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ORIGINAL RESEARCH ARTICLE

Variability in phosphite sensitivity observed within and between seven *Phytophthora* species

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Abstract

Phosphite is used to control and manage many phytophthora diseases in horticultural systems worldwide and natural ecosystems in Australia, Africa, New Zealand and parts of Northern America and Europe. Phosphite does not kill *Phytophthora* species, but inhibits growth while also stimulating host defence responses. *Phytophthora* species differ in their underlying tolerance to phosphite and isolates have been shown to acquire tolerance after prolonged exposure. Intra- and inter-specific variability in phosphite sensitivity is of interest to determine the efficacy and sustainability of phosphite for the treatment of phytophthora diseases, which continue to spread globally. Seven *Phytophthora* species were tested for their sensitivity to phosphite in vitro in a mycelial growth experiment. *Phytophthora agathidicida* was the species most sensitive to phosphite, being inhibited by 98.7% on average at the lowest phosphite treatment (15 µg/mL phosphite), followed by *P. aleatoria*, *P. cinnamomi*, *P. pluvialis*, *P. multivora*, *P. kernoviae* and *P. citricola*. Huge intraspecific variability was observed with *P. kernoviae,* which raises the question of whether diseases caused by *P. kernoviae* such as phytophthora needle blight of *Pinus radiata* could be managed effectively with phosphite. Further work is required to determine the phosphite sensitivity of different introduced and native *Phytophthora* species growing in key hosts and whether tolerance observed in vitro is also expressed in vivo.

Keywords Phytophthora · Phosphite · Interspecific · Intraspecific Variability · Mangement

Introduction

Phytophthora diseases are commonly managed using phosphite in agricultural and natural settings (Hardy et al. 2001). In New Zealand, phosphite is widely used in most commercial nurseries and orchards to manage avocado root rot caused by *Phytophthora cinnamomi*. Phosphite has been proposed as a potential management option for red needle cast of *Pinus radiata* caused by *P. pluvialis* (Dick et al. 2014). It is also being explored for use to control kauri dieback caused by *P. agathidicida* (Bradshaw et al. 2020). Understanding the phosphite sensitivity of *Phytophthora* species is important as phosphite is one of few available chemical treatments to manage new and introduced species, which are spreading due to globalisation and trade (Scott et al. 2019).

Phosphite works directly on *Phytophthora* by inhibiting growth and sporulation, and also stimulates host defence responses (Smillie et al. 1989; Guest et al. 2010). *Phytophthora* species differ in their sensitivity to phosphite (Coffey and Bower 1984) and isolates can acquire tolerance to phosphite after prolonged exposure (Wilkinson et al. 2001; Dobrowolski et al. 2008; Ma and McLeod 2014; Hunter et al. 2018; Hunter 2018). This prompts concerns about the future efficacy of phosphite to control Phytophthora diseases, especially in horticultural systems where phosphite has been used for extended periods of time already. Significant research has been conducted into the molecular, genetic and biochemical mechanisms underlying phosphite control of phytophthora diseases (Eshraghi et al. 2011; Lim et al. 2013; Burra et al. 2014). Understanding how different *Phytophthora* species and isolates acquire phosphite tolerance will help determine the mechanisms of phosphite-induced control of *Phytophthora* pathogens.

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This study assessed inter- and intraspecific variability in the in vitro phosphite sensitivity of seven *Phytophthora* species present in New Zealand.

Materials and methods

Experimental design

Phosphite sensitivity was tested across six concentrations of phosphite (0, 15, 40, 80, 200 and 500 µg/mL) using an optical density assay (Hunter 2018).

Phytophthora isolate sampling and culture

Phytophthora isolates from the New Zealand Forest Research Institute Culture Collection (NZFS) at Scion Research (Table 1) were maintained in water vials at 4 °C on carrot agar. Isolates were subcultured into 90 mm Petri dishes containing 20 mL liquid broth of modified Ribeiro's Minimal Medium (RMM) (Ribeiro et al. 1975), modified as outlined below. The glucose concentration was 9.0 g/L and β-sitosterol was omitted. MES hydrate buffer (2-(N-morpholino) ethanesulfonic acid) was added at a final concentration of 0.03 M and the pH adjusted to 6.2 with KOH 3 M. The inoculum plates were stored at 20 °C in the dark.

