Calcium sulphate soil treatments augments the survival of phosphite-sprayed *Banksia leptophylla* infected with *Phytophthora cinnamomi*

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Abstract

The application of either phosphite or calcium salts to plants that are susceptible to *Phytophthora* spp. protects them from infection and the development of disease symptoms. This suggests that there may be an additive protective-effect when they both are applied together. The combined effect of foliar phosphite and soil calcium levels on the health and survival of *Banksia leptophylla* seedlings infected with *Phytophthora cinnamomi* was investigated. Six month-old *Banksia leptophylla* plants grown in sand supplemented with 0, 3, 10 or 30mM calcium sulphate were sprayed with 0, 0.1 or 0.3% phosphite and inoculated with *P. cinnamomi.* Plant survival and health were recorded for 12 months after inoculation. The combination of foliar-phosphite spraying with the supplementation of sand with calcium sulphate significantly increased the survival and health of plants infected with *P. cinnamomi*. There was 2.7% survival of plants with no phosphite or additional calcium, 8.3% survival with 30 mM calcium alone, 53 % survival with 0.3% phosphite alone and 100% survival of plants given 0.3% phosphite and 30mM calcium. The pathogen survived in the sand of all treatments for the 12-month period of the trial. Combining foliar-application of phosphite with addition of calcium sulphate to soil is a cheap and practical way of significantly increasing the efficacy of phosphite in controlling the development and spread of *Phytophthora* dieback disease. A mechanism involving inhibition of calcium-dependent ATPases by phosphite and pyrophosphate, and the subsequent disruption of calcium ion signaling, is discussed.

Keywords dieback. disease control. gypsum . phosphonate

Introduction

Phytophthora cinnamomi is a soil-borne plant pathogen destroying billions of dollars worth of agricultural and horticultural crops worldwide, and is a major threat to natural ecosystems (Zentmyer, 1980; Erwin and Ribeiro, 1996). Disease caused by *P. cinnamomi* (often referred to as *Phytophthora* dieback) is difficult to control in horticultural or natural environments. The pathogen infects a very broad range of species (Cahill *et al.*, 2008), and spreads easily in warm, wet conditions (Erwin and Riberio, 1996). It is a root pathogen that invades and destroys the lateral and fine root systems, and collars of trees in successive rounds of infection that leave the plant vulnerable to abiotic stresses such as drought, heat and poor nutrition (Zentmyer, 1980; Shearer and Tippett, 1989). *Phytophthora* is particularly damaging in the biodiverse regions in the South West Botanical Province of Western Australia's sand plains and jarrah (*Eucalyptus marginata*) forest (Shearer and Smith, 2000), with 2284 of the 5710 described plant species in the Province being susceptible to *P. cinnamomi* and of these, 800 being highly susceptible to the pathogen (Shearer *et al.*, 2004).

Since the 1970s, control of *Phytophthora* plant pathogens has been achieved by the application of phosphite, a chemical analogue of orthophosphate, which is applied either as a soil drench, trunk injection or foliar spray (Hardy *et al.*, 2001). Phosphite is thought to control disease symptoms and reduce pathogenic lesions by stimulating the plant defense responses and/or directly inhibiting pathogen growth (Fenn and Coffey, 1984; Guest and Grant, 1991; Jackson *et al.*, 2000). The precise mechanism remains unknown, although it is thought likely that phosphite acts primarily by substituting for orthophosphate in cell's phosphate cycle (Martin *et al.*, 1989; Guest and Grant, 1991; Barchietto *et al.*, 1992; Niere *et al.*, 1994).

Calcium ions also affect the interaction between *Phytophthora* species and their plant hosts. When calcium ions are included in soil or irrigation solutions, plants infected with *Phytophthora* show increased resistance to disease as judged by symptom development and time to death (von Broembsen and Deacon, 1997; Sugimoto *et al.*, 2008; Serrano *et al.*, 2012). However, as with phosphite, there is debate over whether the control of disease by calcium ions is primarily a result of calcium stimulating plant defense (defense priming) (Stab and Ebel, 1987; Sugimoto *et al.*, 2008), inhibition of sporangiogenesis in *Phytophthora* spp. (Messenger *et al.*, 2000; Serrano *et al.*, 2012), or a combination of both (Sugimoto *et al.*, 2008). This is because there is no significant relationship between inhibition of pathogen growth rate *in vitro* and disease reduction (Wilkinson *et al.*, 2001; Sugimoto *et al.*, 2008), and the fact that removal of extracellular calcium abolished an elicitor-mediated phytoalexin-response in soybean cells (Stab and Ebel, 1987).

The rapid and dramatic changes in cytosolic calcium ion concentrations, known as calcium signatures, are a vital part of the signal transduction pathways that enable cells to respond to the environmental and developmental cues necessary for survival (Tuteja, 2008). These calcium signatures are responsible for initiating physiological events as diverse as stomatal closure in response to abscisic acid (ABA) in plants

(Hirayama and Shinozaki, 2007), and exocytosis due to stimulation of phospholipase D (PLD) in neuroendocrine cells (Vitale *et al.*, 2002).

As cytosolic calcium levels reflect external concentrations (Jackson and Heath, 1989; Xu and Morris, 1998; Connolly *et al.*, 1999) it is possible that calcium signatures may be influenced by manipulation of the external environment. The initial transient rise in cytosolic free calcium required to induce cytokinesis in zoosporangia in *P. cinnamomi* (Jackson and Hardham, 1996) is subverted by either a higher internal (or lower external) background calcium ion concentration. High extracellular calcium concentrations are known to interfere with zoosporogenesis in *P. parasitica* and reduce the ability of zoospores to infect plants (von Broembsen and Deacon, 1997) and Messenger *et al.* (2000) have shown that sporangial production and inoculum load of *P. cinnamomi* in gypsum (calcium sulphate) amended soil is significantly reduced. Additionally Irving *et al.* (1984) found that successful zoospore encystment required excretion of 30% of internal calcium suggesting that co-ordinated regulation of intracellular calcium ion concentration is important at various stages of the pathogen life cycle.

Despite significant differences in charge, size and function, calcium ions and phosphite ions have similar effects on *Phytophthora* spp. as well as various fungi and plants (Table S.1, supplementary information), suggesting that there may be some interaction in the biochemical pathways they perturb. For example, both calcium ions and phosphite inhibit sporangiogenesis in *Phytophthora* spp. (Farih *et al.*, 1981; Coffey and Joseph, 1985) and cause increased phytoalexin production in plants (Blume *et al.*, 2000; Suddaby *et al.*, 2008). While both calcium ions and phosphite salts alone reduce disease symptoms caused by *Phytophthora* spp., the interactions between the two treatments have not been examined. The similar antipathogenic-effects of calcium and phosphite suggests that some mechanistic synergy may exist that could be exploited by treating plants with both chemicals simultaneously.

We investigated the disease control of *P. cinnamomi* by foliar application of phosphite in *Banksia leptophylla*, growing in pots of sand supplemented with various levels of calcium. The information gained from investigating the combined effect of applying these two chemicals simultaneously may result in improved disease control, and may also indicate the mode of action of phosphite, and hence illuminate the pathogenic processes, in *P. cinnamomi*.

Materials and methods

Experimental design

The independent variables were calcium ion concentration supplementation of soil and the concentration of phosphite applied to foliage. Dependent variables were: time to first symptom and death; plant height (for non-inoculated, treated plants) throughout the trial; plant dry weight and root mass and gross morphology at the end of the trial. The trial was conducted in an evaporative cooled glasshouse over 18 months from

April 2008 until November 2009. Plants were inoculated in November 2008. On average, daily minima were between 9 – 10^{0}C and maxima were 27^{0}C (+/-6^{0}C) with a median daytime temperature of 25^{0}C. The total number of pots was 120 and there were six pots per treatment. A factorial nested pot design (Doncaster and Davey, 2007) was used in which there were 20 combinations of soil calcium supplementation and foliar phosphite application (Table 1). Each treatment was represented once in each block, by a pot containing six seedlings, and there were six blocks. The null hypothesis predicts that the joint application of both calcium and phosphite to *P. cinnamomi* infected plants will not result in enhanced growth or survival when compared to the growth and survival of plants treated with either phosphite or calcium alone.

