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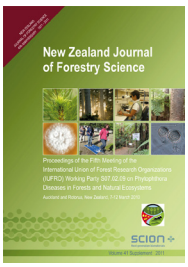

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Application of phosphonate to prevent sudden oak death in south-western Oregon tanoak (*Notholithocarpus densiflorus*) forests†

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Abstract

We conducted four experiments to evaluate the effectiveness of phosphonate application to tanoak (*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S.H.Oh) forests in south-western Oregon: (1) aerial application to forest stands; (2) trunk injection; (3) foliar spray of potted seedlings; and (4) foliar spray of stump sprouts. We compared aerial spray treatments: (1) no treatment (unsprayed); (2) low-dose (17.35 kg a.i. ha^{-1}); and (3) high dose (34.5 kg a.i. ha^{-1}), applied by helicopter in a carrier volume of 188 L ha^{-1} to 4-ha treatment plots. Treatments were applied in November 2007, in May 2008, and in December 2008 and May 2009 (double treatment). At the same time as the aerial application we injected phosphonate into the trunk of nearby mature tanoak trees at the standard label rates of 0.43 g a.i. cm-dbh^{-1}. We used three different biological assays to measure uptake of phosphonate: (1) canopy twig dip in zoospore suspension; (2) *in situ* bole inoculation with *Phytophthora gonapodyides* (Petersen) Buisman; and (3) laboratory inoculation of log bolts with *Phytophthora ramorum* S. Werres, A.W.A.M. de Cock & W.A. Man in 't Veld and *P. gonapodyides*. We also simulated an aerial spray of potted seedlings, comparing an untreated control, a low dose (2.9 kg a.i. ha^{-1} applied in 935 L spray solution ha^{-1}), and a high dose (17.35 kg a.i. ha^{-1}applied in 187 L spray solution ha^{-1}).

Aerial spray with phosphonate consistently resulted in smaller bole lesions on trees challenge inoculated with *Phytophthora gonapodyides in situ* and in logs inoculated with *P. ramorum*. This effect persisted for 18 months post treatment. Results from detached canopy twig assays were variable and showed only small treatment effects. Trunk injection consistently reduced bole lesion size in trees and logs, but gave inconsistent results in the canopy twig assay, possibly due to the twig assay methodology. In the spring and autumn trunk injection treatment, canopy twig lesion length was reduced by 32 percent compared to untreated controls, indicating that trunk-injected phosphonate was mobilised to the outer twigs of the tree crown. Trunk injection with phosphonate resulted in a greater reduction in bole lesion area than aerial spray. Spray application of phosphonate to tanoak seedlings did not protect them from infection when exposed to artificial or natural inoculum of *P. ramorum*. Foliar application of phosphonate to stump sprouts reduced lesion length by 44% of control in a shoot-dip assay three months post-treatment.

Keywords: Oregon; phosphonate; *Phytophthora gonapodyides*; *Phytophthora ramorum*; sudden oak death; tanoak.

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Introduction

Phytophthora ramorum S. Werres, A.W.A.M. de Cock & W.A. Man in 't Veld is a recently established non-native invasive pathogen in the forests of coastal California and south-western Oregon, United States of America (USA) (Goheen et al., 2002, Hansen et al., 2008, Rizzo et al., 2002, Rizzo & Garbelotto, 2003). *Phytophthora ramorum* spreads via airborne propagules produced under wet conditions and mild temperatures. The main host for *P. ramorum* in Oregon is tanoak (*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S.H.Oh)) which is killed by the pathogen but also supports disease spread (Hansen et al., 2005). In Oregon, there has been an intense effort to eradicate or slow the spread of the pathogen by early detection, and by cutting and burning of infected and nearby host plants. The eradication treatments have eliminated the pathogen from many sites, but the overall effectiveness of the program is limited not only by difficulties in early detection but also delays in completing various treatments, which are labour-intensive. Infected trees often remain untreated for months when weather is conducive to sporulation and disease spread. As a result, the pathogen continues to spread slowly through Oregon's tanoak forests in the direction of prevailing wet season winds (Kanaskie et al., 2009). As the extent of the infestation increases and cost of containment mounts, alternative treatments need to be explored.

One possible alternative to the current treatment approach is to create a host-free barrier well ahead of the leading edge of infestation. Given the scale of the Oregon infestation this would take years and would have considerable cost. Another option is to aerially apply a fungicide over large areas. The aim here is to prevent spread of *Phytophthora ramorum* either by protecting trees from infection or by reducing spore production in trees that become infected. *Phytophthora ramorum* initially infects foliage and twigs in the crown of tanoaks. Subsequent sporulation on these tissues is the basis for natural long distance and local spread (Hansen et al., 2008). Treatments that prevent infection of these tissues or reduce sporulation on them could be part of an integrated approach to controlling pathogen spread. An effective fungicide treatment could be used locally to prevent expansion of new infestations or to treat large areas of forest in advance of the disease, much like a host-free zone but at much lower cost.

