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Selection for decreased sensitivity to phosphite in *Phytophthora cinnamomi* with prolonged use of fungicide

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To test the hypothesis that resistance in *Phytophthora cinnamomi* to control by the fungicide phosphite (phosphonate) would arise in sites with prolonged use of phosphite, 30 *P. cinnamomi* isolates were collected from a range of sites with different phosphite-use histories, including phosphite-treated and untreated avocado orchards, and phosphite-treated and untreated native vegetation sites. The colonizing ability of these isolates was tested by different inoculation methods against a range of host tissues, treated and untreated with phosphite, including mycelial stem inoculation on clonally propagated *Leucadendron* sp., mycelial root inoculation of lupin seedlings and zoospore inoculation of *Eucalyptus sieberi* cotyledons. Isolates from avocado orchards with a long history of phosphite use were, on average, more extensive colonizers of the phosphite-treated *Leucadendron* sp., lupin seedling roots and *Eucalyptus sieberi* cotyledons. These isolates did not colonize untreated plant tissue (*Leucadendron* sp.) more extensively than isolates from sites with no history of phosphite use and no isolates were resistant to control by phosphite. Analysis of all isolates with microsatellite markers revealed the majority were from a single clonal lineage. Selection for decreased sensitivity to phosphite *in planta* has taken place within asexual clonal lineages of *P. cinnamomi* in sites with prolonged use of phosphite.

Keywords: avocados, fungicide resistance, oomycetes, phosphonate, selection

Introduction

Phytophthora cinnamomi is a soil- and water-borne plant pathogen of woody plants. It has a worldwide distribution and an extremely wide host range, with susceptible species from many plant families and genera. *Phytophthora cinnamomi* invades the roots or collars of its hosts, causing disease symptoms such as necrosis of roots, cankers, and necrotic lesions in the trunk and stem, often leading to shoot-dieback or crown-death (Zentmyer, 1980; Shearer & Tippett, 1989). It is a pathogen of both horticultural crops such as avocados, chestnuts and pineapples, and of forests and native plants (Zentmyer, 1980). In Western Australia alone, 2285 plant species of the 5710 described in the South-West Botanical Province are susceptible to *P. cinnamomi* impacts (Shearer *et al*., 2004). In this and other regions of the world, it is a serious pathogen affecting ecosystem structure and diversity.

Chemical control of the disease caused by *P. cinnamomi* is possible with phosphite, a neutralized solution of the phosphonate anion (Roos *et al*., 1999; Hardy *et al*., 2001). Phosphite is applied to the plant as an aqueous solution with a wetting agent or injected into the trunks of trees and is taken up by the plant via the phosphate pathways (Guest & Grant, 1991). Phosphite is not metabolized and so remains in the plant tissue for a considerable time, months to years depending on the plant species and how quickly it is growing or losing leaves (Guest & Grant, 1991; Hardy *et al*., 2001). Invading *P. cinnamomi* mycelium and other pythiaceous pathogens may be controlled by the direct fungistatic action of phosphite. However, given that phosphite concentrations found in plant tissues are often well below concentrations found to be fungistatic *in vitro*, an indirect mechanism of action must also be invoked (Guest & Grant, 1991). With the indirect actions, phosphite is hypothesized to cause the pathogen to produce elicitors or inhibit its production of suppressors, allowing plant defence responses to halt invasion by the pathogen (Guest & Grant, 1991). Supporting evidence for an indirect action has been demonstrated in histopathological studies in *Eucalyptus marginata* (Jackson *et al*., 2000) and *Xanthorrhea australis* (Daniel *et al*., 2005).

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The probability that high levels of resistance to phosphite will emerge in pathogens is reduced by the multi-site action of phosphite (Guest & Grant, 1991). Numerous cases of fungicide resistance have arisen in systemic fungicides, whose mechanism of action targets specific fungal metabolism, whereas there are few reports of resistance to protectant fungicides, which are characterized by non-specific mechanisms of action (Peever & Milgroom, 1995). Phosphite, although systemic, has similarities to the protectant fungicides, with its hypothesized indirect mechanisms of action rather than a mechanism targeting specific oomycete metabolism. However, there are some reports of insensitivity to this fungicide developing in oomycete pathogens. Californian isolates of *Bremia lactucae*, the lettuce downy mildew pathogen, have become insensitive to fosetyl aluminium, a chemical whose active ingredient is phosphite (Brown *et al*., 2004). Isolates of *P. cinnamomi* obtained from avocado orchards treated with fosetyl aluminium and phosphite were less inhibited by phosphite *in vitro* than control isolates (Duvenhage, 1994). *In vitro* insensitivity to phosphite has also been observed in isolates of *P. citrophthora* and *P. cinnamomi*, but this was within the range observed in natural populations of isolates and *in vivo* control of these isolates with phosphite was possible (Guest & Grant, 1991). Indeed, the *in vitro* sensitivity may not correlate with the *in vivo* sensitivity of the pathogen in phosphite treated hosts (Guest & Grant, 1991; Wilkinson *et al*., 2001c).

