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Twig assay refinement for use in phosphite trials

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EXECUTIVE SUMMARY

Twig assay refinement for use in phosphite trials

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July 2017

Phosphite has been successfully used to treat kauri trees infected with *Phytophthora agathidicida*. However, it is difficult to determine required re-treatment intervals. Excised twig or leaf assays were considered as a potential tool for indirectly determining biologically active concentrations of phosphite within kauri tissues, thus aiding decisions on re-treatment. It was hoped that differential lesion growth rates on phosphite-treated and untreated trees would help determine when phosphite concentration had dropped below biologically useful levels.

Living twigs and leaves were collected from kauri trees 3 and 18 weeks after trees were treated with various phosphite applications. In laboratory assays these twigs and leaves were then inoculated with *P. agathidicida*, and subsequent lesion growth was measured by direct observation and tissue plating. There were no significant differences in lesion growth rates between tissues collected from untreated control trees or any of the phosphite-treated trees. This was the case for both twig and leaf assays, at both time periods. Variability was very high, and outweighed any potential treatment effects.

Twig age was considered as a potential source of variation or confounding factor. Analysis of twig age data showed that in the 3 week post-treatment assessment there was significantly slower lesion growth in older twigs, but this accounted for only 15% of the observed variability. There was no twig age effect in the subsequent test, 18 weeks post-treatment.

The girth, canopy health and percentage trunk girdling of the source trees were considered as potential sources of variation, but their effects were either not evident or inconsistent.

Thus, we have concluded that excised twig or leaf bioassays are not going to be a simple or useful determinant of phosphite concentrations in kauri trees. Attached twig and leaf assays are likely to be more accurate, but would be difficult to carry out and problematic with an unwanted organism such as *P. agathidicida*.

Laboratory testing of phosphite residues was of little value. Although these assays confirmed the presence of phosphite remote from application points following all of the various application methods, the concentrations measured were very low, and bore no correlation to the amount of phosphite applied in the various treatments.

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1 INTRODUCTION

Phosphite has been successfully used to treat kauri trees infected with *Phytophthora agathidicida*, although there are still many unknowns regarding suitable treatment regimes (Horner et al. 2015). An issue that needs to be addressed regarding future use of phosphite for treatment of kauri dieback is determining the periodicity of retreatment. This is relevant both to current experiments and to any future roll-out of phosphite. Rather than waiting until tree health regresses or *Phytophthora* lesions reactivate, it would be useful to have a predictive tool that might help determine when phosphite concentrations have dropped to such a level that re-treatment might be necessary.

There was interest in testing to see if a twig assay system could be used to determine when trees required re-treatment with phosphite. By inoculating *P. agathidicida* onto excised twigs or leaves collected from treated trees and from comparable untreated trees, differential growth rates of the pathogen could potentially be used to determine if there is residual phosphite activity.

Investigation of a twig assay in the Omahuta phosphite trial gave inconclusive results (Horner & Hough 2014b). Although lesion growth on excised twigs from phosphite treated trees was less than that on untreated trees, the differences were small and the variability was high. These samples were from a highly disturbed and variable site, which may have confounded the studies.

The current report summarises trials to determine the feasibility of twig or leaf assays for assessing phosphite activity in kauri trees, and attempts to determine some of the factors that might contribute to variability in results.

2 METHODS

2.1 Plant material source

In March 2016, trials investigating low phosphite rates and trunk sprays were established in two neighbouring Dargaville sites (Horner et al. 2017). Only kauri ricker trees with dieback symptoms were selected for the trial. Tree girth and canopy health (on a 0–5 scale where 0=healthy and 5=dead) and the percentage of basal trunk girdling was recorded, then trees were treated with various phosphite applications as summarised below:

A Untreated control

B 7.5% phosphite trunk injection, 20 mL every 20 cm

C 4% phosphite trunk injection, 20 mL every 20 cm

D 4% phosphite trunk injection, 20 mL every 40 cm

E 10% trunk spray with bark penetrant (Pentrabark™)

F 10% trunk spray without bark penetrant.

In April and August 2016, 3 and 18 weeks respectively after phosphite application, live twigs and leaves were collected from branches within 6 m of the ground on selected trees from each treatment. Samples were directly transported to the laboratory for processing within 24 h.

2.2 Twig inoculation

Within 24 h of collection, twigs and leaves were inoculated with *P. agathidicida*, using the assay of Horner and Hough (2014a).

