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Phospite resistance in *Phytophthora cinnamomi* isolates within NZ Forestry Nurseries

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Report information sheet

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Executive summary

To determine if prolonged phosphite exposure leads to decreased phosphite sensitivity in *Phytophthora* pathogens within New Zealand production forestry, *Phytophthora cinnamomi* isolates were collected from plots with past phosphite treatment at the Scion and Tokoroa arborgen forestry nurseries and screened for tolerance using a mycelial growth inhibition assay. Phosphite inhibition on mycelial growth and sporangia production was tested *in vitro* in 5 concentrations. Most isolates were phosphite sensitive and did not produce any mycelial growth or sporangia when exposed to any phosphite treatment. However, 2 isolates from the Tokoroa nursery had clear phosphite resistance, expressed by mycelial growth. Phosphite resistance may have resulted from repeated phosphite exposure. Further work is required to determine if phosphite treatment within New Zealand nurseries and globally will result in increased tolerance within *Phytophthora* populations and how this may affect long term plant production.

Phospite resistance in *Phytophthora cinnamomi* isolates within NZ Forestry Nurseries

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Introduction

Phosphite is one of the most effective chemical control agents for managing *Phytophthora* diseases. Current phosphite application rates within forest nurseries may be contributing to selection for phosphite resistance within *Phytophthora* populations (Reglinski et al. 2009; Reglinski et al. 2010). Phosphite has a complex mode of action. It works directly on the pathogen by inhibiting hyphal growth and preventing sporulation while also stimulating host defence responses in the plant (Smillie et al. 1989; Guest and Grant 1991). Phosphite is phloem mobile so it is also transported down to the root system of the tree which is the infection point for the causal agent of avocado root rot, *Phytophthora cinnamomi*. Induced phosphite resistance in *P. cinnamomi* after prolonged exposure has been clearly demonstrated *in vitro* (Bower and Coffey 1985; Duvenhage 1994; Kaiser et al. 1997; Wilkinson et al. 2001; Dobrowolski et al. 2008).

Methods

Isolate sampling and culture

In December 2017 to January 2018, *P. cinnamomi* isolates were collected from wilting and unhealthy *Pinus radiata* seedlings growing in bare earth nursery beds at the Scion and Tokoroa Nurseries by baiting (Khaliq et al. 2018). In a plastic container 140 x 85 x 70 mm, 15-20 mm depth of soil and fine feeder roots were placed and slowly flooded with 400 mL of distilled water. Two leaves of a cultivated *Rhododendron* species and five Himalayan cedar (*Cedrus deodara*) needles were floated on top of the water. Baits were checked daily for 'water-soaked' lesions on the leaves and brown lesions on the needles. Three days after commencing baiting the leaves and needles were removed, washed with tap water, and patted dry with paper towels. Plant material was then plated onto CRNH selective agar, containing blended frozen carrots 100 g/L, agar 15 g/L, $CaCO_3$ 3 g/L, pimaricin 4 mg/L, ampicillin 200 mg/L, rifampicin 10 mg/L, nystatin 50 mg/L and hymexazol 50 mg/L modified from (Tsao 1983). *Phytophthora*-like colonies were sub-cultured onto CAD agar containing blended frozen carrots 100 g/L, agar 15 g/L, $CaCO_3$ 3 g/L and grown at 17.5 °C in the dark. *Phytophthora i*solates were identified morphologically. Isolates were stored in water vials, within vials of sterile de-ionized water.

In vitro phosphite sensitivity

Growth inhibition

Seven *Phytophthora cinnamomi* isolates from the Scion nursery and six *P. cinnamomi* isolates from the Tokoroa nursery, were grown for five days in 90 mm petri dishes containing a modified version of the chemically defined agar medium, Ribeiros' synthetic medium (RMM) (Fenn and Coffey 1984), containing 0.084 mM phosphate, buffered with 0.03 M MES which was added to the medium prior to pH adjustment (pH 6.2) with KOH 3 M. Mycelial mat plugs, 3 mm diameter, were taken from the growing margin and added to 90 mm petri dish plates containing RMM that had been amended with five phosphite concentrations (0, 15, 40, 80, and 200 µg phosphite/mL) diluted with deionised water. Agri-Fos® 600 (Agrichem, Yatala QLD, Australia), a commercial potassium phosphate containing 600 g/L phosphorous acid, present as mono- and di-potassium phosphonate, was used. Five replicate plates were made for each of the five phosphite concentrations (including the control) and each *P. cinnamomi* isolate. Plates were grown in the dark at 17 °C in a Panasonic Incubator (VWR International). The radial growth rate was recorded on 2–5 days after plating, along two lines intersecting the centre of the inoculum at right angles.

Inhibition of sporangia formation

The efficacy of phosphite to inhibit sporangia formation was tested for each *P. cinnamomi* isolate. A 5 mm plug, from the actively growing margin of culture on 10% carrot agar, was placed into a 90 mm agar plate so the upper surface of the plug was on its side. Plugs were submerged in 6 mm depth of filtered non-sterile pond water that had been amended with 0, 15, 40, 80 and 200 µg phosphite/mL. Plates were incubated at 20 °C in the light. After 24 hours, the water was changed. Mature, full sporangia produced on the upper surface of the inoculum plug were counted at 4x magnification, 48 hours after inoculation. Five replicates per treatment were randomised in a split-plot design.

Statistics – Analysis

Phosphite sensitivity was measured using mycelial growth and sporangia production tests to measure inhibition in the presence of phosphite. The inhibition values calculated are used to predict the amount of phosphite required to inhibit growth by 50% relative to the control, known as the Effective Concentration 50 (EC50). EC50 was calculated using the R dose response package, using a four-parameter log-logistic model (Ritz et al. 2015).

Results

Radial Growth

All isolates from the Scion and Tokoroa nurseries produced radial growth above 6 mm/day when they were not exposed to phosphite (0 µg phosphite / mL) (Fig. 1). Most isolates did not produce mycelial growth when exposed to phosphite treatments. Growth was recorded for five isolates when exposed to 15 µg phosphite / mL, including isolates Scion isolate 1 and Tokoroa isolates 1, 3, 5 and 6. However, Scion isolate 1 and Tokoroa isolates 1 and 3 did not produce growth across all replicates when exposed to 15 µg phosphite / mL, and the mean growth was less than 0.1 mm/day. Tokoroa isolates 5 and 6 produced mycelial growth when exposed to 15, 40 and 80 µg phosphite / mL. Tokoroa isolate 5 produced mycelial growth when exposed to 200 µg phosphite / mL.