Phosphite amendment

Agri-Fos® 600 (Agrichem, Yatala QLD, Australia), a commercial potassium phosphite fungicide containing 600 g/L phosphorous acid present as mono- and di-potassium phosphonate, was used as the phosphite source for this study. The phosphite was filtered using 0.22 µm pore filters (Millex®-GV, Millipore Corporation, Bedford, MA, USA) then added to autoclaved media that had cooled to approximately 50 °C.

Phosphite medium

Growth experiments were conducted in 24-well microtiter plates (Corning, New York, United States), containing 2 mL of RMM amended with phosphite in each well. The wells were randomised for inoculation in each replicate block.

Table 1 *Phytophthora* species, hosts and collection details for isolates used in this study

Phytophthora species	NZFS[a]	Host	Substrate	Crosby region[b]	Collection date
P. agathidicida	3118	*Agathis australis*	Rhizosphere soil	Auckland	11/03/2009
	3813	*A. australis*	Rhizosphere soil	Coromandel	30/01/2014
	3815	*A. australis*	Rhizosphere soil	Coromandel	30/01/2014
P. aleatoria	4037	*Pinus radiata*	Root Collar	Nelson	14/08/2014
	4040	*P. radiata*	Branch	Nelson	14/08/2014
P. cinnamomi	3034	*P. radiata*	Cuttings	Bay of Plenty	26/06/2008
	3750	*P. radiata*	Soil and roots	Nelson	9/01/2013
	3784	*P. radiata*	Soil and roots	Nelson	19/01/2013
P. citricola[c]	4460	*Persea americana*	Soil and roots	Bay of Plenty	15/2/2017
	4461	*P. americana*	Soil and roots	Bay of Plenty	15/2/2017
	4462	*P. americana*	Soil and roots	Bay of Plenty	15/2/2017
P. kernoviae	3610	*P. radiata*	Needle	Auckland	22/06/2011
	3680	*P. radiata*	Needles	Bay of Plenty	17/05/2011
	4053	*P. radiata*	Needles	Bay of Plenty	9/10/2014
	4470[d]	*P. americana*	Soil and roots	Coromandel	27/2/2017
P. multivora	3866	*A. australis*	Soil	N/A	9/05/2014
	3871	*A. australis*	Soil	N/A	9/05/2014
	3913	*A. australis*	Soil	N/A	9/05/2014
P. pluvialis	4019	*P. radiata*	Needles	Gisborne	25/08/2014
	4234	*P. radiata*	Needles	Nelson	12/08/2015

a = New Zealand Forest Service, culture collection reference number

b = Crosby regions are regions of New Zealand with similar biological and climatic characteristics (Crosby et al. 1998)

c = *Phytophthora citricola* isolates were isolated from an avocado orchard (Hunter 2018) which has used phosphite to manage avocado root rot for 32 years via annual injection of all trees with Agrifos 600. Declining trees received an additional injection

d = *Phytophthora kernoviae* isolate 4470 was isolated from an organic avocado orchard which has no recorded use of phosphite

A 2 mm diameter circle was cut from the growing margin of a five-day-old mycelial mat in liquid RMM broth and used to inoculate the wells. A control well was included on each plate, containing the amended broth with no isolates. Optical density measurements at 620 nm (OD620) were taken 13 days after inoculation using the Polar Star Galaxy Microplate Reader (BMG Lab Technologies, Offenburg, Germany). For each well, 32 measurements were taken at consistent locations, and the average OD620 was used as the final value. The plates were stored at 20 °C in the dark.

Data analysis

Data analysis was carried out in R (version 3.6.3). The phosphite response data of the isolates were analysed using a four-parameter log-logistic model with the R package drc (Ritz et al. 2016). The Effective Concentration (EC) to inhibit growth by 50% (EC50) and 90% (EC90) were predicted. The isolates *P. agathidicida* (3118, 3813 and 3815), *P. aleatoria* (4040) and *P. kernoviae* (4470 and 4053), did not fit any of the models in the drc package; however, all isolates were used for the cluster analysis.

A K-means clustering analysis was used to determine the variability in phosphite tolerance within and between the *Phytophthora* isolates (package 'cluster', Maechler (2019)). Using the percentage growth inhibition at 200 and 500 µg/mL phosphite, we specified three clusters, seeking to force a classification into three groups (phosphite sensitive, intermediate and tolerant).

