A direct chemical method for the rapid, sensitive and cost effective detection of phosphite in plant material

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Abstract Phosphite application mitigates diseases caused by oomycete plant pathogens. Tissue concentrations of phosphite above 1 mM are generally required for disease protection. Determining the concentration of phosphite in plant material requires extensive extraction and derivatisation procedures prior to separation by gas-liquid chromatography (GLC). This paper describes a direct chemical method to estimate the concentration of phosphite using a silver nitrate reagent. Glass fiber filter papers were saturated with a 1 M aqueous solution of silver nitrate (adjusted to pH 2.5 with nitric acid) and dried for two hours at 60^0C. 20 µL of polyvinylpolypyrrolidone treated aqueous plant extract was adsorbed onto the filter paper and incubated in the dark at room temperature (25^0C) for one hour. The presence of phosphite in the extract reduces the silver ions to elemental silver resulting in a grey-black precipitate that is clearly visible. The method is rapid, sensitive and inexpensive, and can detect phosphite at concentrations of 1 mM in 20 µl of aqueous extract from 100 mg of fresh plant material. Samples analysed by this method gave similar results to analysis by GLC, indicating the method can be used in the field or the laboratory to determine uptake and distribution phosphite in the plant, the retention of phosphite over time and the timing of phosphite reapplication.

Keywords Phosphite detection . *Phytophthora cinnamomi* . Silver nitrate

Introduction

Phosphite, and phosphite esters (phosphonates) are systemic fungicides used to control the spread and impact of diseases caused by oomycete plant pathogens, particularly within the genus *Phytophthora* (Hardy *et al.,* 2001; Shearer, 2007). In Australia, phosphite is marketed as Agri-fos 600 (Agrichem) and is used extensively to protect susceptible native and agricultural plant species (Hardy *et al.*, 2001). Phosphite or phosphonate derivatives such as ethyl-phosphite (Aliette, Bayer) are used to control oomycete diseases of many horticultural crops including both herbaceous and woody species (Brown *et al.*, 2003; Leonardi *et al.,* 1999; Yandoc-Ables *et al.*, 2007), bok-choy and cabbage (Abbasi *et al.*, 2006).

Phosphite is applied to plants in the form of a neutralized soluble potassium phosphite salt by foliar application, trunk injection or a soil drench (Hardy *et al.*, 2001). The use of phosphite for these applications leads to increased levels of phosphonates (phosphite esters) in the environment and in the tissues of plants and animals (White *et al.*, 2007). However, when applied at the recommended rates that result in tissue concentrations of approximately 1 mM, phosphite is considered to be of low toxicity to organisims other than oomycetes (Guest *et al.*, 1995). In some agricultural species phosphite is said to have nutritional and developmental benefits (Rickard, 2000). At the higher rates of application (which are generally not used) and under phosphate limiting conditions, phosphite may have general phytotoxic effects, such as leaf burn and exfoliation, as well as numerous adverse effects on metabolism. For example, phosphite enhances programmed cell death in cell cultures of *Brassica napus* (Singh *et al.*, 2003) and at high concentrations can be toxic to non-pathogenic microorganisms (Hardy *et al.*, 2001; Barrett *et al.,* 2001). It is therefore important to apply the minimum amount of phosphite necessary to avoid negative side effects, whilst maintaining sufficient phosphite in plant tissues to control disease symptoms and spread.

Little is known about the cycling of phosphite in plants or in the environment. In plants, phosphite is systemically translocated and follows a source-sink relationship between the various growing points such as roots, leaves or fruit (Guest *et al.*, 1995; Danova-Alt *et al.*, 2008). However, it is not known how long it remains in the root tissue in different species, or the rate at which it is lost from the plant. We do not know how phosphite accumulation or loss is affected by drought, fire or water logging. The *in planta* concentration of phosphite needed to control *Phytophthora* diseases is also difficult to determine. This is mainly due to the species-specific differences amongst plants and the sensitivities of various *Phytophthora*

species to phosphite (Barrett *et al.*, 2001), as well as limitations imposed by the current methods for measuring phosphite (Roos *et al.*, 1999). These limitations include high cost, low throughput, use of toxic reagents and technical complexity (Fenn and Coffey, 1989; Roos *et al.*, 1999; Saindrenan *et al.*, 1985). All current methods are laboratory based and are not widely available. This paper describes a simple, inexpensive method for estimating phosphite concentration in plant tissues.

