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Phosphonate (PO_3^-) Effectiveness Against *Phytophthora cinnamomi* Rands on *Thryptomene calycina*, *Banksia grandis* and *Banksia spinulosa*

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Abstract: The present study shows that Potassium phosphonate has been proven to slow down the growth rate of *P. cinnamomi* in *in vitro*. Phosphonate drench as low as 1 g L^{-1} was effective in protecting *Thryptomene calycina*, *Banksia grandis* and *B. spinulosa* in pot and field trials. In glass house trials, concentrations as low as 1 g L^{-1} (drench) significantly suppressed the *P. cinnamomi* population. Concentrations over 2½ g L^{-1} were phytotoxic to all plant species tested. The most sensitive species was *B. spinulosa*. Phosphonate (5 g L^{-1}) killed all *B. spinulosa* plants in seven weeks, therefore it must be used with a great care. Phosphonate treatment alone was effective protecting plants from disease in the field, but did not result in high plant health. Despite new root growth in pot trials after seven weeks, poor growth was commonly observed on *T. calycina* after 14 months in field trials. This suggests that phosphonate is not suitable as sole application particularly for the long term. A combination of phosphonate with compost as well as antagonist as an integrated management will be a good alternative for *P. cinnamomi* management in the future.

Key words: Phosphonate, *Phytophthora cinnamomi*, *Thryptomene calycina*, *Banksia grandis*, *Banksia spinulosa*, phytotoxic, Integrated control

INTRODUCTION

Phosphonates are the fourth major group of systemic fungicides which have good activities against plant diseases caused by Peronosporales and Pythiales[1]. These fungicides include a range of salts (Na^+, Mg^{++}, Cu^{++}, Zn^{++}, Al^{+++}) of phosphorus acid and its esters. More recently, a range of formulations based on mono, di-potassium phosphonate, have become available[2].

Whether the chemical works directly to the fungus or not, there are several arguments about mode of action of it in controlling plant disease. Coffey and Joseph[3] found that phosphorous acid (4.1 to 6.2 μg ml^{-1}) inhibited the mycelial growth of *P. cinnamomi* in *in vitro* studies. Fenn and Coffey[4] suggested that phosphate metabolism may be one target of phosphonate toxicity in Oomycetes. On the other hand, Phosphonates were shown to enhance plant defense responses, including lignification, phytoalexin accumulation and hypersensitive cell death[5,6].

Several trials on different plants have been reported to give a good control of *P. cinnamomi* such as: on Azaleas Benson[7]; on avocado Coffey *et al.*[8]; on *Leucodendron* Marks and Smith[9] and on *Xanthorrhoea minor* and *X. australis* Ali and Guest[10]. In addition, Cooke and Little[11] found a significant control of phosphonate against *Phytophthora infestans* on potato. In natural ecosystem, phosphonate also reported has been successfully protecting native plants from *P. cinnamomi* in Western Australian forest[12].

In spite of that, there is no study on the use of phosphonates for controlling *P. cinnamomi* on phosphorus susceptible species of plants. Therefore, this study was investigating the effectiveness of phosphonate in controlling *P. cinnamomi* on three Australian native plants i.e. *Thryptomene calycina*, *Banksia grandis* and *B. spinulosa*.

MATERIALS AND METHODS

***In vitro* assay:** *In vitro* trial on the growth inhibition of *P. cinnamomi* by potassium phosphonate was carried out on potato dextrose agar (PDA) plates. The final concentration of filter sterilized potassium phosphonate incorporated in the medium were : 1, 2.5 and 5 μg mL^{-1} after sterilization separately. Plates were made by

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dispensing 15 mL melted PDA into ten centimeters plastic petri dishes. After the plate cool at room temperature inoculation with five day old *P. cinnamomi* grown on PDA was conducted. Ten replicates were made for each treatment dose. The plates were incubated at 20°C, the mycelial growth was observed and the diameter of colony was measured after five days.

Glasshouse trial

Pot trial with *Thryptomene calycina*: Two ages of *T. calycina* were used in this trial i.e one year and three month-old. River sand was used as potting mix after being sieved through three millimetre sieve, then washed under running tap water for 15 min. The potting mix was inoculated with 10% sand-bran inoculum of *P. cinnamomi* before being transplanted with *T. calycina*.

