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Efficacy of phosphonic acid, metalaxyl-M and copper hydroxide against *Phytophthora ramorum in vitro* and *in planta*

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The ability of metalaxyl-M, phosphonic acid in the form of phosphonate, and copper hydroxide to inhibit different stages in the life cycle of *Phytophthora ramorum*, the causal agent of sudden oak death (SOD), was tested *in vitro* using 12 isolates from the North American forest lineage. In addition, experiments were conducted *in planta* to study the ability of phosphonic acid injections and metalaxyl-M drenches to control pathogen growth on saplings of California coast live oak (*Quercus agrifolia*), and of copper hydroxide foliar sprays to control infection of California bay laurel (*Umbellularia californica*) leaves. *Phytophthora ramorum* was only moderately sensitive to phosphonic acid *in vitro*, but was highly sensitive to copper hydroxide. *In planta* experiments indicated the broad efficacy of phosphonic acid injections and of copper hydroxide sprays in preventing growth of *P. ramorum* in oaks and bay laurels, respectively. Finally, although metalaxyl-M was effective *in vitro*, drenches of potted oak trees using this active ingredient were largely ineffective in reducing the growth rate of the pathogen *in planta*.

Keywords: disease management, phenotypic variability, *Quercus agrifolia*, sudden oak death, *Umbellularia californica*

Introduction

Phytophthora ramorum (Werres *et al*., 2001) is a generalist pathogen capable of infecting over 100 plant hosts both in wildlands and in commercial nurseries (Anon., 2007). Infection of several oak (*Quercus*) species and of the related tanoaks (*Lithocarpus densiflorus*) generally results in large lethal lesions girdling the phloem and occluding the outer xylem of the main bole, and is referred to as sudden oak death (SOD) (Garbelotto *et al*., 2001; Rizzo *et al*., 2002). However, infections on other hosts consist mostly of foliar lesions and twig dieback, not necessarily resulting in death of the host (Davidson *et al*., 2003; Garbelotto *et al*., 2003a). Noticeably, stem cankers of oaks and tanoaks do not appear to be infectious, while, for example, tanoak twigs, rhododendron twigs and leaves, California bay laurel (*Umbellularia californica*), camellia, fuchsia and redwood (*Sequoia sempervirens*) leaves are all reported to support production of sporangia (Tooley *et al*., 2004; Davidson *et al*., 2005). Due to their sporulation potential, California bay laurels, tanoaks and rhododendrons appear to be the most infectious and epidemiologically important hosts in natural plant communities in California (Rizzo & Garbelotto, 2003; Davidson *et al*., 2005), Oregon (Goheen *et al*., 2002) and the United Kingdom (Brasier *et al*., 2004a,b), respectively.

Phytophthora diseases are often managed through chemical treatments, with successful outcomes reported not only from commercial nursery settings or strictly agricultural operations (Cohen & Coffey, 1986; Guest *et al*., 1995; Stein & Kirk, 2002), but also from natural ecosystems (Aberton *et al*., 1999; Fernandez-Escobar *et al*., 1999). In California, treatments employing the phosphonate Agrifos400® are currently registered for the most susceptible hosts of *P. ramorum*, namely a few oak species and tanoak. The efficacy of phosphonates has been shown in controlled experiments on at least two oak species (*Q. agrifolia* and *Q. parvula* var. *shrevii*) and tanoak (Garbelotto *et al*., 2002, 2003b, 2007). No treatment has yet been tested or approved for other epidemiologically important hosts of *P. ramorum* such as bay laurels. To date, most studies have focused on treatment of the disease in nursery situations rather than in forests (Jeffers, 2003; Chastagner *et al*., 2004; Heungens *et al*., 2005; Tjosvold & Chambers, 2005).

Although not all *Phytophthora* species are equally sensitive to all active ingredients (Coffey & Bower, 1984; Cohen & Coffey, 1986; Stein & Kirk, 2002), there is only limited information regarding the relatively recently described *P. ramorum*. Furthermore, nothing is known

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about the differential effect of specific fungicides on the various stages of its life cycle, although this has been shown in other *Phytophthora* spp. (Coffey & Joseph, 1985; Wilkinson *et al*., 2001a; 2001b). *Phytophthora ramorum* spreads through airborne deciduous sporangia, infects plants through motile zoospores released from the sporangia, and causes disease by growing within plant tissues. While plant colonization by the pathogen may occur throughout the year, sporangia production is observed during the rainy season (December-June), and plant infection occurs mostly when temperatures are mild or warm in the spring months (Rizzo & Garbelotto, 2003). It is not known if formulations containing systemic fungicides such as phosphonic-acid or metalaxyl-M will be equally effective on all life stages of *P. ramorum*, thus potentially protecting treated hosts and slowing down the epidemic. It is also unknown whether formulations containing a contact fungicide such as copper hydroxide provide an effective and durable barrier against zoospores, thus preventing new infections.