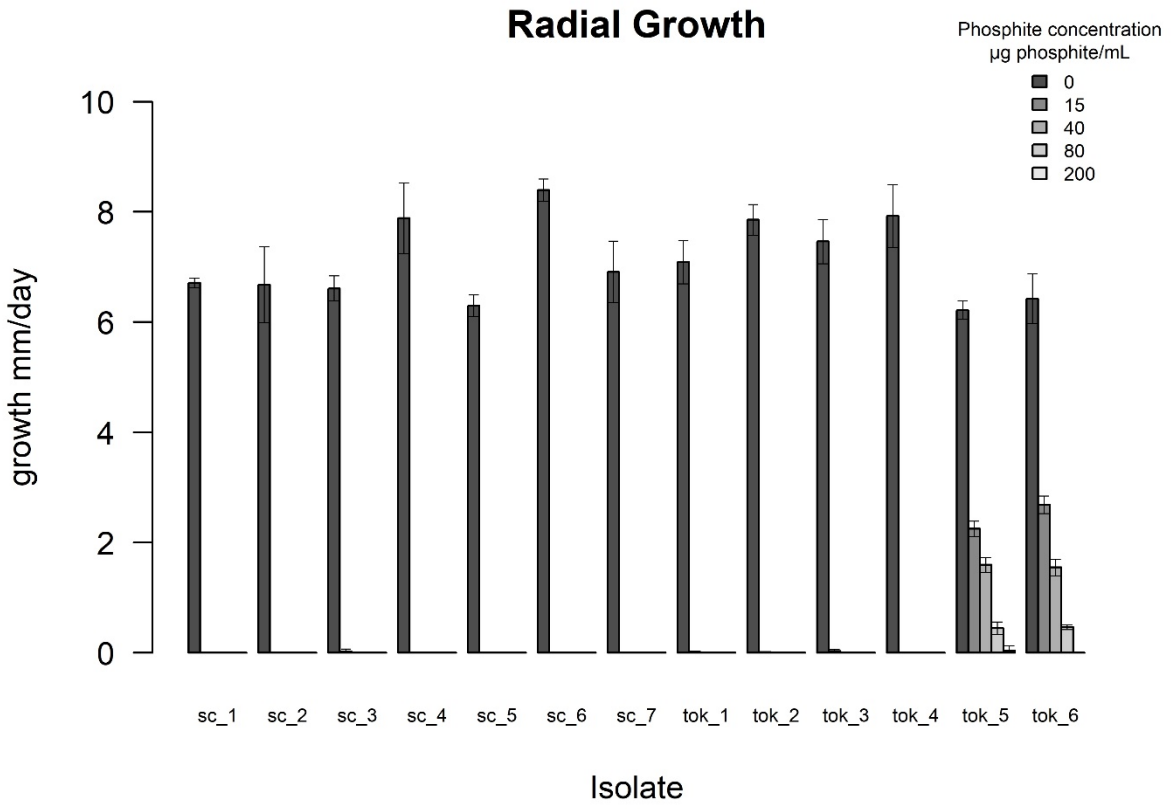

Figure 1: Average radial growth rate (mm/day) (± standard error), of *Phytophthora cinnamomi* isolates from the Scion (sc) and Tokoroa (tok) arborgen nursery in Jan 2018, grown in Riberio's selective agar media (Fenn and Coffey 1984) amended with 0, 15, 40, 80 and 200 µg phosphite/mL.

Effective concentration rates 50, could only be calculated for Tokoroa isolates 5 and 6 (Fig. 2). All Scion isolates and Tokoroa isolates 1, 2, 3 and 4 did not produce sufficient growth when exposed to phosphite treatments to fit the four parameters log-logistic model or any other dose-response models Ritz et al. (2015). Tokoroa isolate 5 had an EC50 concentration of 13.1 µg phosphite/mL, while Tokoroa isolate 6 had an EC50 concentration of 14.5 µg phosphite/mL. Tokoroa isolate 5 had more variable growth when exposed to phosphite than Tokoroa isolate 6.

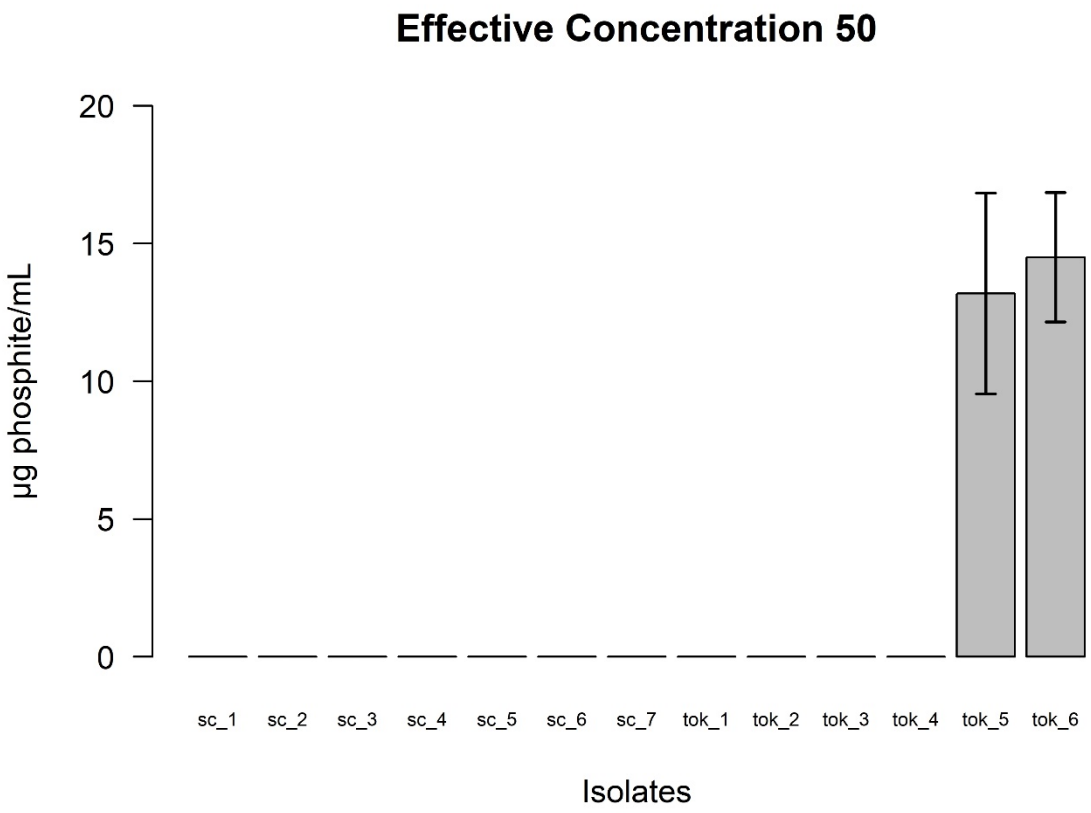

Figure 2: Mean effective concentration rate 50 µg phosphite/mL (± standard error), of *Phytophthora cinnamomi* isolates from the Scion (sc) and Tokoroa (tok) arborgen nursery in Jan 2018, grown in Riberio's selective agar media (Fenn and Coffey 1984) amended with 0, 15, 40, 80 and 200 µg phosphite/mL. EC50 was calculated using the R dose response package, using a four-parameter log-logistic model (Ritz et al. 2015).

Sporangia production

Sporangia production was more variable between isolates and phosphite treatments compared to radial growth measurements. All isolates from the Scion and Tokoroa nurseries produced more than 5 sporangia when they were not exposed to phosphite µg phosphite / mL) (Fig. 2). No isolate produced more than 2 sporangia when exposed to phosphite treatments. Effective concentration rates 50 could not be calculated for any isolates as not enough sporangia where produced when exposed to phosphite treatments to fit the models stated above.

