical staining method was demonstrated to be effective although it is affected by compounds in the plant. The results obtained depend very much on the standardisation of the conditions used in the assay. Quantitative determination of phosphite using this assay may be possible. This will require more rigorous assay conditions and precise proportions of reacting ingredients. We will continue to refine this assay to make it a more robust and commercially viable technique. We aim to develop a protocol that can be carried out in the field to monitor sites previously treated with phosphite, in order to identify when they require to be resprayed with phosphite to ensure their on-going protection from the pathogen. These studies are now in progress in PhD projects.

We have also developed a phosphite assay based on the phosphite dehydrogenase gene of *Pseudomonas stutzeri*. This is a simple, low cost, high throughput spectrophotometric assay, which takes advantage of the enzyme's ability to convert phosphite to phosphate. The technique can be carried out on small amounts of plant tissue. In future experiments, we will be comparing this method with the current chemical method (WA Chemical Laboratories) for sensitivity and robustness. We will also assess its performance on a wide variety of native plant species.

The advantages of the two methods are that they are cheap and rapid. Once they have been refined they will allow study of phosphite uptake and distribution in the plant under different physiological conditions. The silver nitrate test was used successfully in the experiments in section 19.2.2.11.

Sub Project 19.2.2.5
Development of lupin as a model system of intact plants for use in assessment of the effects of the addition of surfactants to phosphite spray

INTRODUCTION

One way of improving the efficacy of phosphite is to enhance its uptake. Historically, adjuvenants used in foliar sprays have been limited to 'sticker' agents (vegetable oil based; Barrett *et al.* 2003, CALM 1999), but penetrants are now favoured (for example, non-ionic alcohol alkoxylate; S Barrett, *pers. comm*.). Between-species differences in phosphite concentration in plants treated with the same surfactant under similar conditions can be 2 - 10 fold (Pilbeam *et al*. 2000, Wilkinson *et al.* 2001, WA Dunstan, unpublished data). To our knowledge, a systematic assessment of the effectiveness of adjuvenants, and their compatibility with phosphite, has never been completed.

Surfactants are wetting agents that lower the surface tension of a liquid, allowing easier spread and reducing spray droplet size. The surfactants allow phosphite to stick to and spread over the leaf, and may thus increase the amount of phosphite that is absorbed by the plant.

An efficient way to approach the screening of surfactants is to initially test a range of surfactants *in vitro* on a model plant species before applying the best treatments to native species in the glasshouse. A screening protocol, using lupin seedlings, is described that is ideal for initial testing of spray treatments. Lupins are fast growing, occupy little space and are susceptible to *Phytophthora cinnamomi* in the first 3 weeks of their growth. Preliminary experiments using this system showed that there was no improvement in phosphite efficacy with inclusion of the surfactants BS1000 (Crop Care, Murarrie, Qld), or Synertrol Oil (Organic Crop Protectants, Lillyfield, NSW) at the manufacturer's recommended concentrations, while Pulse appeared to reduce the phosphite concentration at which lesions were contained (Appendix I). Pulse is an organosilicate penetrant/surfactant commonly used with herbicides for improved penetration of several agricultural pesticides by improving the spread of spray droplets on leaves. The active constituent of this non-ionic wetter is 1020 g/L polyether modified polysiloxane (http://www.nuturf.com.au).

Further information suggested that standard rates of Pulse given by the manufacturer may not be high enough to achieve improvements in efficacy of phosphite, and that a level of 0.25% is used in the field on Australian native species (J Moore *pers. comm*.). We report here results of a comparison of the addition of 0.1 and 0.25% Pulse to phosphite sprays.

AIM

This research aims to achieve additive or multiplier effects in the strength and/or duration of host responses to *Phytophthora* by increasing the efficiency of uptake of phosphite through use of previously untested surfactants, and by

using phosphite in combination with other compounds that elicit plant defense responses.

METHODS

Lupin seeds were bubbled in water overnight before sowing. This increases uniformity of germination. Seeds were then placed on a bed of damp absorbent paper (24 x 38.5 cm), sandwiched between two layers of clear plastic, then rolled up and placed in a jar of water at 5 cm depth. Rolls were cultured under a 12/12 h light/dark cycle at 25°C (Figure 17).

Figure 17

Lupin seedlings growing in 'rolls' placed in jars containing water

After 6 days, seedlings with radicles longer than 5 cm and well-developed leaves were selected and grouped into 4 replicates of 10 seedlings per treatment (Figure 18). On day 6, plants were infected with a 0.5 cm disc of culture of *P. cinnamomi* isolate MP94-48 on V8 agar which consists of 10% cleared V8 juice (Campbell's Soups Australia P/L) and 2% Difco bacteriological agar (Bacto Laboratories P/L, Liverpool, NSW). The *P. cinnamomi* colonised disks were placed on the tip of the radicle with the mycelium side in contact with the root. Seedlings were sprayed on day 7 with water or 0.5% phosphite (Agri-Fos 600, Agrichem, Loganholme, Qld), with or without the addition of Pulse at 0, 0.1% (recommended by manufacturer) and 0.25% (recommended by J Moore, Agwest). Each treatment consisted of 4 replicates of 10 seedlings. Lesion lengths in roots were measured on day 13 to determine if the surfactant had influenced the effect of phosphite on plant resistance (Figure 18).

Lesion shown with arrows

Figure 18

➢ Infected lupin seedlings arranged on moist paper towel for lesion measurement at 3 days post spraying

➢ Measurement of lesion length at 3 days post spraying

RESULTS

Phosphite (0.5%) significantly reduced the length of lesions in this model system using lupin roots. There was no evidence of a significant reduction of lesion length induced by phosphite with the inclusion of Pulse at 0.1 or at 0.25% Pulse (Figure 19).

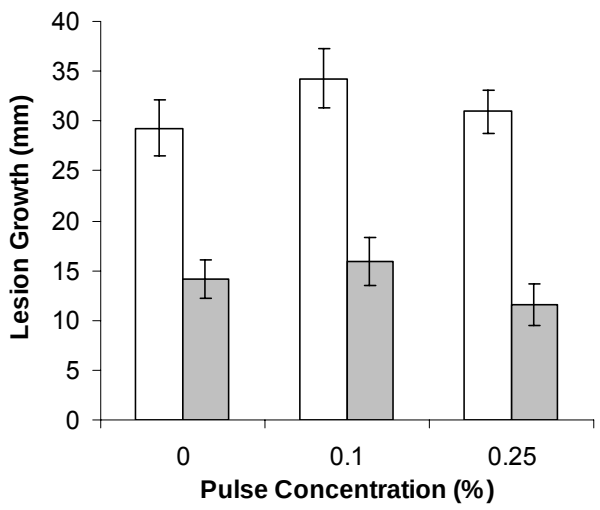


Figure 19

Lesion length (mm) in *Phytophthora cinnamomi* infected lupin roots 6 days after spraying foliage with 0 (□) or 0.5% phosphite (▣) at different Pulse concentrations (0, 0.1 and 0.25%). Vertical bars indicate two standard errors of the mean

DISCUSSION

There is little evidence from the assessments of lesion lengths in the model plant species, lupin, that surfactants greatly increase phosphite efficacy. While there was no increase in efficacy in the model species, which is an herbaceous species and was used at the seedling stage, the native species of interest are woody shrubs that may show a different response to the surfactants. The protocol selected for further experiments was the treatment of plants with 0.5% phosphite and 0.25% Pulse as this is what is currently being used in the field in Western Australia.

Sub Project 19.2.2.6
Screening of surfactants to improve the efficacy of phosphite in native plants

INTRODUCTION

Leaf surfaces vary between plant species, and certain surfaces avoid moistening, particularly those on waxy and hirsute (hairy) leaves. Leaf orientation can also affect the time that a spray is in contact with the leaf surface, for instance vertically hanging leaves of eucalypts would have a reduced contact time compared to horizontal leaves in other species. Surfactants can increase the spread and contact time by reducing the surface tension of the treatment upon the leaf.

To test whether the addition of a surfactant affects uptake of phosphite into leaf tissue, two species with different leaf surfaces, *Banksia baxteri* (hairy surface) and *Eucalyptus staeri* (glabrous surface), grown in the glasshouse were sprayed with phosphite with or without the addition of Pulse surfactant. Apart from providing information on species differences, the results will indicate whether Pulse increases the rate and/or time period of phosphite uptake and the optimum time to harvest leaves in future experiments when a measure of uptake is needed.

AIM

This experiment measured the uptake of phosphite into leaves with different surface structure and the effect of the inclusion of Pulse at different concentrations in the spray solution.

METHODS

Experimental design

Glasshouse grown 12-month-old *B. baxteri* and *E. staeri* plants were sprayed to run-off with 0.5% phosphite with the addition of 0, 0.1 or 0.25% Pulse. There were 10 plants in each treatment.

Phosphite analysis

At 0, 15, 30 and 45 min. after spraying, leaves were harvested from the plants. Two leaves were removed from each plant, at each time point, and a random mixture of leaf sizes and ages were selected. They were washed in a solution of Deconex phosphate-free detergent (Borer Chemie AG, Zuchwil, Switzerland), rinsed twice in water, blotted dry, and allowed to air-dry at 37^{o}C for 7 days. The leaves were ground using a rotary grinder and their phosphite content was analysed by the WA Chemistry Centre.

RESULTS

Pulse increased the internal phosphite concentration of the leaves of both plant species, at most increasing the concentration in *E. staeri* 6-fold and in *B. baxteri* 4-fold. *E. staeri* had a higher internal phosphite concentration at the highest Pulse concentration (Figure 20a), while this was not so in *B. baxteri* (Figure 20b). More phosphite accumulated in the leaves of *E. staeri* more

rapidly than in *B. baxteri*. In both species uptake was virtually complete within 15 minutes. Uptake was greatest in *E. staeri* using 0.25% Pulse while in *Banksia* the 0.1% Pulse gave slightly higher uptake.

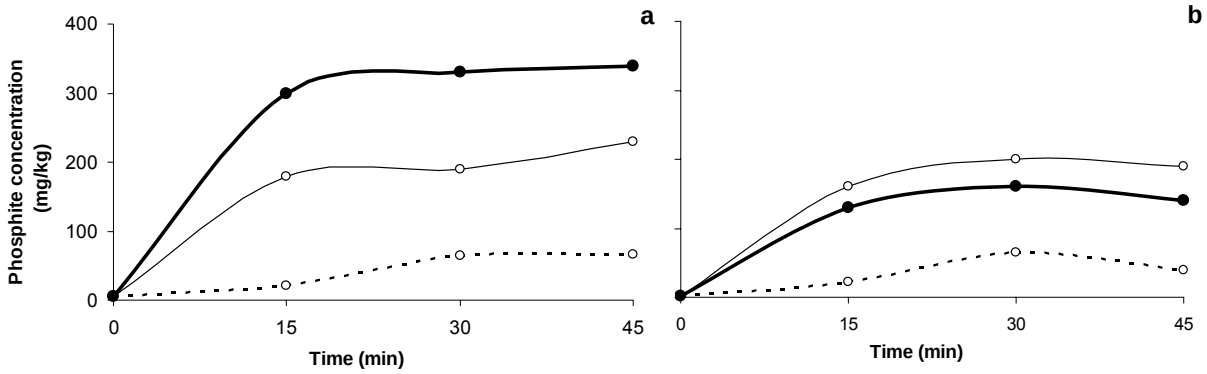


Figure 20 Phosphite concentration in leaves of **a**) *Eucalyptus staeri* and **b**) *Banksia baxteri* plants sprayed to run-off with 0.5% phosphite containing 0 (dashed line), 0.1 (solid line, open symbol) or 0.25% Pulse (solid line, filled symbols).

DISCUSSION

The addition of Pulse greatly increased the concentration of phosphite in leaves of both plants tested compared to the controls, and the final concentration of phosphite in the leaf was greatest in *E. staeri*, especially when 0.25% Pulse was used.

The higher concentrations of phosphite in the leaves of *E. staeri,* compared to *B. baxteri,* may be due to either of two scenarios. Firstly, *E. staeri* may take up more phosphite than the *Banksia* because *Eucalyptus* leaves have stomata on both surfaces, while in *Banksia* stomata occur on the top surface of the leaf only. Secondly, both species may take up similar amounts of the compound, but *Banksia* translocates it from the leaf more quickly than *Eucalyptus.*

In summary, the results clearly demonstrate the need to apply a penetrant such as Pulse when treating native plant species with phosphite to control *P. cinnamomi.* Pulse at 0.25% would be recommended over 0.1%.

Sub Project 19.2.2.7
Screening of plant defence activators to improve the efficacy of phosphite using the lupin model plant

INTRODUCTION

Plant defense activators induce non-specific forms of disease resistance against a wide range of pathogens. They act by inducing the production of plant hormones (jasmonic acid, salicylic acid and ethylene) which are the major regulators of induced resistance. Jasmonic acid dependant defense responses are generally considered to provide a defense against necrotrophic pathogens (Pieterse and Van Loon 2007). There are a number of plant defense activators, which have been studied in a range of plant-pathogen systems that have potential for use in controlling *Phytophthora* diseases.

Analogs of salicylic acid, including Acibenzolar-S-methyl (benzothiadiazole, BTH; 'Bion ®', Syngenta) and benzoic acid (BZA), activators of systematic acquired resistance (SAR; Lawton *et al.* 1996), have been demonstrated to provide protection to some plant species against a range of pathogens (Benhamou *et al.* 1998a, Colson-Hanks and Deverall 2000, Iriti and Faoro. 2003), including some Oomycetes (Benhamou and Belanger 1998b, Godard *et al*. 1999). Bockshi *et al.* (2003) found that below-ground organs of potato (but not including roots) were protected by foliar applications of BTH, indicating basipetal movement of the compound. In some experiments, health of avocado trees treated with phosphite plus BTH (Bion®) was shown to be better than those treated with phosphite alone (Whiley *et al.* 2001). In contrast, our own experiments with *Eucalyptus marginata* showed reduced protection against *Phytophthora cinnamomi* in combined phosphite + BTH treatments (WA Dunstan, unpublished data). High volume foliar application of BZA has been shown to protect *Banksia attenuata* against *P. cinnamomi* (Williams *et al.* 2003). Jasmonic acid (JA) has also been found to induce systemic resistance to *Phytophthora* in potato and tomato (Cohen *et al.* 1993). The induced systemic resistance pathway (ISR) is partially independent of the SAR pathway (Hammond Kosack and Parker 2003). Transport of JA from shoots to roots, mainly via phloem, has been demonstrated by Pu *et al*. (2003). JA application may also result in an increase in photosynthetic partitioning to roots (Babst *et al*. 2004), therefore there is potential for JA application to be of benefit in two ways, by induction of ISR, and indirectly by creating a sink (increased root growth) for movement of phosphite from shoot to roots. BTH, BZA and JA (and derivatives) have demonstrated protective effects in foliar sprays at concentrations between 10 - 250 mg/L, indicating aerial application of these compounds may be economically feasible.

Three plant defense activators were tested in the present study. Methyl jasmonate (MeJ) Methyl ((1R,2R)-3-Oxo-2-(2Z)-2-pentenyl-cyclopentane-acetate) along with jasmonic acid acts as a signalling compound for the production of phytoalexins, which are involved in plant defense. The jasmonate signal often spreads systemically throughout the plant and is a major component of systemic acquired resistance.

DL-ß-amino-n-butyric acid (BABA) is reported to induce resistance to subsequent infection by *Phytophthora capsici* of pepper plants (Sunwoo *et al*. 1996). BABA also had a curative effect by inducing systemic resistance in lettuce against downy mildew (Pajot *et al*. 2001).

Benzothiadiazole (BTH; Acibenzolar-S-Methyl) is a known systemic activator of salicylic acid-dependent *PR-1* induction in plants (Borges *et al*. 2003). The response is manifested by increased tolerance to infection by the virulent pathogen *Phytophthora palmivora*, by increased B-1,3-glucanase and chitinase activities, and by increased accumulation of a PR1 mRNA. Infection of untreated papaya by *P. palmivora* also induced B-1,3-glucanase and chitinase activities but at much lower levels. This response to *P. palmivora* is characteristic of a compatible interaction (Zhu *et al*. 2003).

The three defense activator compounds were tested in combination with phosphite for their ability to increase the efficacy of phosphite in controlling infection caused by *P. cinnamomi. Lupinus angustifolius* cv. 'tanjil' was used as a model plant.