Efficacy of chemical control agents is also influenced by variation in their persistence over time, in their behaviour on different plant hosts, and in sensitivity among different genotypes of the pathogen. Variation in sensitivity to chemical compounds among *Phytophthora* isolates belonging to the same species has been reported (Hunger *et al*., 1982; Coffey & Bower, 1984; Coffey *et al*., 1984; Dolan & Coffey, 1988; Ferrin & Kabashima, 1991; Wilkinson *et al*., 2001b), but such variation has yet to be studied among individuals belonging to the US lineage of *P. ramorum* (known as US1 or NA1). The extent of variation in sensitivity to a fungicide has important implications not only in determining the potential for its long-term use, but also in indicating if the pathogen populations have been previously exposed to such chemicals (Cohen & Coffey, 1986; Gisi & Cohen 1996).

The goals of this study were to analyse the effects of three chemicals *in vitro* on the mycelial growth, sporangia production and zoospore germination of 12 isolates of *P. ramorum*, and to determine their potential efficacy in controlling the disease caused by this pathogen on California coast live oaks and California bay laurels.

Table 1 Isolates of *Phytophthora ramorum* employed in this study, plant host, location, year of isolation, and genotype as determined by amplified fragment length polymorphisms (AFLP) analysis

Isolate[a]	Source	Location	Year	AFLP genotype[b]
Pr1	*Quercus agrifolia*	Marin	2000	7
Pr2	*Lithocarpus densiflorus*	Marin	2000	1
Pr27	*Q. agrifolia*	Marin	2000	1
Pr36	*Q. agrifolia*	Sonoma	2000	3
Pr52	*Rhododendron sp.*	S. Cruz	2000	5
Pr57	*L. densiflorus*	S. Clara	2001	1
Pr70	*Vaccinium ovatum*	Marin	2001	1
Pr71	*Q. agrifolia*	Sonoma	2001	2
Pr75	*Q. agrifolia*	Monterey	2001	1
Pr102	*Q. agrifolia*	Marin	2001	1
Pr106	*Umbellularia californica*	Sonoma	2001	8
Pr159	*L. densiflorus*	Curry, OR	2001	4
Pr345	*Sequoia sempervirens*	Sonoma	2002	6

[a]Pr isolate numbers are from culture collection maintained at University of California, Davis.
[b]AFLP genotypes described in Ivors *et al.* (2004).

Table 2 Fungicides and their concentrations used to test the inhibition of hyphal growth, zoospore germination and sporangial production of isolates of *Phytophthora ramorum*

	Active Ingredient (mg L^{-1})		
	Phosphonic acid	Copper hydroxide	Metalaxyl-M
Hyphal growth	1·32	20	$4{\cdot}66^{-5}$
	53	34	0·002
	2133	56	0·07
Zoospore germination	45	1	0·0105
	67·5	1·5	0·021
	108	2	0·042
	135	3	0·105
Sporangia production	42	na[a]	na
	84	na	na
	337	na	na

[a]Not assessed.

Materials and methods

Inhibition of hyphal growth

Isolates used in this study are described in Table 1 and were selected to be representative of different hosts, geographic provenance and AFLP genotypes of the US forest lineage NA1 (Ivors *et al*., 2004). Before each experiment (*in vitro* or *in planta*), isolates were inoculated on bay laurel leaves as described by Anacker *et al.* (2007). Leaves were incubated at room temperature in mist chambers for 14 days prior to re-isolation of each isolate by plating the margins of visible lesions onto the selective medium P_{10}ARP (Erwin & Ribeiro, 1996).

Three different products were tested: Champ® (0·55 g mL^{-1} of copper hydroxide, Nufarm Americas Inc.), Agrifos400® (0·62 g mL^{-1} of phosphonic acid in the form of phosphonate, Agrichem) and Subdue MAXX® (0·24 g mL^{-1} of metalaxyl-M, Syngenta Crop Protection) (Table 2). All tests were performed in 60 mm diameter Petri dishes filled with V8 juice (Campbell, USA) agar (Granulated agar- Fisher Chemicals, diluted to contain 10% of the amount of V8 juice prescribed by the standard recipe (Erwin & Ribeiro, 1996). The pH was adjusted to 6·2 using NaOH, and chemicals were added once the media cooled to 50 °C. In negative controls, sterile water was added instead of the chemical (Garbelotto *et al*., 2002). Plates were inoculated by placing 5 mm plugs from actively growing edges of colonies at the centre of each Petri dish; for each isolate all plugs came from the edges of the same colony. Five replicate plates per isolate per

concentration were used and inoculated plates were incubated at 20 °C for 10 days in the dark before calculating the area of each colony. Inhibition tests were performed in two stages. First, isolate Pr1 was grown on V8 juice agar containing five different concentrations of each product and approximate ED10, ED50 and ED90 values were determined by plotting probit percentage inhibition values of the estimated dose (ED) against the logarithmic concentration (Coffey & Bower, 1984). Secondly, the inhibition of growth on these estimated ED10, ED50 and ED90 concentrations for each product was determined for a set of 12 isolates.