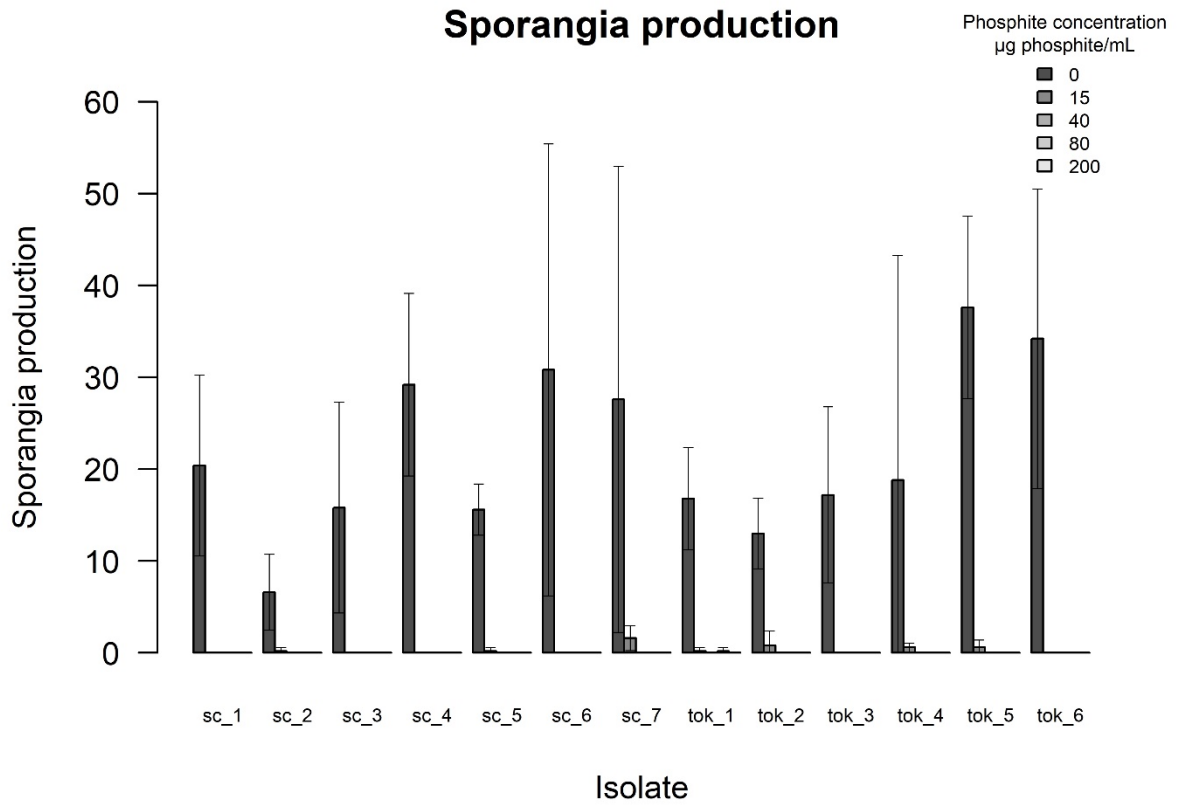

Figure 3: Mean sporangia production (± standard error), of *Phytophthora cinnamomi,* isolates from the Scion (sc) and Tokoroa (tok) arborgen nursery in Jan 2018, grown in non-sterile pond water that had been amended with 0, 15, 40, 80 and 200 µg phosphite/mL.

Discussion

Phosphite treatments were effective at suppressing nearly all mycelial growth in 9 of the 13 isolates tested. Only Tokoroa isolates 5 and 6 appeared to have any tolerance to phosphite exposure and provided a measurable EC50. Mycelial growth in Tokoroa isolates 5 and 6 produced typical dose-response curves, suggesting that some phosphite resistance is occurring. Phosphite treatment effectively suppressed sporangia production across all isolates, although 7 out of 13 isolates produced some sporangia when exposed to phosphite. There was no clear relationship in mycelial growth and sporangia production between isolates with some phosphite tolerance. Sporangia production was highly variable between isolates and phosphite treatments. Sporangia production requires more complex metabolic process compared to direct mycelial growth and is likely to be more sensitive to variations in environmental conditions. Mycelial growth measurements may, therefore, provide a more realistic measure of phosphite sensitivity.

Further work is required to determine if the observed phosphite resistance in *P. cinnamomi* isolates has resulted from phosphite exposure within the nursery or is within the natural variation of *P. cinnamomi*. However, acquired phosphite resistance within *Phytophthora* species has been observed within other plant production systems (Hunter et al. 2018a; Hunter et al. 2018b); and may have serious implications for long term plant production and biosecurity. Wide-scale phosphite application is critical for plant production within many nurseries within New Zealand, including many bare earth forestry nurseries. Increased phosphite resistance within *Phytophthora* species may affect nursery viability and increase productions costs. Many *Phytophthora* species have been shown to

be spread from nurseries where they are suppressed, but not eradicated, through phosphite treatment (Jung et al. 2009). Phosphite is also an important tool to help manage long established *Phytophthora* diseases in horticulture, including the avocado industry (Coffey 1987), and within natural ecosystems, including the management of *Phytophthora* disease in kauri (Horner et al. 2013). *Phytophthora* isolates with phosphite resistance caused within nurseries, may, therefore, cause significant problems when spread into the surrounding environment.

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