Despite the advances in understanding the mechanisms of fungicide resistance and their underlying genetics in some pathogens, there are few experimental studies that have examined the factors controlling the evolution of resistance (Peever & Milgroom, 1995). In planning experiments to evaluate these factors, an appreciation of the population biology of the pathogen is needed both to ensure an adequate sampling strategy and to examine the effect of recombination on resistance evolution (Peever & Milgroom, 1995). *Phytophthora cinnamomi* reproduces asexually, with three clonal lineages known in Australia and limited clonal lineages worldwide (Dobrowolski *et al*., 2003).

The objective of this study was to test the hypothesis that *P. cinnamomi* would develop resistance to chemical control by phosphite in areas of prolonged phosphite use such as horticultural farms. Isolates of *P. cinnamomi* were collected from a range of sites with different histories of phosphite use, and their ability to colonize a number of plant host tissues, phosphite-treated and untreated, under controlled experimental conditions, was assessed. These isolates were assayed at microsatellite loci to determine their clonal lineage in order to assess the affect this may have on the observed pathology.

Materials and methods

Experimental plan

In order to test the broad effectiveness of phosphite in controlling *P. cinnamomi* isolates *in vivo*, different host species, plant tissues and inoculation methods were used in three distinct experiments. The *Leucadendron* experiment (performed in 2001 with 28 isolates and repeated in 2002 with 30 isolates) involved stem inoculation with *P. cinnamomi* mycelium. The lupin root experiment (performed in 2002 with 30 isolates) involved root inoculation of *Lupinus angustifolius* seedlings with *P. cinnamomi* mycelium, and the cotyledon experiments (performed four times in 2001 with 28 isolates) involved zoospore inoculation of detached *Eucalyptus sieberi* cotyledons.

Isolate sampling and culture

In 2000, *P. cinnamomi* isolates from four avocado orchards with a history of phosphite use were sampled in the southwest of Western Australia (Table 1). In addition, phosphite-treated native vegetation sites on the south coast were sampled, in which plants were still succumbing to *P. cinnamomi*. Other isolates that had no history of exposure to phosphite were obtained from a culture collection of *P. cinnamomi* isolates sampled between 1992 and 1997. These originated from a range of locations in southwest Western Australia (Table 1). All these isolates made up the group of 28 used in the first *Leucadendron* experiment and the cotyledon experiments. An avocado orchard that had never been treated with phosphite was sampled in 2001, contributing the additional two isolates for the second *Leucadendron* experiment and the lupin root experiment.

Phytophthora cinnamomi was isolated from root (avocado orchards) and collar (native vegetation) samples by rinsing the material in sterile water then placing it on NARPH (nystatin, ampicillin, rifampicin, pentachloronitrobenzene, hymexazol) medium (Hüberli *et al*., 2000) which is selective for *Phytophthora* spp. Isolates of *P. cinnamomi* were subcultured within 2 to 4 days. All isolates were microscopically checked for *P. cinnamomi* morphological characters, including coralloid hyphae and sporangial characteristics induced by the method described by O'Gara *et al*. (1997). Cultures were maintained on corn meal agar at 16°C. All isolates were passaged through host plant material and re-isolated prior to their use in experiments to ensure pathogenicity was not lost during culture storage.

Leucadendron experimental trials

A range of host plants were investigated for use as a model host in order to satisfy the following three criteria: (i) the plant could be propagated clonally thereby reducing the variation due to different plant genotypes; (ii) sufficient clonal material could be obtained for large experiments and could be guaranteed free of previous phosphite exposure; and (iii) the plant should be susceptible to disease caused by *P. cinnamomi* but show some degree of resistance, allowing discrimination between limited and extensively colonizing isolates. From preliminary investigations of approximately 20 host species, in which

Table 1 Sites from which *Phytophthora cinnamomi* isolates of indicated clonal lineage were obtained