AIM
To determine whether defense activator compounds used in combination with phosphite can increase the effectiveness of phosphite to control *P. cinnamomi.*

METHODS

Lupin seedlings were grown in paper rolls as described above**.** On day 6, plants were infected with a 0.5 cm disc of culture of *P. cinnamomi* isolate MP94-48 on V8 agar which consists of 10% cleared V8 juice (Campbell's Soups Australia P/L) and 2% Difco bacteriological agar (Bacto Laboratories P/L, Liverpool, NSW). The *P. cinnamomi* colonised disks were placed on the tip of the radicle with the mycelium side in contact with the root. Seedlings were sprayed with water or 0.5% phosphite (Agri-Fos 600, Agrichem, Loganholme, Qld), with or without the addition of a plant defense activator on day 7. Each treatment consisted of 4 replicates of 10 seedlings. Lesion lengths in roots were measured on day 13 to determine if the activator compounds had influenced the effect of phosphite on plant resistance.

The plant defense activators selected were sourced as follows: β-amino butyric acid (BABA) (Sigma-Aldrich, Australia), methyl jasmonate (Sigma-Aldrich, Australia), and Acibenzolar-S-Methyl (BTH) (Novartis Crop Protection). Application rates were 0.1 mM and 1 mM for methyl jasmonate (based on Desmond *et al.* 2006), 4.85 mM and 48.5 mM for BABA (based on Cohen 2002) and 0.12 mM and 1.2 mM for BTH (Zhu *et al.* 2003).

In a further experiment designed to test for the presence of interactions between activator compounds, plants were treated with 0.5% phosphite and a

combination of plant defense activators (BABA 0.5 mg/L plus methyl jasmonate 1 mM).

In a third experiment, lupin seeds were soaked overnight with aeration in solutions of the activators to test if germinating the seeds of lupins by soaking them in solutions of the defense activators would have an effect on the efficacy of sprayed phosphite in controlling infection by *P. cinnamomi*. The seeds were then placed in rolls of paper for germination (Figure 17). At 6 days old they were infected with *P. cinnamomi* and sprayed with 0.5% phosphite on day 7. Seeds soaked in methyl jasmonate displayed significantly reduced germination and hence methyl jasmonate was not used in the experiment.

RESULTS

There was no significant ($p > 0.05$) decrease in lesion length (i.e. increase in the efficacy of phosphite) induced by addition of any of the plant defense activators (Figures 21 to 23) in the presence or absence of phosphite in lupins. In the case of BTH at 1.2 mM combined with phosphite, an increase ($p = 0.09$) in lesion length was observed (Figure 23). Furthermore, there was no increase in the efficacy of phosphite when seeds were germinated by soaking overnight in solutions of the activators (Figure 24).

Combining plant defense activators (BABA 0.5 mg/L plus methyl jasmonate 1 mM) also showed no enhancement over the effect of phosphite (data not shown).

DISCUSSION

None of the defense activators increased the efficacy of phosphite to control *P. cinnamomi* in the model plant species, lupin. Therefore, subsequent trials were conducted using native Australian species in a field trial and a glasshouse trial. While there was no increase in efficacy in the model species, which is a herbaceous species and was used at the seedling stage, the native species of interest are woody shrubs that may show a different response to the activator compounds. These trials are described in the next sections.

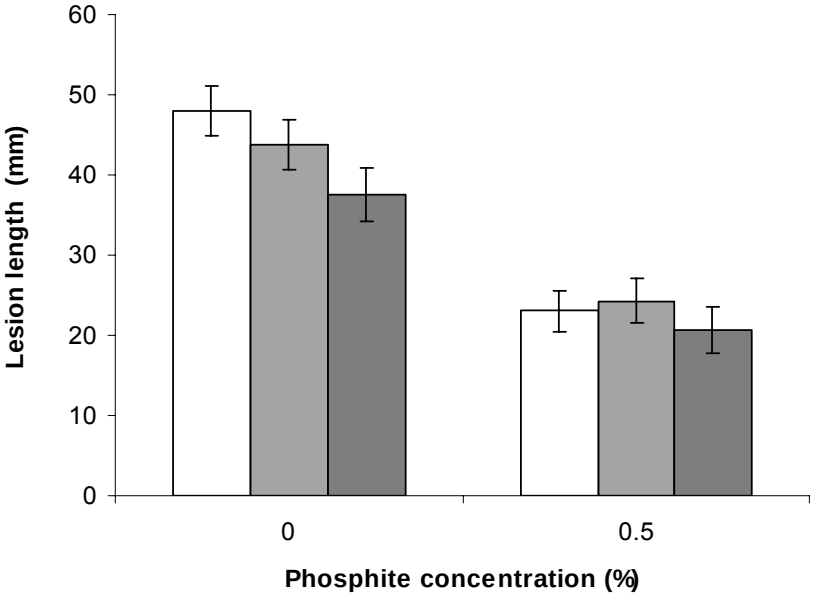
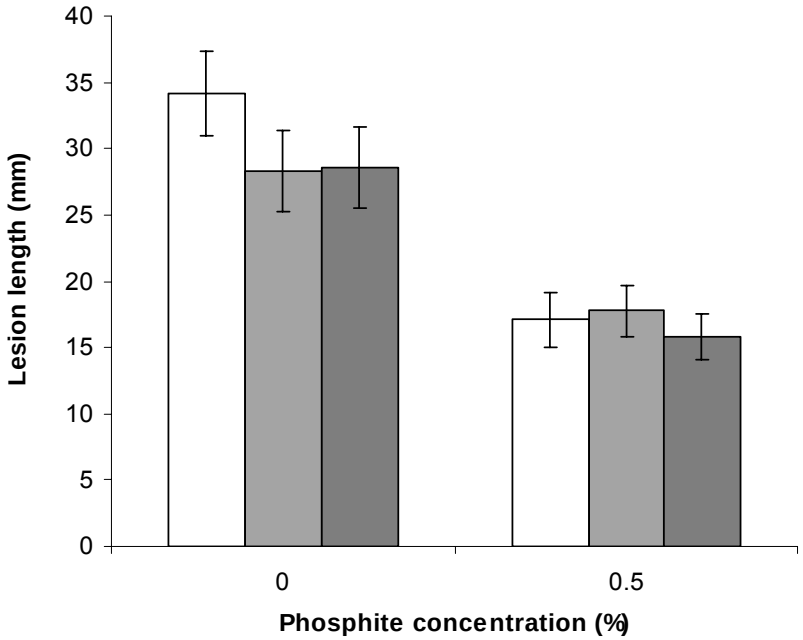


Figure 21

Lesion lengths in lupin roots infected with *Phytophthora cinnamomi* 6 days after spraying foliage with methyl jasmonate at 0 (□), 0.1 mM (▣) and 1 mM (▩) with 0 and 0.5% phosphite. Vertical bars represent two standard errors of the mean.

Figure 22

Lesion lengths in *Phytophthora cinnamomi* infected lupin roots 6 days after spraying foliage with β-amino butyric acid (BABA) at 0 (□), 4.85 mM (▣) and 48.5 mM (▩) with 0 and 0.5% phosphite. Vertical bars represent two standard errors of the mean.



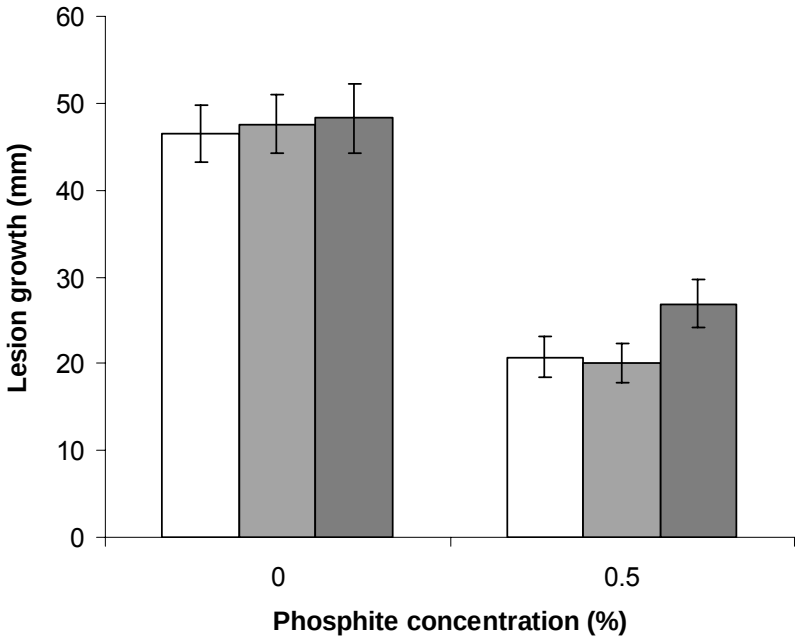
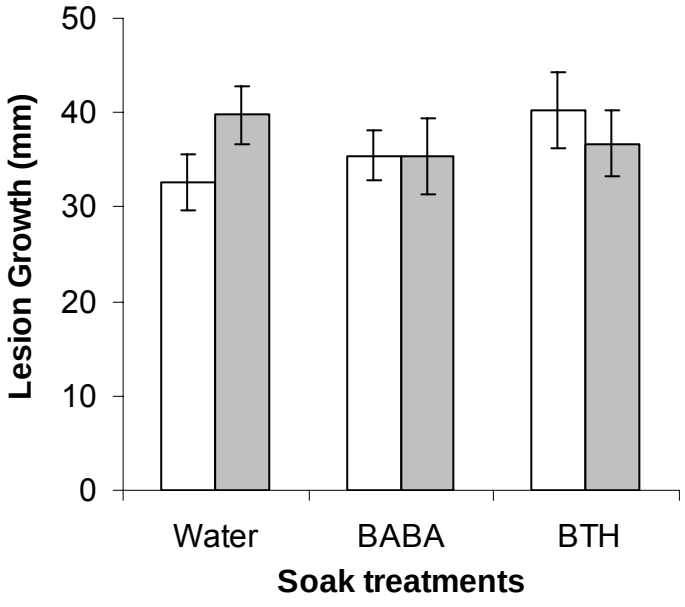


Figure 23

Lesion lengths (mm) in *Phytophthora cinnamomi* infected lupin roots 6 days after spraying foliage with Acibenzolar-S-Methyl (BTH) at 0 (□), 0.12 mM (▣) and 1.2 mM (▦) with 0 and 0.5% phosphite. Vertical bars represent two standard errors of the mean.

Figure 24

Lesion lengths (mm) in *Phytophthora cinnamomi* infected lupin roots 6 days after spraying with water (□) or 0.5% phosphite (▣). Seeds were soaked before germination in water, 5 g/L β-amino butyric acid (BABA), or 1 mM Acibenzolar-S-Methyl (BTH) for 24 h. Vertical bars represent two standard errors of the mean.



Sub Project 19.2.2.8
Screening of plant defence activators to improve the efficacy of phosphite using native plant species – a field trial

INTRODUCTION

The three plant defense activators selected for the primary lupin screening trial; β-amino butyric acid (BABA), methyl jasmonate (MeJ) and Acibenzolar-S-Methyl (BTH) were tested on *Banksia attenuata*, in the field. This species occurs near Perth, WA as well as on the south coast sand plain of WA.

AIM

The aim of this study was to examine whether plant defense activators can increase the efficacy of phosphite in field grown *B. attenuata* challenged with *P. cinnamomi*.

METHODS

A field trial to screen the defense activators was conducted at Jandakot Airport bushland, approximately 9 km south-east of Murdoch University, WA. using *B. attenuata*. Approximately 200 suitably sized plants were mapped (Figure 25). The plants were under-bark inoculated with *P. cinnamomi* isolate DP55 (Figure 26) on branches of < 1.5cm diameter at points at least 10 cm above ground level. The bark flaps were replaced over the inoculum disc and the wound was covered with a 1 cm^3 piece of moist cotton wool. The area was wrapped with a strip of Parafilm and then covered with water-proof tape. Eighty-five plants were inoculated with 5 mm discs of *P. cinnamomi* grown for 7 days on V8 agar and 25 were inoculated with uncolonised V8 agar discs as controls.

Phosphite and enhancer treatment

Eleven days after being inoculated, the plants were sprayed with solutions of 0 and 0.5% phosphite containing 2.5 g/L BABA, 1 mM MeJ, 2 mM BTH or water. All solutions contained 0.1% Pulse penetrant (Monsanto, Australia). The MeJ was dissolved in 5% ethanol while the other treatments were in aqueous solutions. The solutions were applied using a 1 L hand-pressurised spray bottle and sprayed to run-off, covering as much of the foliage as possible. Spraying was carried out in the early morning to take advantage of low wind conditions.

There were 8 treatments with 10 experimental replicates and 3 control replicates per treatment.

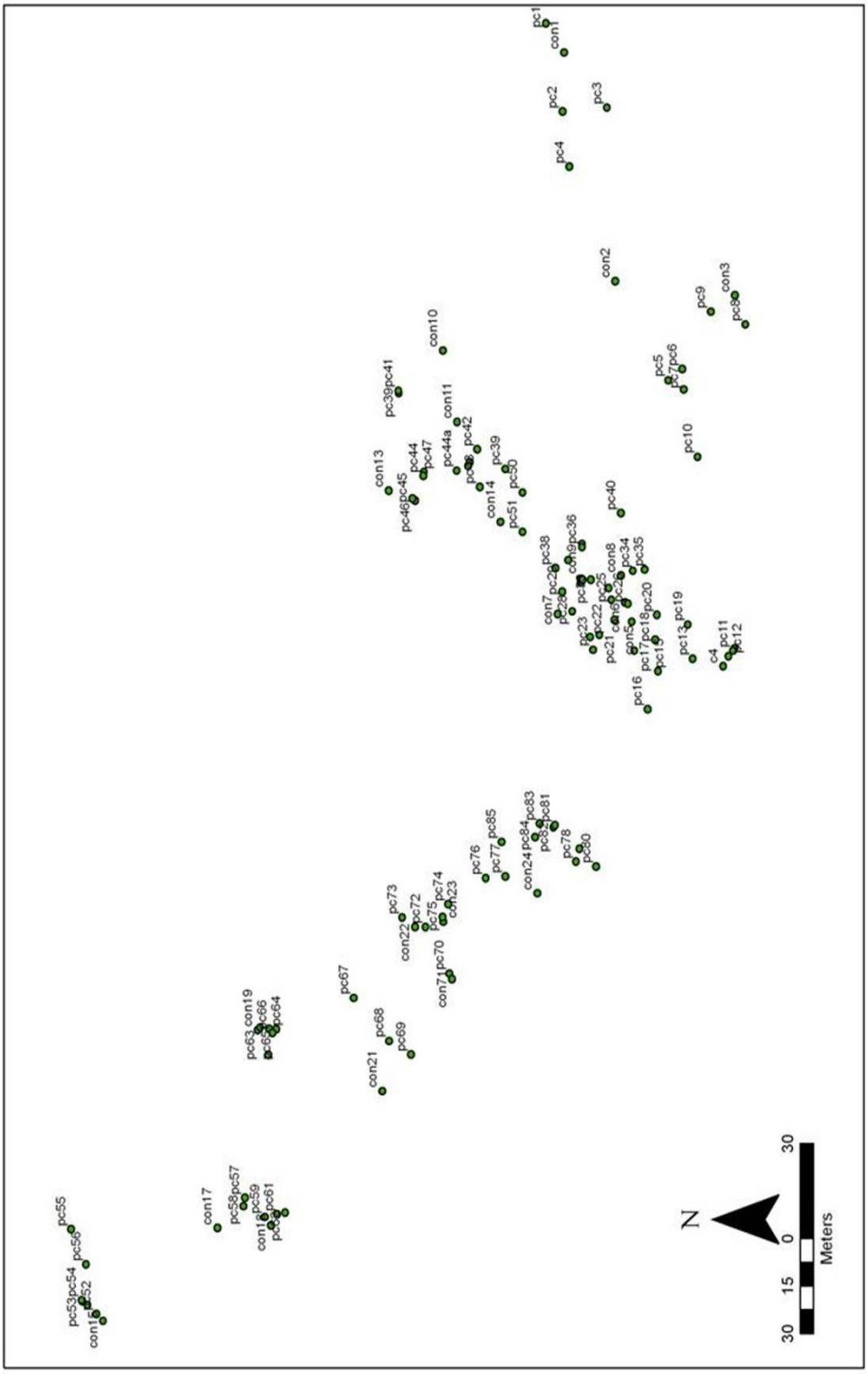


Figure 25 *Banksia attenuata* mapped at Jandakot Airport for screening the plant defense activators

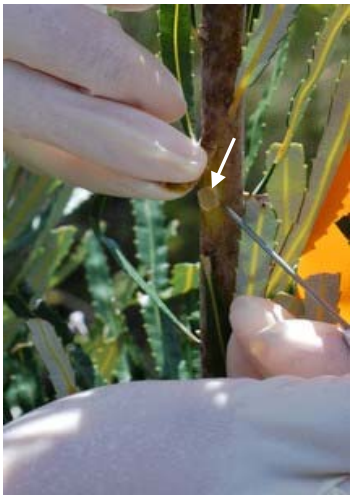


Figure 26

Underbark inoculation of *Banksia attenuata* in the field. White arrow indicates inoculum plug

Harvesting, data collection and analysis
At 4 weeks after inoculation, the inoculated side branches were removed and returned to the laboratory. Visible lesion lengths were measured in the laboratory and the presence of *P. cinnamomi* was confirmed by plating 3 x 1 cm stem sections, from the wound-inoculation point itself and from the inner edges of the lesion boundary, onto *Phytophthora* selective media (NARPH; Hüberli *et al.* 2000). To determine the extent of spread of *P. cinnamomi* in the branch, five 1 cm sections of the stem from each end of the lesion front were plated onto NARPH. Plates were incubated and monitored for 10 days to detect the presence of *P. cinnamomi*.