Phosphonate was applied either as a soil drench or a foliar spray. Soil drenches were applied by dipping the base of each inoculated pot for few seconds in a bucket filled to two centimeter depth with a stock solution of potassium phosphonate. A piece of nylon mesh was put inside the base of the pot to trap the sand in the pot. Control pots were dipped the same way except in deionised water. Foliar sprays were applied using 500 mL plastic hand sprayer to spray until the whole plant became wet. Care was taken to minimize the leaking of the solution from the stem into the potting mix.

Five replicates were used for the one year-old plants, while seven replicates were used in the three months-old plant. Observations on the first trial were carried out by counting the total number of dead branches weekly for seven weeks. In the second trial, plant deaths were counted after seven weeks.

P. cinnamomi populations were estimated by using plate counting method with selective medium (PCH agar) as described by Shew and Benson[13]. Each was done after four weeks following transplanting. The dead pants were tested for the presence of *P. cinnamomi* by plating the root pieces onto PCH medium.

Pot trials with *Banksia grandis* and *Banksia spinulosa*: A similar trial was also conducted using *B. grandis* and *B. spinulosa*. Due to the limited number of seedlings, plants in this trial were only treated with soil drenches. Five replicates were used for both *Banksia* species. Observations were conducted for seven weeks by counting the number of dead plants weekly. To assure the plant was killed by *P. cinnamomi*, its presence was confirmed by direct isolation onto PCH.

Statistical analysis: *In vitro* inhibition results and total number of dead branches were analysed using a one way analysis of variance (anova) which is provided in MINITAB 11 computer software (Minitab Inc., Pennsylvania, USA). Total deaths of whole plants were analysed by using logistic regression analysis (MINITAB 11).

RESULTS

***In vitro* assay:** All three concentrations of potassium phosphonate (1, 2.5 and 5 g L^{-1}) significantly inhibited the mycelial growth of *P. cinnamomi* (Table 1). These data were recorded on the fifth day after inoculation of the agar medium. The treatments did not inhibit fungal growth completely. The highest rate (5 g L^{-1}) inhibited colony diameter by 48%. The fungus kept growing but was slower than the non-treated control. Phosphonate affects colony morphology; colonies growing on phosphonate - amended media are more sparse than colonies on unamended agar.

Glasshouse trials

Pot trial of *T. calycina*: Potassium phosphonate (1 and 2.5 g L^{-1} soil drench or 2.5 g L^{-1} foliar spray) significantly reduced plant die back in one year-old *T. calycina* on the first trial (Fig. 1). This figure shows that both drenches and sprays delay the onset of symptoms and reduce their rate and frail severity.

With three month-old *T. calycina* in the second trial, drench technique (1 g L^{-1}) and spray technique (5 g L^{-1}) significantly protected the seedlings against *P. cinnamomi* as estimated by counting total number of plant deaths out of seven replicates (Table 2). An opposite pattern was observed in this trial, i.e deaths are reduced by drenches of 1, but not 2.5 and 5 g L^{-1}. On the other hand, sprays of 5 g L^{-1} reduced deaths, while 1 and 2.5 g L^{-1} did not.

Surviving plants treated with soil drenches of 1 g L^{-1} from this trial produced new and healthy white coloured roots growing back after the first infection by *P. cinnamomi* (Fig. 2). This kind of root regeneration was very distinctive and was not found on the uninfected plants. Non-infected roots were smaller and light brown in colour. Infected root systems were mostly dead and roots were dark brown to black and rotten in the absence of phosphonate. All dead plants were positive with *P. cinnamomi* after plating the root pieces on PCH medium.

Quantitative data of *P. cinnamomi* population from two investigations are presented on Fig. 3. Both results show that potassium phosphonate significantly reduced *P. cinnamomi* populations over four weeks. All treatments except a spray of 1 g L^{-1} caused significantly lower