Data analysis

For each treatment, colony sizes were transformed into percentage area values of the size of colonies growing on control plates amended with water. Variation in colony size among different isolates at different ED values was analyzed by multiple analyses of variance (MANOVA), using the software Statistica (StatSoft). One-way ANOVA was used to compare differences among isolates at each ED tested. All data points were log transformed before being analyzed, for normality of distribution of data points and to establish equalized variances among treatments. Simple linear regressions using the least significant difference (LSD) model were run between percentage control values at each chemical concentration and the size of colonies of untreated samples, to identify whether the level of control was associated with growth rate of untreated isolates.

Inhibition of zoospore germination

Media were amended as described above for hyphal growth inhibition assays, except that full-strength corn meal agar, supplied by BBL, was used instead of 10% V8 agar. For each fungicide, four concentrations of active ingredient were selected based on preliminary tests performed on isolate Pr1, and tested on three isolates (Tables 1 & 4). Zoospores were produced as follows: 10 plugs, each of 1 cm^2, were cut from the margins of 2-week-old mycelial colonies and floated on sterile de-ionized water in 100 mm-diameter Petri dishes. Plates were incubated in the dark at 20 °C for 72 h (Erwin & Ribeiro, 1996). At the end of the incubation period, the eluate from each of the 10 plates was poured into a 250 mL wide-mouth plastic bottle (Nalgene). Additional eluate was obtained by rinsing the plates with 10 mL of sterile deionized water and adding this to the bottle. The cap of the bottle was then placed on the bottle opening without tightening it. Sporangia were induced to release zoospores by placing the bottle on ice for 30 min followed by 1 h at room temperature. In order to visually verify zoospore release had occurred, zoospore counts were performed using a haemocytometer 0·1 mm deep (Homusser Scientific). Each Petri dish was inoculated with 100 μL of zoospore suspension and incubated at 20 °C for 72 h, then germination was stopped by tranferring them to 6 °C until germinating zoospores were scored. Where there were too many colonies to score, colonies were marked then scanned and analyzed with the image analyzing software Assess (APS). Five replicates were used for each treatment, and data were analyzed as described above.

Inhibition of sporangia formation

Only phosphonic acid was tested for its efficacy to inhibit the formation of sporangia, because its ability to reduce lesion sizes in infected plants has already been shown (Garbelotto *et al*. 2002, 2003b, 2007) and formulations containing this active ingredient are currently being used to control *P. ramorum* on tanoaks in California. It was therefore important to determine whether phosphonic acid treatments may reduce the inoculum and hence slow the spread from tree to tree, besides protecting treated trees from contracting lethal stem infections. This dual effect would be relevant for tanoaks, trees that are known both to be lethally affected by the pathogen and to spread the disease from sporulating lesions.

As in the hyphal growth inhibition tests described above, four approximate ED values (Tables 2 & 5) were determined using isolate Pr1 as a tester strain and a probit analysis. Petri dishes filled with 10% V8 agar were inoculated, and 14 days later, 3 mm mycelial plugs were taken from the actively growing margins of the same colony and were placed in the centre of each well in a 24-well plastic plate. Plugs were submerged in 350 μL of appropriately diluted phosphonic acid solution using a randomly blocked design for each plate. Sterile water was used in control wells, and in addition, 14% of all wells were filled with water, but not inoculated. These wells were used to monitor possible across-well contamination events. Plates were incubated for 72 h at 20 °C, then further sporangial formation was blocked by pouring cotton blue stain into each well. Finally, sporangia were counted using a microscope (Nikon E400) at 100× magnification. The experimental design included five replicates per treatment (isolate × ED), and data analyses were analogous to those performed with the hyphal growth inhibition assays described above.

Phosphonic acid injection on coast live oak saplings

A total of 120 saplings, each 2–3 m tall and 2–3 cm in diameter, were used in this experiment. Saplings for this and other experiments were 6 years old and grown in 60 L pots filled with equal amounts of fir mulch and sand. Each of the 12 pathogen isolates was inoculated underbark (Garbelotto *et al*., 2007) onto 10 trees, five of which were untreated and five treated with a single phosphonic acid injection one week prior to inoculations. Injections were applied to the main stem of saplings 50 cm above the root collar using Chemjet® injectors (Chemjet Trading Pty Ltd) each containing 10 mL of the phosphonic acid formulation at a concentration of 217 mg mL^{-1}. The size of lesions was measured 6 weeks after inoculation, by removing the outer bark and measuring the extent of the

necrotic lesions. At least four isolations were taken from the edges of the visible lesion and transferred to PARP to confirm if they were caused by *P. ramorum*. For each sapling, the cumulative linear lesion size was calculated by adding the maximum linear extent of the lesion along the stem to the maximum lesion extent across the stem's circumference. The data were then log transformed and a *t*-test was used to compare lesion sizes in treated vs. untreated trees for each *P. ramorum* isolate. Two one-way ANOVA were independently run to compare lesion size across all treatments. The experiment was performed twice, but results were comparable and only data from one experiment are presented.