Insert Table 1

Plant material

Seeds of *Banksia leptophylla*, a woody shrub from calcareous soils in the south-west of Western Australia that is susceptible to *P. cinnamomi* (Shearer, unpub) were obtained from Nindethana Seed Service Pty LTD (Albany, Western Australia). These were surface sterilised for 1 min in 0.5% sodium hypochlorite, rinsed three times in deionised water prior to planting in trays containing washed, pasteurized yellow river sand. Trays were placed in a glasshouse at 27^{0}C, +/- 6^{0}C and watered twice daily. At 8 weeks seedlings were transplanted into individual free-draining 50 mm polyurethane pots containing washed, pasteurized river sand and watered twice daily to container capacity until they were 6 months old.

Calcium sulphate treatment of soil

Six-month old seedlings were transferred to 150 mm diameter free-draining polyurethane pots that had been lined with shade cloth, filled with washed river sand (approximately 6 kg) that had been dried and mixed in a cement mixer with technical grade hydrated calcium sulphate (Sigma) to final concentrations of 0, 3, 10 and 30 mmol $CaSO_4.2H_2O$ kg^{-1} sand. Calcium sulphate was chosen because it is non-toxic, relatively soluble in water (compared to calcium carbonate) and does not alter the pH of the soil (Shainberg *et al.*, 1989). Filled pots were then pasteurized once for 2 h at 60^{0}C. Six *B. leptophylla* seedlings were planted in a circle around three sterile polyurethane inoculation tubes (20 cm long, 2 cm diameter) in each pot. Plants were placed in a glasshouse at 25^0 C, +/- 6^0 C and hand watered to container capacity once daily with deionised water (approximately 500ml per pot).

Calcium sulphate suspension in deionised water was applied twice a week at a rate of approximately 500 ml per pot and at the concentrations of 0, 3, 10 or 30 mM. Five hundred ml of diluted liquid Native Focus (5ml L^{-1}), (Growth Technology, Australia and United Kingdom) was applied per pot twice a month

throughout the trial. The fertilizer treatment replaced the watering regime each time it was applied. At 4 months post inoculation calcium sulphate treatments were reduced to once a week due to minor calcium toxicity symptoms in the 30 mM calcium sulphate treatment groups. When plants were 12 months old (6 months after inoculation) watering was reduced to alternate days and calcium sulphate treatments maintained at once a week. This was continued until 11 months post-inoculation, when watering was reduced to every third day in order to further stress the plants. Plants were harvested 12 months post-inoculation.

Phosphite treatment of plants

Eight-month old plants were treated with phosphite 2 weeks prior to inoculation. Phosphite (600g L^{-1}, Agrifos) was prepared to a final concentration of 0.1% and 0.3% in deionised water containing 0.1% Pulse (Nufarm, Australia) as a penetrant and applied to plants foliage using a handheld sprayer. Non-phosphite control plants received 0.1% Pulse in deionised water only. Plants were hand-sprayed to the point of run-off (approximately 3.3 ml per plant) and care was taken not to contaminate the sand. After phosphite application plants were not watered for 48 h to allow absorption of phosphite. The 0.3% phosphite spray equates to 7.5 Kg of phosphite ha^{-1} applied at a rate of 18L per 100 m^2.

Preparation of inoculum and inoculation of plants

Pine (*Pinus radiata*) plugs were prepared according to the method of (Shearer *et al.*, 2004) and were inoculated with recently passaged cultures of *P. cinnamoni*, isolate MP 94.48 (Murdoch culture collection) grown on V8 agar for 1week. The isolate had been passaged through "Granny Smith" apples prior to use in order to ensure pathogenicity. This isolate was chosen because it is known to be one of the most pathogenic isolates on susceptible plants (Huberli *et al*., 2000). Inoculated pine plugs were incubated for 8 weeks at 20^oC, +/- 2^oC, in the dark, with occasional shaking. Control plugs were prepared by autoclaving colonised pine plugs for 30 minutes at 121^0C on 2 consecutive days. Prior to inoculation of pots, samples of control and colonised plugs were placed onto a *Phytophthora* selective medium (NARPH) (Huberli *et al.*, 2000) to ensure pathogen viability, and that autoclaved control plugs were sterile.

When plants were 8 months old, the three inoculation tubes were removed from the pots and one colonised pine plug per hole was inserted and covered with pasteurized river sand. Control pots were inoculated with colonised plugs that had been autoclaved. The base of each pot was placed into a water-tight plastic bag, and pots watered to soil saturation with deionised water and left overnight (12 h) to facilitate infection of roots through sporangial production and zoospore release from the pine plugs. Bags were then removed and pots were free draining for the remainder of the experiment. Control non-inoculated pots were placed into an additional pot to prevent cross contamination.

Monitoring of disease symptoms, plant harvests and recovery of pathogen

The development of disease symptoms was assessed at least once a week by recording leaf yellowing, leaf wilting, plant discolouration and plant drying. The time to first symptom is defined as the number of days post-inoculation until the development of leaf yellowing. Disease lesions were not always visible on the stem of the plant. Plant death was deemed to have occurred when the plant turned brown and leaves were crisp. The mean survival time of plants in the different treatment groups was calculated by dividing the sum of the total number of days the plants remained alive by the number of plants in the group. The height of the control, non-inoculated plants was measured five times during the trial. Shoots of dead plants were cut at the base. The basal three centimeters of stem was blotted dry and cut into three, one-cm sections. The stem sections were aseptically cut in half longitudinally and the internal stem surface placed onto NARPH and incubated in the dark at 25^0 C for up to 4 days, for recovery of the pathogen. A minimum of one dead plant per pot was tested in each treatment. At the end of the experiment, 12 months post-inoculation, the height of all surviving and control plants were measured, shoots removed, weighed and dried at 37^o C for 6 weeks until constant weight. Roots were removed from 1-4 pots per treatment, washed gently in water and photographed. It was not possible to extract roots with sufficient accuracy to make weights meaningful but records were made of root structure and the abundance of cluster roots. The size of the plants in relation to the pot size made it inappropriate to continue the trial for longer.

Soil was removed from at least two pots from each treatment group, mixed thoroughly then spread 3 cm deep in a 17 x 11 cm plastic food container and flooded with deionised water to cover the soil to a depth of 3 cm. Soil and water were stirred and sediments allowed to settle prior to baiting for *P. cinnamomi* by floating *Hibbertia scandens* petals and *Eucalyptus sieberi* cotyledons on the surface of the water. This water is referred to as bait-water. Containers were partially covered and incubated in the glasshouse at an average temp of 25^0 C until the petals became translucent and water-soaked and the *E. sieberi* cotyledons changed colour from purple to green. Infected baits were recorded and removed, blotted dry and plated onto NARPH to confirm the presence of the pathogen.

Chemical analysis of plant material

Three independent samples of dried foliage from within each treatment group were pooled, weighed, ground to a fine consistency, and acid digested with nitric acid and hydrogen peroxide and analysed according to the method of Huang *et al.* (2004) for total plant calcium and phosphorus.

Chemical analysis of soil

Soil was analysed for calcium ions and total phosphorus from 3 -10 pots per calcium treatment. As phosphite was applied to leaves and not soil it was valid to bulk results of soil from the same calcium treatments, but differing phosphite applications. For each individual pot, all plant material was removed and soil was mixed thoroughly. A sample of soil from each pot was dried at 37^o C, and 10g of dry soil was

then shaken overnight with 40 ml of deionised water. The filtrate was filtered through a 22 µm filter (Millipore, Australia) and analyzed on a Varian (Vista AX) Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) simultaneously for calcium ions and phosphate (Determination of elements in waters and other appropriate solutions by ICP-AES, Marine and Freshwater Research Laboratory: ICP 001 instrument). Conductivity of the aqueous extract was measured to estimate soil salinity. The pH of the soil was measured regularly during the trial using litmus paper applied to moist soil. pH of the baiting water was also tested.

