

Phosphorous acid for controlling *Phytophthora* taxon Agathis in kauri: glasshouse trials

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Abstract *Phytophthora* taxon Agathis (PTA) is a serious problem in Auckland and Northland kauri forests. Phosphorous acid (phosphate) is a potential treatment for infected or threatened trees. *In vitro* tests on phosphate-amended agar showed that PTA was more sensitive to phosphate than other *Phytophthora* species commonly controlled by this chemical. Before progressing to forest trials, phosphate efficacy was tested on PTA-inoculated kauri seedlings in the glasshouse. Two-year-old kauri seedlings were inoculated with PTA applied directly to trunk wounds or by soil application. Phosphate was applied as a foliar spray, as a trunk injection or as a soil drench either 5 days before or 5 days after inoculation. All untreated control trees died, whether trunk- or soil-inoculated. With phosphate injection, survival was 100% following PTA soil inoculation and 67% following trunk inoculation. Foliar spray and soil drench-applied phosphate treatments were less effective than trunk injection, although some trees survived.

Keywords: phosphate, kauri, *Phytophthora* taxon Agathis.

INTRODUCTION

Phytophthora taxon Agathis (PTA) is a serious problem, killing kauri trees of all ages in forests in Auckland and Northland, New Zealand (Beever et al. 2009). Treatment with phosphate (phosphorous acid, PA) is one of the potential options for treatment of infected or threatened trees. Phosphate has been used successfully for treating a wide range of *Phytophthora* diseases of many plant species. Its predominant use is in horticulture and nurseries, with some use in forest systems (Smillie et al. 1989; Hardy et al. 2001).

The current work includes tests of phosphate inhibition of PTA *in vitro*, and glasshouse trials testing phosphate applications for control of PTA in inoculated kauri seedlings. Ridomil® (metalaxyl) was also included for comparison,

as this compound has been used for many years for *Phytophthora* control in horticulture, and has been demonstrated to reduce kauri seedling root disease and mortality in forests infested with *P. cinnamomi* (Horner 1984; Johnston et al. 2001).

MATERIALS AND METHODS

In vitro tests

Six isolates of *Phytophthora* taxon Agathis and two isolates each of *P. cactorum* and *P. cinnamomi* were grown on V8 agar for 5 days. Agar plugs, 5 mm in diameter, were taken from culture margins and subcultured to V8 agar amended with various concentrations of phosphorous acid (PA; 0, 5, 15, 40, 100 and 250 µg/ml), then incubated at 20°C. There were three replicate

plates of each isolate for each PA concentration. The PA used was a commercial preparation of Agrifos®600 (Key Industries) containing 60% phosphorous acid present as mono- and di-potassium phosphonate. The PA was added to the agar after autoclaving, when it had cooled to approximately 70°C.

One day after subculturing onto the PA-amended media, the margins of all colonies growing from the inoculum plug were marked on the bottom of the Petri plate using a fine marker pen. All subsequent measurements were taken from this reference point. Growth measurements were taken 4 and 7 days after subbing and growth rates per day were calculated. Response curves were fitted to data averaged across each species, using a third order polynomial. From this, EC₅₀ values were calculated.

Microscopic examinations of the cultures were made after 7 days, to observe anomalous growth or sporulation on agar amended with PA.

Glasshouse trials

Two-year-old kauri seedlings, 60–80 cm tall and 8–10 mm in diameter at the base of the trunk, were grown in potting mix in 1.5 litre planter bags in the glasshouse at Plant & Food Research, Havelock North.

Seedlings were inoculated with PTA by either trunk or soil inoculation:

- For trunk inoculation, an oat grain colonised by incubating on a PTA-colonised V8 agar plate was inserted in a small incision in the trunk bark, approximately 10 cm above the soil. The wound and oat inoculum was sealed with grafting tape. Uncolonised oat grains were used as uninoculated controls.
- For soil inoculation, 4 weeks before inoculating the seedlings, two 8-mm diameter wooden dowels were inserted 6 cm into the soil. Immediately before soil inoculation, these dowels were removed, leaving a cavity for applying the PTA inoculum. A 10 ml suspension of a macerated mycelial mat of PTA grown in V8 broth was poured into each of the two holes. The pot was then flooded for 24 h by raising the water level to half-way up the soil profile in the bag (approximately

4 cm deep). After 24 h, trays were drained. For the following 10 weeks, frequent misting in the glasshouse ensured that soil water was maintained at a high, but not flooded state.

