Investigating root phosphite quantification methods for optimising foliar spray application in avocado

Preliminary report

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ABSTRACT

Phosphonates are fundamental to the management of avocado root rot caused by *Phytophthora cinnamomi*. In plants, phosphonates are metabolised to phosphite (phosphonic acid), which is the active compound against *P. cinnamomi.* The main aims of this study were to (i) develop a cost effective method for measuring phosphite in avocado roots, and to (ii) evaluate the efficacy of phosphonate foliar sprays based on root phosphite concentrations. Two methods are being investigated for measuring root phosphite concentrations, which include a fluorescent enzyme assay that is based on a recombinant phosphite dehydrogenase enzyme, and high performance ion chromatography (HPIC). The fluorescent enzyme assay yielded good linear standard curves, and is in the process of being optimised for analyses of phosphite in avocado roots. The HPIC method was optimised by investigating several sample clean-up methods of root extracts, which was a constraint in method development. The detection limit of the HPIC method was 1 ppm, with recovery rates of phosphite spiked root samples being more variable (40-70%) than spiked water samples (60 ± 5%). The HPIC method was used to quantify root phosphite concentrations in two avocado orchard trials receiving different phosphonate treatments, which included three to four foliar sprays at different concentrations (0.5%, 0.75% and 1% a.i.) and a trunk injection applied after summer flush hardened off. All concentrations of the foliar sprays at both orchards resulted in very low root phosphite concentrations (< 3 ppm_{dw}). Future studies will further optimise the HPIC method to improve recovery rates from root samples, and will focus on investigating the critical (minimum) phosphite concentration required in roots for suppressing *P. cinnamomi.*

INTRODUCTION

In South Africa, trunk injections are currently being used in a preventative strategy against Phytophthora root rot, but the cost is increasing due to increasing labour costs. Therefore, alternative application methods must be investigated. Phosphite, including phosphonic acid, are the products of phosphonate fungicides once hydrolysed within plant tissue at physiological pH. These compounds provide control against root rot and are highly mobile in plants, being translocated upward and downward within plants, which allows for various application methods (Cohen & Coffey, 1986; Guest & Grant, 1991; Whiley *et al.*, 1995; Menge *et al.*, 1999). Alternative methods for application of phosphonates in avocado include soil drenches, bark sprays combined with penetrants and foliar sprays (Ouimette & Coffey, 1989; Whiley *et al.*, 2001; Giblin *et al*., 2007). Of these methods, foliar sprays are now most widely used in a preventative strategy on mature avocado trees in Australia (personal communication, W.A. Whiley, Sunshine Horticultural Services Pty Ltd). A key technique that has been used in the Australian studies for evaluating phosphonate application methods is the measurement of root phosphite concentrations (Whiley *et al.*, 2001; Giblin *et al.*, 2005). Therefore, a

commercial root phosphite analysis service has been established in Australia and New Zealand in order to assist growers in their decision making process of applying phosphonates, especially for foliar applications (Thomas, 2008; personal communication A.W. Whiley). The methodology of these phosphite quantification techniques are the intellectual property of commercial laboratories, and are not available publically or in the scientific community.

A few articles have been published on methods that can be used for quantification of phosphite in plant tissue following the application of phosphonate fungicides. Of these methods, gas chromatography (using various detection methods) and high performance ion chromatography (HPIC) have most frequently been used. In most recent literature, gas chromatography was mainly used by researchers working on Australian native tree species (Shearer & Crane, 2009; Barrett *et al.*, 2003; Shearer *et al*., 2012), whereas HPIC was used by Australian researchers working on avocado and Australian native tree species (Whiley *et al.,* 2001; Nartvaranant *et al.,* 2004; Thomas, 2008; Jackson *et al*., 2000; Wilkinson *et al*., 2001). Recently, a new enzymatic based fluorescent assay was also published for measuring phosphite in plants (Berkowitz *et al.,* 2011).