Site, description and location	Phosphite exposure history	Isolate name	Clonal lineage[a]
(a) Buller Reserve	Never exposed	MPD 084	A2 type1
(*Banksia* woodland)		MPD 085	A2 type1
32·87°S 115·82°E		MPD 086	A2 type1
(b) Moore River	Never exposed	MPD 087	A2 type1
(*Banksia* woodland)		MPD 089	A2 type1
31·09°S 115·76°E			
(c) Alcoa mine sites	Never exposed	MP 97	A2 type1
(Jarrah forest)		MP 62	A2 type1
32·60°S 116·09°E &		MP 94-03	A2 type1
32·87°S 116·06°E		MP 94-48	A2 type1
		MP 125	A2 type2
(d) Avocado orchard	Never exposed	JD 2	A2 type1
32·14°S 116·04°E		JD 9	A2 type1
(e) South coast	Two years, aerial spray (fine mist) 1996, 1999	MPD 055	A2 type2
(heath land)		MPD 062	A1 type1
35·00°S 117·99°E		MPD 063	A2 type1
		MPD 065	A1 type1
(f) Avocado orchard	Three years, two injections per year (injections in diseased trees	MPD 001	A2 type1
31·60°S 115·73°E	for 5 years prior)	MPD 003	A2 type1
		MPD 007	A2 type1
		MPD 010	A2 type1
(g) Avocado orchard	Five years, one injection per year and spray in final year	MPD 025	A2 type1
33·83°S 115·88°E			
(h) Avocado orchard	Five years, two injections per year (two isolates from this orchard)	MPD 030	A2 type1
34·48°S 115·94°E	or four sprays per year (two isolates from this orchard)	MPD 039	A2 type1
		MPD 045	A2 type2
		MPD 046	A2 type1
(i) Avocado orchard	Eight years, three injections per year	MPD 016	A2 type1
34·39°S 115·95°E		MPD 019	A2 type1
		MPD 022	A2 type1
		MPD 050	A2 type1
		MPD 052	A2 type1

[a]The clonal lineage assigned to each isolate is based on comparisons of microsatellite profiles compared to standard isolates from which the original clonal lineage naming was defined (Dobrowolski *et al.*, 2003).

host material was inoculated using the wrap method (Lucas *et al.*, 2002), *Leucadendron* 'Safari Sunset', a commercially grown cut-flower variety in the family Proteaceae, was selected.

Cuttings of *Leucadendron* 'Safari Sunset' from a flower orchard which had never been treated with phosphite were made in late autumn and propagated in peat/perlite potting mix with base heat and overhead misting in a greenhouse. The rooting hormone powder (Clonex (indole-3-butryic acid), Yates) used in propagation did not contain phosphite; some rooting hormone gels do. Plants were re-potted in potting mix in 50-mm square by 120-mm deep polyethylene forestry tubes after root development (about 2 to 4 months after cutting) then grown in a greenhouse. To ensure healthy vigorous plants for inoculation, regular feeding with a fish emulsion fertilizer and treatments every 14 days with a prophylactic fungicide were carried out. Manufacturer recommended applications of the fungicides pyrimethanil and iprodione (Scala and Rovral, respectively, Bayer CropScience Australia), neither of which contain phosphite or anti-pythiaceous chemicals, were used alternately for the control of *Botrytis* spp. Almost all plants had a single major stem for inoculation, rather than multiple stems. Plants were 8-months-old for the first experiment and 10-months-old for the second experiment.

Plants were inoculated with the isolates of *P. cinnamomi* using a non-invasive technique (Lucas *et al.*, 2002). An agar plug of newly grown mycelium (less than one-week-old on V8 agar) was placed on the stem of the plant, with the mycelium facing the stem, covered with sterilized wet absorbent cotton wool, and wrapped to the stem with Parafilm M (American National Can). In the second experiment the inoculum was placed on new growth in preference to the original wood of the cutting material, as inoculation was less successful on the original wood in the first experiment regardless of the isolate used. Plants were inoculated in random order. Control plants were identical to those used for normal inoculations except for absence of the pathogen on the agar plug. Inoculated plants were kept in the laboratory for 1 day, since in previous experiments placing plants directly into the growth cabinet

caused the inoculum plugs to dry before colonization could occur.

Approximately 24 h post inoculation, plants were sprayed with one of 0 (no phosphite), 2·5, or 5 g L^{-1} phosphite solutions to run-off. The solutions were appropriate dilutions of Foli-R-Fos 400 (400 g L^{-1} phosphorous acid present as mono-di potassium phosphite; Unitec Group Pty Ltd) and 0·25% (vol/vol) of the adjuvant Synertrol Oil (Organic Crop Protectants Pty Ltd), a wetting agent used to facilitate the uptake of phosphite. Plants were returned to the laboratory for one more day. On day 2 plants where placed in a growth cabinet set at 24°C with 14 h daylight, lit with metal arc lighting.

In the first experiment a total of 476 plants were inoculated with 28 isolates, five plants for each 0 g L^{-1} phosphite by isolate combination, and 6 plants for each 2·5 and 5 g L^{-1} phosphite by isolate combination. In the second experiment a total of 990 plants were inoculated with 30 isolates, 11 plants for each 0, 2·5, and 5 g L^{-1} phosphite by isolate combination. In both experiments the 0 g L^{-1} phosphite treatment was harvested before the other treatments (day 4 in the first experiment and day 43 in the second experiment), as wilting symptoms, indicative of colonization, were observed in them but not the other treatments. Total colonization of the stem by *P. cinnamomi* in all plants of all treatments would not allow the possibility of measuring the differential colonization ability of the isolates. The 2·5 and 5 g L^{-1} treatments in the first experiment were harvested on day 8 and in the second experiment were harvested in two batches, with three plants of each phosphite by isolate combination on day 69 and the remaining eight plants on day 139.