Metalaxyl-M drench and phosphonic acid injection on coast live oak saplings

A total of 60 potted oak saplings (as described above) were used for this experiment. Treatments (each applied to 15 saplings) were as follows: i) negative controls: trees that were mock-inoculated using sterile agar; ii) positive controls: trees that were inoculated with the pathogen, but untreated; iii) trees that were inoculated with the pathogen after injection with phosphonic acid as described above (Garbelotto *et al*., 2007); and iv) trees that were inoculated with the pathogen after a drench treatment with 5 L of metalaxyl-M (72 μg mL^{-1}) per potted tree. A week after treatment, all saplings were inoculated as described above using *P. ramorum* isolates Pr1, Pr2 and Pr52, or sterile agar. Individual treatments were fully randomized within each of five replicate blocks. The size of lesions was measured 6 weeks after inoculation, by removing the outer bark and measuring the extent of the necrotic lesions. For each sapling, a cumulative linear lesion size was calculated as described above. Results were analyzed by ANOVA and Student's *t*-tests were run on log-transformed data to compare lesion sizes of treated vs. untreated saplings, pooling results from the three isolates. The experiment was performed twice, but results were comparable and only data from one experiment are presented.

Copper hydroxide foliar application on California bay laurel

Bay laurel branches approximately 1 m in length were collected using a pole pruner from a single tree on the U.C. Berkeley campus. The branches were taken from the same height and from the same aspect of the tree. Using a spray bottle (ACE), two branches were sprayed until run-off with the copper hydroxide product using the manufacturer's recommended dose of 460 μg mL^{-1}, and two control branches were sprayed with water. Branches were left to dry for 30 min and then cut again and placed in water buckets. The buckets, each containing the two branches from a single treatment to prevent cross-contamination between branches, were then placed in a cool greenhouse with temperature ranging between 18 and 20 °C and relative humidity regulated at 80%. At each inoculation date, 15 leaves were randomly picked from each treatment and were inoculated in the laboratory using a zoospore suspension (2×10^4 zoospores mL^{-1}) of *P. ramorum* isolate Pr52 (produced as described for the zoospore germination test). Leaves to be tested were picked at the following times: 1 day, 1, 2, and 4 weeks after treatment. Leaves were placed petiole side up in 50 mL falcon tubes (Fisher) that had been previously treated using 5 M hydrochloric acid, to prevent encystment of zoospores on the walls of the tube. The zoospore suspension was applied to the bottom of the tubes and leaves were placed tip-first in the tubes and incubated overnight at 19 °C in domes at 100% relative humidity. Tubes were covered with moist paper towels and were placed on holding trays in a random design. One day after inoculation, leaves were placed on trays and sandwiched with moist paper towels for nine days.

Lesions, appearing as dark blotches bordered by a black line and a chlorotic zone, were visible at the end of the experiment, when the leaves were digitally scanned and the lesion size was measured using the image analysis software Assess. Two segments from the margin line of the lesion of each leaf were transferred to modified P_{10}ARP selective medium (Erwin & Ribeiro, 1996) and two segments from symptomless leaf tips were similarly transferred as controls. Two-way ANOVA was used to look at the effects of treatment and of time of inoculations on lesion size. Independent ANOVA were run on log-transformed data for each collection time to allow for the different susceptibility to infection of leaves kept in hydroponic culture for variable periods of time. The experiment was performed twice, but results were comparable and only data from one experiment are presented.

Results

In vitro experiments

Significant differences were observed *in vitro* in hyphal growth of untreated isolates (df = 11, $F = 83{\cdot}1$, $P < 0{\cdot}0001$; Table 3). Regression analyses between growth values of treated and untreated isolates indicated that the percentage control by phosphonic acid, copper hydroxide and metalaxyl-M was not correlated to the differences in growth rate of the untreated isolates (e.g. using growth values at ED50 and growth values of untreated isolates, $R^2 = 0{\cdot}22$, $F = 2{\cdot}5$, $P = 0{\cdot}14$ for phosphonic acid; $R^2 = 0{\cdot}04$, $F = 0{\cdot}4$, $P = 0{\cdot}54$ for copper hydroxide; and $R^2 = 0{\cdot}29$, $F = 1{\cdot}7$, $P = 0{\cdot}26$ for metalaxyl-M).