Phosphonate fungicides are widely used to control diseases caused by *Phytophthora* spp. (Barrett et al., 2003; Guest et al., 1995; Hardy et al., 2001). Their mode of action is complex and mainly involves an enhancement of the plant defense mechanism, with limited direct effect on the pathogen (Guest & Grant, 1991). In central California, various phosphonate products have been shown to limit development of *P. ramorum* in coast live oak (*Quercus agrifolia* Nee) and tanoak when sprayed directly onto or injected into individual tree boles (Garbelotto et al., 2007, Garbelotto & Schmidt, 2009, Garbelotto et al., 2009). Related studies also demonstrated that phosphonate reduces sporulation of *P. ramorum* growing in culture, and suggest that this effect may be independent of its effect on hyphal growth (Garbelotto et al., 2009). These studies clearly demonstrated that phosphonate treatment reduced lesion size in treated trees that were subsequently challenge inoculated with zoopores or mycelia of *P. ramorum*. In Western Australia, aerial application of phosphonate is used to control the soil-borne pathogen *Phytophthora cinnamomi* Rands (Barrett, 2001; Hardy et al., 2001). There have been no tests, however, of aerial application of phosphonate to control *P. ramorum* under forest conditions, or of its efficacy as a broad scale disease protectant in forests.

The aims of our study were: (1) to determine the feasibility of *Phytophthora ramorum* control in tanoak forests through aerial application of phosphonate; and (2) to test the use of biological assays to evaluate uptake and translocation of phosphonate in trees treated by foliar application or trunk injection. Although our main purpose was to evaluate aerial application to forests, simulated aerial application to seedlings allowed us to evaluate treatment of intact living plants under natural inoculum conditions, which was not possible with forest trees. Injection has been shown as an effective method of phosphonate application (Garbelotto et al., 2008) and we included it in our experiments to show that the expected treatment response was detectable by our biological assays. Ideally, our study would have involved treating trees with phosphonate and allowing them to be challenged by natural inoculum as the disease progressed across the landscape. However, *P. ramorum* is a quarantine pathogen and infested areas are subject to mandatory eradication treatments, so it was not possible allow sources of inoculum to remain on the landscape long enough for a meaningful study.

Materials and Methods

The phosphonate product used was Agri-Fos® 400 (Agri-Fos® 400 is 620 g L^{-1} active ingredient of mono- and di-potassium salts of phosphorus (phosphonic) acid, equivalent to 400 g L^{-1} phosphorus acid; Agrichem Manufacturing Industries, Queensland, Australia). The surfactant used was Pentra-Bark® (Pentra-bark® is an organosilicate surfactant, 89% polyalkylene modified heptamethyltrisiloxane and non-ionic surfactants and 10% coupling agents; Agrichem Manufacturing Industries, Queensland, Australia).

Experiments

Four types of experiment were conducted: (1) aerial application; (2) tree injection; (3) natural infection of potted seedlings; and (4) stump sprouts.

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1. Aerial application

Treatment areas for helicopter sprays were located in extensive stands of tanoak north of the sudden oak death infestation region in Curry County, Oregon, approximately 20 km north of the Californian border (42° 10' 54" N, 124° 14' 56 W). The treatments were: no treatment; low-dose phosphonate (17.36 kg a.i. ha^{-1}; using a mixture of 28 L Agri-Fos®, 95 mL Pentra-Bark® and 160 L water ha^{-1}), and; high dose phosphonate (34.72 kg a.i. ha^{-1}; using a mixture of 56 L Agri-Fos®, 95 mL Pentra-Bark® and 132 L water ha^{-1}) (Table 1). The spray formulations were applied at a rate of 188 L ha^{-1} from 10 m above the canopy by helicopter (Bell OH-58 Jet Ranger, Bell Helicopter, Texas, USA) with a spray boom spanning 9.14 m equipped with CP-03 nozzles (CP Products, Arizona, USA) set to deliver 300 – 500 micron droplets. Plots were double-flown, with half of the spray volume applied each time. Treatments were applied on 24 November 2007 (autumn only), 23 May 2008 (spring only), and 16 November 2008 and 20 May 2009 (autumn and spring double). Treatments were replicated three times, with each replication (block) consisting of three 4-hectare square or rectangular treatment plots, one of which was an untreated control. Foliage was dry at the time of application and wind speed was 2.2 m s^{-1} or less. There was no rain for at least 24 hours after treatment. Total helicopter application costs (chemicals, helicopter time, and pilot) for each treatment in Oregon were US$395 ha^{-1} for the low dose and US$558 ha^{-1} for the high dose.

Approximately 6 to 7 months following each treatment, and again18 months after the May 2008 treatment, ten treated trees per treatment plot and replication were felled, and twenty 25-cm long live shoot tips (twigs) from the outer canopy were randomly collected from each and transported to the laboratory for bioassay. Dead or damaged shoot tips were not collected. For the May 2008 treatment only, five trees per treatment plot were felled and a bolt (10 to 15 cm diameter and 1.0 to 1.5 m long) was cut from the trunk. Ends of the bolt were sealed immediately with Anchorseal® wax emulsion (UC Coatings Corporation, NY, USA) and transported to the laboratory.