Statistics
ANOVAs were carried out in Statistica software package Version 5 (Statsoft 1999) to determine whether lesion length and colonisation lengths were affected by the phosphite and/or enhancer treatments.

RESULTS

There were no significant differences in lesion lengths ($p > 0.3$) or colonisation lengths ($p > 0.4$) between the treatments. In this experiment phosphite alone did not reduce lesion length compared to the treatment with water (Figure 27). Ten percent of plants treated with MeJ and BTH in combination with phosphite had no visible lesion or evidence of colonised tissue. These were the only plants that did not form lesions. Power analysis showed a high level of variance between replicates and a higher numbers of replicates were needed to achieve significance.

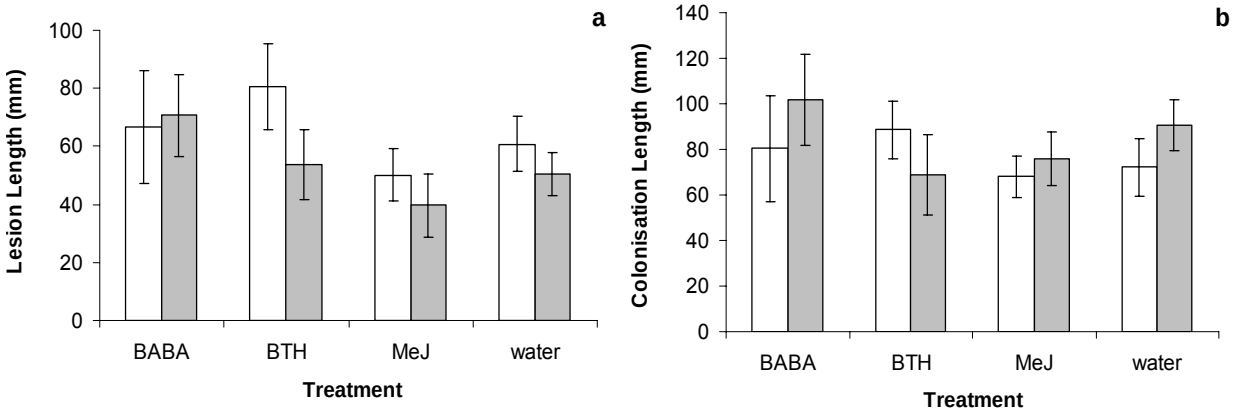


Figure 27 Lesion length (a) and tissue colonisation length (b) caused by *Phytophthora cinnamomi* in *Banksia attenuata* in a field trial treated with 0 (☐) and 0.5% phosphite (■) containing 2.5 g/L β-amino butyric acid (BABA), 1 mM methyl jasmonate (MeJ), 2 mM Acibenzolar-S-Methyl (BTH) or water. All solutions contained 0.1% Pulse penetrant. Vertical bars indicate SE of means.

DISCUSSION

There are a number of reasons why phosphite and the plant enhancers (especially the former) were not as effective as expected based on previous trials. Ideally, the *B. attenuata* should have been sprayed approximately 5 - 7 days after inoculation, allowing enough time for the pathogen to establish in the host tissue without causing necrosis and death, and at the same time ensuring an adequate amount of host tissue to respond and induce defense mechanisms stimulated by phosphite and possibly the enhancers. However, in the current trial, due to extreme weather conditions (high winds and rain), the plants were not sprayed until 11 days after inoculation. It is possible, and likely, that the variability in results is due to the pathogen colonising the branches to such an extent that in some instances the phosphite and plant enhancers were unable to induce the desired defense responses that would otherwise have occurred if the plants had been sprayed earlier. There are also many other factors that can cause variability in results obtained from trials conducted under natural conditions in the field. These include stresses such as weeds, diseases and pests, and competition for water, nutrients and light. In order to avoid such environmental and biotic influences, glasshouse trials can be conducted under controlled conditions to test different treatments without the need to have huge numbers of replicate plants to account for any variation which is normally required in trials conducted in natural environments.

Sub Project 19.2.2.9
Screening of plant defence activators to improve the efficacy of phosphite using native plant species – a glasshouse trial

INTRODUCTION

Experiments on plants in the field pose difficulties due to the variability in size and age of the plants, and environmental variables that may prevent steps of the experimental protocol actually occurring on the days anticipated. It is also not possible to allow infected plants to remain in the field for more than 4 weeks, due to the possibility of escape of *P. cinnamomi* into the environment. These factors may obscure any effects of the treatments applied. To obtain significant effects impossibly large replicate sizes may be needed. Thus, to examine longer term effects of the plant defense activators in combination with phosphite, native plant species under glasshouse conditions were used.

AIM

This experiment examines the ability of three plant defense activators in combination with phosphite to increase the efficacy of phosphite to control disease caused by *P. cinnamomi*. The longevity of the treatment will also be assessed.

METHODS

Experimental design

To determine if the addition of plant defense activators could enhance the efficacy and longevity of phosphite in controlling disease caused by *P. cinnamomi,* the three plant species were treated with eight treatments, and underbark inoculated with *P. cinnamomi* at three times after the treatments had been applied. The plants were randomised in a block design, with 10 replicate plants for each treatment and assessment time (Figure 28).

Plant material and growth conditions

Three native species that occur on the southern sand plain, *Banksia baxteri, Eucalyptus staeri* and *Lambertia inermis* were raised under glasshouse conditions in 150 mm free-draining plastic pots in a peat perlite mix (50:50% vol) with added basal fertiliser (O'Gara *et al*. 1996). Every 6 months they were treated with an additional 15 g of a low phosphate slow release fertiliser (Osmocote Plus Native Gardens, Scotts Australia, NSW). Plants were watered twice daily to container capacity. Plants were 13 months old at the commencement of the experiments.

Immediately before implementing the experimental treatments the *Lambertia* stem diameters and the heights of the *Banksia* and the *Eucalyptus* were measured. Plants were then allocated to the 8 treatments to ensure the plants of different size classes were evenly distributed across the treatments. Significant ($p < 0.05$) influences of height and diameter at 5 cm above ground level were accounted for and corrected in the analysis.

Treatments and inoculations

The six defense activator treatments all contained 0.1% Pulse and 0.5% phosphite and different activators: β-amino butyric acid (BABA) (Sigma-Aldrich Pty Ltd) at 48.5 and 100 mM; Acibenzolar-S-Methyl (BTH) (Sigma-Aldrich Pty Ltd) at 0.15 and 0.3 mM; and methyl jasmonate (MeJ) (Sigma-Aldrich Pty Ltd) at 1 and 10 mM. Two control treatments were applied; a negative control containing 0.5% phosphite and 0.1% Pulse in water, and a positive control containing only 0.1% Pulse in water. Plants were sprayed to runoff at Time 0 (Figure 29).

Figure 28

Plants arranged in the glasshouse, *Banksia baxteri* (left foreground) with *Eucalyptus staeri* behind, and *Lambertia inermis* on the right hand side bench

Figure 29

Plants being sprayed to run-off in the glasshouse

The plants were under-bark inoculated with *P. cinnamomi* (isolate MP94-48) using the method described by Hüberli *et al*. (2001) in three separate inoculation events; at 1 week (Time 1), 6 weeks (Time 2) and 11 weeks (Time 3) after treatment. Ten plants of each treatment were assessed at each inoculation time.

Measurement of efficacy of treatments

Plants were assessed for lesion presence and extension daily after inoculation. Visible lesion lengths and colonisation was measured 17 (Time 1), 14 (Time 2) and 13 (Time 3) days after inoculation with *P. cinnamomi.* The bark layer (if present) was removed to facilitate the observation and measurement of lesions and colonisation above the inoculation point was measured by plating five x

1 cm longitudinally split sections of the stem from beyond the lesion front onto NARPH. Data for lesion length correlated closely with colonisation, so only data for colonisation above the inoculation point were analysed (*Phytophthora* extension).

Measurement of symptoms and phytotoxicity

Plant growth, disease symptoms and phytotoxicity were assessed 11 days after treatment application and immediately before each successive harvest. Plant growth was measured as height above container substrate level. Disease symptoms were rated as: 0, no decline symptoms; 1, decline symptoms < 50% of the plant; 2, decline symptoms > 50% of the plant; and 3, plant death. Decline symptoms included wilting and discolouration.

Phytotoxicity symptoms were rated as: 0, none; 1, on 0 -15% of the plant; 2, 15 - 30% of the plant; and 3, 30 - 45% of the plant. Phytotoxicity was assessed 4 weeks after treatment application (Figure 30).

Statistical analyses

All analyses used the relevant modules of the Statistica software package Version 5 (Statsoft 1999). Where appropriate, data were screened for common assumptions of tests (eg normality of distribution and homoscedascity) following the recommendations of Tabachnick and Fidell (1996) and Milliken and Johnson (1992). Any transformations used are indicated in the appropriate section later in the report.

Where a single dependent variable was involved in an experiment, the data were analysed by ANOVA. Significant main effects and interactions (taken as $p < 0.05$) were further analysed using post hoc LSD tests (Milliken and Johnson 1992). Where repeated measurements were taken on the same plants over time, time was treated as a repeated measures factor and the entire experiment analysed as a repeated measures ANOVA (von Ende 2001). Covariates such as plant height, phytotoxicity or stem diameter were measured in some experiments and included as covariates in the relevant ANOVA.

Data were log transformed to remove correlation between mean and variance. An analysis of variance of the covariates shows that neither phytotoxic burn ($p = 0.40$), diameter ($p = 0.29$) or plant height ($p = 0.91$) had a significant influence on the treatment groups. Analysis of the covariate glass house temperature shows that inoculation 1 week after treatment was significantly ($p < 0.05$) compromised by the high glasshouse temperatures over 40^{o}C. This is reflected in the low recovery of *P. cinnamomi* from inoculated stems at this harvest.

Figure 30

Phytotoxicity rating system shown by symptoms displayed 4 weeks after treatment in *Banksia baxteri.*

Rating 0 (no phytotoxicity)

Rating 1 (0 – 15% of the plant affected)

Rating 2 (15 – 30% of the plant affected)

Rating 3 (30 – 40% of the plant affected

RESULTS

Colonisation by *Phytophthora cinnamomi*

Mean stem colonisation for each species, and at each time and treatment (Figure 31), shows that the colonisation data for the inoculation 1 week after treatment differs substantially to the data for inoculations at 6 and 11 weeks after treatment. The colonisation in plants inoculated 1 week after treatments with and without phosphite did not differ significantly ($p > 0.05$) to each other. In contrast, plants inoculated 6 and 11 weeks after treatment with phosphite, or phosphite in combination with defense activators, had significantly ($p < 0.05$) smaller lesions than plants that were not treated with phosphite. The mean percentage recovery of *P. cinnamomi* onto NARPH selective agar from the stems harvested after the inoculations at 1, 6 and 11 weeks after treatments, corresponded to 27.2, 83.7 and 73.5%, respectively. The mean daily minimum and maximum temperatures in the week following each inoculation were; 16.3 and 45.1ºC (1 week after treatment), 13.6 and 34.5ºC (6 weeks) and 7.1 and 34.1ºC (11 weeks).

The high temperatures in the week following the first inoculation had the potential to reduce inoculum survival, even within the stems. The low recovery of *P. cinnamomi* (27%) from stems confirms this, so to avoid biasing the remaining data (inoculations 6 and 11 weeks after treatment); the data from the first inoculation were removed from the analyses.

There were significant treatment ($p < 0.0000$) and species ($p < 0.0000$) effects but no interaction effects between time, treatment and species (Table 5).

Table 5 Summary of ANOVA of all effects of Time, Treatment and plant species on *Phytophthora cinnamomi* colonisation of *Banksia baxteri, Lambertia inermis* and *Eucalyptus staeri* stems inoculated at 6 or 11 weeks after treatment with 0.5% phosphite (negative control plants), and 0.5% phosphite combined with: 48.5 and 100 mM β-amino butyric acid (BABA); 0.15 and 0.3 mM Acibenzolar-S-Methyl (BTH); and 1 and 10 mM methyl jasmonate (MeJ).

Interactions	p values
Time	0.8806
Treatment	**0.0009**
Species	**0.0067**
Time, Treatment	0.0532
Time, Species	0.0530
Treatment, Species	0.0860
Time, Treatment, Species	0.2880

When the mean colonisation data for the spray treatments were combined with species and inoculations at 6 and 11 weeks, plants that were not treated with phosphite had significantly ($p < 0.05$) longer colonisation than colonisation for all other treatments (Figure 32). None of the defense activator treatments in combination with phosphite reduced colonisation in comparison to when phosphite was applied alone. Although, the application of phosphite combined with 100 mM BABA reduced colonisation more than the other treatments the values were not significantly ($p > 0.05$) different to the phosphite controls (Figure 32).

Phytotoxicity

Analysis of the covariate data shows that the phytotoxicity ($p = 0.0696$) and seedling height ($p = 0.582$) did not significantly ($p > 0.05$) influence colonisation. The diameter at the inoculation height ($p = 0.0122$), did significantly ($p < 0.05$) influence colonisation. This was corrected for in the colonisation datasets.

Although phytotoxicity did not significantly ($p > 0.05$) influence colonisation, the different spray treatments resulted in significantly ($p < 0.05$) different phytotoxicity ratings across the three plant species (Figure 33). Analysis of variance shows that both spray treatments and species treatments had a significant effect on phytotoxicity (Table 6).

All three plant species treated with phosphite and the combinations of phosphite and activators, consistently had higher phytotoxicity ratings than the positive control plants that were not treated with phosphite or with the defense activators (Figure 33). The highest levels of leaf damage were seen for *E. staeri*, followed by *B. baxteri* and *L. inermis*.

L. inermis plants treated with phosphite in combination with 100 mM BABA had the most significant ($p < 0.05$) phytotoxicity compared to the negative control plants that were only treated with phosphite (Figure 33c). *B. baxteri* and *E. staeri* plants treated with 100 mM BABA also had a significant ($p < 0.05$) increase in phytotoxicity compared to the non-phosphite treated plants, although the variation was not significantly ($p > 0.05$) different to the negative control plants treated with phosphite alone (Figures 33a, b).