Phosphonic acid, copper hydroxide and metalaxyl-M treatments were all effective in reducing hyphal growth in all but one isolate *in vitro* (tests for isolate Pr70 failed). Metalaxyl-M was the most active (mean ED90 value 5×10^{-5} mg L^{-1}); copper hydroxide (mean ED75 56 mg L^{-1}) and phosphonic acid (mean ED90 2134 mg L^{-1}). There were significant differences in hyphal growth among isolates at each of the concentrations tested for each of the three compounds (df = 10, $F = 4$–72, $P = 0{\cdot}0004$–$0{\cdot}001$ for phosphonic acid; df = 10, $F = 14$–24, $P < 0{\cdot}0001$ for

Table 3 Mean ($n = 11$) colony diameter values of isolates of *Phytophthora ramorum* growing on media amended with different dosages of three active ingredients, expressed as percentage of the mean colony diameter of control isolates growing on unamended media

Active ingredient	Concentration a.i, μg mL^{-1}	Colony diameter (% of control)	SD[a]	2-way ANOVA summary statistics		
Control	0	18·4[b]	1·9			
					df	*P*
Phosphonic acid	1·32	97·3	3	Isolate	10	0·000001
	53	77·7	12·6	Concentration	3	0·000001
	2134	11	7·9	Isol. × Conc.	30	0·000001
				Error	176	
Copper hydroxide	20	56·3	12·3	Isolate	10	0·000001
	34	43	10·3	Concentration	3	0·000001
	56	35·4	7·6	Isol. × Conc.	30	0·000001
				Error	176	
Metalaxyl- M	5×10^{-5}	101·2	3·2	Isolate	10	0·000001
	0·002	88·6	10·2	Concentration	3	0·000001
	0·07	9·1	9·2	Isol. × Conc.	30	0·000001
				Error	176	

[a]Standard deviation.
[b]Colony diameter in mm.

copper hydroxide; and df = 10, $F = 4{\cdot}7$–68, $P = 0{\cdot}0001$–$< 0{\cdot}0001$ for metalaxyl-M) (Table 3).

Concentrations of copper hydroxide inhibiting zoospore germination were lower than those needed to inhibit hyphal growth. Germination of the three isolates tested was completely inhibited by 1·5 μg copper hydroxide mL^{-1}. However, 1 μg mL^{-1} had no effect on zoospore germination, and actually significantly enhanced growth of one of the three isolates tested (df = 2, $F = 7{\cdot}8$, $P = 0{\cdot}002$). In the case of phosphonic acid, the ED50 and ED90 values were 45 and 135 μg mL^{-1}, respectively, and for treatments at the ED90 concentration, there were significant differences among the three isolates tested (df = 2, $F = 4{\cdot}1$, $P = 0{\cdot}02$). A higher concentration of metalaxyl-M was required to inhibit zoospore germination than was needed to arrest hyphal growth: 0·1 μg mL^{-1} completely inhibited zoospore germination, the ED90 was 0·02 μg mL^{-1} and the ED50 was 0·01 μg mL^{-1}. Variation among isolates was observed only for the ED50 treatments for metalaxyl-M (df = 2, $F = 5{\cdot}6$, $P = 0{\cdot}009$). Isolates showed a steep dose-response to increasing active ingredient concentration for all three compounds tested (Table 4).

There was significant variation (df = 11, $F = 14$, $P < 0{\cdot}0001$) in the production of sporangia among untreated isolates. Sporangial production was on average reduced to 90% compared to the untreated controls by phosphonic acid at 337 μg mL^{-1} (Table 5). This concentration was significantly lower than that needed to reduce hyphal growth to the same extent. At the ED50 and ED90 concentrations there were significant differences in the responses of the isolates (df = 10, $F = 3{\cdot}1$, $P = 0{\cdot}002$; and df = 10, $F = 8{\cdot}3$, $P < 0{\cdot}0001$, respectively). There was no significant correlation ($R^2 = 0{\cdot}12$, $P = 0{\cdot}31$) between the percentage reduction of hyphal growth at the ED50 concentration for hyphal growth and the percentage reduction in sporangial production at the ED50 concentration for sporulation.

In planta experiments

Phosphonic acid injection treatments on coast live oak saplings

In spite of differences in growth rate among isolates on untreated oak saplings (df = 11, $F = 4{\cdot}7$, $P < 0{\cdot}0001$), all isolates were equally affected by the phosphonic acid treatment (Fig. 1). Growth of all 12 isolates was minimal beyond the point of inoculation in oak saplings that were injected with phosphonic acid one week before inoculation.

Table 4 Mean number of colonies from germinating zoospores of three isolates of *Phytophthora ramorum* produced on media containing different concentrations of three active ingredients, expressed as percentage of colonies in relation to control colony numbers of zoospores on unamended media (control). Standard deviations are given in parentheses

	Mean percentage colony numbers compared with control											
	Concentration μg mL^{-1}											
	Control	Phosphonic acid				Copper hydroxide				Metalaxyl-M			
Isolate	Col. mL^{-1}	45	67·5	108	135	1	1·5	2	3	0·01	0·02	0·04	0·1
Pr1	924 (75)	44 (9·7)	33 (14)	18·8 (14)	5·4 (5·2)	120 (32)	0	0	0	35·3 (18)	9·7 (9·5)	0·2 (0·4)	2·6 (8·1)
Pr2	78 (7)	62·6 (19·2)	47·4 (20)	24·5 (18)	16·3 (9·5)	373 (172)	0	0	0	0	0	0·13 (0·4)	0·25 (0·5)
Pr52	723 (65)	62·6 (21)	47·4 (12)	24·6 (7·6)	16·4 (7·3)	103 (50)	0	0	0	22·8 (19)	6·1 (14)	0·04 (012)	0