2. Trunk injection

At the time of each aerial application we selected individual tanoak trees for injection in an area located between 200 and 500 m from the aerial spray plots. For the autumn 2007 and the spring 2008 applications, we injected 10 trees each and selected 10 untreated controls for each. Approximately 6 to 7 months following each treatment, and again 18 months after the spring only treatment, five trees per treatment plot were felled, and twigs and trunk sections were collected as described for Experiment 1. The remaining five trees per treatment were used for *in situ* inoculation as described in the analysis section (an alternate bioassay). For the autumn and spring double application study, we injected 10 trees in November 2008 only, 10 in May 2009 only, and 10 in both November and May, and identified 10 untreated controls trees. In November 2009 (seven months after the last treatment) five trees from each treatment were felled for twig collection for bioassays as described for Experiment 1, and five were left standing for *in situ* inoculation. Trees were in a co-dominant position in the forest canopy and ranged in size from 10 to 25 cm diameter at 1.5 m above ground (dbh). The injection treatment was phosphonate at the label rate of 10 mL injection fluid (1 part Agri-Fos® and 2 parts water) per 15 cm trunk circumference (0.43 g a.i. cm-dbh^{-1}). Injections were made 1 to

TABLE 1: Summary of treatment and exposure dates for the spray application of phosphonate to potted tanoak seedlings. In treatment group 3, the same trees were treated in autumn and spring. Field exposure: plants exposed to natural inoculum beneath infected tanoak trees in Curry County, Oregon. Laboratory exposure: artificial inoculation by spraying a zoospore suspension of *Phytophthora ramorum* onto seedlings.

Treatment group	Treatment date	Exposure	Exposure date	Time between treatment and exposure (weeks)
1	28 October 2008 (autumn)	Field	1 December 2008	5
			5 May 2009	26
		Laboratory	13 December 2008	6
			8 May 2009	26
2	3 January 2009 (spring)	Field	5 May 2009	5
		Laboratory	8 May 2009	5
3	28 October 2008 (autumn)	Field	5 May 2009	5
	& 3 January 2009 (spring)	Laboratory	8 May 2009	5

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1.5 m above ground and penetrated 1 to 2 cm into the sapwood. We used the Sidewinder® injection system (Sidewinder Pt Ltd, Queensland, Australia) and closed the 5 mm diameter injection holes with threaded plastic plugs after treatment.

3. Potted seedling study

We simulated a helicopter application to potted healthy two-year old tanoak seedlings kept in a growth room using a backpack sprayer with carbon dioxide (CO_2) at a pressure of 165 kPa, with a double-nozzle spray boom (30 cm between nozzles) equipped with Cone Jet TXVS-8 hollow cone spray tips (TeeJet Technologies, Illinois, USA). Seedlings were grown without fungicides at Oregon State University from acorns collected two years prior in Curry County, Oregon, USA. Treatments were: an untreated control, a low dose of phosphonate (2.9 kg a.i. ha^{-1} applied at a rate of 935 L spray solution ha^{-1}; using a mixture of 2.49 mL Agri-Fos®, 0.61 mL Pentra-Bark® and 496.9 mL water) and a high dose (17.35 kg a.i. ha^{-1} applied at a rate of 187 L spray solution ha^{-1}; using a mixture of 75 mL Agri-Fos®, 0.61 mL Pentra-Bark® and 424.4 mL water). The low dose was equivalent to the recommended Agri-Fos® label rate for application of phosphonate to nursery plants. The high dose was equivalent to the lowest helicopter application rate. A metronome was used to keep the correct pace as the spray boom was passed over the seedlings, 60 cm above seedling tops.

One set of seedlings was sprayed in either October 2008 or in April 2009, with another set of seedlings sprayed in both October and April. Each treatment, dose and exposure combination contained 13 seedlings. Five weeks post treatment, seedlings were challenge inoculated with *P. ramorum* either in a growth chamber or exposed to natural inoculum in the field. Seedlings sprayed in October 2008 were also challenge inoculated at 26 weeks post treatment (Table 1).

Growth-room inoculation was done using an airbrush (Badger Airbrush Co. model 250, Franklin, IL, USA) to spray 3 mL of a 1×10^4 per mL zoospore suspension of *Phytophthora ramorum* on each seedling. Seedlings were incubated in polyethylene tents to maintain near 100% humidity for the first 48 hours post-inoculation, then incubated at 21 °C for one month with alternating 12 h light-dark periods.

In the field, inoculation was done by placing potted seedlings beneath infected tanoak trees for 4 to 6 weeks. To verify that natural inoculum was present, an 8-L white HDPE plastic bucket lined with a 30 cm x 20 cm x 50 cm 0.0015-gauge polyethylene bag (Pacific Packaging, Oregon, USA) was placed beneath each infected tree. The bucket contained 375 mL de-ionised water and two bait leaves each of Pacific rhododendron (*Rhododendron macrophyllum* G. Don) and tanoak, and was covered with fiberglass mesh window screen to exclude large debris. Bait leaves were retrieved at two-week intervals and replaced with new bait leaves, bucket liners and water. Bait leaves were placed in Zip-loc® bags, transported to the laboratory in a cooler, washed in tap water and blotted dry. Necrotic areas of leaves were plated on *Phytophthora* selective agar (CARP; natamycin (Delvocid, DSM Food Specialties) at 10 ppm, Na-ampicillin at 200 ppm, rifampicin at 10 ppm, and commercial corn meal agar (CMA)). Petiole ends were always included whether necrotic or not. Isolation plates were incubated in the dark at 20 °C for 7 to 10 days, then examined for the growth of *P. ramorum*, which was recognised by a combination of distinctive hyphae, chlamydospores, and sporangia.