HPIC is the most attractive method for analysing large sample numbers. It also has the advantage of simultaneously measuring phosphite and phosphate (Roos *et al.,* 1999; Ouimette & Coffey, 1988; Smillie *et al.*, 1988). Roos *et al.* (1999) optimised a HPIC method that is simple, reliable, high throughput, cost effective and sufficiently sensitive (3-5 ppm). Ouimette & Coffey (1988) also published a HPIC method for determining phosphite in aqueous extracts of plants and soil. Their limit of detection in plant tissue was 2 ppm (Ouimette & Coffey, 1988). In this same time period, Smillie *et al.* (1988) also published a HPIC method that detected phosphite and phosphate.

Berkowitz *et al*. (2011) recently published a high throughput and cost effective micro titer fluorometric enzyme assay for the quantification of phosphite in plant tissue. The assay uses a phosphite dehydrogenase enzyme with NAD^+ as co-substrate to oxidate phosphite to phosphate, which yields the highly fluorescent reaction product resorufin. The assay is very sensitive and has a detection limit of 0.25 nmol (0.41 ppm). However, the assay is only effective when used in some plant species (*Arabidopsis*, wheat and potato), but not others (Australian native tree species). Herbaceous species seem to be most amendable to the assay, whereas the determination of phosphite concentrations in woody plants is problematic (personal communication, O. Berkowitz). Therefore, the assay will have to be optimised and altered for it to be effective for the analyses of avocado root tissue.

In South Africa, Duvenhage (2001) evaluated the efficacy of foliar phosphonate sprays based on root phosphite concentrations. He conducted one trial on 8-year old 'Hass/Duke' trees, where it was found that two 0.75% phosphonate leaf sprays (one after summer flush completion and the other after spring flush completion) applied at 943 L/ha provided adequate phosphite levels (22.6 ppm) in roots 28 days after the second application. This root phosphite concentration was equivalent to root phosphite concentrations obtained with two trunk injections. A 1% foliar spray was also effective and resulted in root phosphite concentrations that remained high (approximately 30 ppm) for a period of 56 days, suggesting that root phosphite levels can be maintained for prolonged periods with foliar applications. Based on this work, Duvenhage (2001) stated that registration trials using the 0.75% foliar potassium phosphonate application are in progress with Ocean Agriculture Pty (Ltd) (the company no longer exists). However, no phosphonate product other than fosetyl-Al has subsequently been registered for foliar applications in South Africa.

In contrast to the high efficacy of two foliar sprays found by Duvenhage in South Africa with a relative low spray volume, work in Australia indicated that a variable number of foliar sprays are required. Whiley *et al.* (2001) found that three foliar phosphonate applications at 0.25%, 0.5% or 1% a.i. (9 L/tree) applied at 6 week intervals, gave similar results than two trunk injections. In other trials up to eight foliar applications were applied from spring flush maturity through to summer flush maturity (Whiley *et al.*, 2001). The variable number of foliar sprays that must be applied to achieve sufficient root phosphite concentrations is due to the fact that the translocation of phosphite to roots of foliar applied phosphonates is influenced by various factors that include crop load, tree phenology, location and spray volume (Thomas, 2001; Whiley, personal communication).

The aims of the current SAAGA and ZZ2 funded project are to (i) develop and validate a cost effective method for quantifying phosphite in avocado roots, (ii) determine the efficacy of phosphonate foliar sprays based on phosphite root concentrations and (iii) determine the critical root phosphite concentration required for suppression of *P. cinnamomi* in roots. The last aim will not reported on in this article, since the research is still in progress.

MATERIALS AND METHODS

High performance ion chromatography

Phosphite standards were prepared from phosphorous acid (Sigma-Aldrich). The HPIC apparatus that was used was a Waters 2515 binary pump with Waters 717 autosampler and conductivity detector. A Waters IC-Pak Anion exchange column was used with a borate-gluconate mobile phase, run at a flow rate of 1.1 ml/min isocratically at room temperature.

Avocado roots were dried at 60°C for three days and ground to a fine powder with an IKA A11 basic mill (Germany). Phosphite was extracted from the roots as described by Roos *et al.* (1999). The root extract was purified by first passing the solution through a C_{18} cartridge (Sep-Pak vac 3cc, Waters, