In vitro sensitivity of South African *Phytophthora cinnamomi* to phosphite at different phosphate concentrations

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ABSTRACT

Phosphonates are very effective for managing avocado root rot caused by *Phytophthora cinnamomi*. In Australia, it has been reported that a threshold concentration of 25-40 µg/ml of phosphite is required for suppressing *P. cinnamomi* in avocado roots. In South Africa, a critical root phosphite concentration has not been established for local *P. cinnamomi* populations. As a first step towards investigating this aspect, a collection of 50 *P. cinnamomi* isolates was isolated from South African orchards and screened *in vitro* (solid agar medium and liquid medium) for sensitivity to phosphite, since *P. cinnamomi* is known to vary in phosphite sensitivity *in vitro.* The isolates showed a range of responses when screened on solid agar medium at 30 µg/ml and 100 µg/ml phoshite and three phosphate concentrations (1 mM, 7 mM and 15 mM). Phosphite tolerant isolates were subjectively classified as isolates with an inhibition of ≤20% at 30 µg/ml and ≤30% at 100 µg/ml. The intermediate isolates (>30% but ≤60%) and sensitive isolates (>60%) were classified based on their response at 100 µg/ml, since the isolates varied in their inhibition (5% to 80%) at 30 µg/ml that did not always correlate with their sensitivity at 100 µg/ml. At 7 mM and 15 mM phosphate, none of the isolates were inhibited by >55% at 30 µg/ml. Phosphate concentration had a marked influence on the phosphite sensitivity of isolates since nine isolates were tolerant at 1 mM phosphate, whereas 19 isolates were tolerant at 15 mM phosphate. A subset of 10 isolates representing the most sensitive and tolerant isolate in the agar test, were also evaluated in liquid medium containing the same phosphite and phosphate concentrations as in the agar test. This showed that only one isolate could be identified as tolerant, with most isolates being sensitive, depending on the phosphate concentration. The relative ranking of isolates in the agar and liquid test did not always correlate.

INTRODUCTION

Phosphonates have long been known as excellent fungicides for the management of *Phytophthora* diseases. Once phosphonates (H_3PO_3) are introduced into plant tissues, they are rapidly hydrolysed to phosphonic acid (strong acid produced by dissolving phosphorous acid in water), which is then ionised to phosphite anions (HPO_3^{-2} and/or $H_2PO_3^{-1}$) with the HPO_3^{-2} anion having activity against *Phytophthora* (Cohen & Coffey, 1986; Ouimette & Coffey, 1989a). In literature, the nomenclature of phosphonates is confusing since the terms phosphonate, phosphorous acid, phosphite and phosphonic acid is often used interchangeably. The terms phosphite and phosphonate are used in literature to refer to phosphonic acid and various derivatives (including the anions) of phosphonic acid, and both terms are acceptable in terms of IUPAC nomenclature (Roos *et al.*, 1999). For the purpose of this article the term phosphite will be used for referring to phosphonic acid and its anions, and phosphonate will be used to refer to the salts and esters of phosphonic acid, i.e. formulated fungicides.

Avocado root rot caused by *Phytophthora cinnamomi* was first reported in Los Angeles County in 1920 and continued to be a devastating disease (Menge *et al.*, 1999), until Darvas discovered phophonate trunk injections in the 1980s that resulted in the complete recovery of severely diseased and defoliated trees (Darvas *et al.*, 1984). Subsequently, phosphonates have remained a major component of managing avocado root rot. Due to the widespread occurrence of *P. cinnamomi* in most avocado production regions of the world, including South Africa, growers often apply a preventative phosphonate application strategy by using either foliar sprays or trunk injections. In Australia, the disease is managed using a preventative phosphonate strategy where the monitoring of root phosphite concentrations is important. A commercial root phosphite

analysis service is available to growers, where growers will only apply more phosphonates if their root phosphite levels are below the critical root phosphite level of 25 µg/g (25 mg/kg) that suppresses *P. cinnamomi* (Thomas, 2008). Peer reviewed scientific data is not available on how this critical root phosphite concentration was established and popular publications from Australia differ somewhat in the amount required, for example Giblin *et al.* (2007) mentioned 25 to 40 µg/ml as the critical root phosphite concentration and cited personal communication with Whiley 2000 (Sunshine Horticultural Services Pty Ltd).