Percentage growth inhibition was calculated for each replicate based on the average growth for the respective control. The average OD value of the control wells (0.084) was subtracted from the growth measurements for all of the replicates.

Results

Phytophthora agathidicida was the most sensitive to phosphite, being inhibited by 98.7% on average at the lowest phosphite treatment of 15 µg/mL phosphite, followed by *P. aleatoria*, *P. cinnamomi*, *P. pluvialis*, *P. multivora*, *P. kernoviae* and *P. citricola*.

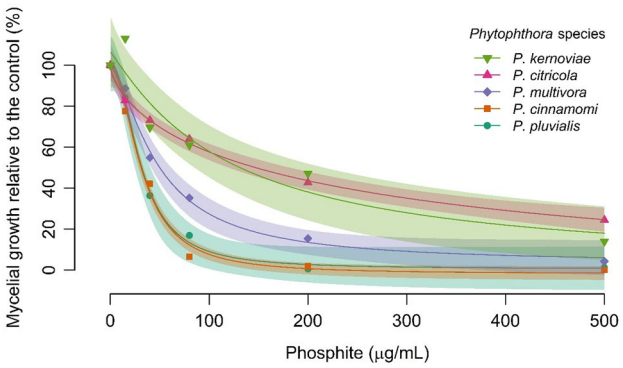

Fig. 1 Response curves showing the average mycelial growth inhibition relative to phosphite concentration of 0 µg/mL for *Phytophthora kernoviae, P. citricola, P. multivora, P. cinnamomi* and *P. pluvialis*, comprising isolates listed in Table 1, at six phosphite concentrations: 0, 15, 40, 80, 200 and 500 µg/mL. Mycelial growth was measured using an optical density assay (Hunter 2018). Bars represent the 95% confidence intervals for a four-parameter log-logistic model, developed using the R package drc (Ritz et al. 2016). Data omitted for *P. agathidicida* and *P. aleatoria* as the model did not converge

Inter-specific variability was observed between *P. kernoviae* and *P. citricola* compared with *P. cinnamomi, P. multivora,* and *P. pluvialis* (Fig. 1). Specifically, isolates of *P. kernoviae* and *P. citricola* were the least sensitive to phosphite, followed by *P. multivora* (Fig. 1). *Phytophthora aleatoria, P. cinnamomi, P. pluvialis* and *P. multivora* had low EC50 and EC90 values compared with *P. citricola* and *P. kernoviae* (Table 2). *P. agathidicida* values would have been even lower, but could not be calculated.

There was no intraspecific variation in the phosphite sensitivity of *P. cinnamomi, P. citricola, and P. pluvialis* based on the isolates used (Fig. 2). There was intra-specific variability in the phosphite tolerance of the *P. kernoviae* and *P. multivora* isolates (Fig. 2). The intra-specific variability was also shown by the K-means clustering analysis (Fig. 3 and Table 3).

Table 2 Mean phosphite EC50 and EC90 values for the five *Phytophthora* species fitted to a four parametric log-logistic response curve (R package drc, Ritz et al. (2016))

	EC50[b] (µg/mL)		EC90[c] (µg/mL)	
***Phytophthora* Species**[a]	**Estimate**	**Standard Error**	**Estimate**	**Standard Error**
P. cinnamomi	32.1	1.85	97.39	9.81
P. pluvialis	33.63	6.20	103.72	39.60
P. multivora	46.68	7.30	204.71	78.56
P. kernoviae	118.04	114.24	864.1	1583.68
P. citricola	146.64	70.83	2118.81	2068.60

[a] *Phytophthora agathidicida* was omitted because the sensitivity was so high it did not converge with the model. One of the *P. aleatoria* did not converge with the model so no average could be calculated at the species level