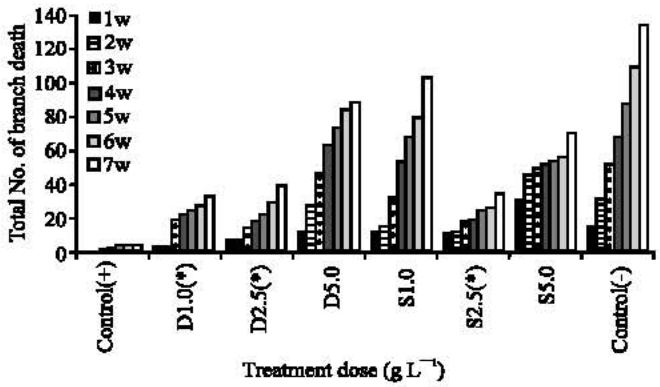

Fig. 1: Potassium phosphonate effect on 1 year-old *T. calycina* over 7-weeks infected with *P. cinnamomi* (D = Drench; S = Spray; w = week) [Stars (*) indicate a significantly different result from the control α =0.05]

Table 1: *In vitro* inhibition assay of potassium phosphonate on *P. cinnamomi* growth after 5 days

Treatment (μg ml^{-1})	Diameter of colony (cm)
Control	6.42a
1.0	5.26b
2.5	4.23b
5.0	3.37b

(Different letters after the data indicate significancy at α =0.05)

Table 2: Potassium phosphonate pot trial against *P. cinnamomi* in 3 months-old *T. calycina* over 7 weeks

Treatment (g L^{-1})	Percentage of death
Control + (non-infected)	0.0a
Drench 1.0	14.3a
Drench 2.5	57.1b
Drench 5.0	85.7b
Spray 1.0	85.7b
Spray 2.5	57.1b
Spray 5.0	14.3a
Control-(infected)	100.0b

(Letter after the data indicate significancy at α =0.05)

Table 3: Pot trial of potassium phosphonate against the disease in *B. grandis* and *B. spinulosa* over 7-weeks (Plants were infected with *P. cinnamomi*)

Plant	Treatment (g L^{-1})	Percentage of death
Banksia grandis	Control	80
	Drench 1.0	0
	Drench 2.5	0
	Drench 5.0	80
Banksia spinulosa	Control	40
	Drench 1.0	0
	Drench 2.5	60
	Drench 5.0	100

Table 4: Phytotoxicity of potassium phosphonate in *B. grandis* and *B. spinulosa* over 7-weeks (Plants were not infected with *P. cinnamomi*)

Plant	Treatment (g L^{-1})	Percentage of death
Banksia grandis	Control	0
	Drench 1.0	0
	Drench 2.5	40
	Drench 5.0	60
Banksia spinulosa	Control	0
	Drench 1.0	0
	Drench 2.5	10
	Drench 5.0	100

Fig. 2: Root growing effect of potassium phosphonate (1 g L^{-1}) on 3 months-old *T. calycina* after 7 weeks infection. (D) dead root; (N) new root

P. cinnamomi populations. As can be seen in this figure, drenches suppress pathogen population more effectively than sprays. The higher the concentration of phosphonate drench, the stronger the suppression of the pathogen population.

Despite positive results against root rot, phosphonate also causes branch and plant death in uninoculated controls. Phytotoxicity effects were observed at a concentrations of 2.5 g L^{-1} as foliar sprays and 5 g L^{-1} as soil drenches (Fig. 4). Spraying seemed to affect the plant more than drenching; more die back was found on sprayed plants compared with drenched plants at the same dose.

Phytotoxicity symptoms were observed mainly as burning of the leaves, sometimes even the entire branch. Stronger symptoms were observed as extensive defoliation. On diseased plants, the colour of the leaf slowly turns dull, somewhat pale and becomes desiccated. In contrast, phytotoxicity symptoms appeared suddenly

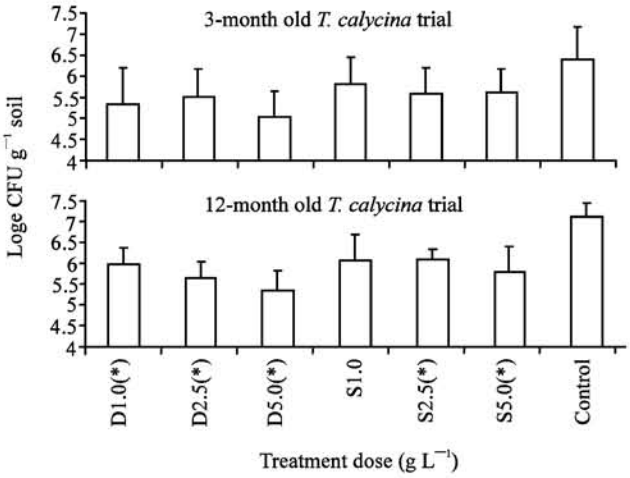

Fig. 3: *P. cinnamomi* populations in phosphonate treated soil on two different repetitions of *T. calycina* pot trial, each assessed four weeks after inoculation (D = Drench; S = Spray). (Stars (*) indicate statistically different from the control, α =0.05)