Several studies have investigated the *in vitro* phosphite sensitivity of *Phytophthora* isolates to determine if resistant or less tolerant isolates are present. Although the ability to generate isolates resistant to phosphite through mutagenesis (Fenn & Coffey, 1989) created concern for the development of resistance under field conditions, this has not been realised in practice under field conditions. To date, only two studies reported that phosphonate resistant isolates occur under commercial agriculture conditions. A *P. cinnamomi* isolate resistant to fosetyl-Al was reported from an ornamental nursery where fosetyl-Al was used intensively for about five years (Vegh *et al.*, 1985; reference within Guest & Grant, 1991). Cohen & Samoucha (1984) reported naturally occurring *Phytophthora infestans* isolates that were resistant to fosetyl-Al. The lack of resistance development under field conditions is most likely due to the complex mode of action of phosphonates involving a direct toxic effect on the pathogen and induced host resistance (Guest & Grant, 1991; McDonald *et al.*, 2001). Whether the direct toxic effect or the induced host resistance contributes most to the suppression of *Phytophthora* in plants is still a highly debated subject.

Different methods can be used to determine the *in vitro* sensitivity of *Phytophthora* isolates to phosphite. Most *in vitro* studies have determined the sensitivity of isolates using radial growth inhibition on solid media. However, the sensitivity of isolates to phosphites might be better determined in liquid cultures, since with radial growth measurements, the effect on density of mycelia cannot be taken into account (Wilkinson *et al.,* 2001a; Grant *et al.,* 1990).

The *in vitro* phosphite sensitivity of *Phytophthora* isolates in artificial media can be influenced substantially by the phosphate content of the media. This is most likely due to the fact that once phosphate becomes limiting, rapid assimilation of phosphite is initiated (Guest & Grant, 1991; Griffith *et al.,* 1993). This results in isolates being more sensitive to phosphite at low phosphate levels than at high phosphate levels. Most studies have used full- or half strength corn meal agar for assessing phosphite sensitivity. This medium is low in phosphate (0.38 mM), as well as Ribeiro's medium that is also often used, which has an even lower phosphate content (0.084 mM). In plants, phosphate concentrations are normally between 5-20 mM (Bompeix, 1989), which is higher than the concentrations that have been used in most *in vitro* phosphite studies. Although a role of phosphate in the *in vivo* phosphite sensitivity of isolates in plants has not yet been shown, it is presumed to be important (Guest & Grant, 1991).

The aim of this study was to evaluate the *in vitro* phosphite sensitivity of 50 *P. cinnamomi* isolates from South Africa, which were collected from several different avocado orchard blocks. The isolates were evaluated at different phosphite and phosphate concentrations to also assess the effect of phosphate on phosphite sensitivity. The assays were conducted using mainly solid agar medium, but liquid medium was also used for testing a subset of ten isolates. The identification of phosphite sensitive and tolerant isolates is important for future studies that will aim to determine the critical root phosphite concentration required for suppression of *P. cinnamomi* isolates from South Africa.

MATERIALS AND METHODS

Phytophthora isolations

Phytophthora cinnamomi isolates were obtained from soil collected from symptomatic avocado trees situated in 15 different orchard blocks in the Tzaneen, Mooketsi and Modjadjiskloof regions in 2007 and 2013. Most, but not all of the orchards, were treated with phosphonates for more than ten years. Isolations from soils were made using a standard soil baiting technique as described by Tsao (1983). Several different baiting materials were used including blue lupins (Greenhalgh, 1978), pears and citrus leaf disks. The baited planting materials were plated onto PARPH media (Jeffers & Martin, 1986) and putative *Phytophthora* colonies were sub-cultured onto potato dextrose agar plus Streptomycin (PDA+). Pure culture isolates were obtained by hyphal tipping each isolate twice onto new medium prior to species identification and *in vitro* sensitivity testing.

Species identification

Isolates were identified to the species level using a species-specific PCR test that targets the ras-related protein, as described by Schena *et al.* (2008), except that the PCR reaction and amplification conditions were slightly modified. The closely-related species of *Phytophthora niederhauseri* was included as negative control and a positive control consisted of a *P. cinnamomi* isolate of which the identity was confirmed through sequence analyses. Amplified fragment sizes were checked on a 3% agarose gel stained with ethidium bromide and visualised under a UV transilluminator. A 100 bp ladder was included on all gels.