[b] The effective concentration to inhibit growth by 50%

[c] The effective concentration to inhibit growth by 90%

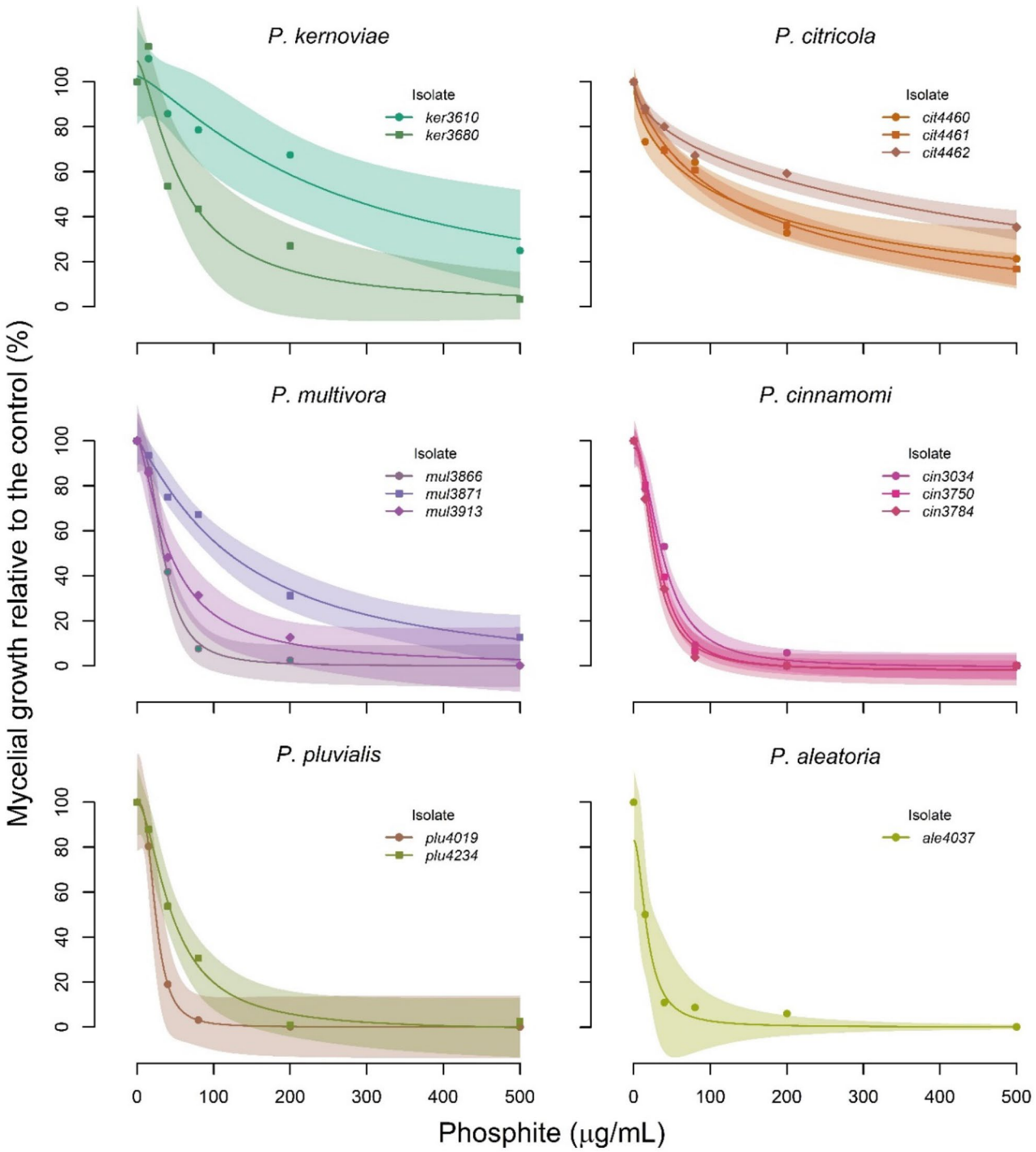

Fig. 2 Response curves showing the average inhibition, or mycelial growth relative to phosphite concentration of 0 µg/mL, for *Phytophthora kernoviae, P. citricola, P. multivora*, *P. cinnamomi, P. pluvialis* and *P. aleatoria*, comprising isolates listed in Table 1, at six phosphite concentrations 0, 15, 40, 80, 200 and 500 µg/mL. Mycelial growth was measured using an optical density assay (Hunter 2018). Bars represent the 5% confidence intervals for a four-parameter log-logistic model (R package drc, Ritz et al. (2016)). *Phytophthora agathidicida* was omitted because it did not converge with the model as it was extremely sensitive

The *P. citricola* isolates with a known history of phosphite exposure (Table 1) grouped as intermediately tolerant in the K-means analysis (Fig. 3). Mycelial growth of isolate 4462 was promoted on some concentrations, resulting in a high EC50 value (Table 3). The *P. agathidicida* isolates were extremely sensitive to phosphite, being inhibited by 98.7% on average by 15 µg/mL and by over 99.8% at the phosphite concentrations of 40–500 µg/mL.