Materials and methods

Principle of the method

The method relies on phosphite reducing silver ions (Ag^{+}) in an acidified silver nitrate solution to elemental silver (Ag^{0}) resulting in the production of a grey-black precipitate that is clearly visible. In the process phosphite (3^{+}) is oxidized to phosphate (5^{+}). The overall equation for the reaction is:

$$Ag^{+} + H_2PO_3^{-} + H_2O \rightarrow Ag_{(s)} + H_2PO_4^{-} + 2H^{+}$$

It is necessary to carry out the reaction in the dark and at room temperature (20-25^{0}C). The oxidation of phosphite to phosphate is a chemical ionization reaction, and not ultra violet or free radical dependent, so excluding light will not affect the reduction of silver by phosphite. However, as most silver salts are light sensitive the presence of these compounds in the reaction mixture will cause the formation of dark precipitates that could be mistaken for elemental silver (Merck Index, 1996). The reducing environment of normal healthy plant cells ensures that phosphite remains in the 3+ oxidation state *in planta* and is not oxidized to 5+ (Schafer and Buettner, 2001). Initially, the silver precipitate produced by phosphite is colloidal and red/brown in colour due to the Tyndall effect caused by the scattering of light by very small particles in suspension (Sienko and Plane, 1966). As the reaction proceeds the precipitate becomes grey-black, the intensity depending on the concentration of phosphite present. Because the apparent colour development is colloidal, its concentration cannot be measured spectrophotometrically.

Plant material and phosphite treatment

A range of Australian native plant species, as well as several exotic species were used to assess the applicability of the silver stain in detecting phosphite in different plant tissues

(Table 1). Species were grown in pots in the glasshouse at 25^0C under natural light conditions and were either untreated (controls) or sprayed to run off with 0.3% (w/v) aqueous solution of phosphite (Agri-fos 600, Agrichem) containing 0.1% (by volume) BS1000® (Cropcare, Australia) as a surfactant. This concentration of phosphite is equivalent to 36.6 mM phosphite and compatible with the concentration of phosphite applied in the field to control *Phytophthora* diseases (Hardy *et al.*, 2001). Plants were left for 48 hours without watering. Plant foliage and soil were then watered on alternate days, for one week until harvest. Leaves, and in some cases roots, were harvested for analysis. The additional species grown in the field (Table 1) were not treated with phosphite, but leaves were collected and extracted.

Extraction of phosphite from plant material

Aqueous extracts of control (unsprayed) tissues and those from plants sprayed with 0.3% phosphite were made by placing a known weight of fresh plant material (approximately one gram) into a small, strong, self-sealing plastic bag (Sandvik, Australia). The bag was sealed, and hit with a rubber coated hammer on a smooth, hard surface until the plant material was finely macerated. Two volumes of deionised water (i.e. 2 mL g^{-1}) was added to the macerated plant material, mixed thoroughly and incubated for 1 hour at room temperature (25^0C). The supernatant containing phosphite was removed to a clean tube (this can be stored at -20^oC). Phenolic compounds were removed by adding polyvinylpolypyrrolidone (PVPP) (Pierpoint, 2004). An amount of PVPP equivalent to half the volume of aqueous extract (or one tenth of its weight) was added to the aqueous extract, mixed and left for 30 minutes at room temperature (25^0C). Samples were centrifuged at 5000 g for 4 minutes. Alternatively, samples were left overnight, or until the supernatant had cleared. For each species, extracts from sprayed and control plants were made alongside spiked samples (1 and 3 mM phosphite) from control plants, and replicated at least three times.

Silver nitrate reagent

Silver nitrate reagent was prepared by adding 5 mL of 1 M nitric acid to 100 mL 1 M silver nitrate solution. The final pH of the solution was 2.5. The reagent can be stored for at least six months in a dark bottle at room temperature (25^0C).

Silver nitrate saturated papers

Glass fiber filter paper discs (0.5 cm, Whatman GF/B) were saturated with the acidified silver nitrate reagent and dried in the dark for approximately 2 hours at 60^0C. Dried, saturated discs were stored in a cool, dry, dark place for up to 6 months. 20 µl (one drop) of PVPP treated aqueous plant extract was adsorbed onto the middle of a dried silver nitrate treated disc, and incubated in a Petri dish in the dark at room temperature (25^0C) for one hour. Phosphite in the plant samples was estimated by visual comparison with standards that had been prepared in the same way.

Preparation of phosphite standards

Standards were prepared either in deionised water, or by adding known concentrations of phosphite (0.3 – 10 mM) to samples of untreated control plant material of the species to be tested. Macerated plant material was extracted in either 2 x volume by weight (i.e. 2 mL g^{-1}) of 0.5 mM or 1.5 mM phosphite in deionsed water to give phosphite standard extracts equivalent to plant material containing 1 mM and 3 mM phosphite, respectively. This assumes that all phosphite is soluble in the aqueous extract. Standards of plant extracts were treated with PVPP as described above.

Detection of phosphite in *Phytophthora cinnamomi* mycelia

Phytophthora cinnamomi (isolate MP 94.48) colonised GF/B filter paper discs were placed in 24-well microtitre plates and grown for 3 days in V8 medium (Ribeiro, 1978) containing phosphite at concentrations of 0, 1, 3 and 10 mM. After 3 days growth at 25^0C, V8 medium was completely removed from the wells and the remaining mycelia dried *in situ* for 24 hours at 60^oC. The dehydrated mycelia was covered in the silver nitrate reagent (0.5 mL) and incubated for 2 hours in the dark at room temperature (25^0C).

Detection of phosphite by Gas-Liquid Chromatography

Plant samples were washed in a 1% solution of Deconex 15-E® phosphate-free detergent (Borer Chemical Ltd Switzerland) and dried at 40 °C for several days. An electric grinder with a 1-mm sieve was used to grind dried samples, the grinder being cleaned with compressed air and a fine brush between samples. One 2 g sample of ground material was placed in screw-cap containers and sent to the Western Australian State Chemistry Centre where phosphite was determined as the methyl ester by Gas-Liquid Chromatography (GLC) with flame photometry detection (Barrett *et al*., 2003). Samples were analysed along with two

control samples of known phosphite content per 50 samples. Concentrations were confirmed for 10-15% of samples by replicate analysis. The limit of detection was 0.1 μg/g dry weight material (Barrett *et al*., 2003).

Results and Discussion

The development of the silver stain was dependent on phosphite concentration

The reduction of silver ions in the silver nitrate reagent, and the subsequent development of a grey–black precipitate, is dependent on the concentration of phosphite in the applied solution (Fig.1). This indicates that the acidified silver nitrate reagent can be used as an indicator for the presence and estimated concentration of phosphite.

Fig. 1 Series of phosphite standards (0 - 10 mM) in deionised water. Aqueous phosphite solution (20μL) was adsorbed onto dried silver nitrate reagent saturated Whatman GF/B disks and incubated in the dark for 1 hour at room temperature (25^0C).

The reaction between phosphite and excess silver nitrate was also found to be dependent on time and pH (data not shown). However, there was no observable difference between aqueous phosphite standards incubated in the dark for one hour, compared to 24 hours, indicating that incubation at room temperature (25^0C) for one hour was sufficient for the detection of phosphite at concentrations of 0.3 -10 mM. The optimum pH for the silver nitrate reagent was pH 2.5. At higher pH, the reduced silver remained in solution and the precipitate produced by phosphite was less intense.

Plant material may contain compounds that could interfere with the accurate detection of phosphite which were not removed by PVPP: for example, the chemical analogue orthophosphate, the commonly used herbicide glyphosate (glycine phosphonate) and the reducing agents glutathione, salicyclic acid and ascorbic acid. Addition of exogenous orthophosphate (100 mM) or glyphosate (100 mM) to aqueous standards of phosphite had no effect on the rate of reduction of silver nitrate, nor did the addition of glutathione (100 mM)

or salicyclic acid (100 mM) (data not shown). The interaction between ascorbic acid and silver nitrate demonstrated that the presence of ascorbic acid at concentrations as low as 0.3 mM will reduce the silver nitrate reagent and would therefore interfere with the colour development of the reaction (data not shown). The reducing (antioxidant) effect of ascorbic acid could be abrogated by the addition of an oxidizing agent, hydrogen peroxide (100 mM); however this concentration of hydrogen peroxide also severely affected the detection of phosphite below 30 mM.

Detection of phosphite in plant tissue

The silver nitrate reagent can be used to detect phosphite in leaves and roots of plant material sprayed with 0.3 % phosphite (equivalent 36.6 mM) (Table 1). The concentration of phosphite detected in the foliage of exotic and various Australian native plant species treated with phosphite ranged from 1 to 3 mM, and was detectable by the reagent in most plants species tested. Extracts from untreated plants of *Jacksonia sternbergiana, Lupinus angustifolius, Pultenaea reticulata*, *Banksia grandis, Beaufortia* spp. and *Arabidopsis thaliana* showed negligible background staining with the silver reagent (Table 1, column A), whilst the same extracts spiked with 3 mM phosphite (column C) showed an intense staining reaction which could be clearly differentiated from the control extract. The extraction procedure and staining method indicates that some variation in background staining occurs in different plant species. However, provided the phosphite treated sample is compared to an untreated control sample of the same plant species and tissue, the presence of phosphite can be detected at concentrations of 1-3 mM in the majority of plants tested. Where differences between phosphite treated and control samples is not apparent, a different part of the plant, for example roots, may be analysed. This was the case with *Eucalyptus gomphocephala* and *Persea americana*. Extracts of control leaves had a high background staining, whereas the background staining in root tips was low and enabled the detection of phosphite in *E. gomphocephala* at concentrations of about 3 mM in roots of plants in which shoots had been sprayed with 0.3% phosphite (Table 1, column D, E and F).

In some cases, for instance *P. reticulata* and *H. robustum*, the extracts from plants sprayed with 0.3% phosphite (column D) gave an even more intense staining reaction compared to the 3 mM spiked extracts. It is likely that leaf-burn, caused by the sensitivity of these plants to phosphite, resulted in tissue death before translocation of phosphite from the tissues occurred (Barrett *et al.*, 2001). This was confirmed by GLC phosphite analysis (Table 1, column F)

indicating that significant quantities of phosphite (> 35 mM) remain bound-up in damaged leaf tissue.

The leaves of native Australian plants are known to contain high levels of phenolic (flavonoid) and other antioxidant compounds (such as ascorbic acid) that may interfere with the detection of phosphite (Dixon and Pasinetti, 2010). Phenolics can be removed with PVPP (Pierpoint, 2004) and this effectively removed the background from most samples allowing the concentration of phosphite present in the material to be clearly seen and quantified against standards after 1 - 2 hours (data not shown). Extracted samples that showed high background in the absence of phosphite, such as leaves of *E. gomphocephala*, *H. robustum* and *P. americana,* were not improved with double extraction with PVPP (data not shown). Double extraction with PVPP did not reduce the amount of phosphite detected in treated samples indicating that PVPP did not affect phosphite extraction, and that phosphite was not oxidized over the period of time (24 hours) taken to do the assay (data not shown).

When the discs containing the standard aqueous solutions of phosphite (Fig.1) were left in the dark for up to 4 days it was found that the colour did not darken more than it had done over the first two hours. However, both control and phosphite treated extracts of plant material incubated in the dark with the silver nitrate reagent for 24 hours developed colour to the same degree and were indistinguishable from each other, indicating that PVPP does not remove all compounds in plant material capable of reducing silver (data not shown). It is possible that the stain may be improved by separating the interfering compounds with activated charcoal, filtration or dialysis tubing. However, thin layer chromatography foils did not remove background from recalcitrant samples such as *E. gomphocephala* extracts (data not shown).

Insert Table 1

Comparison of the silver nitrate method with Gas-Liquid Chromatography phosphite analysis

The phosphite levels detected by the silver nitrate reagent were consistent with those detected by analysis on GLC (Table 1, column E), with 95% of the samples tested being within the phosphite concentration range estimated by the silver nitrate reagent. The limit of detection of phosphite using GLC is 0.5 µg g^{-1} fresh weight (equivalent to 6 µM) (Ouimette and

240 Coffey, 1990), whereas the lower limit of phosphite detection using the silver nitrate method
241 was 0.3 mM phosphite in 20 µL of aqueous solution (equivalent to 30 µg g^{-1} fresh weight)
242 (Fig.1). There is some debate as to the concentration of phosphite necessary in plant roots,
243 leaves and stems to ensure protection from pathogenic attack by *P. cinnamomi* (Tynan *et al.*,
244 2001). Although it is likely to vary between plant species, it is clear that the 1 mM to 3 mM
245 concentration of phosphite present in the leaves and roots of plants that have been treated
246 with 0.3% solution of phosphite (a concentration frequently used in the field to protect
247 horticultural and native plant species) is detectable by the silver nitrate reagent. This indicates
248 that the method can be used to determine the phosphite-status of treated plants in horticultural
249 and natural ecosystems.

250 Plants that have been treated with phosphite, by either trunk injection or foliar spray, have
251 been shown to accumulate phosphite at concentrations of between 25 - 425 µg g^{-1} dry weight
252 (equivalent to approximately 0.3 – 6.0 mmol phosphite g^{-1} dry weight) depending on the
253 concentration of phosphite applied, length of time after spraying that the tissue is tested and
254 the type of tissue being assayed (Fenn and Coffey, 1989; Hardy *et al.*, 2001; Pilbeam *et al.*,
255 2000; Roos *et al.*, 1999; Fairbanks *et al*., 2000). In the present study, the concentration of
256 phosphite in fresh leaves of *L. inermis, J. sternbergiana, L. angustifolius* and *A. cygnorum*
257 after spraying with 0.3% phosphite was determined by the silver nitrate reagent to be between
258 >3 mM, and was confirmed by GLC analysis. GLC analysis also showed that the leaves of
259 *B. elegans* and *B. squarrosa* that had been sprayed with phosphite did not contain the
260 expected levels (1 – 3 mM) usually found in treated leaves, a fact that was in agreement with
261 the levels determined by the silver nitrate reagent.

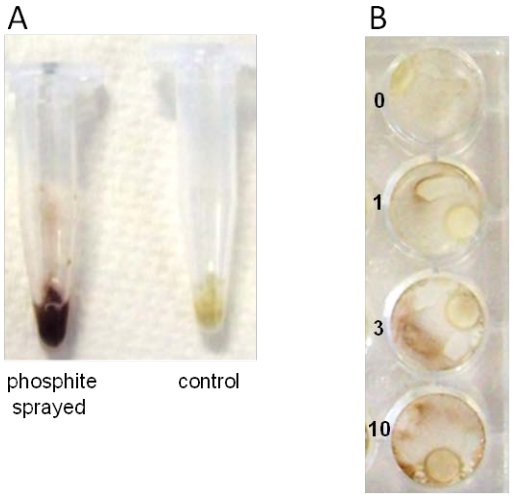
262 Applicability of the method

263 The silver nitrate reagent is straight forward and inexpensive to prepare, and treated glass
264 fiber papers onto which the reagent was adsorbed can be stored in the dark for several weeks
265 prior to use. Sample preparation is minimal when compared to other methods used to detect
266 phosphite (for example Roos *et al*., 1999).

267 Phosphite is water soluble and providing an aqueous extract can be prepared, it should be
268 possible to test for the presence of the anion in almost any aqueous medium derived from
269 plants, animals and microorganisms, including *Phytophthora* species. Providing a negative
270 control is available for comparison, the simplicity and speed of the silver nitrate method

enables it to be used to confirm the presence, or absence, of phosphite under "field" conditions. A better estimate of the phosphite status can be obtained by comparing the sample with standards. It was not possible to use the silver nitrate reagent to reliably detect phosphite in living tissues of *L. angustifolius* or *P. cinnamomi* as it is likely that silver ions are actively excluded by the membranes of viable cells during the incubation time of the experiment (24 hours) (data not shown). However, phosphite treated *L. angustifolius* roots that had been macerated (Fig.2, A) and hyphae of *P. cinnamomi* that had been killed through dehydrated (Fig. 2, B), prior to submersion in the silver nitrate reagent and incubation in the dark for 1-2 hours, showed the presence of phosphite.