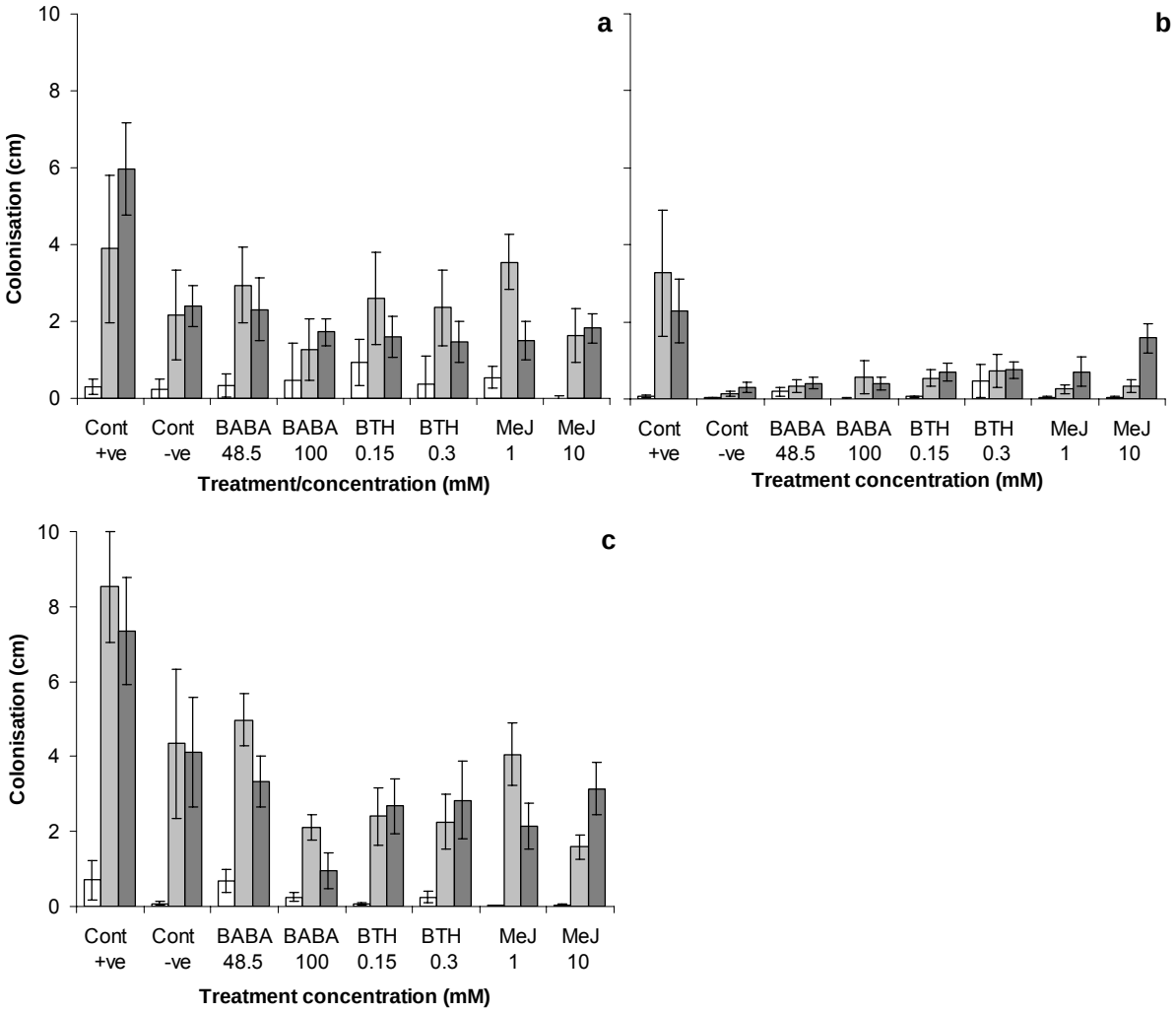


Figure 31 Colonisation of *Phytophthora cinnamomi*, in the stems of **a**) *Banksia baxteri,* **b**) *Eucalyptus staeri* and **c**) *Lambertia inermis* treated with no phosphite (positive control plants), 0.5% phosphite (negative control), and 0.5% phosphite combined with: β-amino butyric acid (BABA) at 48.5 and 100 mM; Acibenzolar-S-Methyl (BTH) at 0.15 and 0.3 mM; and methyl jasmonate (MeJ) at 1 and 10 mM at 1 (□), 6 (▭) and 11 (■) weeks after under bark inoculation with *P. cinnamomi*. All treatments contained 0.1% Pulse. Stems were harvested at 17, 14 and 13 days after inoculation at 1, 6 and 11 weeks, respectively. Vertical bars represent two standard errors of the mean.

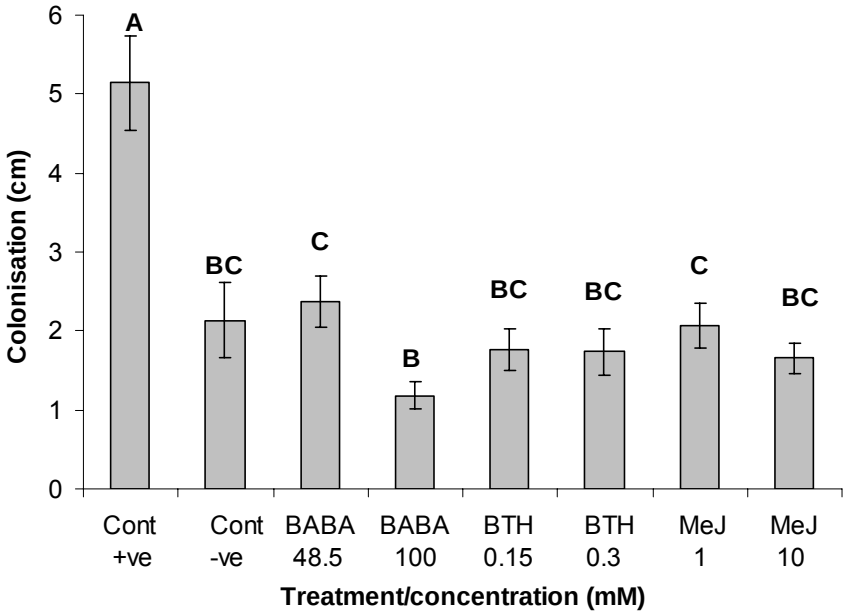


Figure 32 Mean colonisation of plant stems by *Phytophthora cinnamomi* for the spray treatments (combined across Species and Time (6 and 11 weeks) after spray treatment). Treatments identified as not significantly different (α = 0.05) using LSD analysis are shown with common letters. Treatments include no phosphite alone (positive control plants), 0.5% phosphite (negative control), and 0.5% phosphite combined with: β-amino butyric acid (BABA) at 48.5 and 100 mM; Acibenzolar-S-Methyl (BTH) at 0.15 and 0.3 mM; and methyl jasmonate (MeJ) at 1 and 10 mM. Plant species include *Banksia baxteri, Lambertia inermis* and *Eucalyptus staeri*. Vertical bars represent two standard errors of the mean.

Table 6 Summary of ANOVA of all effects of Time, Treatment and plant Species on phytotoxicity in *Banksia baxteri, Lambertia inermis* and *Eucalyptus staeri* after treatment with 0.5% phosphite (negative control plants), and 0.5% phosphite combined with: β-amino butyric acid (BABA) at 48.5 and 100 mM; Acibenzolar-S-Methyl (BTH) at 0.15 and 0.3 mM; and methyl jasmonate (MeJ) at 1 and 10 mM.

Interaction	df Effect	MS Effect	p level
Treatment	7	3.226	0.000000
Species	2	24.878	0.000000
Treatment, Species	14	0.445	0.186243

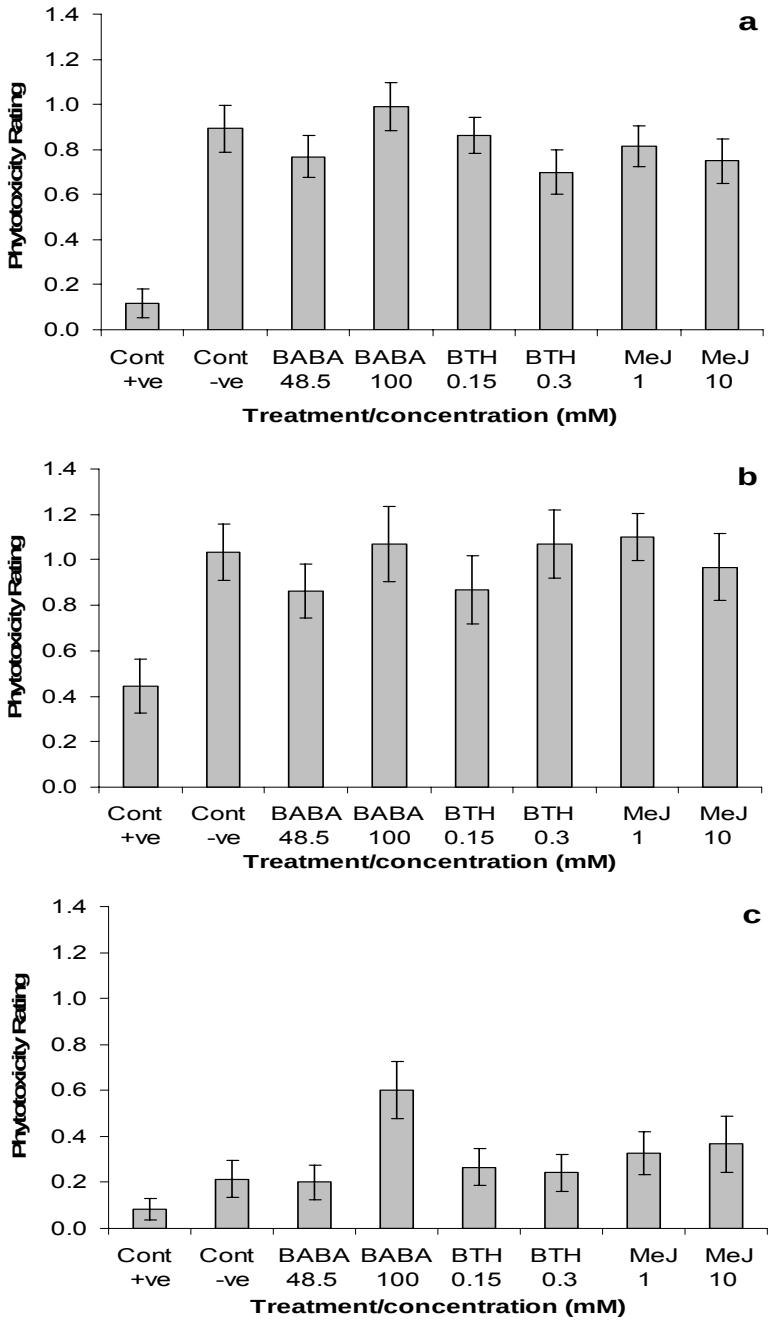


Figure 33 Mean phytotoxicity rating in **a**) *Banksia baxteri*, **b**) *Eucalyptus staeri* and **c**) *Lambertia inermis* treated with no phosphite (positive control plants), 0.5% phosphite (negative control plants), and 0.5% phosphite combined with: 48.5 and 100 mM β-amino butyric acid (BABA); 0.15 and 0.3 mM Acibenzolar-S-Methyl (BTH); and 1 and 10 mM methyl jasmonate (MeJ). All treatments contained 0.1% Pulse. Phytotoxicity symptoms were rated as: 0, none; 1, on 0 -15% of the plant; 2, 15 - 30% of the plant; and 3, 30 - 45% of the plant. Vertical bars represent two standard errors of the mean.

DISCUSSION

This study showed quite categorically that the addition of the defense enhancers to phosphite did not increase the effectiveness or persistence of phosphite compared to the treatments with phosphite alone. This was true for all plant species. Therefore, future studies should consider other chemical defense enhancers, as increasing the concentrations of MeJ, BABA and BTH used here would only increase the levels of phytotoxicity observed. Increased phytotoxicity levels would reduce the effectiveness of phosphite in controlling *P. cinnamomi* by substantially stressing the plant and reducing the ability of the plants to photosynthesise or undergo normal metabolic functions. The levels of phytotoxicity observed in the defense activator treated plants did not reduce the efficacy of controlling *P. cinnamomi* colonisation compared to the negative controls treated only with phosphite. Therefore, a sufficient amount of phosphite was being taken up by the plants to allow the plants to respond to *P. cinnamomi.*

Of all the species treated with any of the phosphite and combination treatments, *E. staeri* was the most susceptible to phytotoxicity followed by *B. baxteri* and *L. inermis*. Plant species belonging to the Myrtaceae (e.g. *E. staeri*) have previously been shown to be more sensitive to phosphite treatments than plants belonging to other families (Barrett, 2001). The lower phytotoxicity observed in the *L. inermis* treated plants compared to *B. baxteri* and *E. staeri* may have occurred as the *L. inermis* plants may have been more efficient at rapidly taking up and redistributing the chemicals throughout the plant. Alternatively, the *L. inermis* plants may have been less able to take up the chemicals as the species produces waxy leaf and stem exudates that may act as a barrier to penetration, and there may have been less uptake of the chemical. However, this is unlikely as *P. cinnamomi* was effectively contained in the stems of treated *L. inermis.* It is also important to note that variation was observed in the waxiness of leaves and stems of *L. inermis* plants used within the trial.

Barrett (2001) showed that Australian plant species very considerably in their leaf morphology. Epicuticular waxes, trichomes, hairs and other protuberances are known to influence the hydrophobicity (wettability) of a leaf, and the uptake of fungicides applied as a sprayed solution (de Ruiter *et al.* 1990, Chung and Kwon 1992, Schreiber and Schonher 1992, Barrett 2001). The amount of leaf waxiness has also been shown to vary between genotypes, and is strongly influenced by leaf age, with young leaves containing more wax than older senescing leaves (Baker 1980). However successful uptake and efficacy of phosphite has been demonstrated on a range of plant species and genotypes with varying degrees of waxiness (Barrett 2001, Hardy *et al.* 2001, Barrett *et al.* 2003, Shearer *et al.* 2004a). Future studies are required to determine how host morphological characteristics such as leaf waxiness,

cuticle thickness, stomatal position and hairs or trichomes may influence deposition of phosphite, its uptake and degree of phytotoxicity.

Glasshouse temperatures unfavourable for *P. cinnamomi* survival and growth invalidated the results of the first inoculation 7 days after treatment. Plants tested 6 and 11 weeks after treatment with phosphite alone or in combination with defense activators, had significantly smaller colonisation than plants that were not treated with phosphite or defense activators.

With regards to *P. cinnamomi* colonisation in the three plant species, there was no significant difference between the defense activator treatments. Future experiments should examine a further range of concentrations of this compound using high replicate numbers due to genotypic variation. *E. staeri* had significantly less colonisation than *B. baxteri* and *L. inermis*. This is consistent with other observations (Shearer and Smith 2000) suggesting that that *Eucalyptus* species are less susceptible to *P. cinnamomi* than other species.

Sub Project 19.2.2.10
Use of defense activators to increase responsiveness of plant species sprayed with low phosphite levels

INTRODUCTION

Although the field and glasshouse experiments could not demonstrate defense activators enhancing the effectiveness of 0.5% phosphite it is possible they may increase the effectiveness of phosphite sprayed at lower concentrations. If so this would be an important step in reducing the possibility of the development of resistance to the fungicide.

AIM

To determine if addition of a plant defense activator together with phosphite will afford plant protection at concentrations of phosphite lower than 0.5%.

METHODS

Experimental design

To determine if low concentrations of phosphite with the addition of a plant defense activator could afford the same protection to *P. cinnamomi* as standard (0.5%) applications of phosphite, *Banksia grandis* seedlings were treated with five different treatments of plant defense activators in combination with four phosphite concentrations. The plants were placed into a temperature-controlled glasshouse in a randomised block design.

Plant material and growth conditions

Eleven month-old *Banksia grandis* seedlings were raised under glasshouse conditions in 150 mm free-draining plastic pots containing a peat perlite mix (50:50% vol) with added basal fertiliser (O'Gara *et al*. 1996). Every six months they were treated with an additional 15 g of a low phosphate slow release fertiliser (Osmocote Plus Native Gardens, Scotts Australia, NSW). Plants were overhead watered to container capacity twice daily and were 11 months old at the commencement of the experiment.

Prior to the experiment commencing plant heights were measured.

Treatments

All plants were sprayed to run-off with a solution of a defense activator, phosphite and 0.1% Pulse. The defense activator treatments were: 0.15 and 0.3 mM Acibenzolar-S-Methyl (BTH); 1 and 10 mM methyl jasmonate (MeJ); and a control with no defense activator added. Each of these treatments was applied in combination with phosphite at four concentrations (0, 0.1, 0.25 and 0.5% - a total of 20 treatments). There were 11 replicates for each treatment of inoculated plants, and six replicate plants for the un-inoculated treatments arranged in a randomised block design. The negative controls were un-inoculated plants treated were BTH (0.3 mM) and MeJ (10 mM) in combination with 0, 0.1, 0.25 and 0.5% phosphite together with 0.1% Pulse, and a positive control with no Pulse.

Inoculation
The containers were inoculated with *P. cinnamomi* (isolate MP94-48) two days after the treatments using pine plugs colonised by the pathogen. Pine (*Pinus radiata*) inoculum plugs were prepared as described by Hardy and Sivasithamparam (1991). The *P. cinnamomi* colonised inoculum plugs were placed near the roots of the plants using inoculum delivery tubes that were in place when the plants were originally potted. At inoculation, the tubes were removed and the colonised pine plugs were inserted into the holes left by removal of the tubes. The holes were then filled with sterile potting mix. In order to stimulate the production of sporangia and the release of zoospores from the inoculum source, the container substrate was flooded for 12 h overnight, immediately after inoculation, and then fortnightly to mimic field conditions after a heavy rainfall event. Flooding was achieved by placing each pot in a water-tight bag inside a second pot.