Discussion

Inter- and intraspecific variability was observed in this study. This has implications for the efficacy of phosphite as a method to control phytophthora diseases. Also, we do not pay enough attention to variation in the biology within different species, including un-identified and non-described species. For example, there is good evidence that established populations of some *Phytophthora* species have more variation in pathogenicity than other species (Hüberli et al. 2001; Vernière et al. 2004). Further research is required to understand the variation in susceptibility to phosphite of different isolates within different species.

Land managers need to consider the biology, ecology and historic exposure to phosphite when determining the value of phosphite for managing phytophthora diseases caused by different species and isolates. Our results showed large error bars for the EC50 values of some isolates, which might reflect the natural variation in phosphite susceptibility between species and within isolates. For some isolates, phosphite increased growth. Further work is required to understand this variation in growth behaviour. Screening a set of isolates that reflect the diversity of *Phytophthora* species within New Zealand and the environments in which they occur, both pathogenic and non-pathogenic, will give a broader understanding of phosphite sensitivity and efficacy.

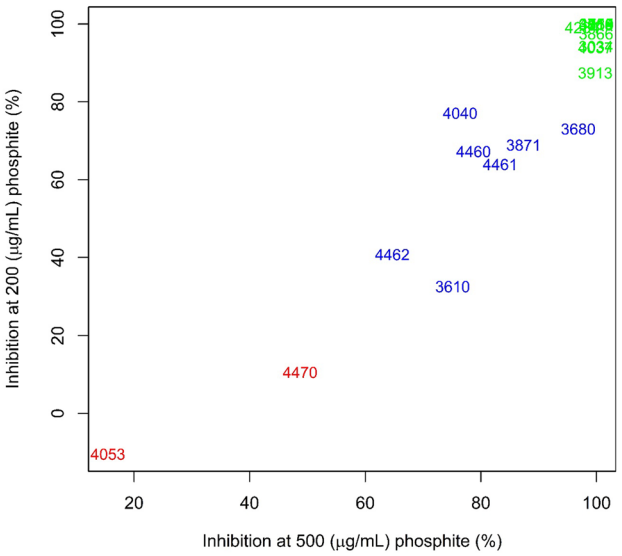

Fig. 3 K-means cluster analysis (R package cluster, Maechler (2019)) of the *Phytophthora* isolates outlined in Table 1, based on percentage growth inhibition at 200 and 500 µg/mL phosphite relative to the control. Groups 1 (red) = tolerant, 2 (blue) = intermediate, and 3 (green) = susceptible

Phytophthora agathidicida was highly sensitive to phosphite, being inhibited almost completely by the lowest phosphite concentration of 15 µg/ml. This validates previous

Table 3 Effective concentration (EC50 and EC90 estimates and standard errors), and Kmeans cluster analysis, of the *Phytophthora* species outlined in Table 1. Effective concentration values fitted to a four-parameter log-logistic model (R package drc, Ritz et al. (2016). NA = no data available and corresponds to isolates where the models did not converge. K-means cluster analysis (R package cluster, Maechler (2019) based on percentage growth inhibition at 200 and 500 µg/mL phosphite relative to the control and represented in Fig. 3