At harvest, leaves were removed from plants and the stems surface sterilized in 70% ethanol, then blotted dry. In the first experiment stems were cut into 0·5-cm sections, up to 10 above, and up to 10 below the inoculation point, depending on the length of the stem. In the second experiment up to 20 sections were cut above, and up to 20 sections were cut below the inoculation point: 10 0·5-cm sections closest to the inoculation point and 10 1-cm sections farthest from the inoculation point. Sections were placed onto NARPH medium in 9-cm Petri dishes and incubated at 26°C for up to 7 days. Each section was scored for *P. cinnamomi* colonization. Individual plant height at inoculation and harvest was recorded to ensure plant growth during the experiment did not have any unforseen effect on colonization by *P. cinnamomi*.

Lupin root inoculation experiment

Lupin seeds (*Lupinus angustifolius* 'Merrit') were germinated in washed river sand held in improvised containers. The containers were made of two polypropylene take-away container lids taped together on the sides and base, with one short edge cut off to form a thin pocket of sand, 12 cm wide, 16 cm high and approximately 1 cm thick. By laying the containers flat and cutting the tape on the sides, the containers could be opened to access the roots of the lupin seedlings for inoculation with minimal disturbance. The 4-day-old lupin seedlings were inoculated with an agar plug of newly grown mycelium by placing the mycelium in contact with the root tip. Ten lupins were inoculated with each of the 30 isolates. The containers were then taped back together. Lupins were sprayed to run-off with 5 g L^{-1} phosphite solution then placed into a growth cabinet set at 24°C and 12 h light/12 h dark. Lupins were harvested 10 days after inoculation – preliminary experiments showed that length of root tissue that different isolates colonized could best be differentiated on this day. Roots were surface sterilized by dipping them in 70% ethanol and blotting dry. Twelve 1-cm sections starting at the inoculation point were cut and placed onto NARPH medium, to determine if *P. cinnamomi* had colonized the root section.

Cotyledon zoospore colonization experiments

Eucalyptus sieberi seeds were germinated in vermiculite in the glasshouse and grown for 3 weeks to cotyledon stage. Different batches of seedlings were sprayed to run-off with 0, 5, and 10 g L^{-1} phosphite solutions. Seedlings were not watered for 48 h to allow phosphite uptake. Residual phosphite was then rinsed off immediately before cotyledons were cut for use in the experiment.

Zoospores were produced in culture as described previously (O'Gara *et al.*, 1997) from 28 isolates and examined microscopically to ensure zoospores had released into the soil filtrate. Three to four millilitres of zoospore suspension from one isolate was added to each of nine Petri dishes containing 10 cotyledons, with three 9-cm Petri dishes for each phosphite level treatment (cotyledons sprayed with 0, 5, and 10 g L^{-1} phosphite solutions). This was the only step in the entire procedure that was not randomized: speed was necessary here to ensure that the zoospores did not encyst prior to their use. The Petri dishes were incubated at 26°C for approximately 20 h to allow zoospores to colonize the cotyledons. Cotyledons from each Petri dish were then surface sterilized by brief immersion in 1% sodium hypochlorite, rinsed in deionised water, pressed dry with paper towels and placed onto 9-cm Petri dishes of NARPH medium (Hüberli *et al.*, 2000). These were incubated at 26°C for up to 7 days. Inoculated cotyledons were examined daily for characteristic colour changes from healthy purple underside to dull green which are typical symptoms of *P. cinnamomi* colonization. The proportion of the 10 cotyledons in each Petri dish colonized by *P. cinnamomi* was recorded, which sometimes required microscopic examination of the cotyledon margins to observe sporangia or other evidence of *P. cinnamomi*. Colonized cotyledons were cut from the Petri dishes as they were identified so that the mycelium did not overgrow other cotyledons not yet showing signs of colonization. Negative controls of cotyledons (incubated with deionised water instead of zoospore suspension) showed no symptoms of colonization by *P. cinnamomi*. This experiment was repeated four times within 2 months.

Table 2 Average colonization of phosphite treated and untreated *Leucadendron* stems (mm), lupin roots (mm), and *Eucalyptus sieberi* cotyledons (counts) by isolates of *Phytophthora cinnamomi* from each site

			Leucadendron experiments					*Eucalyptus sieberi* cotyledon experiments (5 and 10 g L^{-1} treatments combined)			
Sites		Site	1st: 0 g L^{-1} phosphite	1st: 2·5 and 5 g L^{-1} phosphite*	2nd: 0 g L^{-1} phosphite	2nd: 2·5 and 5 g L^{-1} phosphite*	Lupin root experiment*	1st*	2nd	3rd*	4th*
Sites with no history of phosphite use		a	55·0	23·9	17·2	6·0	9·3	6·3	9·2	9·8	9·3
		b	25·5	9·6	12·1	7·1	7·5	5·2	9·7	9·5	6·7
		c	45·9	22·0	19·9	5·9	15·4	8·2	9·7	9·1	9·0
		d			24·3	7·0	8·5				
Sites with a history of phosphite	Native vegetation	e	40·2	33·6[a]	24·0	6·3[a]	16·8[a]	7·0[a]	9·4	9·4	9·7[ac]
	Avocado orchards	f	74·1	24·1[a]	24·6	11·4[ab]	12·2[a]	6·0[a]	9·8	9·2	9·8[a]
		g	59·0	30·4[a]	16·0	8·3[ab]	32·0[a]	7·8[a]	9·7	9·8	8·7[a]
		h	64·5	53·6[a]	36·2	9·7[ab]	13·5[a]	8·6[a]	9·7	9·5	9·1[a]
		i	55·0	30·7[a]	25·4	10·1[ab]	16·6[a]	8·2[a]	9·8	9·9	9·2[a]

*These experiments showed significant ($P < 0·05$) differences among sites by ANOVA, allowing planned comparisons among contrast groups.
[a]Sites with a history of phosphite use showed greater colonization than sites without a history of phosphite use within the experiments indicated ($P < 0·05$).
[b]Avocado orchard sites with a history of phosphite use showed greater colonization than natural vegetation sites with a history of phosphite use within this experiment ($P < 0·05$).
[c]Natural vegetation sites with a history of phosphite use showed greater colonization than all sites without a history of phosphite use within this experiment ($P < 0·05$).

Experimental design and statistical analyses

All experiments were designed as single completely randomized blocks with fully factorial treatments of site, phosphite (*Leucadendron* and cotyledon experiments) and harvest day (*Leucadendron* experiments), except for 0 g L^{-1} phosphite treatments in the *Leucadendron* experiments, which were confounded with harvest day. Thus, these 0 g L^{-1} phosphite treatments were analysed separately. Of most interest was the effect that different phosphite histories had on the colonizing ability of *P. cinnamomi* isolates, that is the effect of sites (and thus the effect of previous phosphite use in sites), rather than differences among isolates within a site. Therefore, the average colonization score for a site was analysed, where each isolate was a replicate within a site (by phosphite by harvest day, as appropriate for the different experiments) treatment using an unbalanced analysis of variance (ANOVA) with Type III sums of squares. Planned comparisons (univariate tests of significance) were made between the following contrast groups: all sites with- and all sites without- a history of phosphite use; natural vegetation sites with- and all sites without- a history of phosphite use; and avocado orchard sites with- and natural vegetation sites with- a history of phosphite use. Homoscedacity of variances was tested with Bartlett's test. Data from the second *Leucadendron* experiment were $\log_{10}(x + 1)$ transformed and data from the cotyledon experiment were arcsin(x) transformed, to make residuals homoscedastic and approximately normal.

Genetic analysis

All isolates were analysed at four microsatellite loci, and by comparison to isolates A15, A2421 and A2412, the three clonal lineages known to occur in Australia, the clonal lineage to which they belonged was determined, as described previously (Dobrowolski *et al.*, 2002, 2003).

Results

In both *Leucadendron* experiments, phosphite was effective in controlling colonization by *P. cinnamomi* as seen by the markedly less colonization by *P. cinnamomi* isolates in treated compared to untreated plants (Table 2). There was no significant difference between 2·5 and 5 g L^{-1} phosphite treatments in terms of the colonizing ability of isolates on average between sites, so these treatments were grouped together (Fig. 1b, Table 2). The differences between treated and untreated plants were statistically significant ($P < 0·01$) even though the 0 g L^{-1} phosphite treatments were harvested 4 or 26 days before the 2·5 and 5 g L^{-1} phosphite treatments, in the first and second experimental trials, respectively. The greater degree of colonization seen in the first experiment in all phosphite treatments (Table 2) can be explained by the age of the plant material inoculated. The plants inoculated in the second experiment were 2 months older than those of the first inoculation experiment hence the plant tissue was more lignified and more resistant to colonization by *P. cinnamomi*. This possible explanation is supported by

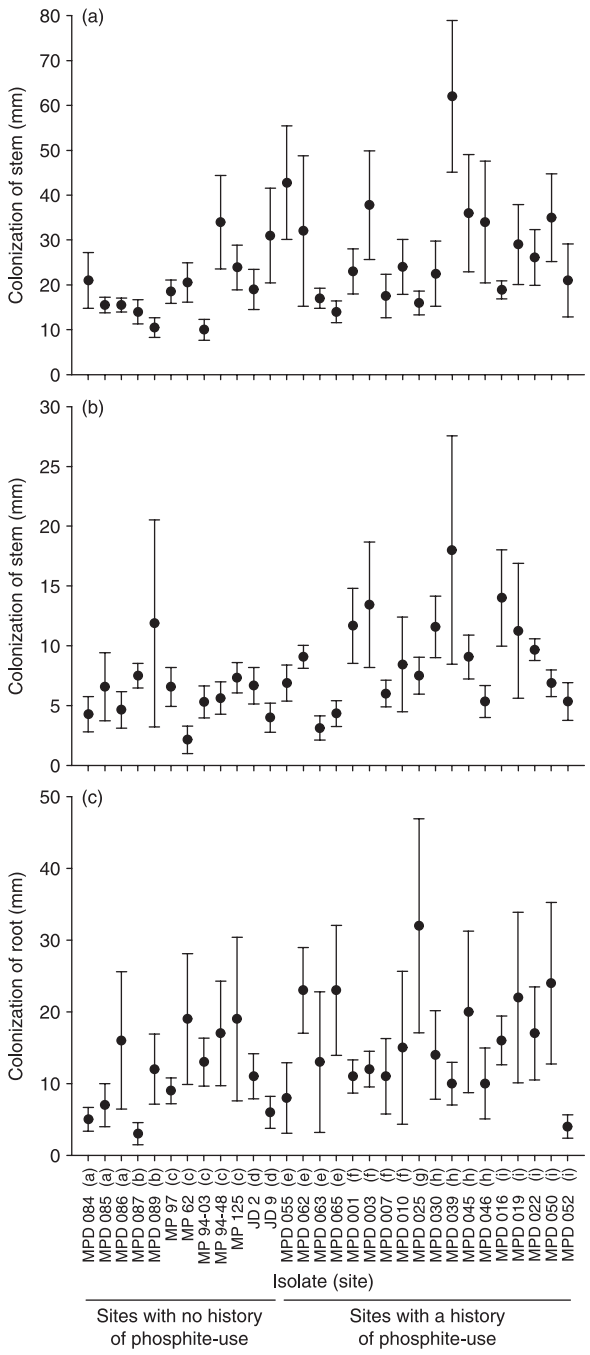

Figure 1 Colonization by *Phytophthora cinnamomi* isolates inoculated on (a) the stems of 0 g L^{-1} phosphite-treated *Leucadendron* 'Safari Sunset' plants of the second experiment, (b) the stems of 2·5 and 5 g L^{-1} phosphite-treated *Leucadendron* 'Safari Sunset' plants of the second experiment (harvest day 139 only), and (c) the roots of lupin seedlings treated with 5 g L^{-1} phosphite. Details of sites, indicated on the x-axis by letters (a) to (i), and isolates are given in Table 1. Bars indicate standard error of the mean.

the wilting symptoms seen in the first but not the second experiment.

Phytophthora cinnamomi isolates from different sites differed significantly in their ability to colonize the 2·5 and 5 g L^{-1} treatments of both *Leucadendron* experiment trials (F statistic significant at $P = 0{\cdot}01$ and $P = 0{\cdot}02$ in the first and second experiments, respectively, Table 2). This was because sites with a history of phosphite use had isolates that were on average able to colonize a greater length of stem tissue of these phosphite-treated plants than sites without such a history (F significant at $P = 0{\cdot}008$ and $P = 0{\cdot}01$ in the first and second experiments, respectively). In addition, the phosphite-treated avocado orchards had isolates that on average were better able to colonize treated plants compared to isolates from phosphite-treated native vegetation sites (F significant at $P = 0{\cdot}02$ in the second experiment). These orchards have a longer history of phosphite use characterized by multiple treatments per year for more than 2 years, using higher dosages than the fine mist sprays employed in native vegetation (Table 1). However, there were no such differences found between sites for untreated *Leucadendron* stems, the 0 g L^{-1} phosphite treatment ($P = 0{\cdot}09$ and $P = 0{\cdot}1$ in the first and second experiments, respectively). Individual isolates varied markedly in their ability to colonize both phosphite-treated and untreated *Leucadendron* stems, with isolates showing limited and extensive colonization found in most sites expressed on both phosphite-treated and untreated hosts (Fig. 1a,b). However, on plants that were phosphite-treated, sites where phosphite had been used had more isolates that showed extensive colonization than sites where phosphite had not been used (Fig. 1b). For example, 8 of 18 isolates from sites with a history of phosphite use colonized on average greater than 10 mm of phosphite-treated stem whereas only 2 of 10 isolates achieved this from sites with no such history (Fig. 1b).

In order to confirm that these results were not restricted to the *Leucadendron–P. cinnamomi* host–pathogen interaction, the range of hosts, plant tissues and inoculation methods that could test the colonizing ability of this set of *P. cinnamomi* isolates was extended. This included root inoculation of phosphite-treated lupin seedlings and zoospore inoculation of *Eucalyptus sieberi* cotyledons.