Statistical analysis

Multivariate analysis of variance (MANOVA) was used to examine the relationships between the categorical predictor (independent) variables of foliar phosphite concentration and rate of soil calcium sulphate application on the dependent variables of time to first symptom, survival, mortality (%) at 41, 84, 157 and 356 days post inoculation, and dry weight of surviving plants at the end of the experiment. Where significant effects (interactions) were found, univariate analysis and post-hoc tests were performed to determine the significance of the results. All analysis used a significance level of 0.05. Statistical calculations were performed using STATISTICA software (StatSoft), using a fitted generalized linear model with an identity link function.

Results

The effect of phosphite and calcium sulphate on the development of dieback symptoms

The application of foliar phosphite in combination with soil calcium sulphate supplementation had a significant effect ($p < 0.001$) on increasing the length of time taken to the development of the first symptom of dieback (i.e. leaf yellowing) (Fig. 1). At all calcium concentrations, the time to yellowing significantly increased with phosphite (Phi) concentration from 28 days (Phi = 0, Ca = 0) to 336 days at Phi = 0.3%, Ca = 30 mM. At each phosphite concentration, time to yellowing increased with increasing calcium concentration; from 28 to 78 days at Phi = 0, and 259 to 336 days at Phi =0.3%. There was no significant difference between the number of symptom-free days in non-inoculated controls and the *P. cinnamomi* inoculated Phi = 0.3%, Ca =30 mM treatment groups at 12-months post inoculation (data not shown).

Plant survival

Treatment of plants with increasing phosphite alone, or increasing soil supplementation with calcium alone, significantly increased the survival time of inoculated plants when compared to untreated inoculated controls (Table 2; Fig. 2). In the absence of calcium treatment, mean survival time post-inoculation increased from 47 days in the untreated infected group, to 320 days in the presence of 0.3% phosphite, and the number of plants surviving at the end of the trial increased from one to 19 (Fig. 2). In the absence of phosphite treatment, soil supplementation with increasing calcium concentration only increased survival

from 47 days at Ca = 0 mM, to 102 days at 30 mM Ca, and did not significantly improve plant survival 12-months post inoculation. The joint application of increasing phosphite and soil calcium sulphate concentration increased the number of *B. leptophylla* plants that survived, and their survival time, after soil inoculation with *P. cinnamomi* when compared to plants that had been treated with phosphite only, or calcium only. At 30 mM calcium supplementation increasing phosphite application to 0.3 % improved the average survival time of infected plants from 102 to 356 days, i.e. 100% survival at the end of the experiment. The survival time of plants sprayed with 0.3 % phosphite and grown in soil amended with increasing amounts of calcium did not improve significantly between treatments, and the number of plants surviving at trial harvest 12-months after soil inoculation was not significantly different from comparable non-inoculated treatment groups.

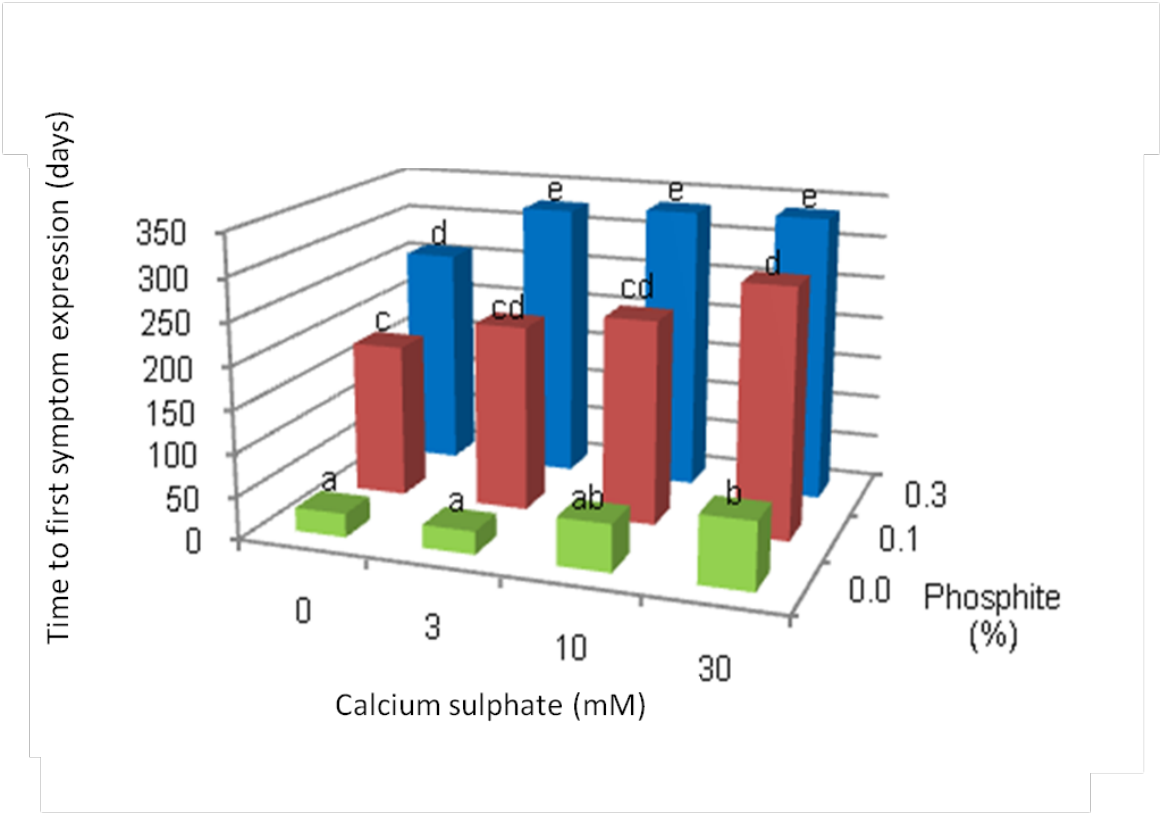

Fig. 1 Mean number of days to development of the first symptom of dieback (i.e. leaf yellowing) following treatment of *Banksia leptophylla* with phosphite and calcium sulphate and soil inoculation with *Phytophthora cinnamomi*. Significant ($p < 0.05$) differences in applying univariate analysis are denoted by lower case letters representing results of the post-hoc test (Tukey) of the combined foliar phosphite and soil calcium sulphate treatments. Treatment block combinations of calcium sulphate and phosphite are indicated by the *X* and *Z* axis, respectively.