Species	Isolate	EC50 (µg/mL)		EC90 (µg/mL)		Kmeans cluster group
		Estimate	Standard Error	Estimate	Standard Error	
P. agathidicida	3118	NA	NA	NA	NA	Susceptible
P. agathidicida	3813	NA	NA	NA	NA	Susceptible
P. agathidicida	3815	NA	NA	NA	NA	Susceptible
P. aleatoria	4040	NA	NA	NA	NA	Intermediate
P. aleatoria	4037	18.11	6.21	55.43	40.42	Susceptible
P. pluvialis	4019	24.38	5.28	51.80	18.09	Susceptible
P. cinnamomi	3784	27.57	3.13	83.59	16.44	Susceptible
P. cinnamomi	3750	31.94	2.11	88.93	10.00	Susceptible
P. multivora	3866	34.46	4.62	81.46	19.45	Susceptible
P. cinnamomi	3034	38.45	3.86	113.52	22.77	Susceptible
P. multivora	3913	42.35	11.15	198.87	130.12	Susceptible
P. kernoviae	3680	44.11	19.42	129.83	137.80	Intermediate
P. pluvialis	4234	46.69	9.43	168.28	74.63	Susceptible
P. multivora	3871	130.87	52.33	813.86	755.58	Intermediate
P. citricola	4461	162.12	105.58	2328.93	2987.53	Intermediate
P. citricola	4460	179.00	235.36	4389.62	11325.11	Intermediate
P. kernoviae	3610	410.73	697.63	3077.09	8391.38	Intermediate
P. citricola	4462	2269.85	7291.39	126490.20	543634.80	Intermediate
P. kernoviae	4053	NA	NA	NA	NA	Tolerant
P. kernoviae	4470	NA	NA	NA	NA	Tolerant

work in which an EC50 value of 4.0 µg/mL phosphite was predicted for *P. agathidicida*, which was also more sensitive than *P. cinnamomi* and *P. cactorum* (Horner and Hough 2013). Forest trials on kauri trees infected with *P. agathidicida* showed dramatic healing of trunk lesions after trunk injections with 7.5 – 20% phosphite (Horner et al. 2015). These studies showed the potential to use phosphite to manage kauri dieback. Caution should be taken against relying on phosphite as the only means to control phytophthora diseases as there is potential for *Phytophthora* species to acquire resistance to phosphite (Dobrowolski et al. 2008).

In our study, *P. kernoviae* showed the greatest intraspecific variation. In the United Kingdom, *P. kernoviae* causes disease in some forest tree and ornamental species (Brasier et al. 2005) and foliar necrosis in native heathland communities of *Vaccinium myrtillus* (Beales et al. 2009). *Phytophthora kernoviae* is believed to have been present in New Zealand for at least 70 years (Ramsfield et al. 2009) and has been isolated infrequently from *Pinus radiata* needles with red needle cast symptoms (Dick et al. 2014). *Phytophthora kernoviae* is thought to be the causal agent of a previously undiagnosed disorder of pine, called physiological needle blight (McDougal and Ganley 2021).

The zoospores of *P. kernoviae* isolates 3610 and 3680 in the current study were also used to inoculate phosphite-treated *Pinus radiata* needles and test mycelial growth inhibition (Rolando et al. 2017). The phosphite treatments were 15, 30, and 60 g/L phosphite of Agrifos 600 (Roland et al. 2017). It is unsurprising that mycelial growth (in an in vitro mycelial inhibition assay) was completely inhibited by the high concentrations tested by Rolando et al. (2017), considering they were inhibited in the current study in which the highest concentration tested was equivalent to 0.5 g/L.

The *P. cinnamomi* isolates in the current study were all from *Pinus radiata* plantations and were consistently sensitive to phosphite. A previous study by Coffey and Bower (1984) also found *P. cinnamomi* to be sensitive to phosphite, relative to other *Phytophthora* species. This is interesting when we consider how several studies have found isolates can gain tolerance to phosphite after prolonged exposure (Wilkinson et al. 2001; Dobrowolski et al. 2008; Ma and McLeod 2014; Hunter et al. 2018; Hunter 2018), emphasising the potential scale of acquired resistance in *P. cinnamomi* from horticultural settings. Further work is required to determine how phosphite resistance persists within an ecosystem after phosphite application has stopped.

Conclusions

Phosphite is a useful management tool for controlling phytophthora diseases in natural and horticultural settings. All of the *Phytophthora* species in this study were inhibited by phosphite, but at different rates. The inter-specific variability of phosphite tolerance may mean that lower phosphite concentrations can be applied to more sensitive species, such as kauri to control *P. agathidicida*. The intra-specific variability shown in *P. kernoviae* and *P. aleatoria* suggests the need for screening isolates in vitro and *in planta* before applying phosphite to a new pathosystem without prior efficacy testing.

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Declarations

Conflicts of interest The authors declare there are no conflicts of interest.

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