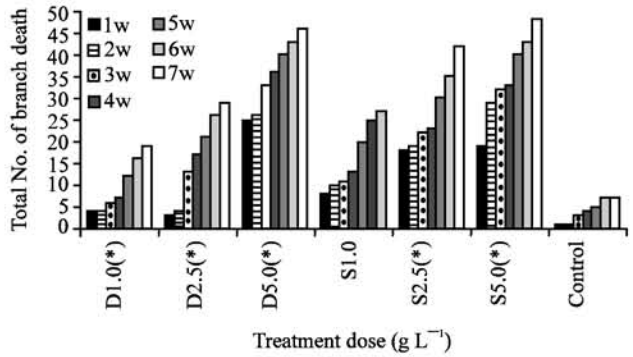

Fig. 4: Phytotoxicity effect of potassium phosphonate on 1 year-old uninfected *T. calycina* after 7-weeks (D = Drench; S = Spray; w = week) [Stars (*) indicate statistically different from the control, α=0.05]

Fig. 5: Phytotoxicity of potassium phosphonate on *B. spinulosa*. Early symptom indicated by arrows at the edge of the leaf (left) and late symptom all leaves are yellow (right)

and appeared as leaf burn and extensive defoliation followed by plant death.

Pot trial with *B. grandis* and *B. spinulosa*: Treatments with 1 and 2.5 g L^{-1} gave the best result in protecting *B. grandis* from *P. cinnamomi* (Table 3). None of the plants died after application of these two different concentrations of phosphonate over seven weeks. On the other hand, four plants out of five died on the control and 5 g L^{-1} dose of phosphonate. Doses of 1 g L^{-1} gave the best protection to *B. spinulosa* compared to the control and the other concentrations. Doses of 2.5 g L^{-1} seemed to be less effective than 1 g L^{-1}, as there were three plants that died at this level of phosphonate.

B. spinulosa is more sensitive to phosphonate than *B. grandis*, but more resistant to *P. cinnamomi*. Five plants out of five (100%) died at 5 g L^{-1} phosphonate concentration over seven weeks. On the other hand, *B. grandis* is less sensitive to phosphonate, as only three plants out of five died at the same level of phosphonate. More plants died at the same concentration for *B. grandis* due to combination of disease and phytotoxicity. Comparison of these results reveal that most plant death on pots treated with 5.0 g L^{-1} soil drench were due to phytotoxicity rather than root rot.

Death caused by phytotoxicity on non-disease infected both *Banksia* species were very obvious as can be seen on the data (Table 4). The phytotoxity symptoms mostly started from the edge or tip of the leaf spreading to the inner part of the lamina until the entire leaf and plant became burnt and died (Fig. 5). The difference between disease and phytotoxic symptom is clearer at later stages. At this stage, it is easy to distinguish plant death due to burning (phytotoxic) or from desiccation (disease). Plants killed by burning turned rusty brown in colour, while plants killed by disease were pale green. Disease symptoms on *B. grandis* also include a blackening of the stem.

DISCUSSION

In an *in vitro* study; potassium phosphonate has been shown to slow down the growth rate of *P. cinnamomi*. The same effect was also found on *P. palmivora* by Grant *et al.*[14] and on *P. cryptogea* and *P. capsici*[15]. Coffey and Joseph[3] also found that 4.1 to 6.2 μg mL^{-1} phosphorus acid could inhibit 50% of mycelial growth of *P. cinnamomi*. A similar level on growth inhibition of *P. cinnamomi* was found at a concentration of 5.0 μg mL^{-1}. In addition, Coffey & Bower[16] also discovered that *P. cinnamomi* together with *P. citricola* and *P. citrophthora* were the most sensitive species to phosphorous acid among nine *Phytophthora* spp. studied. It does not kill the fungus as most chemical agents do. It seems that the physiology of the fungus has been altered, as can be noticed from the mycelial growth rate and the colony morphology. Grant *et al.*[14] found that a low concentration of phosphonate could alter the metabolism of *P. palmivora*; reducing the amount of macromolecular materials of the fungus.