4. Stump-sprout spray

We simulated an aerial application of phosphonate to tanoak stump sprouts that had completed two growing seasons since stumps were created in a clear-felling operation. The dense clumps of tanoak stump sprouts ranged in height from 0.5 to 1.5 m. Treatments were the same as described for the helicopter application; high dose, low dose, and an untreated control. Sprays were applied on December 10, 2007 using a 15 L capacity Field King backpack sprayer (The Fountainhead Group Inc., New York Mills, New York, USA). We treated 10 sprout clumps per treatment plot and each treatment plot was replicated three times. On 8 March 2008 one stem per clump from each of the high dose and control treatments was harvested and returned to the laboratory. The cut ends of shoot tips were immersed 1 cm deep in either sterile distilled water for controls or a suspension of 1×10^4 per mL *P. ramorum* zoospores for 3 days, then incubated in clear polyethylene tents for three weeks at near 100% humidity and 21 °C in a growth chamber with 12 h alternating light and dark periods. Lesion lengths were measured from the cut end of the twig to the distal edge of discolored tissue. Further sampling for this study was not possible because all plots were inadvertently destroyed by an aerial herbicide application in April 2008.

Bioassay Methods

Three separate bioassay methods were used to determine phosphonate uptake in samples collected in the four experiments outlined above.

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Phytophthora gonapodyides (Petersen) Buisman was chosen for inoculation studies because it causes symptoms on tanoak similar to those caused by *P. ramorum,* it is native to forests in the study area, and it is not a quarantine pest. *Phytophthora ramorum* and *P. gonapodyides* isolates for inoculation studies were originally recovered from diseased plants collected in the sudden oak death outbreak area in south-western Oregon. Long-term storage was on agar plugs in vials of water at room temperature. *Phytophthora ramorum* isolates were A2 mating type, North American genotype.

Canopy twig assay

Phytophthora ramorum sporangia for zoospores for inoculations were produced on one-third-strength clarified V8 juice agar (66 mL of clarified V8 juice and 15 g of agar/liter). Plates with 2 week old colonies bearing abundant sporangia were flooded with double-distilled sterile water and chilled for 1 to 3 h, then lightly scraped before decanting. Because of variable characteristics and pathogenicity of the North American genotype, zoospores and sporangia from three to four isolates were mixed. Zoospore concentrations were approximately 1×10^4 per mL. Cut ends of shoot tips from Experiments 1, 2 and 4 were immersed 1 cm deep in either sterile water for controls or a suspension of *P. ramorum* zoospores for 3 days and incubated in clear polyethylene tents for three weeks at 21 °C in a growth chamber with 12 h alternating light and dark periods. Lesion lengths were measured from the cut end of the twig to the distal end of discolored tissue.

In situ *bole inoculation with* Phytophthora gonapodyides

Phytophthora gonapodyides was used in field inoculations instead of *P. ramorum* due to quarantine regulations. Approximately 6 to 7 months following each treatment in experiments 1 and 2, and again 18 months after the May 2008 treatment, *in situ* stem inoculations were made at 1 m above ground on the trunk of 10 standing forest trees located in the central area of each aerial spray treatment plot, and on five trees for each of the injection treatments. Two holes (5 mm in diameter) through the bark to the cambium on opposite sides of the tree were made with a cork borer. A matching plug of agar, either colonised by *P. gonapodyides* or un-colonised for controls, was removed from the growing margin of colonies on clarified V8 juice agar (66 mL V8 juice with 15 mg mL^{-1} $CaCO_3$ clarified by centrifugation or filtration, 934 mL deionised water, 15 g Bacto agar) and inserted into each hole and the bark piece replaced. Inoculation points were covered with wet cheese cloth and then aluminum foil held in place with tape. After 5 weeks, wrappings were removed and the outer bark was scraped away to reveal margins of any necrotic area. The lesion extent was measured horizontally and vertically. Lesion area was calculated as a diamond-shaped area less the inoculation wound: (horizontal measure – 5 mm) × (vertical measure – 5 mm)/2. To confirm that lesions were caused by *P. gonapodyides,* isolations were attempted from the edges of each visible lesion by plating onto CARP agar.