In vitro phosphite sensitivity testing on solid agar medium

The *in vitro* phosphite sensitivity of isolates was tested using a slightly modified Ribeiro's agar medium (defined mineral salts medium) as previously described (Fenn & Coffey, 1984). The phosphate

concentration in the medium was adjusted to a final phosphate concentration of 1 mM, 7 mM or 15 mM. A concentration of 30 µg/ml or 100 µg/ml phosphite was added to the medium for each phosphate concentration by using filter sterilised Phytex 200 SL (Horticura cc, Pretoria, South Africa), a commercial potassium phosphate fungicide containing 200 g/L phosphorous acid. For each isolate an un-amended control plate for each of the phosphate concentrations were also included. The percentage phosphite inhibition of isolates was calculated relative to the un-amended control plates.

In vitro phosphite sensitivity testing in liquid medium

A subset of ten isolates that represented the most sensitive and tolerant isolates were evaluated for their phosphite sensitivity in liquid medium, using the same phosphite and phosphate concentrations and methodology as for the agar test. The exception was that liquid Ribeiro's medium was used with no added agar, and five mycelium plugs from each isolate were inoculated into 50 ml of liquid Ribeiro's medium within a 250 ml Erlenmeyer flask. After 14 days growth, mycelia were harvested and dried and the percentage inhibition was calculated.

RESULTS

Phytophthora isolations and identifications

A total of 50 isolates were obtained from baiting materials, which were preliminary identified as *Phytophthora* based on morphology. The isolates were all identified as *P. cinnamomi* since the expected ~250 bp amplification product (Schena *et al.*, 2008) was obtained for all isolates in the species-specific PCR assay. The negative control isolate of *P. niederhauseri* did not yield any amplification product.

In vitro phosphite sensitivity testing on solid agar medium

The 50 *P. cinnamomi* isolates showed a range of responses at the different phosphite and phosphate concentrations (Fig. 1). Isolates were subjectively classified as tolerant when the percentage growth inhibition was ≤20% inhibition at 30 µg phosphite/ml and ≤30% at 100 µg phosphite/ml. Phosphite intermediate isolates were classified as those having an inhibition of >30% but ≤60% at 100 µg phosphite/ml, and sensitivity isolates as having more than 60% inhibition at 100 µg phosphite/ml. The 30 µg phosphite/ml phosphite concentration could not be included as a parameter for classifying the intermediate and sensitive isolates since these isolates showed a range of inhibitions (~2% to ~80%) at this concentration, which did not always correlate with their inhibition at 100 µg phosphite/ml (Fig. 1). It is important to note that at 30 µg phosphite/ml and at the 7 mM and 15 mM phosphate levels, none of the isolates were inhibited by more than 55%.

The number of phosphite tolerant isolates was markedly influenced by the phosphate concentration with nine (18%), 13 (26%) and 19 (38%) isolates belonging to this group at 1 mM, 7 mM and 15 mM phosphate respectively (Fig. 1). The intermediate isolates consisted of four (8%), nine (18%) and seven (14%) isolates at 1 mM, 7 mM and 15 mM phosphate respectively. Consequently, the largest number of phosphite sensitive isolates was identified at 1 mM phosphate (37 isolates; 74%) followed by the 7 mM phosphate (28 isolates; 56%) and 15 mM phosphate (24 isolates; 48%) concentrations (Fig. 1).

In vitro phosphite sensitivity testing in liquid medium

The ten isolates that were evaluated in liquid medium were in general inhibited more by phosphite than in the agar test, since only one isolate was identified as tolerant using the same subjective classification of tolerant, intermediate and sensitive isolates as