Measurement of infection
Plants were examined daily after treatment application for disease symptoms, including chlorosis, wilting and the development of visible lesions in the stem collars. The time of death was determined to be when all the leaves of the plant were brown and dry. Once plants were considered dead, the outer bark of the stems was removed, and lesioned/necrotic material was plated on NARPH, to confirm the presence or absence of *P. cinnamomi*, and to indicate if death occurred though infection or phytotoxicity. After 64 days, the stems were harvested from all remaining plants and assessed for the presence of lesions. The outer bark layer (if present) was removed to facilitate the measurement of lesions. Five x 1 cm sections of the stem from above the upper extent of the visible lesion were split longitudinally and tested for the presence of the pathogen by plating onto NARPH, a *Phytophthora* selective agar medium (Hüberli *et al.* 2000), to determine the extent of spread of *P. cinnamomi* in the stem. To assess damage roots of five replicate plants from each treatment were washed out and dried at 70^{o}C for 3 days before measuring dry weight. The colonised pine plug was removed from each harvested pot and plated onto NARPH to assess the survival of *P. cinnamomi* within the soil.

Measurement of phytotoxicity
Phytotoxicity was assessed 14 days after treatment application. Phytotoxicity symptoms were rated as: 0, none; 1, on 0 - 15% of the plant; 2, 15 - 30% of the plant; and 3, 30 - 45% of the plant.

Statistical analyses
All analyses were carried out as described in Section 19.2.2.9.

RESULTS

During the experiment, 6 plants died and *P. cinnamomi* was recovered from the stems of all dead plants. On completion of the experiment, *P. cinnamomi was* recovered from all of the pine plugs retrieved from each pot. No lesions were observed in any of the stems harvested from the living plants but *P. cinnamomi* was isolated from the stems of nine of them. These were from a range of treatments so no conclusions can be drawn from their occurrence:

- two positive control plants (without phosphite or defense activator treatments);
- one negative control plant (treated with 0.5% phosphite);
- one plant treated with 0.1% phosphite and 10 mM MeJ;
- one plant treated with 0.25% phosphite and 0.15 mM BTH;
- one plant treated with 0.25% phosphite and 0.3 mM BTH; and
- three plants treated with 0.5% phosphite and 0.3 mM BTH.

None of the spray treatments influenced mean dry root weights (Figure 34). A summary of all effects showed that neither phosphite concentration ($p = 0.40$), defense activator treatment ($p = 0.17$) or the combined phosphite and defense activator treatments ($p = 0.32$) had a significant influence on the dry root weight of harvest plants. Analysis on stem colonisation could not be performed, due to insufficient numbers of isolations of *P. cinnamomi*.

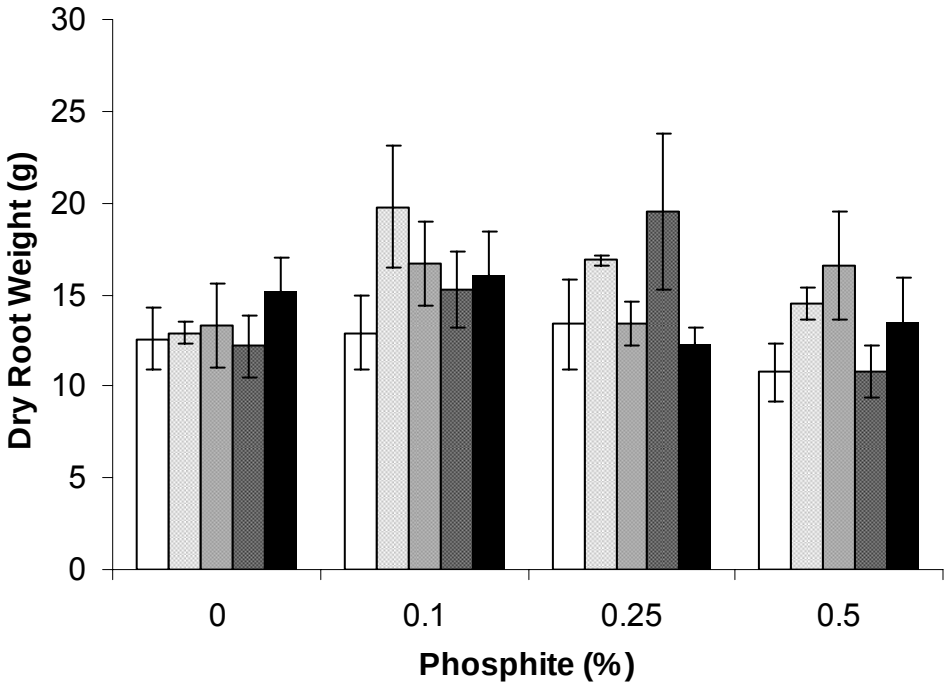


Figure 34 Mean dry weight of *Banksia grandis* roots from plants treated with 0, 0.1, 0.25 and 0.5% phosphite combined with defense activators: no defense activator (☐), Acibenzolar-S-Methyl (BTH) at 0.15 (☐) and 0.3 mM (■); and methyl jasmonate (MeJ) at 1 (■) and 10 mM (■). Vertical bars represent two standard errors of the mean.

Varying levels of phytotoxicity was observed on the *B. grandis* plants treated with the different defense activators (Figure 35). Both phosphite ($p = 0.00003$) and defense activator ($p < 0.00001$) treatments had a significant influence on the level of phytotoxicity compared to the control plants. The treatment interaction between phosphite and defense activator treatments had an emerging significant ($p = 0.0530$) influence on phytotoxicity.

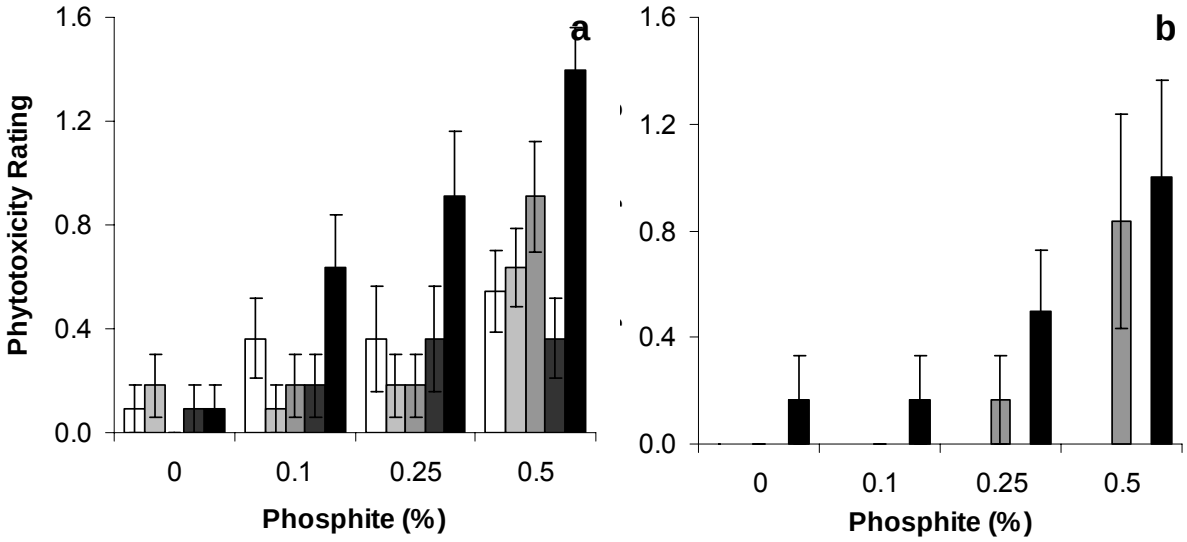


Figure 35 Mean phytotoxicity rating of *Banksia grandis* treated with 0, 0.1, 0.25 and 0.5% phosphite in combination with defense activators: no activator (□), 0.15 (▫) and 0.3 mM (▪) Acibenzolar-S-Methyl (BTH); 1 (■) and 10 mM (■) methyl jasmonate (MeJ). Phytotoxicity shown for **a**) plants inoculated with *Phytophthora cinnamomi* (*n* = 11) and **b**) non-inoculated plants (*n* = 6). Phytotoxicity symptoms were rated as: 0, none; 1, on 0 - 15% of the plant; 2, 15 - 30% of the plant; and 3, 30 - 45% of the plant. All combined treatments contained 0.1% Pulse. Vertical bars represent two standard errors of the mean.

The mean phytotoxicity data, combined across the defense activator treatments, shows that *B. grandis* treated with phosphite at 0.5% had significantly ($p < 0.05$) greater phytotoxicity than treatments of 0.1 and 0.25% (Figure 36). Whilst mean phytotoxicity data combined across the phosphite treatments, shows that plants treated with 10 mM MeJ had significantly ($p < 0.05$) greater phytotoxicity than plants treated with the other defense activator treatments. There was no significant ($p > 0.05$) difference in phytotoxicity between the positive control, and the defense activators, 0.1 mM BTH, 0.3 mM BTH and 1 mM MeJ (Figure 37).

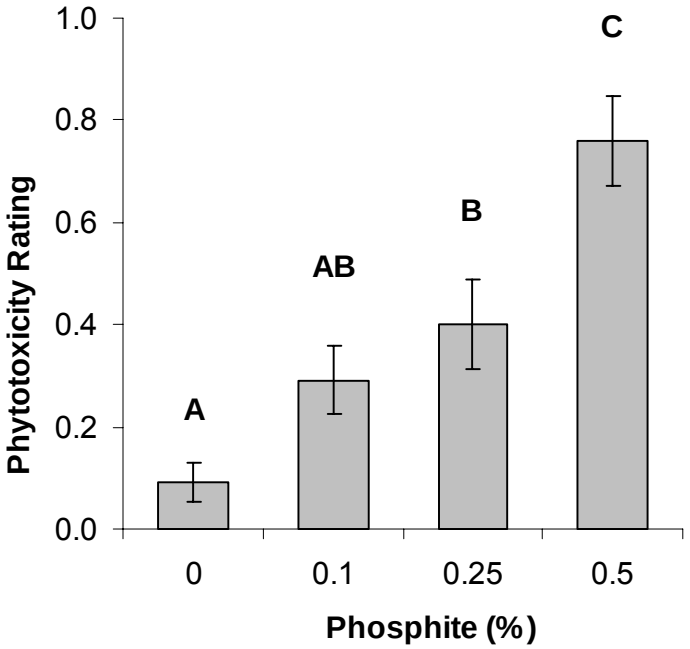


Figure 36

Mean phytotoxicity rating in *Phytophthora cinnamomi* inoculated *Banksia grandis* plants, for phosphite treatments 0, 0.1, 0.25 and 0.5%, combined across defense activator treatments including 0.15 and 0.3 mM Acibenzolar-S-Methyl (BTH); 1 and 10 mM methyl jasmonate (MeJ). All phosphite defense activator treatments contained 0.1% Pulse. Bars represent two standard errors (n = 11). Treatments identified as not significantly different (α = 0.05) using LSD analysis are shown with common letters.

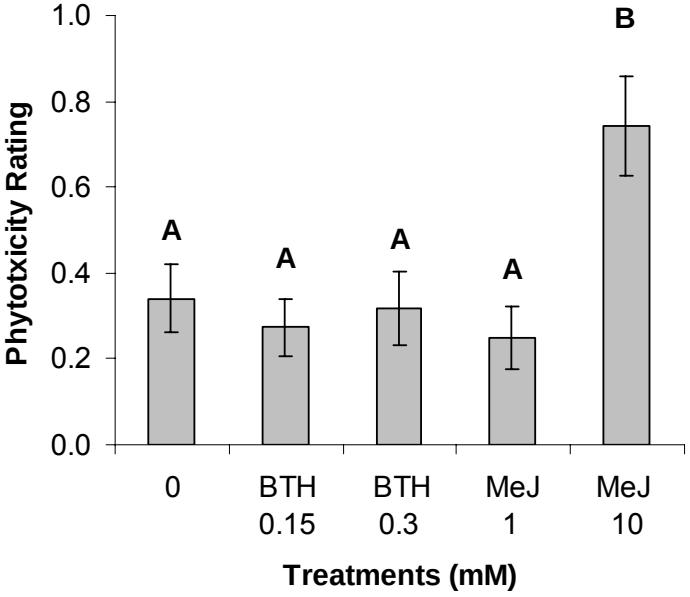


Figure 37

Mean phytotoxicity in *Phytophthora cinnamomi* inoculated *Banksia grandis* plants, for defense activator treatments including 0.15 and 0.3 mM Acibenzolar-S-Methyl (BTH); 1 and 10 mM methyl jasmonate (MeJ), combined across phosphite treatments 0, 0.1, 0.25 and 0.5%. All phosphite defense activator treatments contained 0.1% Pulse. Bars represent two standard errors (n = 11). Treatments identified as not significantly different (α = 0.05) using LSD analysis are shown with common letters.

DISCUSSION

The results did not indicate that addition of a plant defense activator together with phosphite will afford plant protection at concentrations of phosphite lower than 0.5%. Assessment of plant infection through root dry weights, plant death or colonisation by *P. cinnamomi* did not provide any significant differences or clear trends across the different spray treatments. *P. cinnamomi* was successfully recovered from every pine plug from harvested pots, confirming that the pathogen survived within the inoculated pots, and that the roots of infected pots were exposed to viable inoculum. A longer time period allowing assessment of the plants until plant death may have shown a response to treatments.

There was a significant influence of phosphite and defense activator treatments on phytotoxicity. Across the different defense activator treatments the highest mean phytotoxicity rating of 1.4 (between 15 and 30% of the plant being affected), was observed with 0.5% phosphite application combined with the 10 mM MeJ application. Future research, testing the efficacy of phosphite and defense activator treatments on controlling *P. cinnamomi* infection, should not use treatments of 0.5% phosphite combined with MeJ at concentrations higher than 10 mM, as the resultant phytotoxicity could be detrimental. Stem and leaf tissues that have been 'burnt' by phytotoxicity are much more susceptible to opportunistic canker pathogens; and phytotoxicity can reduce flowering and seed set.

Sub Project 19.2.2.11
Use of defense activators to stimulate non-phosphite responsive plant species to respond to phosphite

INTRODUCTION

An additional problem with use of phosphite alone for control of field outbreaks of *P. cinnamomi* is that some species (e.g. some *Lambertia* spp. and *Banksia attenuata* genotypes*)* show little or no protection against infection by *P. cinnamomi* (BL Shearer, unpublished data). Application of other elicitors of plant defense mechanisms may protect these species or may stimulate the protective effects of phosphite.

To identify the genotypes that are not responsive to phosphite it is necessary to infect plants that have been sprayed with phosphite then 'rescue' the non-responsive genotype before it is killed by *P. cinnamomi.* The genotypes valuable for experimentation could then be clonally propagated from cuttings or in vitro. Time did not permit this to be done in this project, but it was possible to test multiple shoots from plants identified as phosphite sensitive or phosphite insensitive using an *in vitro* screening protocol. This technique involves inserting cut shoots into a culture of *P. cinnamomi* on agar and assessing the lesion extension up the stem after 6 - 7 days. It has been shown that genotypes ranked for resistance to *P. cinnamomi* in this way showed the same relative ranking as did the parent plants when roots were infected with *P. cinnamomi*.

This in vitro screening technique permitted testing the resistance to *P. cinnamomi* of cut shoots of the same genotype given different treatments with phosphite alone, or with the addition of plant defense activators.

AIM

To determine if addition of plant defense activators will afford protection to *Phytophthora cinnamomi* in plants that are not responsive to phosphite.

METHODS

Experimental design

To determine if plant defense activator application could increase the effectiveness of phosphite in non-responsive plants, cut shoots from responsive and non-responsive plants were treated with defense activators in combination with phosphite, and inoculated with *P. cinnamomi* using a cut shoot protocol described by Gilovitz (2007) with minor modifications. There were six treatments; four activator (0.15 and 0.3 mM BTH, and 1 and 10 mM MeJ) and two control treatments. All activator treatments contained 0.5% phosphite and 0.1% Pulse. The negative control contained 0.5% phosphite and 0.1% Pulse in water, and the positive control contained only 0.1% Pulse in water. There were five replicate shoots for each treatment, and of these, four shoots were inoculated with *P. cinnamomi* (isolate MP94-48), leaving one un-inoculated control. The un-inoculated controls were harvested after 24 h and the phosphite concentration was determined

Plant material

The four plants of *Lambertia inermis* used in this experiment were selected from the under bark inoculations described under 19-2-2-6 and included: two plants that responded to phosphite and contained *P. cinnamomi* colonisation (Genotype 1), and two plants that did not respond to phosphite (Genotype 2), and *P. cinnamomi* colonisation was not contained. From each plant, 30 shoot tips approximately 10 cm long were removed for the treatments and placed immediately with their bases in deionised water to prevent desiccation and air embolisms occurring in the stems. The stems were supported in polystyrene boats (7 cm in diameter) to keep the foliage dry before the chemical treatments (Figure 38).