Log inoculation

Inoculation with *Phytophthora ramorum* and *P. gonapodyides* was done on 5 logs from each treatment plot for the May 2008 aerial application and trunk injection plots, 7 months after treatment. Inoculations were performed in the Laboratory at Oregon State University. Holes (5 mm in diameter) through the bark to the cambium were made with a cork borer. A matching plug of agar, either colonised by *P. ramorum, P. gonapodyides,* or un-colonised for controls, was removed from the growing margin of colonies on clarified V8 juice agar and inserted into each hole and the bark piece replaced. Inoculation points were covered with damp cheese cloth and then aluminum foil held in place with tape. Logs were incubated in clear polyethylene sleeves for 5 weeks at 21 °C in a growth chamber. Wrappings were removed and the outer bark was scraped away to reveal margins of any necrotic area. The lesion extent was measured as described for the *in situ* inoculation. To confirm that lesions were caused by *P. ramorum* or *P. gonapodyides,* isolations were attempted from the edges of each visible lesion by plating onto *Phytophthora* selective agar (CARP). Cultures were identified from morphological features.

Seedling infection (Experiment 3 only)

After exposure to natural inoculum for 4 – 6 weeks (based on results from baited rainfall buckets), seedlings were returned to the laboratory and isolations were attempted from each necrotic lesion on leaves and stems by plating onto CARP medium. Only lesions from which *Phytophthora ramorum* was recovered were counted. For the October 2008 exposure only, incidence of infection (percentage of seedlings infected) was recorded. For the April 2009 exposure and the October – April double exposure both incidence of infection (percentage of seedling infected) and severity of infection (measured proportion of total stem length with necrosis) were recorded.

Analysis of results

Analysis of Variance was used to test the main effects of treatment and block (site) on lesion size for the various assays, and Fisher's Least Significance Difference (LSD) test ($P < 0.05$) was used to compare differences among treatment means (Statgraphics Plus 5.0, Statpoint Technologies, Virginia, USA). Individual twig lesion lengths were combined into single tree means before analysis. For Experiment 4, treatment effects were evaluated using the Student's t-test.

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Results

Experiment 1 – aerial spray treatment

November 2007 treatments

Six months after the November 2007 treatment, bioassays were undertaken using both the canopy twig assay with *Phytophthora ramorum* and the *in situ* bole inoculation assay with *P. gonapodyides.* Results of these assays indicated that aerial application of phosphonate at either the low or the high dose tested reduced lesion size in canopy twigs and in tree boles compared to untreated controls. Mean twig lesion length for the low dose and high dose did not differ from each other but were significantly smaller than those in untreated controls (Table 2).

May 2008 treatments

There was no significant treatment difference in lesion length on canopy twigs 7 months following the spring aerial application with phosphonate. There was a significant block (site) effect (P = 0.03); twigs from one treatment block having a smaller mean lesion length than those from the other two blocks. Examined separately, one of the blocks showed that both the high and low doses of phosphonate reduced lesion length in canopy twigs compared to controls, but this effect was not apparent in the other two blocks. Considerable variation in the high dose plots may relate to high-temperature injury from unusually warm weather during the first few weeks following treatment. Damage was evident not only in the treated areas but also in nearby untreated forests. The injury may have caused bias when collecting canopy twigs for the assay; branch ends most exposed to aerial spray are also the ones that would have been most exposed to sun and heat, and severely damaged twigs would have been excluded from collection.

Aerial treatment with phosphonate reduced *Phytophthora gonapodyides*-induced bole lesion size 7 months post-treatment. Mean lesion area for the low and high application rates did not differ from each other but were significantly smaller than those of untreated controls (Table 2). The treatment effect was still significant 18 months post treatment (Table 2).

In the log assays, differences among treatments were significant for *Phytophthora ramorum* but not for *P. gonapodyides*, although the trend was similar

TABLE 2: Mean lesion sizes resulting from challenge inoculation with *Phytophthora gonapodyides* or *P. ramorum* following treatment with phosphonate. For the canopy twig assay, lesion size is expressed as length, mm. For the *in situ* bole inoculation and the log inoculation, lesion size is expressed as area, mm^2 and cm^2, respectively. Numbers with the same letters within a row for each application method are not significantly different (Fisher's LSD, P < 0.05).

Treatment date	Assay date and lession type	Aerial spray treatment			Injection treatment		
		Control	*Low dose*	*High dose*	*Control*	*Treated*	*% Change*
November 2007 (autumn)	**May 2008 (6 months post-treatment)**						
	Canopy twigs (mm)	39.1a	32.1 b	33.9 b	40.3a	37.6a	7
	In situ bole (mm^2)	1959 a	1027 b	832 b	1065 a	268 b	75
May 2008 (spring)	**December 2008 (7 months post-treatment)**						
	Canopy twigs (mm)	46.5a	43.9a	45.8a	47.6a	45.8a	4
	In situ bole (mm^2)	1535 a	787 b	707 b	2366 a	37 b	98
	Log – *P. ramorum* (cm^2)	249.2a	148.1 b	201.4ab	205.8a	81.8 b	60
	Log – *P. gonapodyides* (cm^2)	55.8a	36.4a	45.3a	63.6a	29.1 b	54
May 2008 (spring)	**December 2009 (18 months post-treatment)**						
	In situ bole (mm^2)	2645 a	719 b	623 b	4741 a	104 b	98
November 2008 (autumn) and May 2009 (spring)	**December 2009 (7 months post-treatment)**						
	Canopy twigs (mm)	40.7a	39.2a	35.8a	55.2a	37.6 b	32
	In situ bole (mm^2)	2607 a	549 b	539 b	1637 a	37 b	98

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(Table 2). The *P. gonapodyides* assay was confounded by excessive variation among the treatment blocks. Lesions caused by *P. ramorum* were approximately 5 times larger than those caused by *P. gonapodyides*. *Phytophthora ramorum* lesion size was smallest for the low dose plots, however, there was no difference between the high dose and controls (Table 2).