Being able to determine whether or not phosphite is present in different plant tissues is an integral part of any phosphite management strategy. Previous work has shown that phosphite concentrations of 1-3 mM and higher are required to maintain resistance and protection against *Phytophthora* species, for example avocados (El-Hamalawi and Menge, 1995) and Australian native plant species (Wilkinson *et al.*, 2001). To maintain these levels, different plant species will require different frequencies of application as the persistence of phosphite within the plant will vary depending on a combination of factors such as metabolism, leaf drop, fruiting or seed fall, climate and soil conditions (Tynan *et al.*, 2001). Translocation of phosphite within the plant will also vary according to species and is likely to be dependent on similar factors, as well as the time of the year that the plant is treated, and needs to be assessed on an individual species basis. The silver nitrate detection method would be a useful tool to assist with decisions on the timing and frequency of phosphite application.



294 **Fig. 2 A**. Roots of *Lupinus angustifolius* seedlings (100 mg) harvested from 10 day-old plants 48 hours after
295 spraying with 0.3% phosphite solution (left) and control unsprayed plants (right), were macerated and
296 submerged in silver nitrate reagent (50 µL) and incubated for one hour at room temperature (25^0C). **B.**
297 *Phytophthora cinnamomi* colonised discs grown in 24-well microtitre plates for 3 days in V8 medium
298 containing 0, 1, 3 and 10 mM phosphite.

299

300 In conclusion, this paper describes a rapid, inexpensive and sensitive direct chemical
301 detection method to estimate the amount of phosphite in plants. The phosphite detection limit
302 of the reagent was 0.5 - 1 mM (depending on background staining) and phosphite could be
303 detected in as little as 20 µl of aqueous extract from 100 mg of fresh plant material. This is
304 equivalent to 82 µg phosphite anion g^{-1}fresh weight or 20 nmol phosphite per sample. We
305 anticipate that this method will provide farmers, land managers and conservationists with a
306 quick and easy test to determine the timing of their phosphite applications. The test may also
307 be of interest to the food industry to test fruits, vegetables and nuts produced by plants that
308 have been treated with, and suspected to contain, residual phosphite, for instance avocados
309 (Pegg *et al.*, 1987) and apples (Malusa and Tosi, 2005). The test would also be of use in
310 plant nurseries, and research laboratories.

311

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319 **References**

320 Abbasi, P. A. & Lazarovits, G. (2006). Seed treatment with phosphonate (AG3) suppresses *Pythium*
321 damping-off of cucumber seedlings. Plant Disease 90(4): 459-464.

322 Barrett, S. (2001). Phytotoxic effects of phosphite in native plant communities in southern Western
323 Australia. PhD Thesis, Murdoch University, Western Australia.

324 Barrett, S., Shearer, B. L. and Hardy, G. E. St. (2003). The efficacy of phosphite applied after

inoculation on the colonization of *Banksia brownii* stems by *Phytophthora cinnamomi*. Australasian Plant Pathology 32: 1-7.

Brown, S., Koike, S., Ochoa, O., Laemmlen, F. & Michelmore, R. (2003). Insensitivity to the fungicide Fosetyl-Aluminium in Californian isolates of the downy mildew pathogen *Bremia lactuca*. Plant Disease 88: 502-508.

Danova-Alt, R., Dijkema, C., Dewaard, P. & Kock, M. (2008). Transport and compartmentation of phosphite in higher plant cells – kinetic and 31P nuclear magnetic resonance studies. Plant Cell and Environment 31: 1510-1521.

Dixon, R. A. & Pasinetti, G. M. (2010). Flavonoids and isoflavonoids: From plant biology to agriculture and neuroscience. Plant Physiology 154(2): 453-457.

El-Hamalawi, Z. A. & Menge, J. A. (1995). Seasonal fluctuations in the extent of colonization of avocado plants by the stem canker pathogen *Phytophthora citricola*. Journal of the American Society for Horticultural Science 120(2): 157-162.

Fairbanks, M., Hardy, G. & McComb, J. (2000). Comparisons of phosphite concentrations in *Corymbia* (*Eucalyptus*) *calophylla* tissues after spray, mist or soil drench applications with the fungicide phosphite. Australasian Plant Pathology 29(2): 96-101.

Fenn, M. & Coffey, M. (1984). Studies on the *in vitro* and *in vivo* antifungal activity of fosetyl-Al and phosphorous acid. Phytopathology 74: 606-611.

Fenn, M. & Coffey, M. (1989). Quantification of phosphonate and ethyl phosphonate in tobacco and tomato tissues and significance of the mode of action of two phosphonate fungicides. Phytopathology 79: 76-82.