Table 5 Average number ($n = 11$) of sporangia of *Phytophthora ramorum* produced on media amended with different amounts of phosphonic acid (Agrifos400), expressed as percentage of sporangia produced by the same isolate on unamended media. Numbers shown are averages of mean values from 11 isolates

	Concentration a.i, μg mL^{-1}	No. of sporangia (% of control)	SD[a]	2-way ANOVA summary statistics		
Control	0	5406[b]	2365			
					df	*P***
	42	85·3	13·6	Isolate	10	0·000001
Phosphonic acid	82	44·6	16·8	Concentration	3	0·000001
	337	16·5	10·3	Isol × Conc.	30	0·03
				Error	347	

[a]Standard deviation.
[b]Number of sporangia per mL.

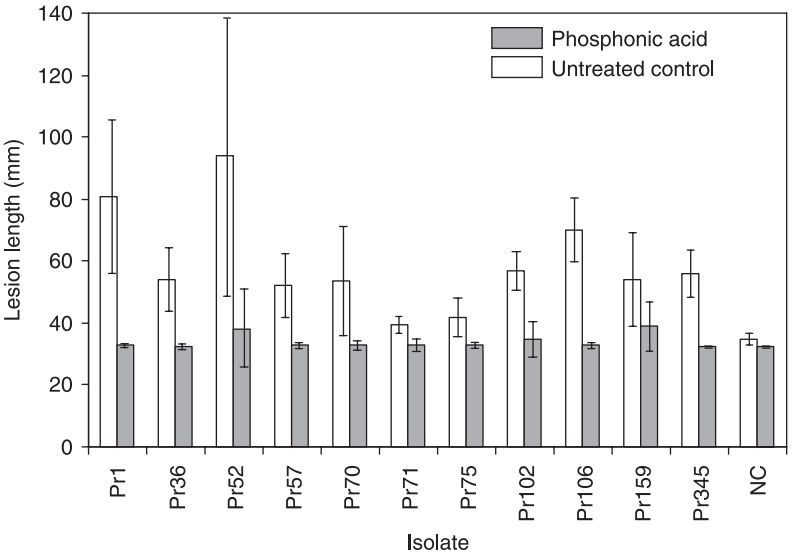

Figure 1 Lesion length (with bars showing standard deviation) caused by *Phytophthora ramorum* isolates and by a mock inoculation (NC) 6 weeks after inoculation on potted *Quercus agrifolia* saplings either treated preventively with a single injection of phosphonic acid 1 week prior to inoculation, or untreated.

Re-isolation of the pathogen was successful from all inoculated trees, independent of treatment.

Metalaxyl-M drench and phosphonic acid injections on coast live oak saplings

Oak saplings that were drenched with the metalaxyl-M formulation a week before inoculation with one of three *P. ramorum* isolates, had cumulative linear stem lesions (mean = 92·1 mm, SD = 47) undistinguishable ($F = 0·1$, $P = 0·7$) from those of the untreated controls (mean = 97·7 mm, SD = 39). Conversely, cumulative linear lesions (mean = 43·7 mm, SD = 18) on oaks injected with phosphonic acid were significantly shorter than those of untreated saplings ($F = 23·2$, $P = 0·0001$) (Fig. 2). Negative controls developed no lesions and were excluded from the analysis. The pathogen was re-isolated from all inoculated trees, independent of treatment.

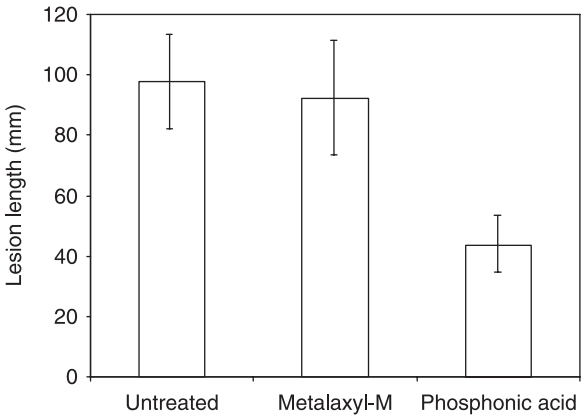

Figure 2 Lesion length (with bars showing standard deviation) caused by three *Phytophthora ramorum* isolates inoculated underbark in the phloem of potted coast live oak saplings, either untreated, treated with metalaxyl-M drench, or by phosphonic acid injection. Each treatment was performed on 15 saplings one week before inoculation; the experiment was terminated 6 weeks after inoculation.