November 2008 and May 2009 treatments

There was no significant difference among treatments in lesion length on canopy twigs 7 months following the second of two aerial applications of phosphonate. Bole lesions from *in situ P. gonapodyides* inoculation 7 months post-treatment were significantly smaller in treated trees compared to controls, but there was no difference between the high dose and low dose (Table 2). Re-isolation from lesion margins confirmed the presence of the *Phytophthora* species used in the inoculation, independent of treatment.

Experiment 2 – tree injection treatment

The autumn-only (November) trunk injection of phosphonate had no significant effect on lesion size in canopy twigs. Trunk injection however, significantly reduced the area of subsequent *Phytophthora gonapodyides*-induced bole lesions compared to untreated controls (Table 2).

Spring-only (May) trunk injection had no significant effect on lesion size in canopy twigs 7 months following treatment, but it reduced the area of subsequent *Phytophthora gonapodyides*-induced bole lesions by 98 percent compared to untreated controls (Table 2). The treatment effect remained significant 18 months after treatment (Table 2). In the log assays, mean lesion size differed significantly among treatments for both *P. ramorum* and *P. gonapodyides* inoculations. The spring only treatment reduced mean lesion area from 64 cm^2 (control) to 29 cm^2 (treated) for *P. gonapodyides* and from 206 cm^2 (control) to 82 cm^2 (treated) for *P. ramorum*.

Spring and autumn injection (the same trees injected each time) reduced mean lesion size in *P. gonapodyides in situ* bole inoculations by 98% compared to controls and in and detached canopy twig assays by 32% compared to controls (Table 2). Re-isolation from lesion margins confirmed the presence of the *Phytophthora* species used in the inoculation, independent of treatment.

Experiment 3 – potted seedlings

Foliar application of phosphonate did not prevent infection by *P. ramorum* in any of the seedling treatments and the percentage of seedlings infected did not differ significantly among treatments (Figure 1). The proportion of stem length with lesions was much greater in seedlings inoculated in the growth chamber than in seedlings exposed to natural inoculum beneath infected tanoak trees, but there was no significant difference between treatments in either the field

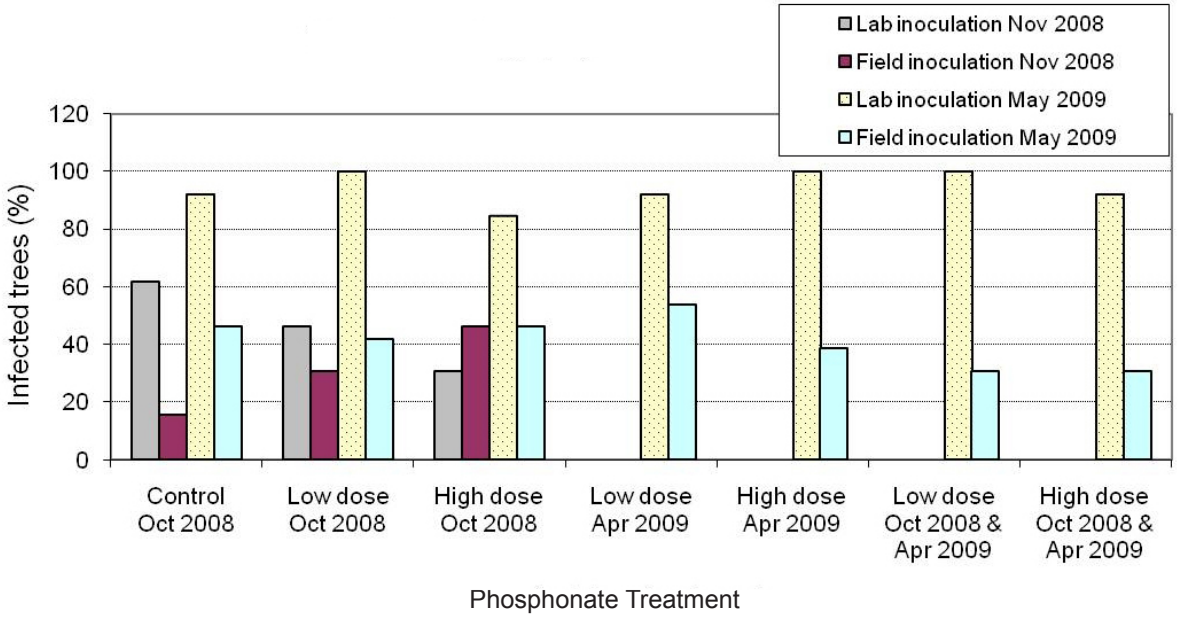

FIGURE 1: Incidence of infection (percentage of seedlings infected) for tanoak seedlings exposed to *Phytophthora ramorum*, either as an artificial inoculum (in growth chamber with zoospore suspension) or as a natural inoculum (beneath infected tanoak trees) after treatment with phosphonate at low dose (2.9 kg a.i. ha^{-1} equivalent), high dose (17.35 kg a.i. ha^{-1} equivalent), or unsprayed. Each combination of treatment and exposure consisted of 13 seedlings.