The following treatments were applied to trees, either 5 days before or 5 days after inoculation with PTA:

1. Untreated control
2. Phosphite spray (low rate, 2 ml/litre phosphorous acid)
3. Phosphite spray (medium rate, 4 ml/litre phosphorous acid)
4. Phosphite spray (high rate, 8 ml/litre phosphorous acid)
5. Phosphite injection (low rate, 0.16 ml of 150 ml/litre phosphorous acid)
6. Phosphite injection (high rate, 0.32 ml of 150 ml/litre phosphorous acid)
7. Phosphite soil drench (low rate, 20 ml of 2 ml/litre phosphorous acid)
8. Phosphite soil drench (high rate, 20 ml of 8 ml/litre phosphorous acid)
9. Ridomil® 2.5G, 1.33 g granules (33 mg metalaxyl/plant).

The phosphite formulation used was Agrifos600. Phosphite sprays were applied to foliage using a hand held mist sprayer until runoff. To avoid PA spray or runoff entering the soil, thick newspaper was used to cover the soil surface during spray application and left in place until leaves were dry. Phosphite injections were carried out by drilling a 0.45 mm diameter hole on a 45° downward angle, two-thirds of the way through the trunk, 2–5 cm above soil level. A hypodermic needle (25 gauge, 0.5 mm) containing the required volume of phosphorous acid was immediately inserted into the hole. To aid pressurising of the syringe, approximately 0.4 ml of air was included above the PA in the syringe. The plunger was manually depressed and held in place with rubber bands, compressing the air behind the PA solution and thus pressurising the syringe. In most cases, the syringes emptied in 1 to 12 h.

Soil drenches were applied by adding the required volume of PA to 20 ml of water, and pouring it over the soil surface.

Metalaxyl was applied by sprinkling the required volume of Ridomil granules to the

soil surface, gently working them in to the top few millimetres of soil, then drenching the soil surface with 20 ml of water per pot.

There were five replicate trees of each fungicide treatment, for each PTA inoculation method (trunk or soil) and for each timing (treated pre- or post-inoculation). The high rate of phosphite injection was not included in the treatments before inoculation. Uninoculated controls were included for comparison, with five uninoculated trees of each treatment.

Throughout the experiment, the aim was to maintain glasshouse temperature below 28°C, as this is regarded as the upper limit of activity for PTA (I.J. Horner, unpublished data). Soil temperatures remained below this threshold, but air temperatures in the canopy occasionally exceeded 30°C, though never for prolonged periods. To prevent cross-contamination between treatments and to minimise contamination of glasshouse benches, after inoculation and treatment all seedlings were maintained in individual aluminium trays.

The first disease assessment was made 10 weeks after inoculation. Foliage health was scored by dividing each tree into three sections (Top=upper 10 cm, Base=lower 10 cm, Middle=remainder). Each section was rated from 0 to 3, where 0=healthy, 1=slight wilt symptoms, 2=severe wilt symptoms, and 3=dead, then averaged to give an overall foliar health score. For trees that had been soil-inoculated, root health was assessed on a 0 to 5 scale, where 0= 0–5% diseased, 1= 5–25%, 2= 25–50%, 3= 50–75%, 4= 75–95%, 5= 95–100% of feeder roots diseased. A selection of diseased feeder roots was plated onto PARPH Phytophthora-selective medium (Jeffers & Martin 1986) to determine whether PTA had colonised the roots. Live trees were re-potted into the same soil. For trees that had been trunk-inoculated, lesion expansion was assessed by measuring the length above and below the oat-grain inoculation point, and by estimating the greatest percentage girdling of the trunk. Trunk tissue samples from within and outside lesion boundaries were plated onto the selective medium.