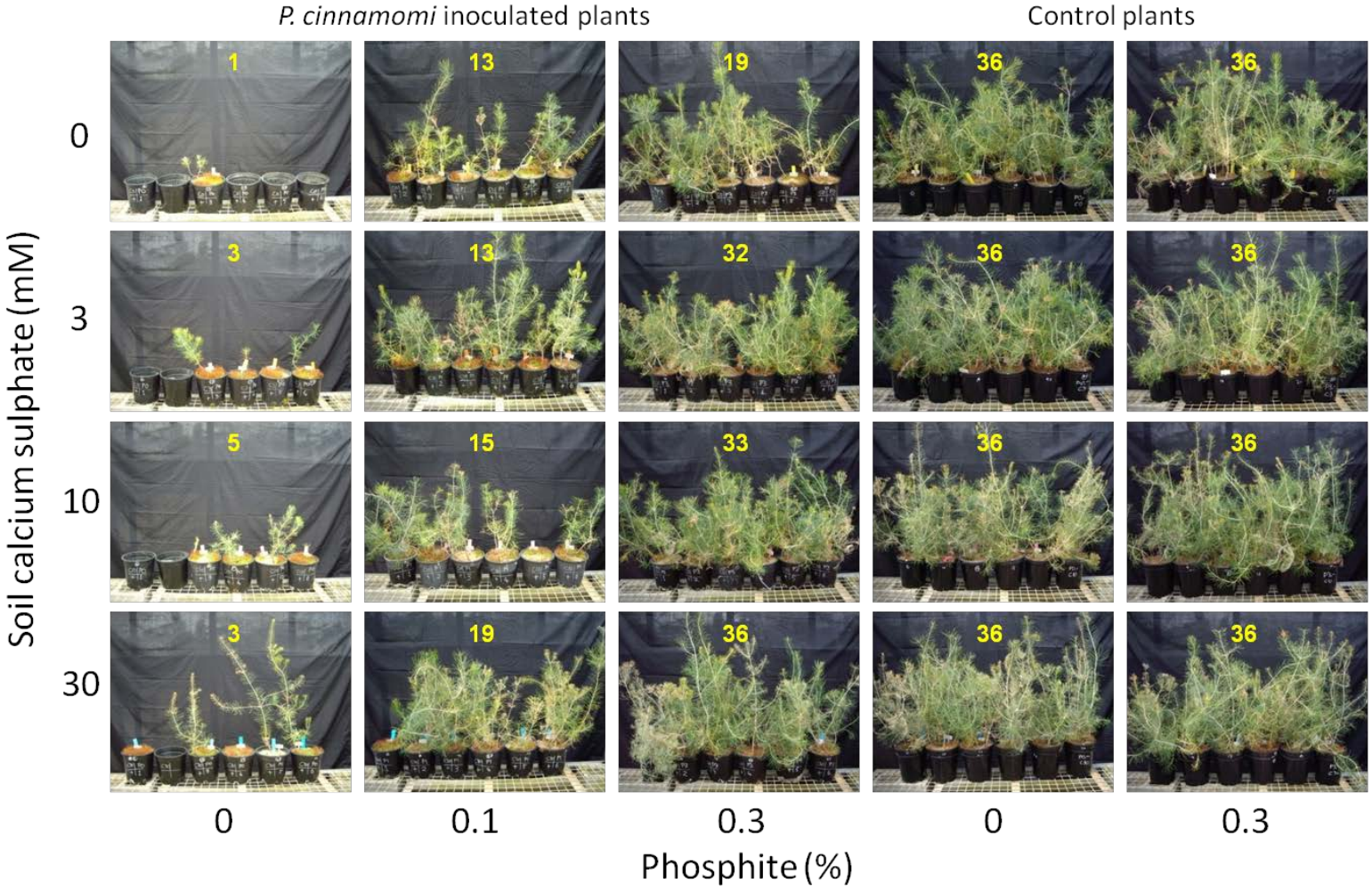

Fig. 2 Foliage of surviving inoculated and control *Banksia leptophylla* plants *in situ* 12-months after soil infestation with *Phytophthora cinnamomi* in each combination of treatment of phosphite and calcium. Soil was amended with calcium sulphate at concentrations of 0, 3, 10 and 30 mM in combination with phosphite spray concentration at 0, 0.1% and 0.3% as indicated. From left to right, columns 1 – 3 are inoculated plants and columns 4 and 5 are non-inoculated control plants. The number in the top-centre of each treatment block is the number of survivors (out of a total of 36 plants) at the time of harvest at 12-months post-inoculation.

Insert Table 2

Shoot health and growth of plants

The above ground condition of plants that had survived 12-months post-inoculation clearly demonstrated the effect of both calcium and phosphite treatments on plant health and survival (Fig. 2). The surviving foliage of infected plants that had not been treated with phosphite was obviously stunted and little difference between calcium treatments could be observed. Treatment with 0.1% phosphite and increasing soil calcium improved the condition of the foliage *pro rata*. The foliage of infected plants that had been treated with 0.3 % phosphite and increasing soil calcium appeared normal and was comparable with the foliage of non-inoculated similarly treated control plants. The condition of the surviving foliage in the phosphite-only treatment groups improved with phosphite application and the number of surviving plants increased from one plant at no phosphite treatment, to 19 plants when treated with 0.3% phosphite.

Plant height and dry weight at harvest

There was no significant difference in plant height or dry weight of foliage in the non-inoculated control treatment groups of plants 12-months post-inoculation of the trial (data not shown), demonstrating that any increase in size of the inoculated plants given higher calcium levels was not a response to calcium or phosphite treatments *per se*. There were insufficient survivors in all the inoculated treatment groups at the end of the experiment to perform a meaningful statistical analysis.

The effect of *P. cinnamomi* on root growth and condition

Accurate measurement of root dry weight was not possible due to the presence of cluster roots, soil compaction, and the fragility of the fine and lateral root system of *P. cinnamomi* infected and control plants across treatments, i.e. soil washing resulted in an unavoidable and unacceptable loss of roots. At the end of the experiment the roots of dead or infected plants were found to be smaller, darker, and had fewer lateral branches and fine roots than uninfected plants or plants that had been grown in soil amended with 30 mM calcium and sprayed with 0.3% phosphite (Fig. 3 and 4). Neither calcium soil amendment nor phosphite treatment had any observable effect on the size of the root mass of non-infected control plants when compared to untreated uninfected plants (data not shown). Uninfected control plants from pots treated with 30 mM calcium sulphate contained a greater number of cluster roots than with no calcium supplementation, but when plants were sprayed with 0.3% phosphite this increase in cluster roots with higher calcium was not observed (data not shown).

Recovery of *P. cinnamomi* from dead plants, pine plugs, and baiting of infected soil 1-year post-inoculation

Phytophthora cinnamomi was recovered from 70% of dead plants from infested soil across all treatments when stems were placed onto NARPH. Three plants from a single pot in the control group died towards the end of the trial, however there was no evidence of root rot and no *P. cinnamomi* was recovered on the selective medium. *Phytophthora cinnamomi* was recovered from all pine plugs tested 12-months post-inoculation when plated onto NARPH indicating that the pathogen was still viable at the end of the experiment.

Phytophthora cinnamomi was detected in the bait-water from soil from infested pots with all combinations of calcium and phosphite treatments except for soil from the 0.3% phosphite and 30 mM calcium treatment. The number of baits infected after 48 h incubation showed that infection rate decreased with increasing calcium amendment and with increased phosphite spraying (Table 3). Plants treated with either 0.1 % or 3 % phosphite in the absence of calcium did not reduce the number of baits infected after 48 h.

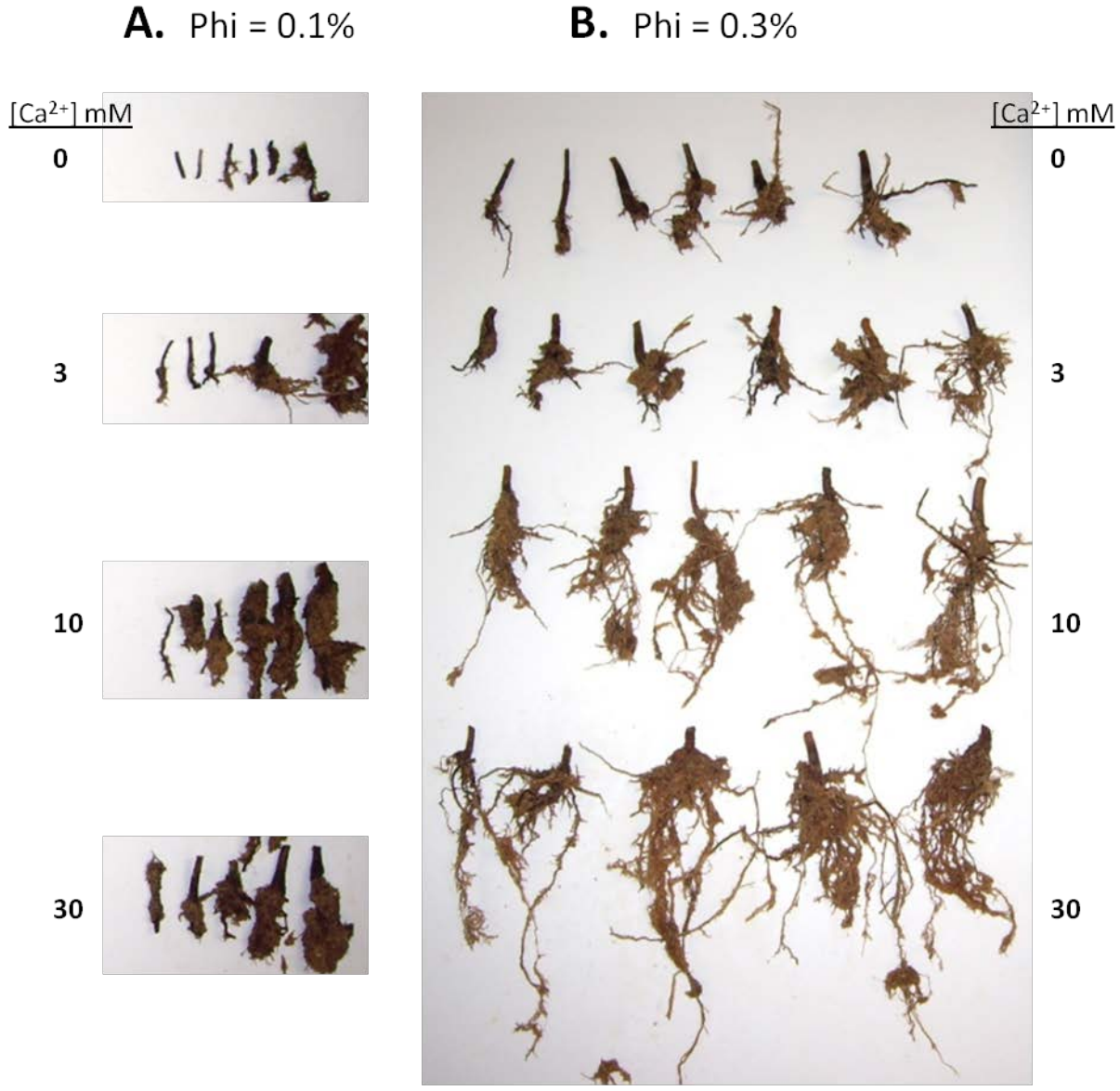

Fig. 3 Root development from *Banksia leptophylla* plants sprayed with (**A**) 0.1% phosphite and **(B**) 0.3% phosphite and grown in soil amended with 0 – 30 mM calcium sulphate, at 12-months after soil inoculation with *Phytophthora cinnamomi.*

Fig. 4 *Banksia leptophylla* roots from plants sprayed with 0.3% phosphite and grown in soil treated with **A**. no addition of calcium sulphate and **B**. supplementation with 30 mM calcium sulphate, 12-months after soil inoculation with *Phytophthora cinnamomi*. The sizes of samples A and B are directly comparable. Ruler (left) is 50 cm long.

Insert Table 3

Chemical analysis of soil

The mean calcium ion concentration in the calcium amended soil at the end of the experiment ranged from 0.62 to 4.3 mmol kg^{-1} dry soil supplemented with 3 mM and 30 mM calcium sulphate respectively (Table 4). The concentration of calcium ions released from the soil during into the bait-water is consistent with the concentration of calcium ions in the amended soil. Bait-water from soil that had been amended with 30 mM calcium contained 1.7 mM of exchangeable calcium ions and inhibited infection for longer when compared to bait-water from soils with lower calcium amendments (Table 4).

Insert Table 4

All samples except for the unamended soils showed less than 2 mg L^{-1} soluble phosphorous (P). Baiting solutions of the unamended soil samples contained between 0.1 and 0.04 mg L^{-1} P and soil extractions of the unamended soils in deionised water contained <0.02– 0.03 mg L^{-1} P. Soil salinity remained low for the duration of the experiment ranging from 0.2 mS/cm in control soil up to 0.8 mS/cm in soil treated with 30 mM calcium sulphate.

Chemical analysis of plant material

The concentration of total extractable calcium in pooled plant material (foliage and small stems) in the 30 mM calcium treatment group was approximately twice the level found in plants that had not been treated with calcium (Table 5). Total extractable phosphorus was slightly lower in the Ca = 0 and Ca = 30 mM plants that had been treated with phosphite compared to those plants that received only calcium.

Insert Table 5

Calcium toxicity

Seventy to 80% of plants in the 0.3% phosphite treatment group showed mild phytotoxic symptoms, i.e. chlorosis of lower leaves or 2 mm of leaf burn at the leaf tip. Calcium toxicity was evident in the 30 mM calcium treatment pots at 6-8 weeks post-inoculation. Calcium toxicity could be differentiated from *Phytophthora* dieback symptoms which manifest as yellowing starting from the bottom of the plant and base of the leaf, whereas calcium toxicity started at the top of the plants and tip of the leaf. A white precipitate was observed on the surface of the soil of most of the pots with the highest calcium treatment. The pH of all the soils in all treatments was neutral (pH 7.0 – 7.5) for the duration of the experiment.

Discussion

We investigated the disease control of *P. cinnamomi* by foliar application of phosphite in *B. leptophylla* growing in pots of sand supplemented with various levels of calcium. When foliar phosphite was used concurrently with soil supplementation with calcium, infected plants showed improved health, better root morphology and survived longer than plants that received only one or neither of the treatments. There was 100% survival of plants grown in soil amended with 30 mM calcium sulphate and sprayed with 0.3% phosphite at 12-months post-inoculation, while only one stunted plant survived in the absence of calcium or phosphite treatments.

Spraying plants with 0.3% phosphite improved plant survival from 47 days to 320 days after soil inoculation with the pathogen, and the average time taken to develop above ground symptoms improved from 28 days to 259 days. However, despite the apparent ability of foliar phosphite to reduce above ground symptoms, the roots of plants sprayed with 0.3 % phosphite but in soil without the calcium amendment showed a dramatic decrease in mass. This is in agreement with Scott *et al.* (2012) who found in *Eucalyptus gomphocephala* that although infected plants may appear healthy there was significant root damage.

The rate of infection of baits floated on flooded soils was considerably slower for soils that had been supplemented with 30 mM calcium, suggesting that increasing soil calcium either reduced the inoculum load in the soil or the pathogenicity of the organism. The average concentration of soluble calcium contained in the 30 mM amended soil was about 4.3 mM and is in agreement with Messenger *et al.,* (2000) who showed that sporangia size, zoospore production and colony forming units of *P. cinnamomi*, but not zoospore motility, were reduced in soils that had been amended with 1 – 5 % gypsum (equivalent to 4 – 5 mM exchangeable calcium).

The pathogen was not killed by either calcium or phosphite or the combined treatments, as it was recovered from all pine plugs in the soils 12-months after inoculation.

The concentration of calcium in non-inoculated plants that had been treated with 30 mM calcium sulphate was twice that of plants that had been grown in its absence. These treated plants when infected had double the survival time of non-treated plants. Non-inoculated control plants on soil with 30 mM calcium sulphate had more cluster roots than plants with no calcium supplementation, but the increase did not occur when calcium treated plants were also sprayed with phosphite. This is possibly because phosphite suppressed the phosphate starvation response (Varadarajan *et al*., 2002), and the presence of higher levels of exchangeable calcium in the soil may alter the availability of phosphate (Shainberg *et al.,* 1989).

Foliar application of calcium phosphite as a 0.3% solution to *Persea americana* (Cervera *et al.*, 2007) did not significantly improve the final number of leaves or the root density of infected plants compared to *P. cinnamomi* infected plants that had been treated with 0.3% potassium phosphite. The relative immobility of calcium ions compared to phosphite in the plant, as well as the insolubility and high pH of calcium phosphite may explain these results. This indicates that the presence of calcium ions in the soil at the plant pathogen interface is necessary to increase the efficacy of foliar phosphite in controlling disease caused by *P. cinnamomi*.

Use of both foliar phosphite and increasing soil available calcium may improve the survival of some species facing extinction in the wild, or at least prolong the time available for developing protocols for *ex situ* conservation. In most situations addition of gypsum to soils of natural ecosystems as a means of controlling *P. cinnamomi* will require initial study of the impacts on local soil characteristics and the microflora. Use of phosphite in plant nurseries is not encouraged as it could lead to distribution of apparently healthy plants in infested soil, but in many domestic and amenity horticultural situations where phosphite is used, the enhanced effect gained by simultaneous addition of soil calcium will be very valuable.