In the glass house study, phosphonate reduced the population of *P. cinnamomi* at concentrations as low as 1 g L^{-1} (drench) or 2.5 g L^{-1} (spray). It is interesting that the population of *P. cinnamomi* is also reduced by foliar sprays. This perhaps supports the complex mode of action of this fungicide as been proposed by Guest and Bompeix[17]. It seems that the lower population of *P. cinnamomi* compared with the control is due to plant reaction. Perhaps the plant absorbed the phosphonate through leaves and stems, then used and released some metabolic product as a defense mechanism. This metabolic product may inhibit the formation of reproductive structure such as sporangium or may also abort the germination of chlamydospore of *P. cinnamomi*. Vegh *et al.*[18] found that fungicidal activity of phosphonate breakdown product (ie H_3PO_3) may persist in soil for several months. Another possibility is that the remnant of phosphonate on the leaves and stems may be washed into the soil during watering and encounter the fungus directly. This is possible to happen in pots, since the volume of the pot is small. As a result, the run-off of phosphonate does not go any where. This small amount of phosphonate may be enough to inhibit the growth or the formation of reproductive structures of *P. cinnamomi*. As a comparison, the formation of *P. clandestina* sporangia were 50% inhibited by as low as 1.4 ppm phosphonate in sterile pond water[19].

The glass house trial showed that a concentration of potassium phosphonate as low as 1 g L^{-1} effectively protected *T. calycina, B. grandis* and *B. spinulosa* from root rot caused by *P. cinnamomi*. Phosphonate was also found to be effective against *P. cinnamomi, P. nicotianae* and *P. palmivora* infecting lupin, tobacco and paw-paw when applied as a drench[20]. Marks and Smith[9] found that phosphonate was most effective against *P. cinnamomi* on *Leucodendron* when applied as a prophylactic. The fact that many new, healthy and vigorous roots grew from *T. calycina* treated with 1 g L^{-1} drench of phosphonate partly explains the reduced severity of symptoms.

Concentrations as high as 5 g L^{-1} or above were found to be phytotoxic to all species tested. The most sensitive species was *B. spinulosa*. Phosphonate killed all five plants at 5 g L^{-1} and one plant out of five at 2.5 g L^{-1} concentration in seven weeks. At the latter concentration,

all the remaining plants were suffering severe phytotoxic effects; and it would eventually die within a few weeks. Therefore, it can be concluded that 2.5 g L^{-1} phosphonate is phytotoxic to *B. spinulosa* and 5 g L^{-1} is phytotoxic to *T. calycina* and *B. grandis*. Ali and Guest[10] found that 5 g L^{-1} of phosphonate spray was also phytotoxic to *Xanthorrhoea australis* and *X. minor* in a glass house study.

Phytotoxicity symptoms were found to be different from disease symptom. In general, phytotoxicity shows as leaf burning while disease symptom typically involve desiccation of the plants. The colour of the leaves in late stages of phytotoxic symptoms were bright rusty brown while in the disease symptoms are pale green. On *B. grandis*, the disease symptom was also associated with a blackening of the stem base which was not common on the toxicity symptoms. In the early stages of disease symptoms, the leaf changed its colour from green to bright yellow starting from the center of the leaf (from the veins) towards the edge. In phytotoxic symptoms, the spread of the symptoms scattered randomly and there was no gradual change of colour as such; the leaves became burnt in a relatively shorter time.

On *B. spinulosa* clear phytotoxic symptoms were observed at an early stage. Phytotoxic symptoms included a colour change from green to rusty yellow, starting from the edge of a leaf moving in towards the center part. Stems normally remain alive (yellow greenish), even though all leaves are already burnt and look dead. This did not happen in disease symptoms, where the whole plant wilted, then died.

On *Thryptomene*, defoliation is the obvious symptom of toxicity, while diseased plants usually retain their leaves. The colour of the basal leaves usually changed from green to yellow as a result of phytotoxicity. Disease symptoms appeared as desiccation of the whole plant, a single leaf or one or many branches, but leaves did not drop as in the toxic symptom.

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