Briefly, twigs were cut to 10–12-cm lengths, leaves were removed, and twig age was determined (based on annual growth scars). The numbers of twigs in various age categories are summarised in Table 1. A small wound (2 mm square) was made 1 cm from the base of each 10-cm length and a plug of *P. agathidicida*-colonised agar was placed on the wound. Five twigs from untreated control trees were similarly inoculated with uncolonised agar plugs. Twigs were then placed on a moist tissue in a plastic tray, sealed in a plastic bag and incubated on the laboratory bench at 20–22°C (Figure 1). After 17 days, twigs were cut into 1-cm lengths and plated onto *Phytophthora*-selective agar, ensuring the orientation of each segment allowed subsequent delimitation of colonisation. After 24, 48 and 72 h, agar around each segment was microscopically examined to determine the emergence of *P. agathidicida* hyphae. In this manner, the extent of *P. agathidicida* colonisation in the segment and the twig at the time of plating could be determined. Subsequent checks of colony identity were made after 10 days (Figure 2).

2.3 Leaf inoculation

Nine leaves were selected from some of the above twigs, targeting undamaged leaves from the past year's growth. Leaves were inoculated by placing a *P. agathidicida*-colonised agar plug over a small wound made using a hypodermic needle 5 mm from the base of the leaf. A 10th leaf from each twig was similarly inoculated with a sterile agar plug. Leaves were placed on moist tissues in a plastic tray, sealed in a plastic bag and incubated on the laboratory bench at

20-22°C. After 7 and 10 days, lesion distance from the edge of the agar plug inoculation point was measured (Figure 3). After 10 days (April samples only), 5 of the 9 leaves from each twig were cut into strips and portions were plated onto selective agar in a manner that allowed determination of the extent of *P. agathidicida* colonisation. Emerging *P. agathidicida* hyphae were recorded 24, 48 and 72 h after plating.

2.4 Analyses

Lesion growth data for both twig and leaf assays in April and August were analysed using mixed effect models to determine differential growth rates between treatments. To assess possible influences tree girth, tree health and percentage of the tree trunk girdled on variability of results in the excised leaf and shoot assays, data from April and August assessments were merged. Samples were from the same tree each time, and it was assumed any consistent tree effect would be seen over both dates. Girth and percentage trunk girdling were treated as continuous variables, and canopy score was treated as a categorical variable.

2.5 Residue testing

In April and August 2016, 3 and 18 weeks after phosphite treatment application, twig and leaf samples were collected from representative trees of each treatment in the two Dargaville sites, and pooled for analysis for phosphite residues. The samples were a subset of the material used in the twig and leaf assays described above. Samples were sent to Hill Laboratories Limited in Hamilton, and analysed for phosphonate content following aqueous extraction and Liquid chromatography-mass spectrometry (LC-MS/MS). The detection threshold was 0.4 ppm.

Table 1. Numbers of excised twigs and leaves inoculated with *Phytophthora agathidicida* in experiments in April (top) and August (bottom) 2016, 3 weeks and 18 weeks, respectively, after treatment of kauri trees using various phosphite applications. Twig age indicates the predominant age of tissue in the 10-cm portion tested. '1/2' indicates a both 1- and 2-year-old wood in the sample. PB=Pentrabark™.

Treatment	Twig Age (years)												Leaves
	1	1/2	2	2/3	3	3/4	4	4/5	5/6	6/7	7/8	total	
Control	4	9	3	8	1	5	2	3	1	1	1	38	63
7.5% injection/20cm	4	3	1	2	1	0	0	1	0	0	0	12	27
4% injection/20cm	3	5	2	2	2	1	0	0	0	0	0	15	27
4% injection/40cm	2	4	2	3	1	2	0	1	0	0	0	15	27
10% TrunkSpray+PB	0	2	1	0	0	0	0	0	0	0	0	3	9
10% TrunkSpray	2	7	4	1	2	1	0	1	0	1	0	19	36
Total	15	30	13	16	7	9	2	6	1	2	1	102	189

Treatment	Twig Age (years)										Leaves
	1	1/2	2	2/3	3	3/4	4	4/5	5	6	total	
Control	5	12	4	8	2	5	0	2	2	0	40	63
7.5% injection/20cm	1	5	4	5	0	2	1	0	0	0	18	27
4% injection/20cm	0	4	0	5	2	3	0	2	1	0	17	27
4% injection/40cm	2	6	3	4	2	1	0	0	0	0	18	27
10% TrunkSpray+PB	1	2	1	1	1	0	0	0	0	0	6	9
10% TrunkSpray	1	7	2	6	2	3	0	2	0	1	24	36
Total	10	36	14	29	9	14	1	6	3	1	123	45

Figure 1. Set-up of excised twig assays. Twigs are 10-cm long, inoculated with a *Phytophthora agathidicida*-colonised agar plug 1 cm from the base (left hand side of photo).

Figure 2. Growth of *Phytophthora agathidicid*a from kauri twig segments cut and plated onto agar 17 days after inoculation at the point marked by the red arrow. Segments are place sequentially from bottom left to top right.

Figure 3. Leaf assay set-up, 10 days after inoculation with a *Phytophthora agathidicida*-colonised agar plug placed near the base of each leaf.

3 RESULTS

None of the leaves or twigs inoculated with sterile agar developed lesions. In contrast, lesions developed in most leaves and twigs inoculated with *P. agathidicida*.

Visual demarcation of lesion margins on twigs was difficult, necessitating the plating of twig segments to accurately determine lesion spread. However, leaf lesions margins were usually obvious. In the comparison between visually assessed lesion measurements and those calculated following plating of leaves, there was a strong correlation between the results (R^2=0.66). This indicated that for the leaf assay, visual assessment of the lesion is adequate and plating is not necessary. Data from leaf plating was not further considered in analyses.

3.1 Treatment effects

There were no significant differences in lesion growth among tissues taken from trees treated with various phosphite applications or untreated controls. This was the case for both leaf and twig assays, in both April and August assessments (Figures 4 and 5). There was considerable variability within treatments, as can be seen from the wide scatter of points on the plots. This variability far outweighs treatment differences, potentially confounding any attempts to use such assays to assess biologically useful concentrations of phosphite in tissues.

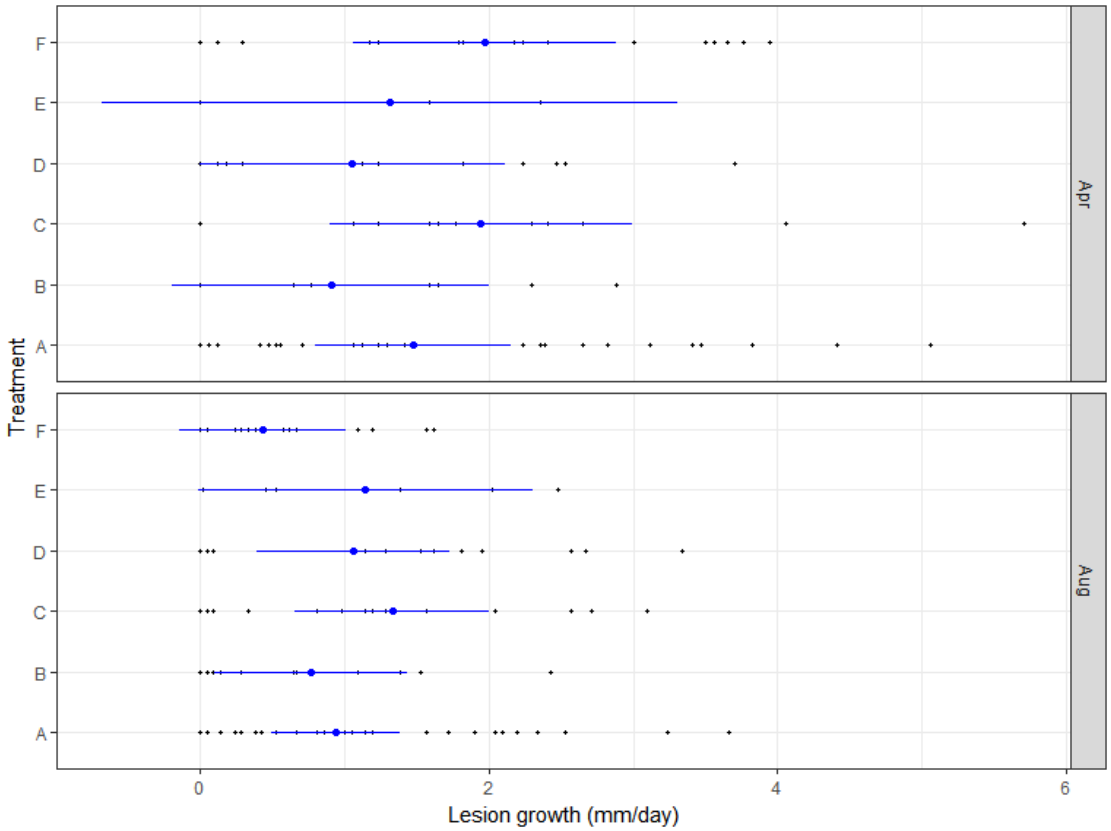

Figure 4. Lesion growth (mm/day) on kauri twigs excised and inoculated with *Phytophthora agathidicida* 3 weeks (Apr) or 18 weeks (Aug) after source trees were treated with various phosphite formulations (Treatments B to F) or left untreated (Treatment A). 95% confidence intervals (+/-) are shown in blue. Black points are the raw data.

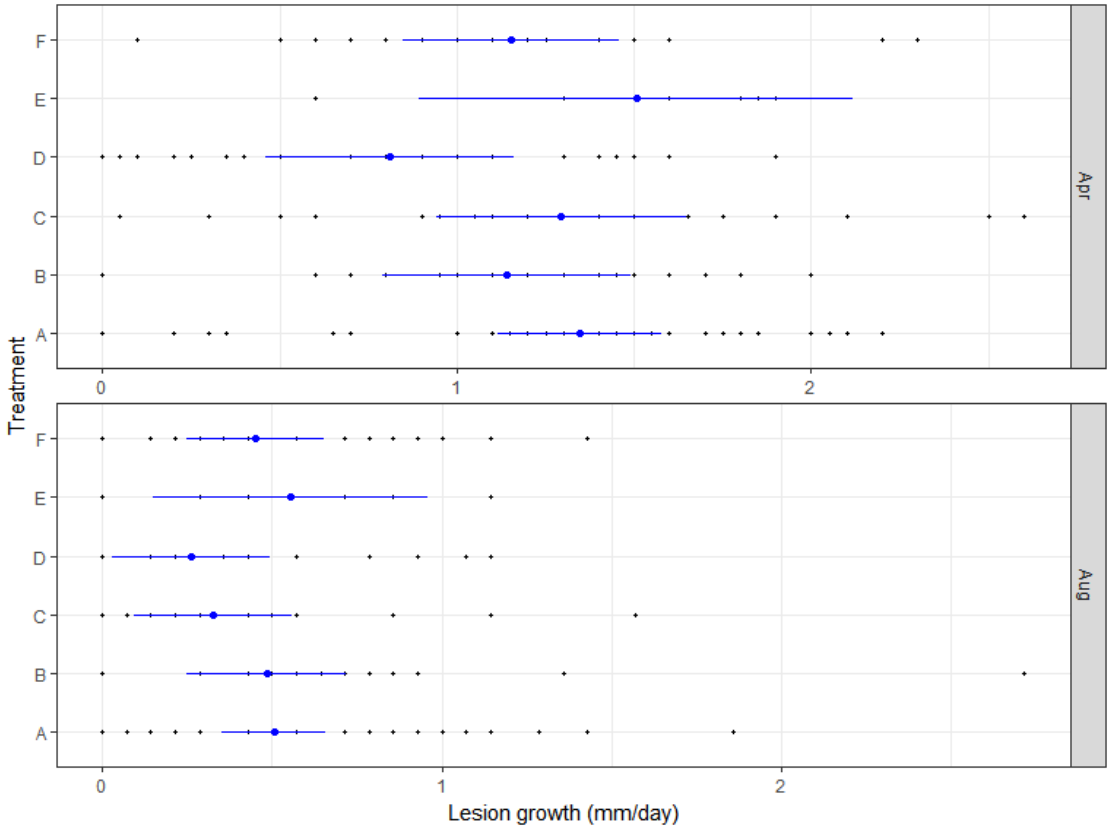

Figure 5. Lesion growth (mm/day) on kauri leaves excised and inoculated with *Phytophthora agathidicida* 3 weeks (Apr) or 18 weeks (Aug) after source trees were treated with various phosphite formulations (Treatments B to F) or left untreated (Treatment A). 95% confidence intervals (+/-) are shown in blue. Black points are the raw data.

3.2 Potential sources of variability

In an attempt to determine factors that may contribute to the variability in results, mixed effects models were used to investigate factors such as twig age, tree girth, tree health and percentage of the tree trunk girdled.

3.2.1 Twig age

In the April assessment, twig age had a significant (P<0.001) effect on lesion growth, with greater growth on younger twigs (Figure 6). However, in the August assay there was no such relationship. Even though the relationship was significant in April, age only accounted for 15% of the observed variability.

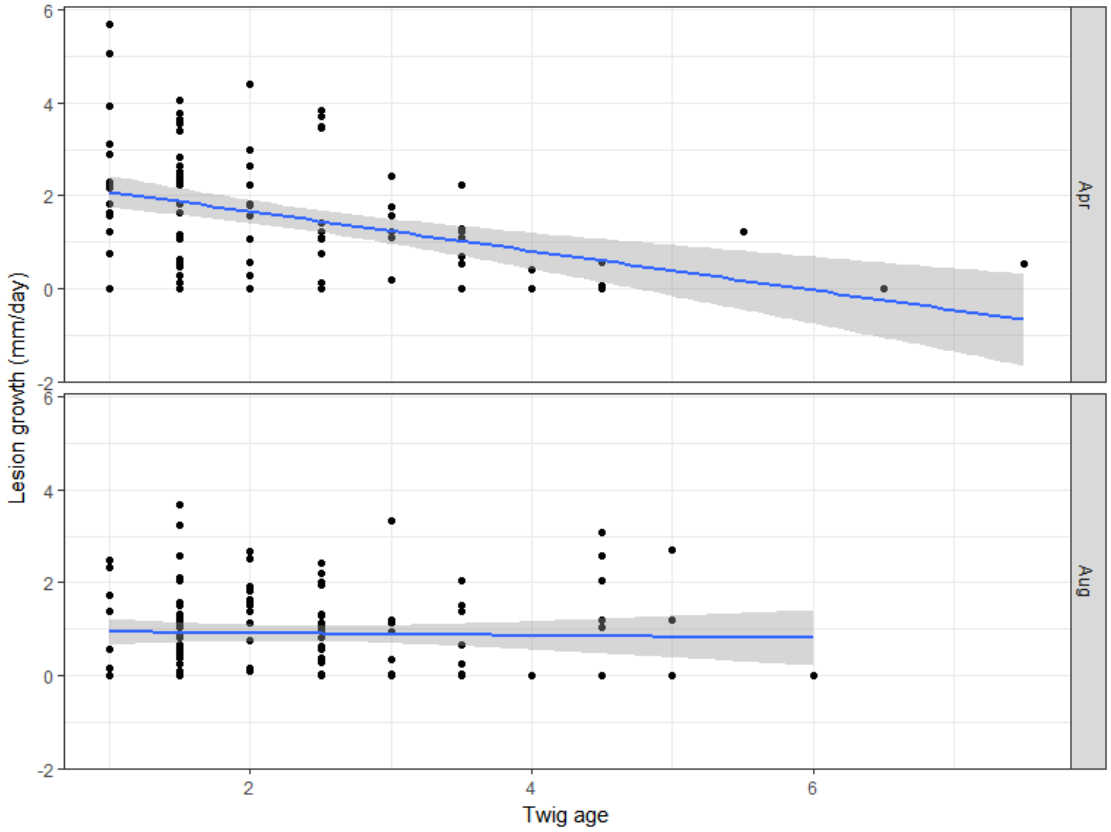

Figure 6. The effect of kauri twig age on lesion growth. Twigs were excised and inoculated with *Phytophthora agathidicida* 3 weeks (Apr) or 18 weeks (Aug) after source trees were treated with various phosphite formulations or left untreated. Grey areas indicate predicted effects +/- 95% confidence intervals. Black points are the raw data.

3.2.2 Tree parameters: trunk girth, canopy health rating, percentage trunk girdling

There was a slightly significant effect of tree girth on average lesion advance on twigs, although this was strongly influenced by two trees with large girth (Figure 7). There was no such relationship with the leaf assay.

Neither canopy health score nor percentage of trunk girdling on the source tree had any significant effect on results for either twig or leaf assays (Figures 8 and 9).

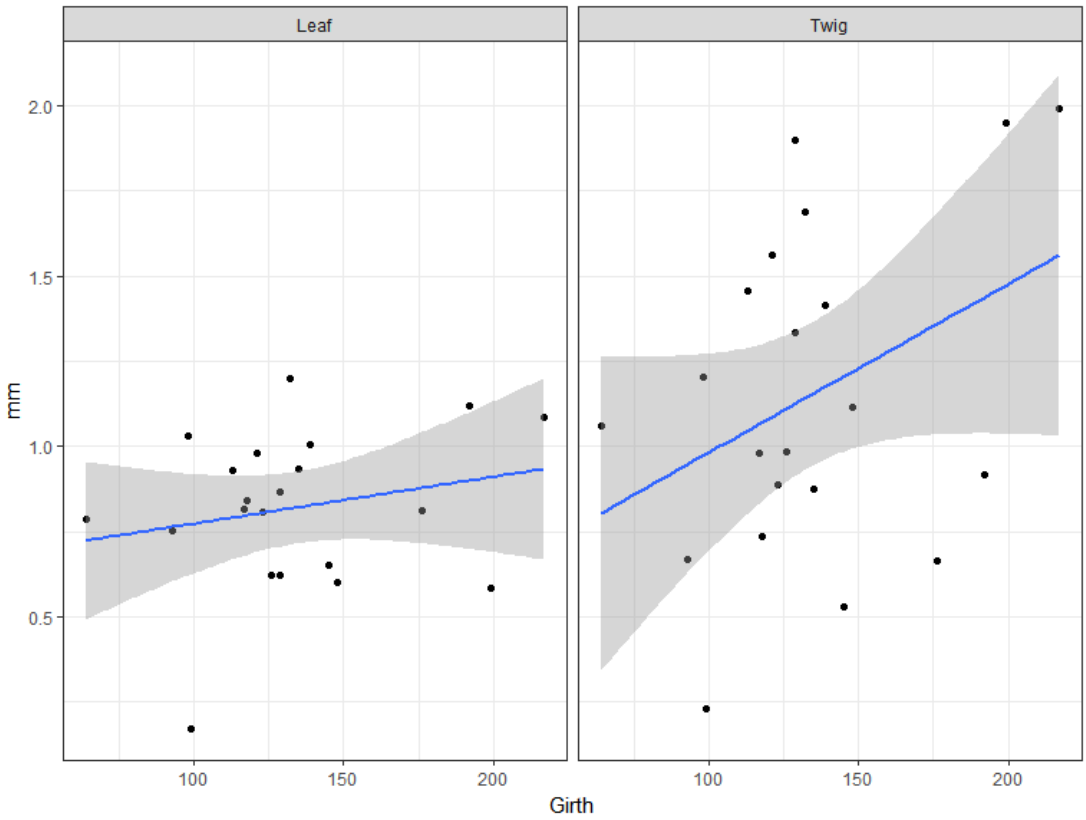

Figure 7. The effect of source tree girth on average leaf and twig lesion progression following inoculation with *Phytophthora agathidicida*. Data for tissues collected in April and August 2016 have been averaged. Grey areas indicate predicted effects +/- 95% confidence intervals. Black points are the raw data.

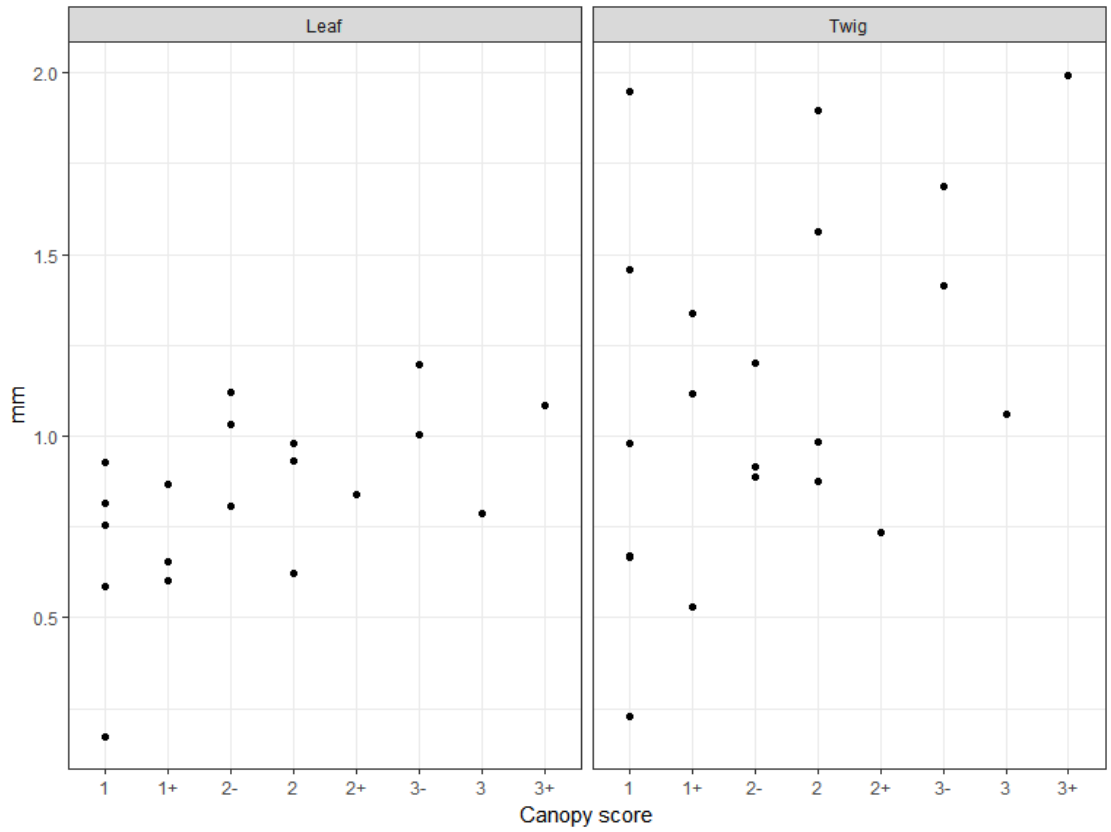

Figure 8. The effect of source tree canopy health score (on a 1–5 scale where 1=healthy and 5 =dead) on average leaf or twig lesion progression following inoculation with *Phytophthora agathidicida*. Data for tissues collected in April and August 2016 have been averaged. Black points are the raw data.

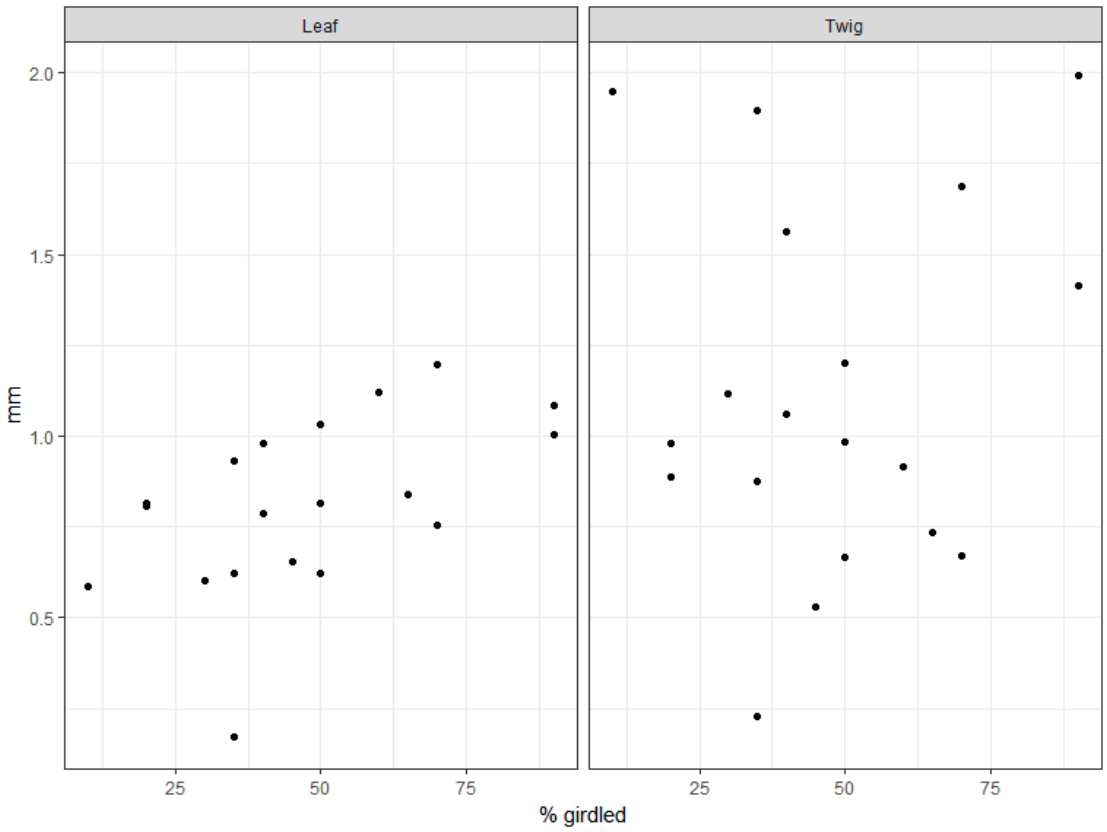

Figure 9. The effect of percentage trunk girdling on average leaf or twig lesion progression following inoculation with *Phytophthora agathidicida*. Data for tissues collected in April and August 2016 have been averaged. Black points are the raw data.

3.3 Residue testing

Results of residue testing showed that regardless of application method (trunk injection or bark spray), phosphite could be detected in tissues remote from the point of application (Table 2). However, residue concentrations were very low (less than 3 ppm), and were not obviously correlated with concentration or dose applied.

Table 2. Phosphite residues detected in kauri leaves and shoots collected 3 or 18 weeks after application of phosphite treatments.

Treatment	Mean phosphite residue detected (ppm)	
	3 weeks	18 weeks
Untreated control	<0.4	<0.4
7.5% phosphite injection/20 cm	<0.4	1.2
4% phosphite injection/20 cm	1.2	1.0
4% phosphite injection/40 cm	2.4	0.7
10% phosphite trunk spray plus Pentrabark™	<0.4	0.5
10% phosphite trunk spray	0.6	0.4

4 DISCUSSION

Results of this work suggest that excised twig or leaf assays may not be useful for determining biologically active concentrations of phosphite in treated kauri trees. While it had been hoped that such assays could help determine when retreatment is necessary, the wide variability in lesion growth and failure to discriminate between phosphite-treated and untreated trees would preclude such assays being a useful predictive tool. While it could be suggested that the results reflected the actual status of phosphite activity in the trees, i.e. that it wasn't present in biologically active concentrations, field observations on the same trees over the sampling period (Horner unpublished) indicate that natural lesions on treated trees were in the process of healing and drying up, suggesting that phosphite was indeed active in these trees.

Twig age and source tree size were identified as factors that had a weak influence on lesion growth in excised twigs, potentially confounding results. However, the responses were not consistent, and are of little value in helping to discriminate a predictive assay.

Similarly, residue testing results were of little value. Although these assays did at least confirm presence of phosphite remote from application points following all of the various application methods, the concentrations measured were very low, and bore no correlation to the amount of phosphite applied in the various treatments.

It is suggested that excised kauri leaf and twig assays are abandoned as a potential technique for determining *in planta* concentrations of phosphite, or predicting retreatment times. Because of the mode of activity of phosphite (i.e. stimulating host defence responses (Smillie et al. 1989)), it is possible that assays on attached leaves and twigs on trees in the field may be more discriminating and reliable. But such assays could be problematic when working with an unwanted organism such as *P. agathidicida*.

5 REFERENCES

Horner I, Hough E, Horner M. March 2017. Trunk sprays and lower phosphite injection rates for kauri dieback control. A Plant & Food Research report prepared for: Ministry for Primary Industries. Milestone No. 66672. Contract No. 33523. Job code: P/345160/04. SPTS No. 14471.

Horner IJ, Hough EG, Horner MB 2015. Forest efficacy trials on phosphite for control of kauri dieback. New Zealand Plant Protection 68:7-12.

Horner IJ, Hough EG 2014a. Pathogenicity of four *Phytophthora* species on kauri: in vitro and glasshouse trials. New Zealand Plant Protection 67: 54-59.

Horner IJ and Hough EG 2014b. Phosphorous acid for controlling *Phytophthora* taxon Agathis in kauri: field trials 2½ years on, June 2014. A Plant & Food Research report prepared for MPI. Milestone No. 47655. Contract No. 28262. Job code: P/345061/01. PFR SPTS No. 10397.

Smillie R, Grant BR, Guest D. 1989. The mode of action of phosphite: Evidence for both direct and indirect modes of action on three *Phytophthora* spp. in plants. Phytopathology 79: 921–926.

6 ACKNOWLEDGEMENTS

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