The decision to use calcium sulphate over other calcium salts, such as calcium nitrate, phosphate or carbonate, was determined by several criteria such as cost effectiveness, high soil infiltration rate, neutral pH, low salinity, a tolerance of plants to sulphate ions, and the fact that gypsum elevates the level of calcium in soil solution more efficiently than lime (Hetman, 2007; Shainberg *et al.*, 1989). Gypsum has been used for centuries as a soil conditioner and in the reclamation of sodic and acid sub-soils. This is achieved by increasing the amount of exchangeable calcium and the breaking up of clay soils, increased soil permeability and improvement of the availability of ions such as phosphate through electrolyte and exchange effects (Shainberg, 1989). The application of 30 mM calcium sulphate is equivalent to about 5 g of calcium sulphate L^{-1} of soil (density 1.68 g).

This rate is comparable to the rates of 10 t ha^{-1} gypsum commonly applied as a soil conditioner (Shainberg, 1989).

The precise way that phosphite controls oomycete pathogens is not known, but proposed models of its action include enhanced plant defense or a decrease in pathogen viability, all of which rely on the similarity between phosphite and its chemical analogue orthophosphate. The fact that the effect of phosphite is augmented at lower concentrations of calcium suggests that there may be some commonality in the underlying mechanism(s) responsible for the similarity of their effects. However, the apparent synergy of their combined use at 30 mM calcium and 0.3% phosphite infers that there may be additional factors, such as positive feedback mechanisms, that enhance the individual actions of phosphite and calcium ions on plant defense and the ability of *P. cinnamomi* to infect plants, reproduce and survive. When these data are considered together with the inhibition of phosphatases by phosphite (Martin *et al.*, 2001), and the subsequent accumulation of pyrophosphate (as has been found in *P. palmivora* treated with phosphite by Niere *et al.*, 1990), the results suggest a biochemical model involving the inhibition of calcium-dependent ATPase pumps in the plasma membrane. A mechanistic overlap between calcium and phosphate signaling could occur at this point.

Calcium homeostasis involves maintaining the concentration of calcium ions in the cytosol at micromolar levels (Tuteja, 2009). Calcium ions flow into the cytosol from either outside the cell or from internal stores/vacuoles via voltage-gated ion channels and/or calcium binding proteins. Plasma membrane Ca-dependent ATPases (PMCAs) then pump calcium out of the cell or into storage (such as into the endoplasmic reticulum or mitochondria) and return the calcium level to its normal resting concentration. In *Phytophthora* spp. and plants it is likely that there are three main types of ATPase: P-type ATPases which undergo phosphorylation during cation transport and include the PMCAs which pump Ca^{2+} out of the cytosol and maintain Ca^{2+} at micro molar levels; F-type ATPases which are reversible ATP-driven proton pumps that use ATP hydrolysis to drive protons up a concentration gradient, and use the proton gradient to generate ATP from ADP and Pi; and V-type ATPases that pump protons uphill to maintain the pH of the cytosol at physiological levels and acidify vacuoles (Stokes and Green, 2003; Kuhlbrandt, 2004; Toyoshima *et al*., 2004). All these enzymes are phosphohydrolase mechanoenzymes that produce orthophosphate as a reaction product. The direct impact of phosphite on these reactions in either plant or pathogen is not known, however given the similarity of phosphite to orthophosphate it is likely that there will be some effect. Inhibition of the Ca^{2+} dependent ATPase reaction by either phosphite or pyrophosphate, both of which are competitive inhibitors of the ATPase reaction, is likely to result in an increased cytosolic concentration of calcium due to its reduced rate of removal from the cytosol. This in turn will affect, *inter alia*, the calcium "signal" responsible for the co-ordinated response of pathogen to plant, and possibly *visa versa* (Fig. 5).

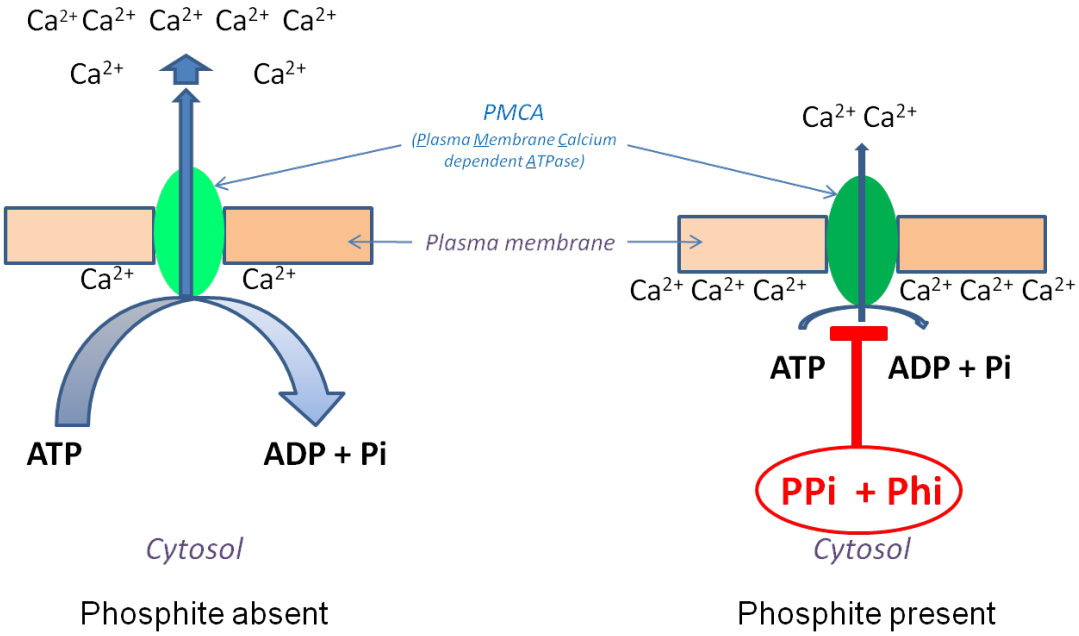

Fig. 5 Schematic representation of plasma membrane calcium dependent ATPase (PMCA) and its interaction with phosphite (Phi) and pyrophosphate (PPi). PMCAs are calcium dependent ATPases (phosphatases) – they hydrolyze ATP to ADP + Pi and in the process move calcium ions from one cellular compartment to another, or remove it from the cell altogether. They control the concentration of calcium ions in the cytoplasm. Many developmental processes in oomycetes are sensitive to and dependent on calcium signaling (Table S1). Left: normal functioning PMCA in the absence of phosphite. Right: PMCA inhibition in the presence of phosphite and pyrophosphate. As PPi and phosphite are analogues of ADP and phosphate respectively, the rate of removal of calcium from the cytosol PMCAs can be expected to be affected by the application of phosphite.

Perturbing pathogen calcium signaling in a *P. cinnamomi* - *L. angustifolius* pathosystem with exogenously applied calcium salts (30 mM), calcium channel blockers (100 µM) or EGTA (3 mM) reduced the pathogenicity of *P. cinnamomi* on lupin seedling roots (Stasikowski, 2012). When the calcium channel blockers ruthenium red (100 µM) and lanthanum chloride (1 mM), or EGTA (3 mM) were used in conjunction with phosphite (3 mM), the inhibitory action was greater than additive (Stasikowski, 2012). The need for a particular calcium ion concentration has been previously noted when producing axenic zoospores (Erwin and Riberio, 1983) and also for normal growth of *Phytophthora* spp. (Boughton *et al.*, 1978). Like phosphorus, calcium ions are important in metabolism and play a role in many aspects of cell signaling in terms of calcium signatures generated by first messengers. Several primary messengers have been elucidated for calcium ion signaling such as inositol bisphosphates (IP_2P), cADP ribose, nicotinic acid adenine dinucleotide phosphate (NAADP) and calcium ions themselves (calcium ion induced calcium ion release) all of which bind to the cognate receptors and function as ligand-gated Ca^{2+} channels (Tuteja, 2009). Calcium ions also play a role in remodeling the actin cytoskeleton via actin binding proteins whose activity is regulated by calcium, and the actin cytoskeleton itself may alter the intracellular calcium releasing mechanisms from the endoplasmic reticulum and other internal stores.