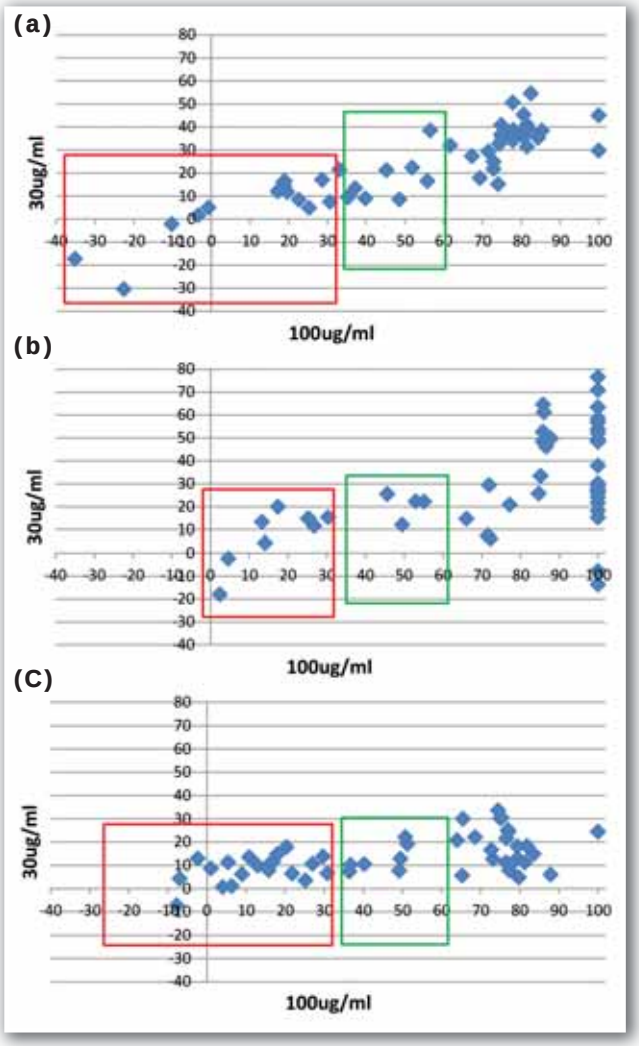

Figure 1. Percentage inhibition of 50 *Phytophthora cinnamomi* isolates grown on Ribeiro's solid agar medium containing phosphite concentrations of 30 µg/ml or 100 µg/ml at three different phosphate concentrations including (a) 1 mM, (b) 7 mM and (c) 15 mM. Isolates that were subjectively classified as phosphite tolerant (<20% inhibition at 30 µg phosphite/ml and <30% inhibition at 100 µg phosphite/ml) are enclosed in a red rectangle and those that were intermediate tolerant (>30% to <60% inhibition at 100 µg phosphite/ml) are enclosed in a green rectangle.

described for the agar test (Fig. 2). Furthermore, only one isolate was inhibited by more than 90% at all the different phosphite and phosphate levels. The effect of phosphate on phosphite sensitivity was similar than what was observed in the agar test, with the highest number (nine isolates; 18%) of sensitive isolates being present at the 1 mM phosphate level, and only five (10%) sensitive isolates being present at the 7 mM and 15 mM phosphate concentrations (Fig. 2).

DISCUSSION

The current study was able to show that the *in vitro* phosphite sensitivity of *P. cinnamomi* isolates from South Africa is influenced by the phosphate concentration, as well as the hardness (liquid or agar) of the *in vitro* growth media. Isolates furthermore also showed a continuum of phosphite sensitivity at a specific phosphate level in the agar test. In the agar test, at the lowest phosphate concentration (1 mM), more isolates (74%) were classified as sensitive than at the 15 mM phosphate concentration (48%). Consequently less isolates were classified as tolerant at 1 mM (18%) than at 15 mM (38%) phosphate. This trend of increasing phosphate levels, resulting in isolates being more tolerant to phosphite, was also evident in the liquid medium test. The effect of the hardness of the medium was that isolates were more sensitive in the liquid medium than in the agar medium. This is most likely due to the higher surface area of *P. cinnamomi* mycelia being exposed to phosphite in the liquid test than in the agar test.