One week after the initial disease assessment, all remaining inoculated trees were retreated

with the same fungicide treatment they received initially, and retained in the glasshouse at between 15 and 25°C. These trees were re-assessed 10 weeks later, scoring overall foliar health of all trees using the same scale as above. Root health was scored for soil-inoculated trees only, and lesion activity or healing was noted for trunk-inoculated trees.

Data were analysed using regression analysis for an unbalanced design in Genstat (Version 13, 2010, VSN International Ltd, Hemel Hempstead, UK). Lesion length data were log-transformed before analysis, but no other data required transformation.

RESULTS

In vitro tests

Phytophthora colony growth on PA-amended media is summarised in Figure 1. Mycelial growth of all three *Phytophthora* species was significantly inhibited by phosphorous acid. PTA was the species most sensitive to phosphorous acid, with a 50% reduction in growth (EC_{50}) at 4.0 µg/ml phosphorous acid, compared with EC_{50} values of 25.2 and 37.9 µg/ml for *P. cinnamomi* and *P. cactorum* respectively.

Microscopic examination showed an increasing delay in sporulation of PTA with increasing

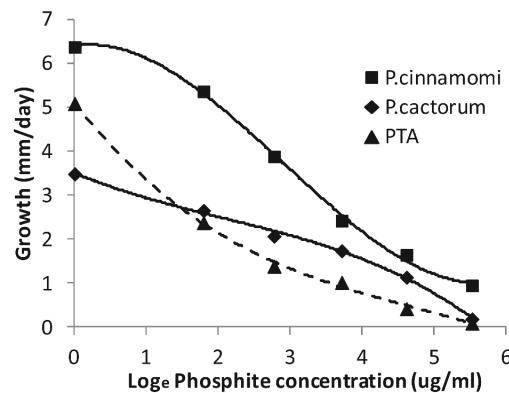


Figure 1 Growth of mycelial cultures of *Phytophthora cinnamomi*, *P. cactorum* and *Phytophthora* taxon Agathis (PTA) on V8-agar amended with various concentrations of phosphorous acid. Growth data are averaged for each species.

phosphorous acid concentration in the medium. In un-amended medium, oogonial formation occurred on hyphae that were approximately 1.5 days old. At concentrations of 5 and 15 µg/ml phosphorous acid, oogonial formation occurred on hyphae 2–3 and 3–6 days old, respectively. At 40 µg/ml, two of the six PTA isolates had not produced oogonia within 8 days. The remaining four isolates had produced a small number of oogonia after 4–6 days, but these oogonia did not appear to mature, and in many cases were plasmolysed.

Glasshouse trials

Soil-inoculated trees

For trees soil-inoculated with PTA, there was a significant ($P<0.001$) increase in root and foliar symptom scores compared with un-inoculated controls. In general, only inoculated trees showed foliar symptoms. There were no significant differences in disease scores between the various treatments in un-inoculated trees (data not shown).

When trees were inoculated, there were significant differences in root score between treatments ($P<0.001$) at both 10- and 20-week assessments (Figure 2). Whether the treatment

was applied before or after inoculation had no significant effect on root or foliar disease scores, and the dose rates within the application method did not have a major effect, so these factors have been combined in the analyses.

Trees injected with phosphite had, on average, significantly lower root disease scores than all other treatments. Trees treated with a soil drench of phosphite had the next lowest root disease scores, significantly lower than the foliar phosphite, metalaxyl or untreated trees after 10 weeks. This difference was not apparent after 20 weeks. The order of symptom severity based on the average foliar score among the treatments was similar to that with root scores, but the statistical significance was marginal ($P=0.062$) at 10 weeks, although higher ($P<0.001$) at 20 weeks (Figure 2).

After 20 weeks, all 15 phosphite-injected trees were still alive, with the next best rate of survival occurring in the phosphite drench (9/20), metalaxyl (3/10) and phosphite spray (6/30) treatments. All the 14 untreated trees were dead within 20 weeks (Table 1, Figure 4).

When feeder roots from a selection of trees were plated onto *Phytophthora*-selective medium

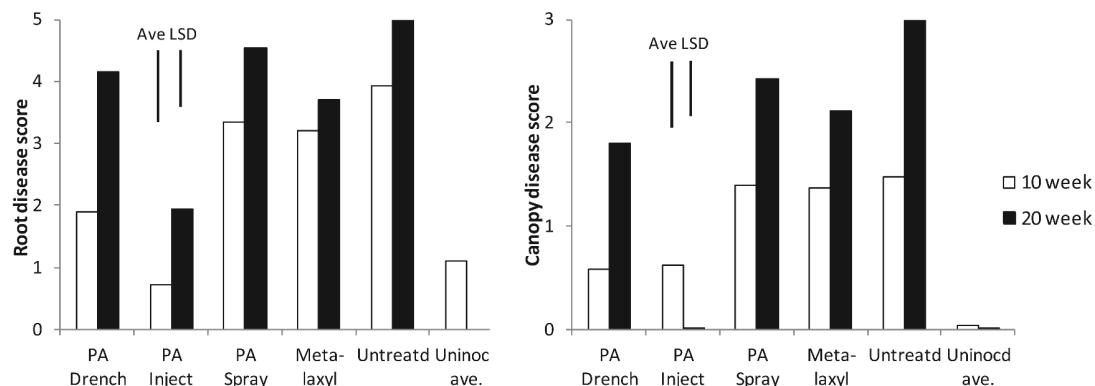


Figure 2 Average root (left) and canopy (right) disease scores assessed 10 or 20 weeks after soil inoculation with *Phytophthora* taxon Agathis (PTA). Kauri trees were treated with either metalaxyl or phosphite (PA) applied at various rates and three application techniques, either 5 days before or 5 days after inoculation. For simplicity, data for 'before' and 'after' treatments have been combined, as have different rates of the same treatment. 'Root score' is the average of root disease scored on a 0 to 5 scale, where 0 = all healthy, and 5 = all dead. 'Foliar score' is the average of foliar disease symptoms scored on a 0 to 3 scale, where 0 = healthy, and 3 = dead. Week 10 data are averages of scores for top, middle and bottom sections of the tree. Week 20 data are from a single overall tree health score. LSD bars are least significant differences ($P<0.05$) averaged across 10-week (left bar) and 20-week (right bar) data.

Table 1 Number of kauri seedlings remaining healthy 10 or 20 weeks after inoculation with *Phytophthora* taxon Agathis (PTA), either to soil or on trunks. Trees were treated with either metalaxyl (Ridomil®) or phosphite (PA) applied at various rates and three application techniques (foliar spray, trunk injection or soil drench), either 5 days before or 5 days after inoculation. There were 5 trees for each treatment combination and 14 trees for the untreated, inoculated controls. All of the five uninoculated controls within each fungicide treatment remained alive and healthy after 20 weeks.

	Soil inoculation with PTA				Trunk inoculation with PTA			
	Treat before		Treat after		Treat before		Treat after	
	10	20	10	20	10	20	10	20
Untreated control			7	0			0	0
PA spray, 2 ml/litre	2	1	3	1	0	0	0	0
PA spray, 4 ml/litre	1	1	2	0	0	0	0	0
PA spray, 8 ml/litre	2	0	3	3	0	0	1	0
PA inject, 0.16 ml	5	5	5	5	5	5	3	3
PA inject, 0.32 ml	-	-	5	5	-	-	3	2
PA drench, 2 ml/litre	5	4	4	3	0	0	0	0
PA drench, 8 ml/litre	4	2	3	0	1	0	0	0
Ridomil® granules	3	3	2	0	1	1	1	1

after 10 weeks, PTA was isolated from all trees except those treated by trunk injection and from un-inoculated trees. The isolation frequency was highest in untreated control trees.

Trunk-inoculated trees

Where sterile oat grains were inserted in the trunk, kauri seedlings remained healthy, with no lesion above the oat insertion point and, on average, lesions of less than 4 mm below the oat insertion point. When trees were trunk-inoculated with PTA-colonised oat grains, lesions established and progressed rapidly in untreated trees and in some of the trial fungicide treatments, in many instances girdling and killing the trees. In contrast, lesions established but were limited in trees injected with phosphite. Differences between the phosphite-injected treatments and untreated controls were highly significant for lesion length (Figure 3), percentage girdling of the trunk (data not shown) and foliar disease symptoms (Figure 3). For other treatments, differences from the untreated controls were small or non-significant.

Most of the trees that were alive at the 10-week assessment were still alive after 20 weeks. All five trees injected with phosphite before

trunk-inoculation with PTA were still alive and apparently healthy when assessed after 20 weeks (Table 1, Figure 4). Five of the ten trees injected with phosphite after trunk-inoculation were still alive. The only other survivors at 20 weeks were two of the ten metalaxyl-treated trees. All untreated controls (14), phosphite-sprayed (30) and phosphite-drenched (20) trees were dead after 20 weeks (Table 1, Figure 4).

Lesion extension was not measured at the 20-week assessment, but the extent of lesion healing was noted. In all cases where trees were still alive at 20 weeks (predominantly phosphite-injected trees) the lesions appeared to have stopped expanding and had callused over. Pieces of lesion tissue from these apparently healed lesions failed to yield PTA when plated onto selective agar media. This was consistent with earlier isolations 10 weeks after inoculation, where PTA was readily isolated from lesion tissue in all but the phosphite-injection treatments. No PTA was isolated from outside the lesion margin.

DISCUSSION

The *in vitro* studies, in which PTA, *P. cinnamomi* and *P. cactorum* were grown on agar medium amended with phosphite, demonstrated that

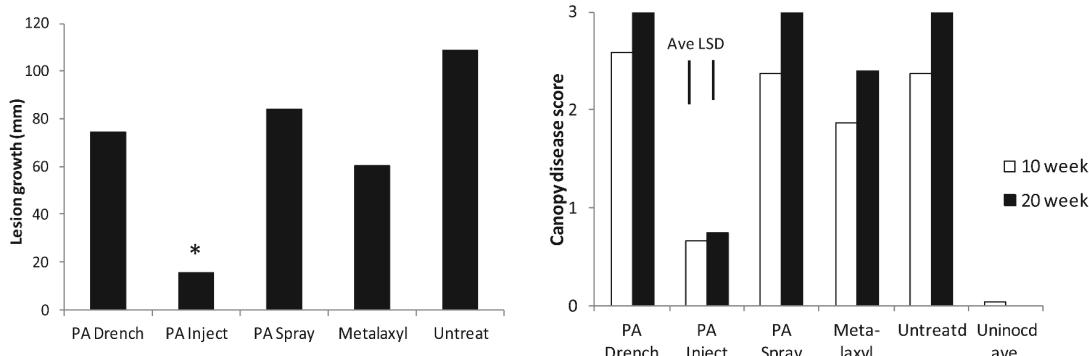


Figure 3 Average lesion extension (left) and canopy disease scores (right) following kauri trunk inoculation with *Phytophthora* taxon Agathis (PTA). Trees were treated with either metalaxyl or phosphite (PA) applied at various rates and three application techniques, either 5 days before or 5 days after inoculation. For simplicity, data for 'before' and 'after' treatments have been combined, as have different rates of the same treatment. Lesion measurements are upwards from the inoculation point 10 weeks after inoculation, and data are back-transformed following analyses of log-transformed lesion length data. The asterisk indicates lesion growth significantly ($P<0.05$) less than in the untreated control. 'Foliar score' is the average of foliar disease symptoms scored on a 0 to 3 scale, where 0 = healthy, and 3 = dead. Week 10 data are averages of scores for top, middle and bottom sections of the tree. Week 20 data are from a single overall tree health score. LSD bars are least significant differences ($P<0.05$) averaged across 10-week (left bar) and 20-week (right bar) data.

PTA is highly sensitive to this chemical. PTA's sensitivity *in vitro* appeared even greater than that of other *Phytophthora* species commonly controlled by phosphite in horticultural systems. The glasshouse trials with kauri seedlings were seen as a necessary step before treating larger trees in the forest. Although slightly artificial in that the size and age of the trees was substantially different from trees likely to be treated in the forest, it was considered an effective 'low risk' way of determining potential efficacy and toxicity on kauri without jeopardising large trees.

The two different PTA-inoculation techniques gave some insight into infections in different parts of the trees and how they might react to various treatments. The soil inoculation and subsequent watering regime was considered to provide a realistic representation of root infection. The fact that all the untreated control trees died under this regime demonstrates just how virulent PTA is towards kauri, and also gives confidence that the observed survival of trees in some treatments reflects a real treatment effect on the disease. The wounding and trunk

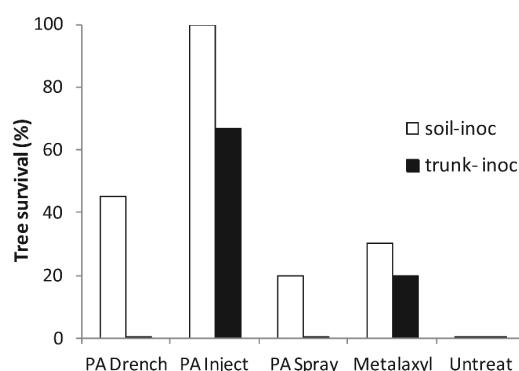


Figure 4 Percentage of kauri seedlings remaining healthy 20 weeks after inoculation with *Phytophthora* taxon Agathis (PTA), either to soil or on trunks. Trees were treated with either metalaxyl or phosphite (PA) applied at various rates and three application techniques, either 5 days before or 5 days after inoculation. For simplicity, data for 'before' and 'after' treatments have been combined, as have different rates of the same treatment. Full data are presented in Table 1.

inoculation with PTA was a severe treatment for such small trees, and a very rigorous test of the experimental control treatments. Disease lesions developed in all cases. All untreated control trees, plus many treated trees, were girdled and dead within 10 weeks, again demonstrating the strong pathogenicity of PTA on kauri.

By far the most effective treatment for controlling PTA was phosphite applied as a trunk injection. All trees in the soil-inoculated and two-thirds of the trees in the trunk-inoculated treatments were still alive and healthy after 20 weeks. The only trees to die were those that were trunk-inoculated 5 days before phosphite treatment. It is likely that lesions had established and rapidly girdled the thin trunks before the treatment had a chance to be effective. Trunk lesions in these surviving trees had, in all cases, healed and callused over. The 100% survival in root-inoculated trees injected with phosphite demonstrates that the chemical was translocated through the tree and into the roots in sufficient quantity to suppress the pathogen effectively. The failure to isolate PTA from these trees also reflects this suppression.

Phosphite applied as a soil drench had a positive impact on root and foliar health and tree survival when trees had been soil-inoculated with PTA. This might reflect either direct suppression of the pathogen in the soil or absorption of phosphite by roots giving internal protection. The application of PA as a soil drench had minimal impact on lesion extension or tree health when applied to trunk-inoculated trees, suggesting that insufficient chemical had been absorbed by the roots and translocated to shoots in time to inhibit lesion development and prevent girdling.

Phosphite applied as a foliar spray was not effective in this trial. The rates applied were comparable to those used in commercial application of phosphite in horticultural tree crops, but had minimal, if any, impact on PTA. It is possible that the anatomy of the kauri leaf is not conducive to absorption of phosphite, or that the phosphite was not translocated from leaves to the trunks and roots. The addition of an agent such as Pentra-bark™, normally used to aid phosphite absorption through bark, may be beneficial and is worthy of further investigation.

These trials demonstrate that phosphite has potential for controlling PTA in kauri trees. Field trials with kauri rickers and slightly larger trees are currently in progress.

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