In conclusion, the combination of soil supplementation with calcium sulphate and spraying plants with phosphite significantly increased the survival time of plants infected with *P. cinnamomi*, decreased the development of dieback symptoms above ground and reduced root damage. The synergistic effect of calcium ions and phosphite can be explained in terms of a model that involves the inhibition of calcium-dependent ATPases by phosphite and pyrophosphate, and the subsequent disruption of calcium ion homeostasis and signaling in the cell. The information gained from investigating the combined effect of applying these two chemicals simultaneously may result in improved disease control, and may also indicate the mode of action of phosphite, and hence illuminate the pathogenic processes, in *P. cinnamomi*.

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Table 1 The 20 treatment combinations used in the glasshouse trial where each treatment consisted of six 150 mm diameter pots each containing six *Banksia leptophylla* plants.

	Phosphite (%)				
Calcium supplement (mM)	**0**	**0.1**	**0.3**	**0**	**0.3**
	Pots inoculated with *P. cinnamomi*			**Control pots**	
0	1	2	3	4	5
3	6	7	8	9	10
10	11	12	13	14	15
30	16	17	18	19	20

Table 2 Mean survival time (days) of *Banksia leptopylla* in days post–inoculation with *Phytophthora cinnamomi* (SE shown in parenthesis) in each combination treatment of phosphite and calcium

	Phosphite		
$CaSO_4.2H_2O$ (mM)	0%	0.1%	0.3%
0	47 (24)	258 (87)	320 (41)
3	69 (64)	270 (93)	351 (9)
10	114 (90)	312 (45)	347 (16)
30	102 (96)	329 (32)	356 (0)

Table 3 Percentage of total baits visibly infected after 48 hours incubation at 25^0C (ten *Eucalyptus seiberi* cotyledons and six *Hibbertia* sp. petals per baiting tray). The pathogen was recovered from all infected baits on *Phytophthora* selective medium (NARPH).

	Phosphite		
$CaSO_4.2H_2O$ (mM)	0%	0.1%	0.3%
0	50	62	68
3	62	56	50
10	31	68	37
30	31	12	0

Table 4 Calcium ion analysis of soil and the amount of calcium released into water during baiting of soils that had been amended with 0, 3, 10 and 30 mM calcium sulphate, 12 months after inoculation with *Phytophthora cinnamomi.*

Ca^{2+} supplementation of soil (mmol kg^{-1} dry soil) at time zero	Ca^{2+} concentration of soil in pots (mmol kg^{-1} dry soil) at 12 months	Ca^{2+} concentration of bait-water of soil (mM) at 12 months
0	0.065[a]	0.0045[a]
3	0.62[b]	0.063[b]
10	2.2[b]	0.33[b]
30	4.3[c]	1.7[c]

[a] mean of 5 samples; [b] mean of 3 samples; [c] mean of 10 samples.

Table 5 Total extractable calcium and phosphorus in the foliage of *B. leptophylla* at harvest, one year after treatment with calcium sulphate (0 and 30 mM) and phosphite (0 and 0.3%). Results are the average of three independent samples comprised of material pooled from at least 20 plants in each treatment group.

Calcium (mM)	Phosphite (%)	Total extractable calcium (mg/Kg)	Total extractable phosphorus (mg/Kg)
0	0	4830	530
30	0	8300	580
0	0.3	3960	490
30	0.3	9000	555

Supplementary information:

Table S1: Comparison of the effects of phosphite application with the disruption of phosphate signaling and the perturbation of calcium signatures, in **A**. *Phytophthora* and related species , and, **B**. Plants.

A. EFFECT on *Phytophthora* spp, oomycetes & fungi	**CAUSE**				
	Phosphite anion application	**Inhibition of phosphatase activity or alteration of kinase activity**	**$[Ca^{2+}]_{external}$**	**$[Ca^{2+}]_{cytosolic}$**	**Calcium channel modification**
Reduction of pathogenicity	Application of 3 mM phosphite to *P.cinnamomi* colonised discs reduced pathogenicity on lupin seedlings (Stasikowski 2012)	Inhibition of PP2A phosphatase in *Sclerotinia sclerotiorum* reduced asexual development and pathogenesis (Erental *et al*., 2007).	Ca^{2+} in soil suppressed root rot caused by *Aphanomyces* in *Pisum sativum* (Heyman *et al.*, 2007) Calcareous soils suppressed disease caused by *Phytophthora* spp., except *P. multivora* (DEC dieback map)	Inhibition of calcineurin, a Ca^{2+} & calmodulin dependent phosphatase, in *S. sclerotiorum* decreased pathogenesis (Harel *et al.*, 2006)	Application of 100 uM RR or La3+ reduced pathogenicity on lupin seedlings (Stasikowski 2012)
Inhibition of growth	Phi inhibited *Phytophthora* spp.growth (Fenn & Coffey, 1984) Phi wass not highly inhibitory to *Phytophthora* growth (Farihi *et al*., 1981) ED_{50} for growth of *Phytophthora* spp. ranged from 5 – 224 ug/ml (Coffey and Bower, 1984). See Erwin & Ribeiro (1996)	Inhibition of a Ras G-protein in *Aspergillus fumigatus* reduced growth and virulence (Fortwendle *et al.*, 2005)	*In vitro* mycelia growth of *Aphanomyces* was optimal between 0.5 -5 mM Ca^{2+} (Heyman *et al.*, 2007). No apparent relationship between reduction in growth rate and disease reduction in *P sojae* (Sugimoto *et al.*, 2008) 200mg/L (2 mM) $CaCl_2.2H_2O$ inhibited growth of *P. palmivora* (Singh, 1975)		Plant defensins block Ca^{2+} channels and inhibited growth of pathogen (Spelbrink *et al*, 2004).
Induction of a bumpy, highly branched, "bunch of grapes" morphology	Fosetyl-al caused severe morphological changes (knot like swellings) to *Phytophthora* spp. (Dercks & Buchenauer, 1987) (King *et al.*, 2010)	Inhibition of a Ras G-protein in *Aspergillus fumigatus* caused irregular hyphal morphology and increase hyphal branching (Fortwendle *et al.*, 2005) Inhibition of a Ca^{2+} dependent phosphatase in *N. crassa* caused increased hyphal branching (Harel *et al.*, 2006)	Low external Ca caused irregular shaped hyphae in *Saproleginia ferax* (Jackson and Heath, 1989)	Irradiation caused an increase in $[Ca^{2+}]_{cyto}$ in *Saprolegnia ferax* and resulted in increased hyphal branching (Grinberg & Heath, 1997).	In *N. crassa* the ionophore A23187 raised cytoplasmic Ca^{2+} and induced profuse hyphal branching. This effect was antagonized by cAMP (Reissig & Kinney, 1983)
Inhibition of sporangiogenesis	Phi inhibited sporulation in Phytophthora spp. (Farih *et al*., 1981). Phi reduced *in planta* zoospore production (Wilkinson *et al*., 2001) Phosphate (50 mM) inhibited	Expression of zoosporangenesis-induced genes coding for proteins with phosphatase activity are inhibited by 2-APB (Walker & Van West, 2007) Inhibition of Cdc14, a	Suppression of zoospore production at > 1 mM in the Oomycete *Aphanomyces* (Heyman *et al*, 2007). Ca^{2+} (2-5 mM) partly suppressed germination of *P infestans* sporangia. Chelation of Ca^{2+} also		La inhibited germination of *P infestans* sporangia (Hill *et al.*, 1998) Zoospore cleavage blocked by 2-APB, an IP3-receptor gated Ca^{2+} channel antagonist (Walker & Van West, 2007)