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(P = 0.24) or growth chamber (P = 0.29) inoculations (Figure 2). The difference between the growth chamber and field inoculations most likely reflects the very high concentration of zoospores used in the artificial inoculation. It is unlikely that such high concentrations exist in rainfall or canopy drip from infected tanoak trees. There was no evidence of phytotoxicity in any of the treatments.

Experiment 4 – stump-sprout spray

Three months following treatment there were significant differences (P < 0.001) in the response of sprouts to challenge by inoculation; lesion length on sprouts sprayed at the high dose averaged 44 mm compared to 78 mm for unsprayed controls. There were no obvious symptoms of phytotoxicity resulting from the spray treatments. We were unable to complete further sampling and analysis for this trial as the plots were prematurely destroyed.

Discussion

Aerial application of phosphonate, at all rates and times assessed, to the canopy of a tanoak forest had a consistent and significant effect on size of bole lesions produced by *Phytophthora gonapodyides in situ* and by *P. ramorum* but not *P. gonapodyides* in log assays. These results demonstrate clearly the uptake of chemical into stems. Aerial treatment of tanoak trees reduced subsequent induced bole lesion size from between 48% and 79% compared to controls, depending on the test. The effect of treatment persisted for 18 months following a single spring aerial application. There were no consistent differences between the high and low fungicide doses.

Aerial application was not consistently effective at reducing lesion size in detached canopy twigs. Of the three aerial spray treatments, only one (November 2007 spray) demonstrated that treatment reduced canopy twig lesions, but the maximum reduction in lesion length was small (18%).

Both the growth room and field tests of sprayed seedlings were discouraging because none of the foliar treatments prevented seedlings from becoming infected with *Phytophthora ramorum*. Also, there were no differences between treatments in the proportion of seedling stem length with lesions. The culture assays used to assess seedling infection were not as refined as for the bole lesion area or twig assays, and we did not have as many replications, but the seedlings clearly were not protected by phosphonate.

The most effective treatment involving foliar application was the spraying of stump-sprouts. In this experiment, the high dose treatment reduced lesion length on detached spouts by 44% compared to controls. Stump sprouts differ from trees, consisting entirely of vigorous, rapidly elongating succulent

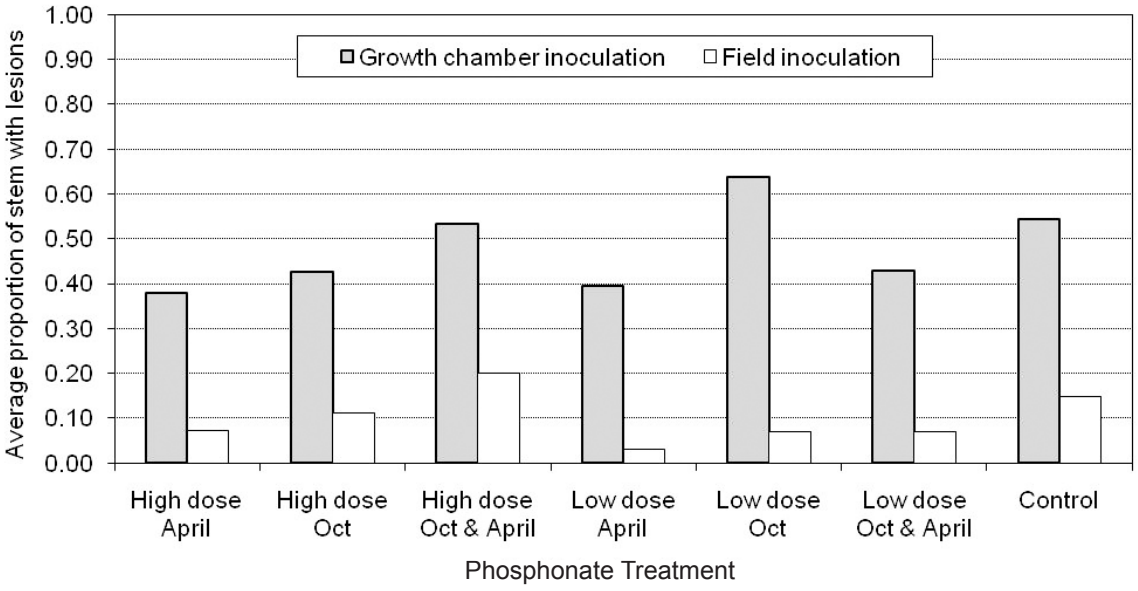

FIGURE 2: Mean proportion of stem length with lesions for tanoak seedlings exposed to *Phytophthora ramorum*, either as an artificial inoculum (in growth chamber with zoospore suspension) or as a natural inoculum (beneath infected tanoak trees) after treatment with phosphonate at low dose (2.9 kg a.i. ha^{-1} equivalent), high dose (17.35 kg a.i. ha^{-1} equivalent), or unsprayed. Each combination of treatment and exposure consisted of 13 seedlings.