Treatment application

The shoots were sprayed to runoff and left for at least 30 min to allow the treatments to dry. Shoots from each genotype which either (i) responded to phosphite or (ii) did not respond to phosphite were kept in separate polystyrene boats. Shoots were then transferred to inoculation tubes. The inoculation tube (4 cm dia. x 15 cm) contained moistened, sterilised filter paper. A 9 mm diameter plug of V8 agar containing a 7-day old culture of *P. cinnamomi* was placed in the bottom of each tube on the filter paper. The cut end of a single shoot was placed into the inoculum plug and the tubes were sealed with Parafilm. The plants were incubated at 20^{o}C under a 12/12 h daylight/darkness cycle.

Phosphite analysis

The uninoculated controls were left for 24 h within the inoculation chambers to allow phosphite uptake, then washed in Deconex phosphate-free detergent (Borer Chemie AG, Zuchwil, Switzerland), rinsed twice in water and blotted dry. Shoots were tested for the presence of phosphite using the silver nitrate test (19-2-2-4). Samples of the mother plants before the cuttings were excised were washed as above, then dried at 60^{o}C for 4 days, ground to 1 mm and sent to the WA Chemistry Centre (Perth) for phosphite analysis. To 0.5 g ground sample, 5 mL of 0.1 M sulphuric acid was added and extractions occurred overnight on a roller-shaker. Following 20 min centrifugation at 6970 g, 100 µL of the clear acid extract was added to 1 mL of 50 µg/mL methyl phosphonic acid in methanol (internal standard solution). A phosphite standard curve was prepared by adding 100 µL of solutions containing from 0.05 to 100 µg/mL phosphite to 11 tubes containing 1 mL of internal standard solution. The solutions were mixed and diazomethane was added to 400 µL of the samples in excess until a persistent yellow colour was observed. Excess diazomethane was neutralised with a few drops of 2% acetic acid, then the dimethyl phosphite content was determined by gas chromatography. A splitless injection with a D.B-Wax column (J & W Scientific, Salsam, CA) and a phosphorous-specific flame photometric detector (Hewlett Packard, USA) were used. The limit of quantitation was 0.5 µg/g dry weight. A replicate sample was taken every 10 samples to provide a control during the analysis. Two control samples of known phosphite content were included in each batch of 40 samples analysed.

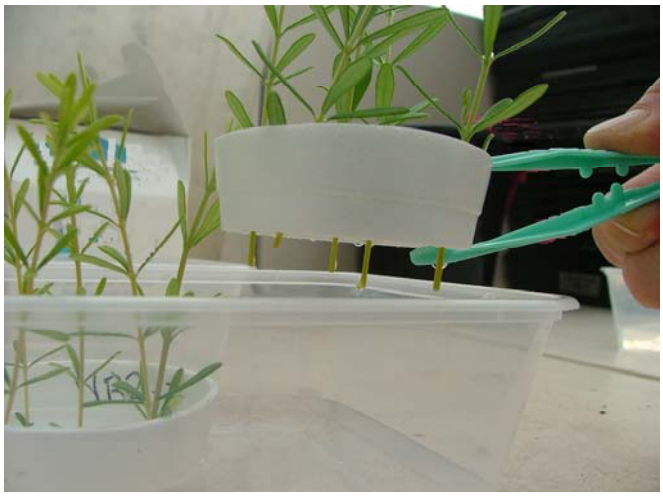


Figure 38

The cut shoots after treatments. The shoots were placed into polystyrene boats to ensure the base of the shoots were in water to prevent them drying out.

Treatment Assessment

Five days after inoculation, shoot length, lesion lengths and phytotoxicity symptoms were measured (Figures 39 and 40). Symptoms of phytotoxicity were rated as 0 (0 – 20% of plant surface showing symptoms), 1 (20 – 40%), 2 (40 – 60%), 3 (60 – 80%) and 4 (80 – 100%).

Figure 39

Shoot material with typical phytotoxic symptoms. Phytotoxicity symptoms included significantly discoloured and distorted tissue, typically at the apical tips of the shoots and leaves

Figure 40

P. cinnamomi lesion extending along a shoot extending from the inoculation point at the base up the shoot stem. The lesion is indicated by adjacent red line

To recover *P. cinnamomi* from the shoots to confirm infection, the leaves were removed from the stem and the outer bark layers of the stem were carefully scraped away prior to plating onto NARPH selective agar.

Statistical analyses
All analyses were carried out as in 19.2.2.9.

RESULTS

Mother plants were selected from plants sprayed with phosphite 12 weeks earlier for use in experiments described in 19.2.2.9. The analyses of the phosphite concentrations in roots and shoots of these plants before cuttings were taken for the in vitro experiments showed that phosphite was present in all genotypes but in NR1 its concentration was at the limit of sensitivity of the analysis (0.5 mg/kg) (Table 7). This indicates that all genotypes could take up phosphite. Staining of treated cut shoots using the silver nitrate stain showed that all had taken up phosphite.

Three *L. inermis* genotypes (R1, R2 and NR2) showed a reduction in colonisation by *P. cinnamomi* ($p < 0.05$) when treated with phosphite, compared with the positive controls (not treated with phosphite) (Figure 41). There was no additional advantage of inclusion of a defense activator in the spray, indeed some treatments caused an increase in lesion length compared with phosphite alone. One of the non-responsive genotypes responded to phosphite and phosphite combined with defense activators in a similar way to the two responsive genotypes. The other non-responsive genotype (NR1) did not respond to phosphite alone, and again the addition of defense activators did not give increased protection from *P. cinnamomi* (Figure 41). Least colonisation occurred in the genotype NR1 when it was treated with phosphite in combination with 0.15 mM BTH, but this was not statistically different from treatment with phosphite alone.

The summary of treatment interactions showed that Genotypes ($p = 0.02$) and Treatment ($p < 0.0001$), and the interaction of Genotypes and Treatments ($p = 0.006$) had a significant influence on colonisation.

Analysis of covariates stem length ($p = 0.21$) and phytotoxicity ($p = 0.2$) do not significantly influence colonisation of the shoots from all *L. inermis* genotypes.

All shoots treated with phosphite, or phosphite in combination with an activator treatment exhibited various degrees of phytotoxicity (Figure 42). There was no correlation between degree of phytotoxicity and lesion length combined across all genotypes ($r = -0.43$) nor for each genotype (NR1 $r = -0.44$, NR2 $r = -0.81$, R1 $r = -0.9$, and R2 $r = -0.2$).

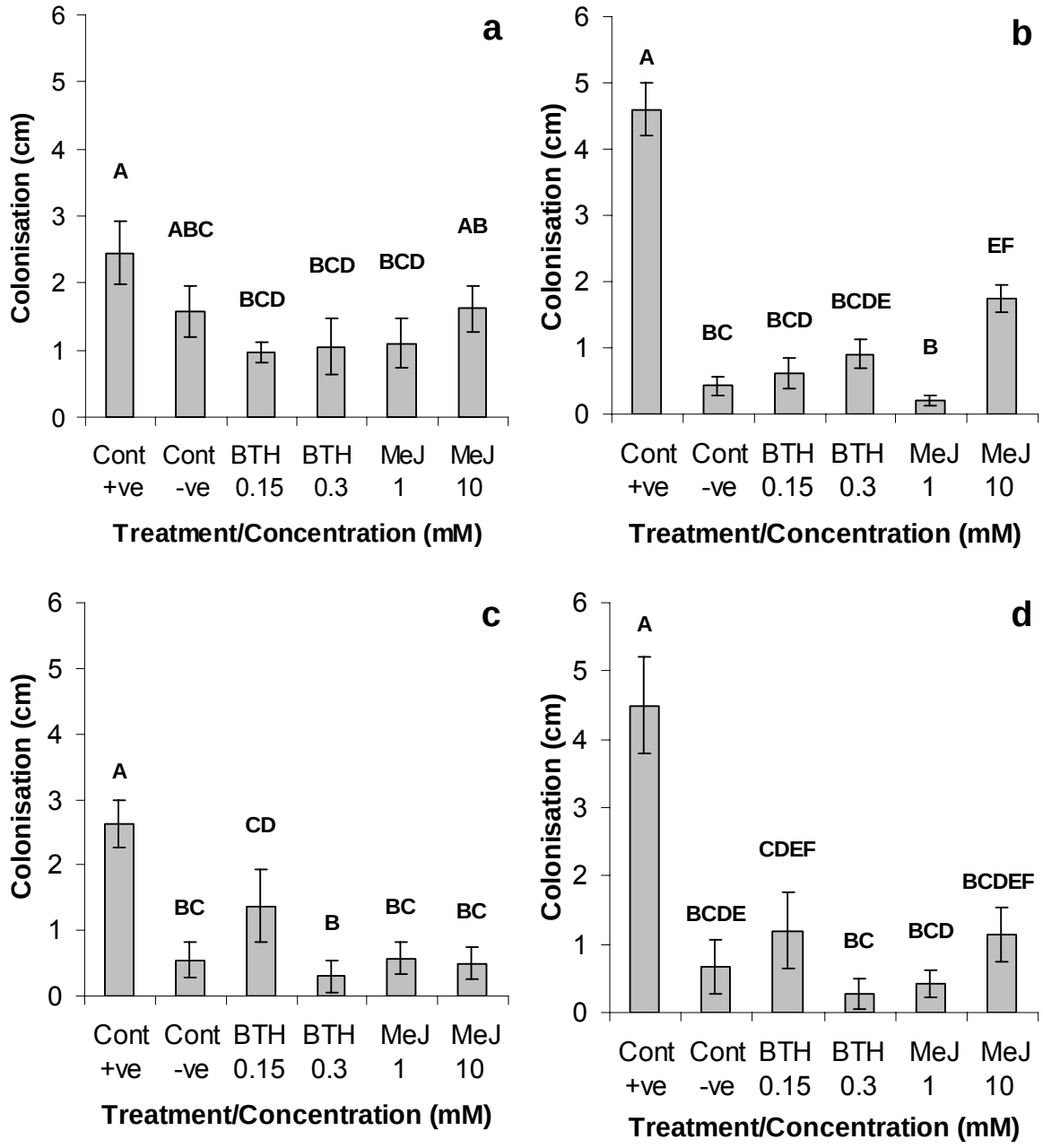


Figure 41 Mean colonisation of *Phytophthora cinnamomi* in inoculated *Lambertia inermis* cut shoots. **a**) non responsive (genotype NR1), **b**) non responsive (NR2), **c**) phosphite responsive (R1) and **d**) phosphite responsive (R2). Treatments include: no phosphite (positive control plants), 0.5% phosphite (negative control plants), and 0.5% phosphite combined with; Acibenzolar-S-Methyl (BTH) at 0.15 and 0.3 mM; and methyl jasmonate (MeJ) at 1 and 10 mM. All treatments contained 0.1% Pulse. Vertical bars represent two standard errors of the mean. Treatments not identified as significantly different (α = 0.05) using LSD analysis are shown with common letters.

Table 7 Phosphite concentrations (as Phosphorous acid, mg/kg) in roots and shoots of phosphite responsive (R1 and R2) and non-responsive (NR1 and NR2) *Lambertia inermis* genotypes harvested for phosphite analysis prior to treating excised shoots with phosphite and defense activators.

Genotype	Phosphorous Acid (mg/kg)	
	Shoots	Roots
Non-responsive 1	< 5	< 8
Non-responsive 2	12	0.5 to 35*
Responsive 1	10	7
Responsive 2	99	30

* sample size too small for greater accuracy

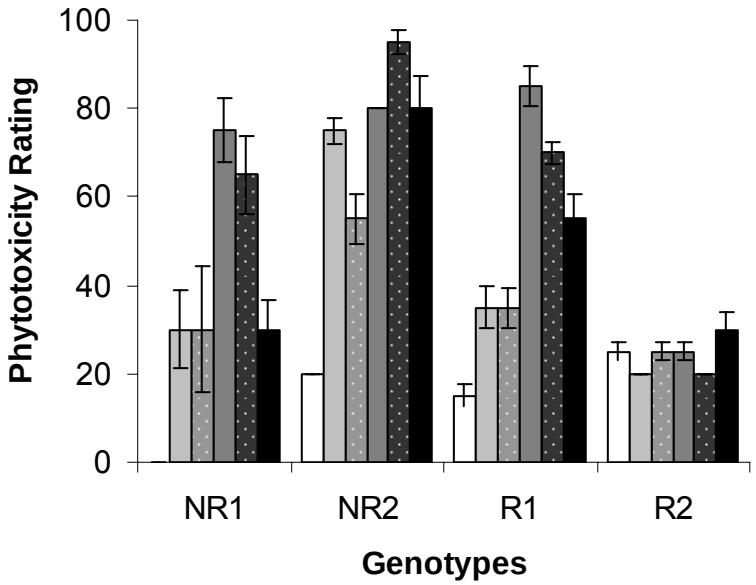


Figure 42 Mean phytotoxicity rating in *Lambertia inermis* cut shoots from genotypes: non-responsive 1 (NR1), non-responsive 2 (NR2), responsive 1 (R1) and responsive 2 (R2) to phosphite treated with: no phosphite (positive control plants □), 0.5% phosphite (negative control plants □), and 0.5% phosphite combined with; Acibenzolar-S-Methyl (BTH) at 0.15 (■) and 0.3 mM (■); and methyl jasmonate (MeJ) at 1 (■) and 10 mM (■). All treatments contained 0.1% Pulse. Phytotoxicity symptoms were scored as the percentage of the cut shoot exhibiting burn. Vertical bars represent two standard errors of the mean.

DISCUSSION

L. inermis genotypes (R1 and R2) shown to be responsive to phosphite in a glasshouse trial using underbark inoculation with *P. cinnamomi*, also responded to phosphite when cut shoots were treated with phosphite and inoculated with *P. cinnamomi* using an in vitro protocol. Plants R1 and R2 are therefore confirmed to be responsive to phosphite application, and the protocol of testing excised shoots in vitro shown to be valid.

Inoculated excised shoots from one of the non-responsive genotypes (NR2) when tested in vitro also had significantly smaller colonisation when treated with phosphite. This indicates that the plant (NR2) is responsive to phosphite and was incorrectly selected as non-responsive. The reason it did not respond when initially treated in the glasshouse may have been because the phosphite concentrations in the roots and shoots of this plant were not high enough to initiate a response. Possibly it did not receive adequate phosphite, or uptake of phosphite in this plant was impaired, due to environmental or physiological factors. However, in the cut shoot trial it did take up phosphite and responded by reducing colonisation to levels similar to the phosphite responsive genotypes (R1 and R2).

Inoculated shoots from the non-responsive (NR1) plant treated with phosphite were colonised to the same extent as shoots that were not treated with phosphite. This experiment therefore confirms genotype NR1 as a non-responsive genotype. Such genotypes are of considerable importance to future research on the mode of phosphite action. The reduction of colonisation of NR1 after treatment with phosphite combined with 0.15 mM BTH, although not statistically significant, suggests that more analyses using defense activators are desirable to further check whether defense activator treatments in combination with phosphite, can make non-responsive plants responsive. Plant material from NR1 and the responsive genotypes have been retained and these are currently being processed for the production of clonal material (P Barua, PhD student) for further experiments.

REFERENCES

Ali Z, Guest DI (1998) Potassium phosphonate controls root rot of *Xanthorrhoea australis* and *X. minor* caused by *Phytophthora cinnamomi*. *Australasian Plant Pathology* 27, 40- 44.

Babst BA, Ferrieri RA, Thorpe M and Orians CM (2004) Herbivory and jasmonic acid increase carbon partitioning to roots in Populus: can herbivory induce enhanced tolerance? 12th New Phytologist Symposium. Functional Genomics of Environmental adaptation in Populus. Gatlinburg, Tennessee, USA. 10-13 October 2004.

Baker EA (1980) Effects of cuticular components on foliar penetration. *Pesticide Science* 11, 367-370.

Barrett SR (2001) Phytotoxic Effects of Phosphite in Native Plant Communities in Southern Western Australia. PhD. thesis, Murdoch University.

Barrett SR, Shearer BL and Hardy GEStJ (2003) The efficacy of phosphite applied after inoculation on the colonisation of *Banksia brownii* stems with *Phytophthora cinnamomi*. *Australasian Plant Pathology* 32, 1-7.