This same trend was evident in the lupin root inoculation experiment, with differences among sites being significant ($P = 0{\cdot}05$) and sites with a history of phosphite use having isolates that were on average able to colonize a greater length of root tissue than sites without such a history (F significant at $P = 0{\cdot}003$, Table 2). Individual isolates again varied in their colonizing ability but the more extensively colonizing isolates came from sites with a history of phosphite use (Fig. 1c).

A less consistent result was seen in the colonization of *E. sieberi* cotyledons by *P. cinnamomi* zoospores (Table 2). In experiments 1 and 4 the same trend was seen, with differences among sites being significant ($P = 0{\cdot}001$ and $P = 0{\cdot}02$ in experiments 1 and 4, respectively) and sites with a history of phosphite use having more extensively colonizing isolates than sites without (F significant at $P = 0{\cdot}04$ and $P = 0{\cdot}02$ in experiments 1 and 4, respectively). In contrast, there were no significant differences between isolates from different sites in experiment 2, and in experiment 3 those significant differences ($P = 0{\cdot}03$) were

not reflected in the sites with and without a history of phosphite use. However, in these experiments most cotyledons from all phosphite treatments were colonized by *P. cinnamomi* (Table 2), so it appears that conditions heavily favoured the pathogen, hence differences among isolates from different sites could not be distinguished. In all cotyledon experiments almost all the 0 g L^{-1} phosphite treated cotyledons were colonized by each *P. cinnamomi* isolate and so this treatment was excluded from the ANOVA. This also indicated conditions that heavily favoured the pathogen.

Although the number of colonized cotyledons did not differ significantly between the 5 and 10 g L^{-1} phosphite treatments in any of the experiment runs, it was observed that individual cotyledons treated with 10 g L^{-1} phosphite had more healthy uncolonized tissue remaining than individual cotyledons treated with 5 g L^{-1} phosphite. This was possible to observe because the characteristic purple underside of the *E. sieberi* cotyledons turns pale green and becomes flaccid when colonized by *P. cinnamomi*. These observations may also explain why the differential ability to colonize plant tissue between populations of *P. cinnamomi* was not observed in all cotyledon experiments. Zoospore encystment and penetration to even a small degree, which is what the test essentially measured, could occur in most cotyledons regardless of the phosphite treatment, given the conditions heavily favoured the pathogen.

In the initial sampling of *P. cinnamomi* isolates from areas of prolonged phosphite use, the majority came from avocado orchards, whereas sites with no phosphite-use history were mainly native vegetation. Hence the factor of prolonged phosphite use was statistically confounded with avocado orchard derived isolates. This problem was addressed by the sampling of isolates from a long running avocado orchard with no history of phosphite use and inclusion of these in the second *Leucadendron* experiment and the lupin root experiment. The pathology of these isolates fit the pattern of being limited colonizers compared to isolates derived from sites with a history of phosphite use (Table 2). This further supports the observation that more extensively colonizing isolates are derived from such sites rather than the common factor being derivation from avocado orchards.

All but two isolates were identified as being members of the A2 type 1 clonal lineage, the most commonly found lineage in southwest WA, based on their microsatellite banding patterns compared to standard isolates used in the initial definition of the clonal lineage hypothesis in *P. cinnamomi* (Table 1). None of the 30 isolates' alleles differed in base pair size at more than two loci compared to the standard isolate of the respective clonal lineage. All allele size differences, compared to the standard isolate of the respective clonal lineage, could be explained by microsatellite length mutation of one or two repeat lengths of the microsatellite motif. There was no evidence to suggest any of the 30 isolates were the result of sexual recombination. Significantly, isolates of the A2 type 1 clonal lineage used in this study were found both in sites exposed and never exposed to phosphite. There was no evidence to suggest that isolates of different clonal lineage were predominant among the limited or extensively colonizing populations of *P. cinnamomi* (Table 1; Fig. 1).

Discussion

The results indicate that selection for isolates of *P. cinnamomi* less sensitive to phosphite *in planta* has occurred in sites where phosphite had been used. The population of isolates sampled from sites of prolonged phosphite use were more extensive colonizers of phosphite treated plant tissue, compared to the populations of isolates from sites with no history of phosphite use. This response was elucidated using a range of host plant tissues, including clonal woody stems, seedling roots and cotyledons. The implication of these results is that the prolonged use of phosphite has caused the selection of isolates of *P. cinnamomi* less sensitive to phosphite *in planta*. The sites sampled that had prolonged use of phosphite were all avocado orchards. The prolonged use of phosphite could therefore be confounded with other avocado orchard horticultural practices. This problem was addressed by including isolates from an avocado orchard in which phosphite has never been used and also isolates from native vegetation sites that have experienced much lower use of phosphite. The results indicate a dose response, that is, isolates from phosphite-treated sites were more extensive colonizers than isolates from untreated sites, including the untreated avocado orchard, and isolates from avocado orchards with prolonged phosphite use were more extensive colonizers than isolates from native vegetation sites with few phosphite treatments. This is firm evidence to support the hypothesis that prolonged use of phosphite has caused the selection of *P. cinnamomi* isolates less sensitive to phosphite.