A. EFFECT on *Phytophthora* spp, oomycetes & fungi	**CAUSE**				
	Phosphite anion application	**Inhibition of phosphatase activity or alteration of kinase activity**	**$[Ca^{2+}]_{external}$**	**$[Ca^{2+}]_{cytosolic}$**	**Calcium channel modification**
	zoospore production in *P. cinnamomi* (Byrt & Grant, 1979) Phi inhibited sporangium production in *P. cinnamomi* & *P. citricola* at aprox. 5ug/mL (Coffey & Joseph, 1985).	phosphatase expressed during & controls sporangiogenesis in *P. infestans* prevented sporulation (Fong & Judelson, 2003). Patent no: WO 03/059936 A2.	inhibited sporangiogenesis (Hill *et al*., 1998). Ca^{2+} inhibited sporangium development (Coffey & Joseph, 1985).		Ca^{2+} channel blocker verapamil & a calmodulin antagonist inhibited zoosporangiogenesis (Judelson & Roberts, 2002)
Inhibition of zoospore release	Release of zoospores from sporangia of *Phytophthora* spp was sensitive to phosphite anions (Cohen and Coffey, 1986) Inhibition of zoospore release in *P. parasitica* and *P. citrophthora* by 10ug/mL efosite (Farih *et al*., 1981) 6 ug/ml phi inhibited zoospore release from *P. cinnamomi* & *P. citricola* (Coffey & Joseph, 1985).		Zoospores releases suppressed in *P. parasitica* by $[Ca^{2+}]_{ext}$ = 10 – 50 mM (von Broembsen and Deacon, 1997). 4-20 mM Ca salts decreased release of zoospores. 0.4 mM stimulated (Sugimoto *et al*., 2008)		
Disruption of zoospore motility		(Latijnhouwers *et al.*, 2002)	Zoospore motility was curtailed by 4-20 mM Ca^{2+} concentrations (Sugimoto *et al*., 2008)		
Disruption of zoospore attachment & encystment		Inhibition of PLD in *P. infestans* reduced zoospore encystment. G-protein activator triggered encystment (Latijnhouwers *et al.*, 2002). No relationship found between degree of root encystment and pathogenicity (Walker & Van West, 2007)	20 mM Ca^{2+} affected zoospore encystment. (Broembsen and Deacon, 1997) External Ca^{2+} (3 mM) promoted *P.cinnamomi* zoospore encystment (Gubler *et al*., 1989)		*P. parastica* zoospore encystment inhibited by La^{3+} which prevented the encystment-associated Ca^{2+} influx (Warburton and Deacon, 1998) EGTA (0.1 mM) inhibited *P.cinnamomi* zoospore encystment (Gubler *et al*., 1989)
Disruption of zoospore germination			Ca^{2+} 10 -30 mM stimulated zoospores to germinate in the absence of an organic nutrient trigger (von Broembsen and Deacon, 1997)	Size of internal Ca stores was a determining factor affecting the developmental fate of *P. sojae* cysts (Connolly *et al*, 1999)	Germination of *P. parasitica* cysts was prevented by TMB-8, which inhibits the release of Ca^{2+} from intracellular stores (Warburton and Deacon, 1998)
Disruption of chlamydospore production	Inhibition of chlamydospore production in *P. cinnamomi* by 15-44ug/ml Phi (Coffey & Joseph, 1985). Inhibition of chlamydospore				

A. EFFECT on *Phytophthora* spp, oomycetes & fungi	**CAUSE**				
	Phosphite anion application	**Inhibition of phosphatase activity or alteration of kinase activity**	**$[Ca^{2+}]_{external}$**	**$[Ca^{2+}]_{cytosolic}$**	**Calcium channel modification**
	formation in *P. parasitica* and *P. citrophthora a* by 50ug/mL efosite. Germination not affected (Farih *et al*., 1981). Phi caused increased chlamydospore production in *P. cinnamomi* (McCarren *et al*., 2006)				
Metabolic effects	Phi increased cellular activity of G-6-P DH, 6-PG DH & UDPG PPiase *in P. citrophthora* (Barachietto *et al*., 1992). Phi caused 35-fold increase in PPi in *Phytophthora* spp. (Niere *et al*., 1994) Phi caused over production of elicitor activity in *P. cryptogea* & *P. capsici* (Saindrenan *et al*., 1990) Phi reduced release of host defense suppressor molecules in *P. palmivora* (Dunstan *et al.*, 1990)	Ca^{2+} inhibited pyrophosphatase activity from *P. infestans* at 0.1 -20 mM (Sysuev *et al.*, 1978)	External calcium ion concentration influenced Ca^{2+} concentration in internal stores (Connolly et al, 1999; Xu & Morris, 1998; Jackson and Heath, 1989)	Soyabean isoflavones triggered a Ca^{2+} influx into encysted zoospores (Connolly *et al.*, 1999)	Soybean PA inhibited Ca^{2+} transport and increased Ca^{2+} leakage from *P. megasperma* membrane vesicles (Giannini *et al.*, 1988)

B. EFFECT on plants	**CAUSE**				
	Phosphite anion application	**Inhibition of phosphatase activity or alteration of kinase activity**	**Alteration of $[Ca^{2+}]_{external}$**	**Alteration of $[Ca^{2+}]_{cytosolic}$**	**Calcium channel modification**
Suppression of infection by *Phytophthora* species	Stem injection protected *Banksia* & *E. marginata* from *P. cinnamomi* for 4 years (Shearer & Fairman, 2007). See Erwin & Ribeiro (1996)	A constitutively active MAPK increased resistance of potato to *P. infestans* (Yamamizo et al, 2006)	Flood irrigation of Vinca seedlings with 10 or 20 mM $Ca(NO_3)_2$ greatly suppressed infection by *P. parasitica* (von Broembsen and Deacon, 1997)		
Alteration of phytoalexin synthesis	Increased PAL in Phi treated and inoculated *Lambertia* spp. (Suddaby et al., 2008) AOA reversed Phi induction of PAs in cowpea and decreased resistance to *P. cryptogea* (Saindrenan *et al.*, 1988) Increased PA & ethylene synthesisin tobacco treated with Phi and infected with *P. nicotianae* (Nemestothy & Guest,1990)	Phosphatase inhibitors caused an increase in PAL activity and phytoalexin accumulation in potato in the absence of an elicitor (Blanco *et al.*, 2008) Protein phosphatase inhibitors increased PA production in soybean (MacKintosh and MacKintosh, 1994) Phosphatase inhibitors mimicked elicitor action and induced hyper phosphorylation of proteins in tomato cell cultures (Felix *et a*l., 1994) Kinase inhibitors inhibited inositol phospholipid turnover & induction of PAL in tobacco suspension culture (Kamada and Muto, 1994)	Ca (1.2 mM) in external medium increased PA induction in soybean cells by a glucan elicitor from *P. megasperma* (Stab & Abel, 1987)	Parsley cells exposed to elicitor exhibited increased Ca^{2+} cyto and increased PA production (Blume *et al.*, 2000)	Accumulation of PAs was inhibited by La^{3+}, but stimulated by A23187 (Blume *et al.*, 2000)
Stimulation of ROS generation by NADPH oxidase	Increased superoxide release in *Lambertia* spp. (Suddaby *et al*., 2008)	Constitutivly active MAPK kinase confered resistance to *P. infestans* in potato and enhanced ROS production (Yamamizo *et al.*, 2006)		Increased Ca^{2+} cyto increases ROS production (Blume *et al,* 2000)	
Altered abiotic stress response		Decreased PPiase activity in *N. benthamiana* caused a wilting phenotype under mild drought stress (George *et al*., 2010)			
Metabolic effects		Decrease PPiase activity in *N. benthamiana* caused decreased starch content (George *et al*., 2010)	Extent of disease reduction in soybean related to increased uptake of Ca^{2+} by plants (Sugimoto et al, 2008)		

Abbreviations: La^{3+} = Lanthanum ions; RR = ruthenium red; A23187 = Ca^{2+} channel ionophore; PA = phytoalexin; TMB-8 = suppresses release of Ca^{2+} from internal stores (Warburton and Deacon, 1998); PAL = phenylalanine oxidase; ROS = reactive oxygen species: 2-APB = IP3 –receptor-gated Ca channel antagonist: MAPK= mitogen activated protein kinase; AOA= : PPiase = pyrophosphatase; Phi = phosphite

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