Guest, D. I., Pegg, K. G. & Whiley, A. W. (1995). Control of *Phytophthora* diseases of tree crops using trunk injected phosphonates. Horticultural Reviews 17: 299-330.

Hardy, G. E. St., Barrett, S. & Shearer, B. L. (2001). The future of phosphite as a fungicide to control the soilborne plant pathogen *Phytophthora cinnamomi* in natural ecosystems. Australasian Plant Pathology 30(2): 133-139.

Leonardi, J., Wiley, A. W., Langdon, P. W., Pegg, K. G. & Cheyne, J. (1999). Progress on the use of foliar applications of phosphonate for the control of *Phytophthora* root rot in avocados. Talking Avocados 10: 18-21.

Long, P. J., Miller, S. A. & Davis, S. K. (1989). Duration of fungicidal effect following injection of

apple trees with fosetyl-Al. Journal of Phytopathology 124: 89-96.

Malusa, E. & Tosi, L. (2005). Phosphorous acid residues in apples after foliar fertilization: Results of field trials. Food Additives and Contaminants 22(6): 541-548.

McKay, A., Floyd, R. & Boyd, C. (1992). Phosphonic acid controls downy mildew (*Peronospora parasitica*) in cauliflower curds. Australian Journal of Experimental Agriculture 32: 127-129.

Merck Index (1996). *An* Encyclopedia of Chemicals, Drugs and Biologicals. Merck Research Laboratories. NJ, USA.

Miller, J. S., Olsen, N., Woodell, L., Porter, L. D. & Clayson, S. (2006). Post-harvest applications of zoxamide and phosphite for control of potato fiber rots caused by oomycetes at harvest. American Journal of Potato Research 83(3): 269-278.

Ouimette, D. G. & Coffey, M. D. (1990). Symplastic entry and phloem translocation of phosphonate. Pesticide Biochemistry and Physiology 38: 18-25.

Pegg, K. G., Whiley, A. W., Langdon, P. W. & Saranah, J. B. (1987). Comparison of phosetyl-Al, phosphorous-acid and metalaxyl for the long-term control of *Phytophthora* root-rot of avocado. Australian Journal of Experimental Agriculture 27(3): 471-474.

Pierpoint,W. S. (2004). The extraction of enzymes from plant tissues rich in phenolic compounds. Protein purification protocols. Methods in Molecular Biology. 244: 65-74.

Pilbeam, R. A., Colquhoun, I. J., Shearer, B. & Hardy, G. E. S. (2000). Phosphite concentration: its effect on phytotoxicity symptoms and colonisation by *Phytophthora cinnamomi* in three understorey species of *Eucalyptus marginata* forest. Australasian Plant Pathology 29(2): 86-95.

Ribeiro, C. A. (1978). *A* Source Book of the Genus Phytophthora. J. Cramer Vaduz.

Rickard, D. A. (2000). Review of phosphorous acid and its salts as fertilizer material. Journal of Plant Nutrition 23(2): 161-180.

Roos, G., loane, C., Dell, B. & J, H. G. S. (1999). Facile high performance ion chromatographic analysis of phosphite and phosphate in plant samples. Communications in Soil Science and Plant Analysis 30(17 & 18): 2323-2329.

Sala, F. C., da Costa, C. P., Echer, M. D., Martins, M. C. & Blat, S. F. (2004). Phosphite effect on hot and sweet pepper reaction to *Phytophthora capsici*. Scientia Agricola 61(5): 492-495.

384 Saindrenan, P., Darakis, G. A. & Bompeix, G. (1985). Determination of ethyl phosphite, phosphite,
385 and phosphate in plant tissues by anion exchange high performance liquid chromatography
386 and gas chromatography. Journal of Chromatography 347: 267-273.

387 Schafer, F. Q. & Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox
388 state of the glutathione disulfide/glutathione couple. Free Radical Biology and Medicine
389 30(11): 1191-1212.

390 Shearer, B. L. & Fairman, R. G. (2007). A stem injection of phosphite protects *Banksia* species and
391 *Eucalyptus marginata* from *Phytophthora cinnamomi* for at least four years. Australasian
392 Plant Pathology 36(1): 78-86.