However, the difference in colonizing ability between isolates from sites with and without a history of phosphite use is relatively minor and is only significant when assessed on phosphite-treated plant tissue. All isolates that were tested were controlled by the effect of phosphite. That is, disease symptoms were not sufficient to kill *Leucadendron* plants treated with phosphite, whereas untreated plants would most likely have died as a direct result of disease caused by *P. cinnamomi* had the destructive assessment of colonization occurred at a later date. Indeed, in the first experiment some plants had started to wilt.

Given that glasshouse treated plants show phosphite levels at much greater concentration than in field treated plants (Wilkinson *et al.*, 2001b) there is reason to believe that the phosphite levels in these experiments may be sufficient for the direct fungistatic effect of phosphite to predominate over an indirect effect. However, phosphite concentration was not measured in the stems inoculated, so the question as to which hypothesized mechanism of action was responsible for the observed differences between isolate populations in their sensitivity to phosphite could not be answered. Perhaps a histopathological investigation, such as done in *Xanthorrhea australis*

(Daniel *et al*., 2005), looking at the defence response induction, of a range of these isolates may indicate whether isolates less sensitive to phosphite induce host defences less quickly, which would suggest evolution of resistance involving an indirect effect of phosphite.

Resistance to control by phosphite has occurred in Californian isolates of *B. lactucae* as measured by sporulation on the lettuce host (Brown *et al*., 2004). Because sporulation rather than colonization was the measured character in those studies, the degree of insensitivity to phosphite measured in *B. lactucae* is difficult to compare to these results here with *P. cinnamomi*. *Phytophthora cinnamomi* is known to continue to infect and produce zoospores from, though not kill, host plants that are treated with phosphite, but at a reduced rate (Wilkinson *et al*., 2001a).

The selection for isolates that are less sensitive to phosphite in these sites of prolonged phosphite use has occurred within a clonal lineage of *P. cinnamomi*. Similarly, the development of phosphite insensitivity in Californian isolates of *B. lactucae* was believed to have occurred in asexually reproducing populations (Brown *et al*., 2004). Reproduction exclusively by asexual means may be an evolutionary impediment. When rated on an evolutionary potential scale, as proposed for the potential of pathogens to overcome host genetic resistance (McDonald & Linde, 2002), which also may be applicable to fungicide resistance, *P. cinnamomi* would have a low potential, given its exclusively asexual reproduction, limited number of clonal lineages, and dispersal by water movement in soil except where human or animal assisted. Perhaps this minor decrease in sensitivity to phosphite is indicative of a low evolutionary potential.

The most likely reason for the minor decrease in sensitivity to phosphite seen with its prolonged use is its multi-site mechanism of action. Those fungicides in which resistance has evolved rapidly are almost exclusively systemic, targeting specific metabolism in the pathogen (Peever & Milgroom, 1995) in which mutation at one or a few loci within the pathogen can cause resistance. Examples include metalaxyl resistance in *P. infestans* (Lee *et al*., 1999), ethirimol, triadimenol, and morpholine resistances in *Blumeria graminis* f. sp *hordei* (Brown, 2002), and numerous examples of resistance to chemicals in curcubit powdery mildew (McGrath, 2001). In contrast, phosphite is not known to target specific metabolic pathways in the pathogen. In addition, phosphite does not kill the pathogen and indeed the pathogen's fecundity may not be reduced sufficiently for phosphite use to exert strong selection pressure, given pathologically significant zoospore production is possible on phosphite treated hosts (Wilkinson *et al*., 2001a). Control by phosphite is also mediated through plant response following elicitor detection caused by leaky membranes due to phosphite action on the pathogen. Plants treated with phosphite are not immune to pathogen invasion, at least with *P. cinnamomi* (Tynan *et al*., 2001; Wilkinson *et al*., 2001b), which may also reduce the selection pressure of phosphite use.

Phosphite is currently the only effective control, other than quarantine, that is available for protecting numerous threatened and endangered plant species from disease caused by *P. cinnamomi* (Hardy *et al*., 2001; Shearer *et al*., 2004). These results showing selection for isolates of *P. cinnamomi* less sensitive to phosphite from areas of prolonged phosphite use, although not dramatic, are of concern to the continuing effectiveness of phosphite. The question is whether the observed incremental decreases in sensitivity will eventually lead to resistance to phosphite.

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