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shoots with foliage throughout their length, so these results may not be comparable to twigs on mature trees. The effectiveness of the stump-sprout spray treatment could also be due to the spray technique used in the experiment. Greater coverage of foliage may be obtained by the backpack sprayer used here compared to a straight overhead and higher elevation of aerial application used in Experiment 1.

Injection of fungicide directly into trees (Experiment 2) consistently and significantly reduced lesion size in the subsequent *Phytophthora gonapodyides* bole assays by 75% to 98% compared to controls, but results from canopy twig assays were variable. Neither the November 2007 nor May 2008 treatments affected canopy twig lesion size, but the November 2008 and May 2009 treatments reduced canopy twig lesion length by an average of 32%. On a per-hectare basis, the amount of phosphonate applied by injection (34.5 kg a.i. ha^{-1}) was slightly greater than the low dose aerial rate, so the results are comparable in that respect. The difference in results between injection and foliar spray probably relates to the more efficient and direct delivery of chemical by injection in contrast to the more variable aerial application to a mature forest. Canopy twig assay results may also be confounded further by the high zoospore concentrations which could override a treatment effect. The high zoospore concentration (1×10^4 per mL) used in the canopy twig assay could overwhelm any fungicidal or defense response induced by phosphonate that might be significant under the much lower inoculum density likely to be present in the upper canopy of a forest. Similarly, a twig dip assay may not yield the same results as a challenge inoculation using sporangia and zoospores applied directly to the surface of leaves and twigs, which would better mimic natural infection in forest trees. Chemical methods have been used to determine phosphonate concentrations in plant tissues (Hardy et al., 2001; Smillie et al., 1989). Using this technology may have improved interpretation of our results.

The ongoing Sudden Oak Death control program in Oregon required prompt eradication of infected trees, and prevented us from testing the efficacy of phosphonate applied aerially to reduce natural infection of tanoak. Further complications to the large scale testing and use of phosphonate arise from ownership patterns, land management objectives, terrain, and forest composition in south-western Oregon. Specifically, industrial landowners have little incentive to protect tanoak which is of low commercial value compared to Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) or other valuable timber species. Small privately-owned properties and rural residential settings do not offer the large treatment areas necessary to make aerial spray applications practical or cost effective. The conservation management objectives of both federal forest management agencies and large non-profit land conservancies are theoretically more amenable to use of phosphonate to protect stands of tanoak, but cultural, and sometimes legal, constraints to fungicide use present obstacles. The steep terrain in coastal south-western Oregon is heavily dissected by streams which would have to be buffered from aerial spraying. Most of the forest is not stands of pure tanoak, but a mixture of tanoak, conifers, and hardwoods of various sizes and canopy positions, all of which limit the amount of forest suitable for an aerial application.

Despite these challenges, aerial application of phosphonate may yet play a role in managing sudden oak death in tanoak and other forests. The results from this study indicate that phosphonate is translocated into the tree following aerial application with some effect on disease development when measured using bioassays. In western Australia, foliar application of phosphonate reduces the impact and spread of *Phytophthora cinnamomi* in a variety of plant species in several natural plant communities (Hardy, 2000; Tynan et al., 2001). Phosphonate treatments do not kill the pathogen, nor do they protect plants from infection, but they do reduce the amount of plant death and can slow disease extension in woodlands (Shearer et al., 2004; Shearer et al., 2006). To be effective at slowing or stopping spread of *P. ramorum* in Oregon forests, phosphonate treatments must prevent or reduce infection or sporulation on tanoak. None of our studies suggest that phosphonate prevented infection, but they did demonstrate that treatment slowed disease development in plant tissues. We did not assess the effect of phosphonate treatment on sporulation, but studies with *P. cinnamomi* in Australia show that phosphonate treatments reduce but do not prevent production of viable zoospores on infected trees (Wilkinson et al., 2001). It is possible the small differences in infection and disease development we measured by challenge inoculation are sufficient to significantly reduce natural spread of disease in the forest, however this has yet to be determined. Additional work is, therefore, necessary to determine if phosphonate can reduce spread of sudden oak death in tanoak forests under natural inoculum conditions.

Conclusion

These results demonstrated uptake of phosphonate applied aerially to the canopy of a tanoak forest. The treatment resulted in reduced lesion size in bole inoculations, but had a small and inconsistent effect on lesion size in canopy twigs. Seedlings treated with phosphonate were not protected from infection by *Phytophthora ramorum* when exposed to artificial or natural zoospore inoculum. Whether aerial applications of phosphonate will significantly affect spread of sudden oak death in tanoak forests has yet to be determined.

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