Copper hydroxide foliar application on California bay laurel

In the foliar treatment of California bay laurel leaves, there was a significant effect of the copper hydroxide

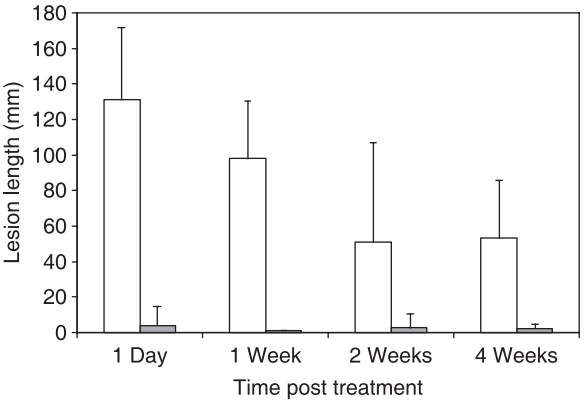

Figure 3 Lesion length (with bars showing standard deviation) assessed on four intervals in California bay laurel leaves inoculated with zoospores of one *Phytophthora ramorum* isolate, with (grey bars) and without (white bars) a single preventive foliar application of copper hydroxide.

treatment ($F = 230$, $P < 0{\cdot}0001$), and only a marginal effect of time ($F = 3{\cdot}8$, $P = 0{\cdot}053$) on size of lesion caused by inoculations of zoospores produced by isolate Pr52. Bay laurel cuttings survived well placed in water, but, as indicated by the data from the untreated positive controls, the lesions that developed after inoculation were smaller as time increased, presumably due to premature senescence of detached branches kept in water for longer periods of time (Fig. 3). Nonetheless, at each inoculation time, the lesions on leaves treated with copper hydroxide were significantly smaller than lesions on untreated leaves (1 day $F = 111$, $P < 0{\cdot}0001$; 1 week $F = 205$, $P < 0{\cdot}0001$; 2 weeks $F = 15{\cdot}2$, $P = 0{\cdot}0005$; 4 weeks $F = 66{\cdot}5$, $P < 0{\cdot}0001$). Hence, preventive copper hydroxide applications were effective in reducing infection by *P. ramorum* up to 4 weeks after treatment (Fig. 3). Negative controls displayed no symptoms and no significant phytotoxicity was associated with the treatment.

Discussion

This study documents the variation in sensitivity of a range of isolates of the US lineage of *P. ramorum* to three chemical compounds traditionally used to treat plant diseases caused by *Phytophthora* species. Variability among isolates in response to chemicals is not uncommon in species of the genus *Phytophthora*, and is not necessarily linked to previous exposure (Hunger *et al*., 1982; Coffey & Bower, 1984; Coffey *et al*., 1984; Dolan & Coffey, 1988; Ferrin & Kabashima, 1991; Wilkinson *et al*., 2001b). In spite of the variation in sensitivity to metalaxyl-M, no metalaxyl-M-resistant isolates were found in this study. In other *Phytophthora* species, such isolates have been identified and are characterized by sensitivity levels that are one or two orders of magnitude higher than those recorded for other conspecific isolates (Hunger *et al*., 1982; Coffey *et al*., 1984; Ferrin & Kabashima, 1991).

With regard to the fungistatic effects of phosphonic acid, results from tests performed *in vitro* highlighted not only the presence of significant variability in sensitivity among isolates, but also that *P. ramorum* isolates were in general less sensitive than *P. cinnamomi* (Wilkinson *et al*., 2001b). *In vitro* sensitivity levels recorded in this study are comparable to those reported for *P. infestans*, a species known to be less sensitive to this compound (Coffey & Bower, 1984). In contrast, complete fungistasis of all isolates was observed *in planta* when testing injections of phosphonic acid; this result is promising as it implies that treatment may lead to the slower progression of girdling lesions in the stems of infected hosts. It has been shown that for *P. cinnamomi*, phosphonic acid has a dual action *in planta* (Guest & Grant, 1991; Jackson *et al*., 2000). When concentrations of phosphonic acid are high in the roots, direct antimicrobial activity appears to be responsible for most of the fungistatic effect, but when concentrations decrease, the fungistatic effect persists owing to activation of plant defences. The differences between results obtained in this study *in vitro* and *in planta* for a range of isolates of *P. ramorum* highlight the fact that the direct fungistatic effect of phosphonic acid on *P. ramorum* may be modest and that most of the efficacy of treatments may be linked to the antimicrobial activity of secondary metabolites triggered *in planta* (Guest & Grant, 1991). This result may also explain why therapeutic treatments have been reported to have only limited efficacy on oaks and tanoaks already infected by *P. ramorum* (Garbelotto *et al*., 2007), while recovery of trees infected by *P. cinnamomi* is reported upon treatment with phosphonates (Cohen & Coffey, 1986; Guest *et al*., 1995).