Barrett SR, Shearer BL, Hardy GEStJ (2004) Phytotoxicity in relation to in planta concentration of the fungicide phosphite in nine Western Australian native species. *Australasian Plant Pathology* 33, 521-528.

Benhamou N, Belanger RR (1998a) Benzothiadiazole-mediated induced resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato. *Plant Physiology* 118, 1203-1212.

Benhamou N, Belanger RR (1998b) Induction of systemic resistance to *Pythium* dampingoff in cucumber plants by benzothiadiazole: ultrastructure and cytochemistry of the host response. *The Plant Journal* 14, 13-21.

Bittner-Eddy PD, Allen RL, Rehmany AP, Birch P, Beynon JL (2003) Use of suppression subtractive hybridization to identify downy mildew genes expressed during infection of *Arabidopsis thaliana*. *Molecular Plant Pathology* 4, 501-507.

Bokshi AI, Morris SC, Deverall BJ (2003) Effects of benzothiadiazole and acetylsalicylic acid on B-1,3-glucanase activity and disease resistance in potato. *Plant Pathology* 52, 22-27.

Borges AA, Cools HJ, Lucas JA (2003) Menadione sodium bisulphite: a novel plant defense activator which enhances local and systemic resistance to infection by *Leptosphaeria maculans* in oilseed rape. *Plant Pathology* 52, 429–436.

Byrt P, Grant B (1979) Some conditions governing zoospore production in axenic cultures of Phytophthora cinnamomi Rands. *Australian Journal of Botany* 27, 103-105.

Cahill DM, Legge N, Grant BR, Weste G (1989) Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. *Phytopathology* 79, 417-424.

CALM (1999) *Phytophthora cinnamomi* and disease caused by it – Volume 3. Phosphite. Operations Guidelines. Department of Conservation and Land Management, Perth.

Chuang H, Hsieh T, Duval M, Thomas TL (2003) Genomic analysis of *Arabidopsis* gene expression in response to a systemic fungicide. In: Genomics of Plants and Fungi: Mycology (Eds RA Prade, HJ Bonhert) p237-253, Marcel Dekker: New York.

Chung BJ, Kwon YW (1992) Relationship between surfactant properties and wettability of rice leaf surfaces for serveral non-ionic surfactants. In: Adjuvants for Agrichemicals (Ed CL Foy) CRC Press: London.

Cohen Y, Gisi U, Niderman T (1993) Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl ester. *Phytopathology* 83, 1054-62

Colson-Hanks ES, Deverall BJ (2000) Effect of 2,6-dichloroisonicotinic acid, its formulation materials and benzothiadiazole on systemic resistance to alternaria leaf spot in cotton. *Plant Pathology* 49, 171-178.

Conn KE, Gubler WD, Mircetich SM, Hasey JK (1991) Pathogenicity and relative virulence of 9 *Phytophthora* species from kiwifruit. *Phytopathology* 81, 974-979.

Daniel R, Guest D (2006) Defence responses induced by potassium phosphonate in *Phytophthora palmivora*-challenged *Arabidopsis thaliana*. *Physiological and Molecular Plant Pathology* 67, 194-201.

Daniel R, Wilson BA, Cahill DM (2005) Potassium phosphonate alters the defence response of *Xanthorrhoea australis* following infection by *Phytophthora cinnamomi*. *Australasian Plant Pathology* 34, 541-548.

de Ruiter H, Uffing AJM, Meinen E, Prins A (1990) Influence of surfactants and plant species on leaf retention of spray solutions. *Weed Science* 38, 567-572.

Dobrowolski MP, Shearer BL, O'Brien PA, Hardy GEStJ (2008) Selection for decreased sensitivity to phosphite in *Phytophthora cinnamomi* with prolonged use of fungicide. *Plant Pathology* Doi: 10.1111/j.1365-3059.2008.01883.x

Dolan T, Coffey M (1986) Laboratory screening technique for assessing resistance of four Avocado rootstocks to *Phytophthora cinnamomi*. *Plant Disease* 70, 115-118.

Environment Australia (2001) Threat Abatement Plan for Dieback Caused by the Root-rot Fungus *Phytophthora cinnamomi.* Environment Australia, Commonwealth Government of Australia, Canberra.

Gilovitz J (2007) Resistance of *Lambertia* taxa to *Phytophthora cinnamomi*. Honours Thesis, Murdoch University

Glazebrook J (2001) Genes controlling expression of defense responses in *Arabidopsis* - 2001 status. *Current Opinions Plant Biology* 4, 301-308.

Godard JF, Ziadi S, Monot C, Le Corre DS, Silue D (1999) CGA 245704, benzothiadiazole (BTH) induces resistance in cauliflower (*Brassica oleracea* var *botrytis* to downy mildew of crucifers caused by *Peronospora parasitica*. *Crop Protection* 18, 397-405.

Groves E (2002) Biochemical mechanisms of induced resistance in plants after treatment with phosphite in the presence or absence of *Phytophthora cinnamomi*. Honours thesis, Murdoch University.

Guest DI, Pegg KG, Whiley AW (1995) Control of *Phytophthora* diseases of tree crops using trunk-injected phosphonates. *Horticultural Reviews* 17, 299-330.

Guest DI, Bompeix G (1984) Fosetyl-A1 as a tool in understanding the resistance response in plants. *Phytophthora Newsletter* 12, 62-69.

Hammond-Kosack KE, Parker JE (2003) Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. *Current Opinion in Biotechnology* 14,177-193.

Hardham AR, Gubler F, Duniec J, Elliott J (1991) A review of methods for the production and use of monoclonal-antibodies to study zoosporic plant-pathogens. *Journal of Microscopy* 162, 305-318.

Hardy GESJ, Barrett SR, Shearer BL (2001) The future of phosphite as a fungicide to control the soilborne plant pathogen *Phytophthora cinnamomi* in natural ecosystems. *Australasian Pant Pathology* 30, 113 - 139.

Hardy GESJ, Sivasithamparam K (1991) Suppression of *Phytophthora* Root Rot by a Composted *Eucalyptus* Bark Mix. *Australian Journal of Botany* 39, 153-159.

Hoagland DR, Arnon DI (1938) The water culture method for growing plants without soil. *California Agricultural Experimental Station Circular* 347.

Holub EB, Benyon JL, Crute IR (1994) Phenotypic and genotypic characterization of interactions between isolates of *Peronospora parasitica* and accessions of *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions* 7, 223-239.

Hüberli D, Tommerup IC, Dobrowolski MP, Calver MC, Hardy GEStJ (2001) Phenotypic variation in a clonal lineage of two *Phytophthora cinnamomi* populations from Western Australia. *Mycological Research* 105,1053-1064.

Huberli D, Tommerup IC, Hardy GEStJ (2000) False-negative isolations or absence of lesions may cause mis-diagnosis of diseased plants infected with *Phytophthora cinnamomi*. *Australasian Plant Pathology* 29, 164-169.

Iriti M, Faoro F (2003) Benzothiadiazole (BTH) induces cell-death independent resistance in *Phaseolus vulgaris* against *Uromyces appendiculatus*. *Journal of Phytopathology* 151, 171-180.

Irwin JAG, Cahill DM, Drenth A (1995) *Phytophthora* in Australia. *Australian Journal of Agricultural Research* 46, 1311-1337.

Jackson TJ, Burgess T, Colquhoun IJ, Hardy GEStJ (2000) Action of the fungicide phosphite on *Eucalyptus marginata* inoculated with *Phytophthora cinnamomi*. *Plant Pathology* 49, 147-154.

Keogh RC, Deverall BJ, MacLeod S (1980) Comparison of histological and physiological responses to Phakopsora pachyrhizi resistant and susceptible soybean. *Transcripts of the British Mycological Society* 74, 329-333.

Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *The Plant Journal* 10, 71-82.

Lu M, Tang XY, Zhou JM (2001) *Arabidopsis* NHO1 is required for general resistance against *Pseudomonas* bacteria. *Plant Cell* 13, 437-447.

Meyerowitz EM, Somerville CR (1994) *Arabidopsis*. Plainview, NY, Cold Spring Harbor Laboratory Press.

Miller P (1955) V-8 juice as a general purpose medium for fungi and bacteria. *Phytopathology* 45, 461-462.

Milliken GA, Johnson DE (1992) Analysis of messy data: Volume 1. Designed experiments. Chapman and Hall, London.

Molina A, Hunt MD, Ryals JA (1998) Impaired fungicide activity in plants blocked in disease resistance signal transduction. *The Plant Cell* 10, 1903-1914.

O'Gara E, Hardy GESJ, McComb JA (1996) The ability of *Phytophthora cinnamomi* to infect through unwounded and wounded periderm tissue of *Eucalyptus marginata*. *Plant Pathology* 45, 955-963.

Pajot E, Le Corre D, Silué D (2001) Phytogard® and DL-β-amino butyric acid (BABA) induce resistance to downy mildew (*Bremia lactucae*) in lettuce (*Lactuca sativa* L). *European Journal of Plant Pathology* 107, 861-869.

Perrone S, Bui F, Sutherland M, Guest D (2000) Gene-for-gene specificity expressed in planta is preserved in cell cultures of *Nicotiana tabacum* inoculated with zoospores of *Phytophthora nicotianae*. *Physiological and Molecular Plant Pathology* 57, 235-242.

Pieterse CMJ, Van Loon LC (2007) Signalling cascades involved in induced resistance. In: Induced Resistance for Plant Defence (Eds D Walters, A Newton, GD Lyon) p65-72 Blackwell Publishing.

Pilbeam RA, Colquhoun IJ, Shearer B, Hardy GEStJ (2000) Phosphite concentration: its effect on phytotoxicity symptoms and colonisation by *Phytophthora cinnamomi* in three understorey species of *Eucalyptus marginata* forest. *Australasian Plant Pathology* 29, 86-95.

Pu M-H, Min L-Z and Jie Z-H (2003) The transport and distribution of JA and ABA in *Malus micromalus* Makino and their relation to water in soil. *Acta Phytophysiologica Sinica* 24, 253-258.

Relyea HA, van der Donk WA (2005) Mechanism and applications of phosphite dehydrogenase. *Bioorganic Chemistry* 33, 171-189.

Ribeiro OK (1978) A Source Book of the Genus *Phytophthora*. Vaduz, Liechtenstein, Cramer.

Robinson LH, Cahill DM (2003) Ecotypic variation in the response of *Arabidopsis thaliana* to *Phytophthora cinnamomi*. *Australasian Plant Pathology* 32, 53-64.

Roetschi A, Si-Ammour A, Belbahri L, Mauch F, Mauch-Mani B (2001) Characterization of an *Arabidopsis-Phytophthora* pathosystem: resistance requires a functional PAD2 gene and is independent of salicylic acid ethylene and jasmonic acid signalling. *The Plant Journal* 28, 293-305.

Roos GHP, Loane C, Dell B, Hardy GEStJ (1999) Facile high performance ion chromatographic analysis of phosphite and phosphate in plant samples. *Communications in Soil Science and Plant Analysis* 30, 2323-2329.

Schreiber L, Schonher J (1992) Analysis of foliar uptake of pesticides in barley leaves, role of epicuticular waxes and compartmentalisation. *Pesticide Sciences* 36, 213-221.

Shearer BL, Smith, IW (2000) Diseases of Eucalypts Caused by Soilborne Species of *Phytophthora* and *Pythium.* In: Diseases and Pathogens of Eucalypts (Eds PJ Keane, GA Kile, FD Podger, BN Brown) CSIRO Publishing.

Shearer BL, Crane CE, Fairman RG (2004a) Phosphite reduces extension of a *Phytophthora cinnamomi* front in *Banksia* woodland, even after fire. *Australasian Pant Pathology* 33, 249 - 254.

Shearer BL, Crane CE, Cochrane A (2004b) Quantification of the susceptibility of the native flora of the South-West Botanical Province, Western Australia, to *Phytophthora cinnamomi*. *Australian Journal of Botany* 52, 435-443.

Shearer BL, Fairman RG (1997a) Foliar application of phosphite delays and reduces the mortality of three *Banksia* species in communities infested with *Phytophthora cinnamomi.* In: Proceedings of the 11th Biennial Conference of the Australasian Plant Pathology Society, Perth p180.

Shearer BL, Fairman RG (1997b) Phosphite inhibits lesion development of *Phytophthora cinnamomi* for at least four years following trunk injection of *Banksia* species and *Eucalyptus marginata.* In: Proceedings of the 11th Biennial Conference of the Australasian Plant Pathology Society, Perth p181.

Shearer BL, Fairman RG, Grant MJ (2006) Effective concentration of phosphite in controlling *Phytophthora cinnamomi* following stem injection of *Banksia* species and *Eucalyptus marginata*. *Forest Pathology* 36, 119-135.

Statsoft (1999) *Statistica*. Statsoft Inc., Tulsa, Oklahoma.

Sunwoo JY, Lee YK, Hwang BK (1996) Induced resistance against *Phytophthora capsici* in pepper plants in response to DL-β-amino-n-butyric acid. *European Journal Plant Pathology* 102, 663-670.

Tabachnick BG, Fidell LS (1996) Using multivariate statistics. Harper Collins Publishers: New York.

Thatcher LF, Anderson JP, Singh KB (2005) Plant defence responses: what have we learnt from Arabidopsis? *Functional Plant Biology* 32, 1-19.

Ticconi CA, Delatorre CA, Abel S (2001) Attenuation of phosphate starvation responses by phosphite in Arabidopsis. *Plant Physiology* 127, 963-972.

Tynan KM, Wilkinson CJ, Holmes JM, Dell B, Colquhoun IJ, McComb JA, Hardy GES (2001) The long-term ability of phosphite to control *Phytophthora cinnamomi* in two native plant communities of Western Australia. *Australian Journal of Botany* 49, 761-770.

van der Merwe M, Kotzé JM, Hall A (1992) Effect of phosphite in avocado roots on the zoospores of *Phytophthora cinnamomi.* South African Avocado Growers' Association Yearbook 1992. 15, 24-26

Van Hemelrijck W, Wouters PFW, Brouwer M, Windelinckx A, Goderis I, De Bolle MFC, Thomma B, Cammue BPA, Delaure SL (2006) The *Arabidopsis* defense response mutant *esa1* as a model to discover novel resistance traits against *Fusarium* diseases. *Plant Science* 171, 585-595.

von Ende CN (2001) Repeated measures analysis: growth and other time-dependent measures. In: Design and analysis of ecological experiments (Eds SM Scheiner, J Gurevitch) p116-157, Oxford University Press: Oxford.

Whiley AW, Leonardi J, Pegg, KG Langdon PW (2001) Use of foliar application of phosphonate to control phytophthora root rot in avocados. Australian and New Zealand Avocado Grower's Conference. 3-7 June, 2001, Bundaberg, Queensland.

Wilkinson CJ, Holmes JM, Dell B, Tynan KM, McComb JA, Shearer BL, Colquhoun IJ, Hardy GEStJ (2001) Effect of phosphite on in planta zoospore production of *Phytophthora cinnamomi*. *Plant Pathology* 50, 587-593.

Williams M, Senaratna T, Dixon K, Sivasithamparam K (2003) Benzoic acid induces tolerance to biotic stress caused by *Phytophthora cinnamomi* in *Banksia attenuata*. *Plant Growth Regulation* 41, 89-91.

Zhu YJ, Qiu X, Moore PH, Borth W, Hu J, Ferreira S, Albert HH (2003) Systemic acquired resistance induced by BTH in papaya. *Physiological and Molecular Plant Pathology* 63, 237-248.

APPENDIX I

Assessment of three surfactants on the efficacy of phosphite using a lupin model system

INTRODUCTION

An efficient way to approach the screening of surfactants or plant defense activators is to initially test a range of surfactants or activators *in vitro* on a model plant species before applying the best treatments to native species in the glasshouse. We have been developing a screening protocol using lupin seedlings which are fast to grow, occupy little space and are susceptible to *Phytophthora cinnamomi* in the first 3 weeks of growth.

METHODS

Glasshouse experiments

Lupin seeds (*Lupinus angustifolius* cv. Tangil) were sown in small containers of sand made from the lids of plastic food containers (15 x 10.5 x 1 cm) and grown in the glasshouse until radicles were 8 cm long, when they were infected using 0.5 cm disc of *P. cinnamomi* (isolate MP94-48) mycelium. The mycelium had been grown on V8 medium, which consisted of 10% cleared V8 juice (Campbell's Soups Australia P/L) and 2% Difco bacteriological agar (Bacto Laboratories P/L, Liverpool, NSW). The next day plants were sprayed with phosphite (Agri-Fos 600, Agrichem, Loganholme, Qld, Australia) at 0.5, 0.1, 0.05, 0.01, 0.005 and 0%. Lesion lengths were measured 2 and 3 days after treatment. Eight repeats of this experiment, incorporating modifications, failed to yield meaningful results due to a range of factors. These included breakdown of the automatic glasshouse watering system, variable percentages of germination, temperature extremes, variable growth rates of the roots in repeat experiments, low percentages of infected roots, and the roots frequently being too long for accurate assessment of lesions.