393 Sienko, M. J. & Plane, R. A. (1966). Chemistry: Principles and Properties. McGraw-Hill, New York.

394 Singh, V. K., Wood, S. M., Knowles, V. L. & Plaxton, W. C. (2003). Phosphite accelerates
395 programmed cell death in phosphate-starved oilseed rape (*Brassica napus*) suspension cell
396 cultures. Planta 218(2): 233-239.

397 Soulie, M. C., Malfatti, P., Demandre, C., Bompeix, G. & Moneger, R. (1995). Phosphonate
398 inhibition in the biosynthetic pathway of polyunsaturated long chain fatty acids of
399 *Phytophthora capsici*. Plant Science 105(1): 53-61.

400 Tynan, K. M., Wilkinson, C. J., Holmes, J. M., Dell, B., Colquhoun, I. J., McComb, J. A. & Hardy,
401 G. E. S. (2001). The long-term ability of phosphite to control *Phytophthora cinnamomi* in
402 two native plant communities of Western Australia. Australian Journal of Botany 49(6): 761-
403 770.

404 White, A. K. & Metcalf, W. W. (2007). Microbial metabolism of reduced phosphorus compounds.
405 Annual Review of Microbiology 61(1): 379-400.

406 Wilkinson, C. J., Shearer, B. L., Jackson, T. J. & Hardy, G. E. S. (2001). Variation in sensitivity of
407 Western Australian isolates of *Phytophthora cinnamomi* to phosphite in vitro. Plant
408 Pathology 50(1): 83-89.

409 Yandoc-Ables, C. B., Rosskopf, E. N. & Lamb, E. M. (2007). Management of *Phytophthora* crown
410 rot in pumpkin and zucchini seedlings with phosphonates. Plant Disease 91(12): 1651-1656.

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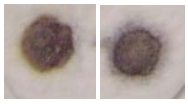
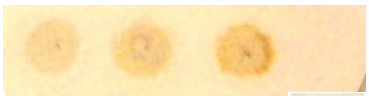
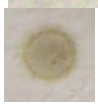
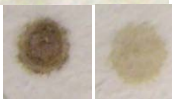
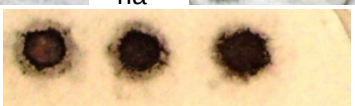
Table 1 Detection of phosphite in plant tissues. **A**. extracts from untreated control plants; **B.** extracts as in A but spiked with 1mM phosphite; **C.** extracts as in A but spiked with 3 mM phosphite; **D.** extracts from plants sprayed with 0.3% phosphite (equivalent to 36.6 mM phosphite) 1 week before analysis; 20 uL of plant extract was adsorbed onto a silver nitrate saturated disc and incubated for 1 hour in the dark at room temperature (25^0C). **E**. Estimated phosphite concentration using the silver nitrate test, on material from phosphite sprayed (0.3%) plants; **F.** GLC phosphite analysis of plant extracts from plants sprayed with 0.3% phosphite and unsprayed controls. All phosphite concentrations shown are mM. [1] = severe leaf burn observed on foliage of phosphite sprayed plants, indicating phosphite accumulation. [2] = Field grown plants. na = not analysed.

Plant species and material	Plant extract standards A Control	B 1 mM Phi	C 3 mM Phi	D 0.3% phi spray	E Estimated Phi concentration (mM) in 0.3% sprayed sample	F GLC phosphite analysis (mM) Control	0.3% sprayed
Jacksonia sternbergiana Leaf					1-3	0.01	1.94
Lupinus angustifolius Leaf					1-3	0.004	1.137
Lupinus angustifolius Root					< 1	na	na
Pultenaea reticulata [1] Leaf		na			> 3	0.008	35
Adenanthos cygnorum Leaf		na			1-3	0.003	2.1
Banksia grandis [2] Leaf					na	na	na
Lambertia inermis Leaf					> 3	0.003	3.15
Beaufortia elegans Leaf					< 1	0.004	0.318
Beaufortia squarrosa Leaf		na			< 1	0.004	0.38
Eucalyptus gomphocephala Leaf					< 1	0.004	0.621
Eucalyptus gomphocephala Root					1-3	0.023	0.683
Hypocalymma robustum [1] Leaf		na			> 3	0.023	54
Persea americana [2] Leaf					na	na	na
Persea americana [2] Root					na	na	na
Arabidopsis thaliana Leaf					>3	na	na
Arabidopsis thaliana Root					< 1	na	na

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