Sporangia act as air-borne dispersal propagules of *P. ramorum* (Garbelotto *et al*., 2003a; Davidson *et al*., 2005). In the wild, sporangia have been observed in large numbers on the leaves of California bay laurels and on the twigs of tanoaks (one of the hosts approved for treatment using the phosphonate Agrifos400®) (Rizzo & Garbelotto, 2003). Results presented here show a significant variation in natural production of sporangia among isolates *in vitro*. No correlation was found between rates of hyphal growth and sporangia production among isolates, whether treated or untreated. This lack of correlation suggests that sporangial production is the result of a pathway independent from that leading to hyphal growth. A strong reduction of sporangial production by isolates grown in media amended with phosphonic acid was measured. Although isolates varied in their responses to treatment, the concentration needed to obtain a 90% reduction in sporangia production was significantly lower than that needed to obtain a 90% reduction in colony size, suggesting phosphonic acid treatments of tanoaks may have a significant effect both on pathogen growth and sporulation. Whether results *in vivo* may parallel those described here for experiments *in vitro* needs to be tested. These results are in general agreement with those of other studies (Coffey & Joseph, 1985; Dolan & Coffey, 1988; Guest & Grant, 1991) that have reported lower ED50 values for sporangial production than for the inhibition of hyphal growth with other species of *Phytophthora*. However, in this study, the range of variation in response among

isolates of *P. ramorum* was greater for sporangial production than for hyphal growth, indicating that treatments involving phosphonic acid may possibly lower, but not completely eliminate, the risk of sporulation. Similarly, a study by Wilkinson *et al*. (2001) showed that zoospore release by *P. cinnamomi* from infected plant material was reduced but not fully suppressed by treatments with phosphonic acid.

Although sporangia are responsible for the spread of the pathogen, it is the motile zoospores produced in the sporangia that actually infect the host (Rizzo & Garbelotto, 2003). The ability to inhibit zoospore germination may thus have important implications in the prevention of infection. Copper hydroxide was the most effective compound in inhibiting zoospore germination and totally inhibited germination of all tested isolates at a very much lower concentration than was needed to inhibit hyphal growth. Similarly, concentrations of phosphonic acid needed to inhibit zoospore germination *in vitro* were lower than those needed to inhibit *in vitro* growth and sporangial production. On the other hand, the concentration of metalaxyl-M needed to inhibit 90% of zoospore germination was higher than that needed to produce a comparable fungistatic effect on hyphal growth. The results of the experiments *in vitro* suggested that the action of metalaxyl-M might be more significant in halting colonization of plant tissue by *P. ramorum* than in preventing the infection process known to be linked to zoospore germination. However, this was not borne out *in vivo*, since metalaxyl-M drench treatments failed to limit the size of lesions caused by *P. ramorum* in oak saplings inoculated with the pathogen. These contrasting results may be linked to lack of effective absorption and/or translocation of the metalaxyl-M in potted oak saplings, but further investigations are needed to confirm this.

The success of preventive treatments with copper hydroxide sprays on California bay laurel leaves confirmed the potential for this compound to prevent infection by *P. ramorum* on this and potentially on other foliar hosts of this pathogen. Infection was prevented in leaves treated with copper hydroxide up to a month after treatment. Because control of infection was still close to 100% after four weeks, the efficacy of this treatment may be longer than one month, and may be sufficient to cover the entire infection season of this pathogen, which is normally limited to the warm and wet months in California (March to June). A further experiment designed to compare persistence of protection between cuttings in the greenhouse and whole plants in the field is needed to confirm the potential use of this treatment in the field. However, while bay laurel and rhododendron leaves may represent suitable candidates for treatments using a contact fungicide such as copper hydroxide, the highly corrugated and irregular bark of oaks and tanoaks may prove harder to protect using a similar contact approach. No phytotoxicity was reported in the course of the experiments described here, but the environmental side effects of copper treatments, including their high toxicity to aquatic organisms, would need to be evaluated before their use could be advocated.

The presence of variability in sensitivity to chemicals among isolates may have important implications for the long term efficacy of these chemicals for the control of *P. ramorum*. Selection of less sensitive individuals within populations of this pathogen could lead to a decrease in the overall levels of sensitivity. The ability of a pathogen to overcome the fungistatic effect of any compound is related to its specific mode of action. For instance, while a single mutation has conferred resistance to metalaxyl on some genotypes of *P. infestans* (Gisi & Cohen, 1996), no true resistance has yet been reported to phosphonic acid, presumably due to the fact that this compound has fungistatic effects based on multiple metabolic processes.

Finally, while the term 'fungicide' is commonly used for compounds employed in the chemical control of plant diseases, these compounds do not always kill the pathogens involved, particularly where treatment of woody plants is involved. In this study, each of the three compounds reduced growth, sporulation and germination of *P. ramorum in vitro*, *in planta* or in both. However, *in planta* in all cases, in spite of a reduction in symptom expression after treatment, the pathogen could be re-isolated at the end of each experiment. Hence, chemical treatments may have the undesirable secondary effect of reducing symptoms caused by a pathogen, without killing it. Thus the possibility that the disease might recur some time after treatment, from symptomless but nonetheless infected plants, needs to be investigated in trials over a longer time period. The risk of this occurring in nursery stock would be particularly problematic since disease might only appear after distribution of the plants to a new location. Indeed, the presence of infected plants or plant parts made symptomless by chemical treatments might allow the introduction of exotic pathogens into new regions.

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