A new protocol was developed which enabled growth of plants under the more uniform conditions of a 25°C growth room.

Growth room experiments

After 6 days, seedlings with radicles longer than 5 cm and well-developed leaves were selected and arranged on the same paper rolls so there were 4 replicates of 10 seedlings per treatment. Plants were then inoculated with a 0.5 cm disk colonised by *P. cinnamomi* on V8 agar by placing the disk on the tip of the radicle with the mycelium side in contact with the root. The day after inoculation, plants were sprayed with 0 - 0.5% phosphite, with and without one of three surfactants applied at manufacturer's recommended concentrations. The surfactants used were; 2 mL/L Pulse (Nufarm Australia Ltd., Laverton

North, Vic), 2 mL/L Synertrol Oil (Organic Crop Protectants, Lillyfield, NSW), and 0.133 mL/L BS1000 (Crop Care, Murarrie, Qld).

Lesion lengths were measured 1 and 2 days after treatments in early experiments but measurements at 3 and 6 days provided better discrimination between treatments. The percentage of infected lupin roots (i.e. a visible lesion was present) varied from 50 - 80%. Data presented represents the increase in lesions length after spray was applied.

RESULTS

The effect of the addition of Pulse to phosphite spray

The lowest concentration of phosphite (without Pulse) that reduced lesion extension was 0.01%, while the addition of 2 mL/L Pulse to the phosphite spray resulted in reduction of lesion length at 0.005% phosphite at 3 days, however the difference was less significant at 6 days. Pulse had a marked effect in the 0% phosphite treatment and was associated with reduced lesion lengths (Figure Ia).

The effect of the addition of BS1000 to phosphite spray

The lowest concentration of phosphite (without BS1000) that reduced lesion extension was 0.05% (6 days data Figure Ib). The addition of 0.133 mL/L BS1000 to the phosphite spray resulted in no reduction of lesion length at 0.01% phosphite compared with the 0% phosphite controls at 6 days (Figure Ib). Further it appeared that in some cases the BS1000 was increasing the lesion length at a particular phosphite level.

The effect of the addition of Synertrol Oil to phosphite spray

Addition of 2 mL/L Synertrol Oil to sprays of phosphite at 0 - 0.5% did not reduce the concentration at which phosphite was effective as phosphite had very little effect at all in this experiment. However, it is interesting to note that Synertrol Oil at each concentration of phosphite either had no effect on lesion length or was associated with increased lesion length (Figure Ic).

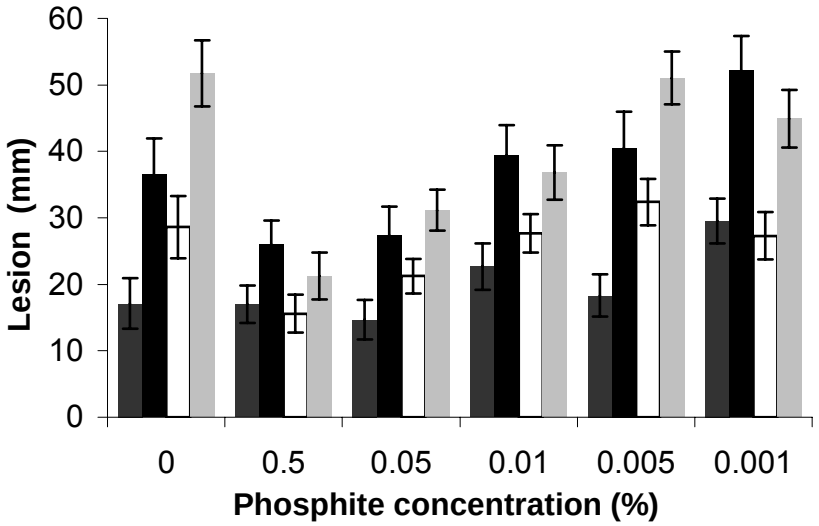


Figure Ia Lesion growth in *Lupinus angustifolius* roots inoculated with *Phytophthora cinnamomi.* Six concentrations of phosphite were sprayed 24 h after inoculation, with or without 2 mL/L Pulse. Phosphite and Pulse: lesion at 3 (▩) and 6 days (▩). Phosphite only: lesion at 3 (□) and 6 days (▩) after spraying. Vertical bars represent two standard errors of the mean.

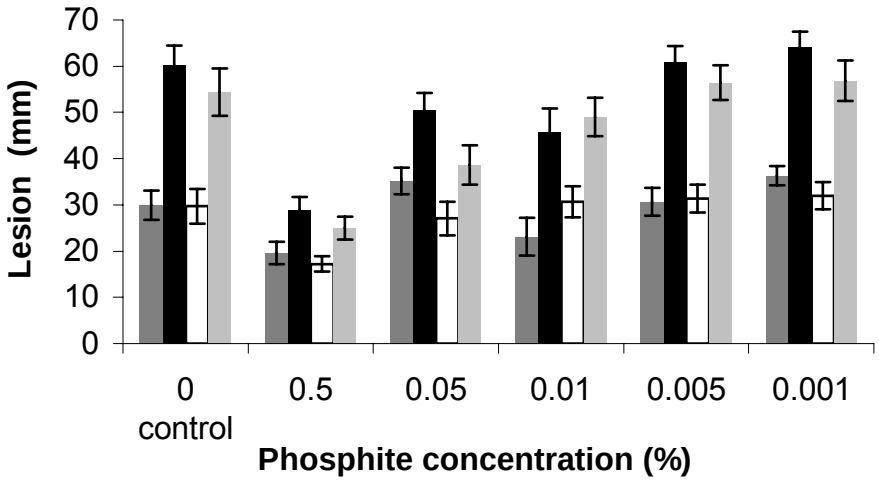


Figure Ib Lesion growth in *Lupinus angustifolius* roots inoculated with *Phytophthora cinnamomi.* Six concentrations of phosphite were sprayed 24 h after inoculation, with or without 0.133 mL/L BS1000. Phosphite and BS1000, lesion at 3 (▩) and 6 days (▩). Phosphite only, lesion at 3 (□) and 6 days (▩) after spraying. Vertical bars represent two standard errors of the mean.

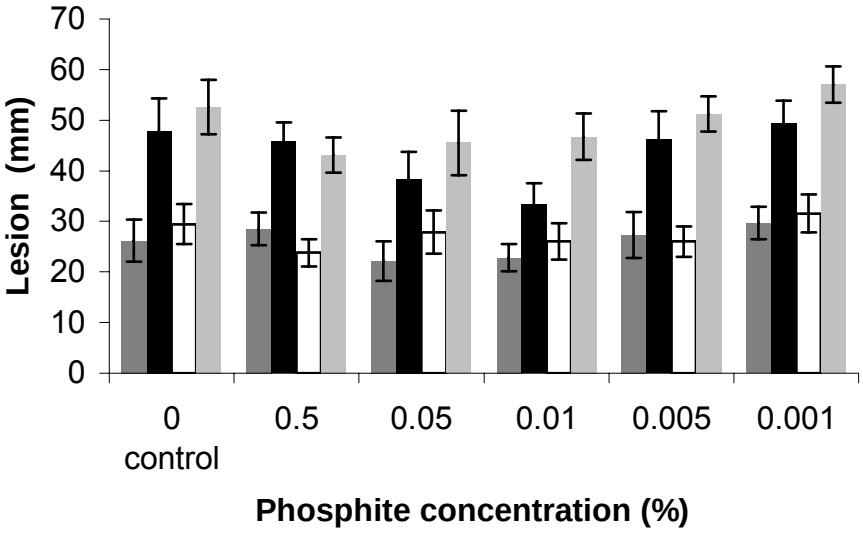


Figure Ic Lesion growth in *Lupinus angustifolius* roots inoculated with *Phytophthora cinnamomi.* Six concentrations of phosphite were sprayed 24 h after inoculation, with or without 2 mL/L Synertrol Oil. Phosphite and Synertrol, lesion at 3 (▩) and 6 days (▩). Phosphite only, lesion at 3 (□) and 6 days (▩) after spraying. Vertical bars represent two standard errors of the mean.

CONCLUSION

There was no evidence that Synertrol Oil or BS1000 enhanced uptake or effectiveness of phosphite, while addition of Pulse allowed a 50% reduction in the concentration of phosphite necessary for the reduction of lesion length.

Further experiments will use as a standard treatment 0.5% phosphite with the addition of Pulse.

APPENDIX II

Publications arising from this research

Submitted to Australasian Plant Pathology in March 2008

Hydroponically grown *Arabidopsis thaliana* is not an ideal host to study root infection by *Phytophthora cinnamomi*

NJ Jardine, P Stasikowski, JA McComb, BL Shearer, PA O'Brien and GEStJ Hardy

In an attempt to identify a compatible host-pathogen interaction to use as a model pathosystem, hydroponically –grown plants of twenty *Arabidopsis thaliana* ecotypes, 10-14 days old, were inoculated with zoospore suspensions from 28 *Phytophthora cinnamomi* isolates. Three weeks after inoculation there were no plant deaths and seedling shoots appeared healthy. In inoculated plants, no root lesions were observed. Although root length was reduced in inoculated plants in comparison to non-inoculated controls, shoot dry weight was either only slightly reduced or unaffected, depending on the ecotype. None of the twenty *A. thaliana* ecotypes screened were considered highly susceptible to the *P. cinnamomi* isolates present in the mixed inoculum. The similarity of inoculated plants to non-inoculated controls after exposure to high inoculum potential heavy zoospore treatment make hydroponically grown *A. thaliana* an unsuitable model to assay the pathogenicity effectiveness of *P. cinnamomi.*

APPENDIX III

Addressing the Aims and Outcomes of Sub Project 19.2.2

AIMS

Achieve additive or multiplier effects in the strength and/or duration of the host responses to *Phytophthora* by using combinations of phosphite and other compounds that elicit plant defense responses. By the use of previously untested surfactants, or modified use of existing surfactants, increase efficiency in uptake and accumulation of phosphite.

Experiment with model herbaceous plants in the laboratory, and woody native species in the glasshouse and field showed that while inclusion of the penetrant Pulse at (0.1 or 0.25%) can be recommended for use with phosphite sprays there is no value in adding either surfactant BT1000 or Synertrol oil. Similarly there is little evidence for a benefit from inclusion of the defense activators Benzothiadiazole (BTH), DL-ß-amino-n-butyric acid (BABA) or Methyl jasmonate (MeJ) except possibly for species that have genotypes that do not respond to phosphite.

OUTCOMES

1. By use of novel combinations of phosphite, other plant defense compounds and surfactants, increase resistance to *Phytophthora* in species that have a poor or no response to phosphite alone.

Three surfactants were tested on a model lupin system to determine whether the addition of surfactant increased uptake so that lesions caused by infection with *P. cinnamomi* were contained by lower levels of phosphite than when no surfactant was used. There was no evidence that Synertrol Oil or BS1000 enhanced uptake or effectiveness of phosphite. Addition of Pulse allowed a 50% reduction in the concentration of phosphite necessary for the reduction of lesion length. However in further experiments when phosphite was used at the operational level (0.5%) the addition of the penetrant Pulse at 0.1 or 0.25% did not enhance the control exhibited by phosphite alone.

However, it was shown that Pulse significantly increased the uptake of phosphite in both *Eucalyptus staeri* and *Banksia baxteri* (a species that takes up phosphite but is still susceptible to *P. cinnamomi* in the field). Phosphite uptake in both species reached a maximum after approximately 15 minutes. Experiments with *B. baxteri* in the glasshouse showed that spraying with 0.5% phosphite containing 1% Pulse effectively reduced lesion lengths from infection with *P. cinnamomi* in tests 6 and 11 weeks after spraying.

2. By extending the duration of protective effects, increase time between treatments.

The three plant defense activators (BTH, BABA and MeJ) were used in combination with phosphite to determine whether they enhanced the effect of phosphite in the lupin model plant system and in the field using *B. baxteri* underbark inoculated *with P. cinnamomi.* They were also tested using native species in the glasshouse which allowed in addition, assessment of whether the plant defense activators increased the longevity of the effect of the phosphite.

None of the plant defense activators increased the efficacy of phosphite in the lupin trials and in some cases they appeared to increase lesion lengths. They had no effect on the development of lesions in *Banksia* in the field, although variability amongst these plants indicated that larger replicate numbers were needed.

The glasshouse trial assessed stem colonisation by *P. cinnamomi* after underbark inoculation of 13-month old *Banksia baxteri, Eucalyptus staeri* and *Lambertia inermis*. Plants were sprayed with phosphite (0.5%) in combination with the defense activators (each at two concentrations), and infected at 1, 6 and 11 weeks after spraying. There was no evidence that the addition of defense activators increased the effectiveness of phosphite over this time-period. The experiment is ongoing and further tests will be made in the future.

3. Lessen the probability of development of resistance to phosphite in *Phytophthora.*

Reducing the concentration of phosphite applied and or increasing the intervals between spraying are ways in which development of resistance to phosphite might be delayed or prevented. The experiments conducted using different surfactants and/ or different plant growth defense activators did not result in enhancement of the effect or longevity of the effect of phosphite

Another way of lessening the probability of the development of resistance, and to allow for less empirical selection of additives is to understand the mechanism of action of phosphite: both the direct effect on the pathogen and the indirect through stimulation of plant defense responses. This was investigated using *Arabidopsis* and lupins as model systems. After demonstrating that intact *Arabidopsis* plants are not a suitable pathosystem with *P. cinnamomi* we showed that detached leaves of accession Landsberg erecta (Ler) are susceptible to *P. cinnamomi* for at least 72 h after infection and that phosphite reduces the effect of the pathogen in this system as assessed by lesion development and the frequency of callose papillae. Using qPCR an increase in the level of expression of the defense gene PRI was quantified. Ongoing research by a PhD student is utilising this system to

determine further gene up and down regulation to help elucidate the mode of action of phosphite on the plant.

A rapid assay was developed to compare the effect of phosphite and of metabolic inhibitors on pathogenicity of *P. cinnamomi.* Filter paper discs overgrown with *P. cinnamomi* were treated with 20 µL drops of phosphite or inhibitors, then tested for pathogenicity (ability to colonise lupin roots), or growth on NARPH plates. It was shown that c-AMP is likely to be involved in the reduction by phosphite of *P. cinnamomi* pathogenicity. The technique provides a means of screening compounds that might enhance phosphite efficacy, and to explain the mode of action of phosphite.

4. Achieve an effective threshold level of phosphite in species that otherwise have poor uptake of phosphite.

Some plants when sprayed with phosphite have relatively low uptake of phosphite and do not show any resistance to *P. cinnamomi*. One such plant selected from a glasshouse screening was a *Lambertia inermis* genotype. Uptake of phosphite by this genotype was shown qualitatively by the silver nitrate staining test (see below) and quantitatively by chemical analysis. Shoots and roots contained less phosphite than genotypes that did respond to phosphite. Cut shoots of this plant were sprayed with phosphite together with BTH or MeJ (at two concentrations) then the cut end of the shoot was exposed to *P. cinnamomi* mycelium in an in vitro assay. BTH (0.15 mM) reduced lesion length compared to phosphite alone, but the protection was not as strong as seen in genotypes that respond to phosphite.

Experiments that require information on phosphite uptake, movement and longevity are hampered by the lack of a quick cheap method of measuring accurately the concentration of phosphite in different plant tissues. Costs of the existing HPLC method (~$30 per sample) prevent such studies, and analyses in the region of $1 - $5 per sample are required. We examined two methods (a silver nitrate assay, and a phosphite dehydrogenase assay as potential methods of accurately and cost effectively measuring phosphite in plant tissues. Both methods proved promising and represent exciting advances in phosphite analysis. Some additional fine tuning is required to ensure that the methods are reliable across a range of plant species from different families. A cheap, accurate and robust analytical method will allow many important questions about phosphite uptake and movement to be investigated.

5. Achieve protection against *Phytophthora cinnamomi* with a reduction in the amount of phosphite applied.

The defense activators BTH and MeJ were tested for their ability to increase the responsiveness of 11-month old *B. grandis* seedlings sprayed with phosphite at 0, 0.1, 0.25 and 0.5 g/L phosphite. At 9 weeks after inoculation there was no evidence that the addition of plant defense activators enhanced

the ability of the lower phosphite concentrations (0.1 and 0.25%) to contain the pathogen.