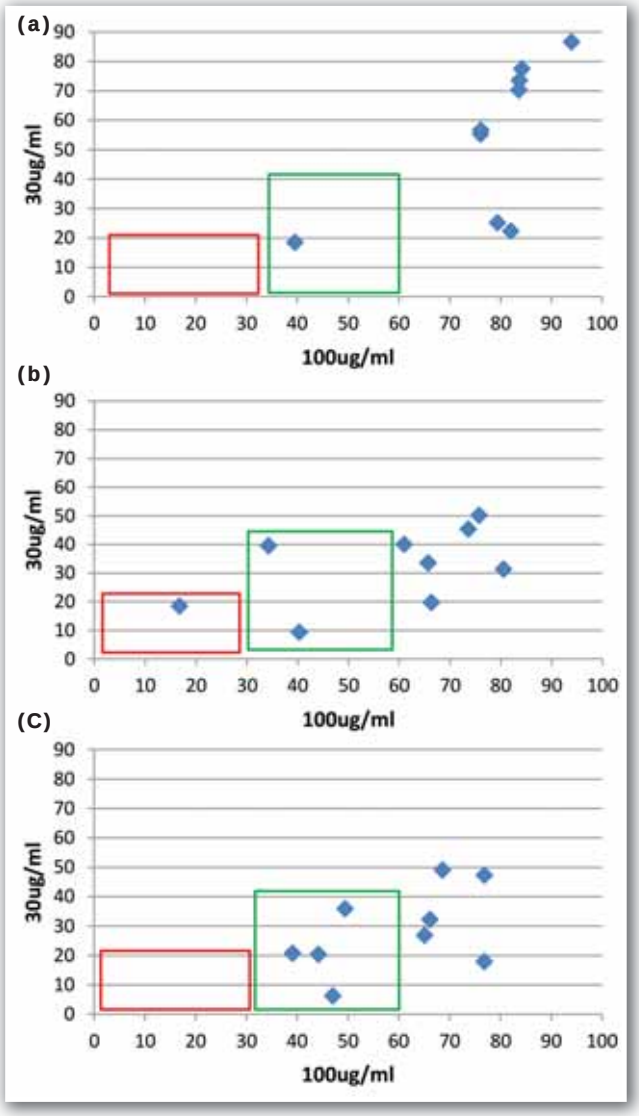

Figure 2. Percentage inhibition of 10 *Phytophthora cinnamomi* isolates grown on Ribeiro's liquid medium containing phosphite concentrations of 30 µg/ml or 100 µg/ml at three different phosphate concentrations, including (a) 1 mM, (b) 7 mM and (c) 15 mM. Isolates that were subjectively classified as phosphite tolerant (<20% inhibition at 30 µg phosphite/ml and <30% inhibition at 100 µg phosphite/ml) are enclosed in a red rectangle and those that were intermediate tolerant (>30% to <60% inhibition at 100 µg phosphite/ml) are enclosed in a green rectangle.

Variation in phosphite sensitivity in *P. cinnamomi* at a specific phosphate concentration has also been reported for *P. cinnamomi* populations in other regions of the world. A range of phosphite sensitivities were reported for 12 *P. cinnamomi* isolates from California, USA (Coffey & Bower, 1984), and 71 isolates from Australia (Wilkinson *et al.*, 2001a). Wilkinson *et al.* (2001) were able to subjectively group their isolates into sensitive (9% of isolates, EC_{50} 4-5 ppm), intermediate (82% of isolates; EC_{50} 9-14 ppm) and tolerant (9% of isolates, EC_{50} 25-148 ppm) isolates. The only reports on the *in vitro* sensitivity of South African *P. cinnamomi* isolates to phosphite consisted of work conducted by Duvenhage (1994, 1999, 2001). In these studies, isolates were collected and tested for phosphite sensitivity from 1992 to 2000 from one orchard that contained phosphonic acid treated, fosteyl-Al treated and control trees. In Australia, Kaiser *et al.* (1997) also evaluated the phosphite sensitivity of isolates collected in avocado orchards that were treated for ten years with phosphonates. These authors identified *P. cinnamomi* isolates from phosphonate treated trees that were only inhibited by phosphite *in vitro* at a concentration of 1000 g/ml (Kaiser *et al*., 1997). It is difficult to compare the results of the current study with the above mentioned studies, since different phosphate concentrations were used for evaluating phosphite sensitivity. The study of Wilkinson *et al.* (2001) used 0.084 mM phosphate, Coffey & Bower (1984) 0.84 mM phosphate, Duvenhage (1994, 1999, 2001) 0.084 mM phosphate and Kaiser *et al.* (1997) an unknown concentration of phosphate. These phosphate concentrations are all much lower than what were used in the current study and those most likely occurring in avocado roots. This could have resulted in isolates exhibiting higher sensitivities to phosphite in the other studies, than what might be experienced during pathogen infection of roots. Consequently, the direct toxic effect of phosphite to *P. cinnamomi* might have been overestimated in these studies, which contributes to the debate on the mode of action of phosphite where the direct toxic effect might be less than previously envisaged, due to higher phosphate levels occurring in

plants than what has been used in most *in vitro* studies. In the current study, even the highest phosphate level (15 mM) might be less than what is present in avocado roots. Studies on the concentration of phosphate in avocado roots is required to further draw conclusions, since to the best of our knowledge, no studies have reported on the concentration of phosphate in avocado roots.

A subset of the phosphite sensitive and tolerant isolates identified in the current study will be used in future studies to determine the critical root phosphite concentration required for suppressing South African *P. cinnamomi* isolates. A root bioassay will be used for investigating this aspect. In future studies the *in vitro* sensitivity of the 50 isolates will have to be re-tested in repeat experiments, since the current study only evaluated sensitivities in one experiment. Preliminary analyses showed that for some isolates the sensitivities were not reproducible (data not shown) and it will therefore be important to re-test all 50 isolates in the agar test, and also the subset of isolates in the liquid test.

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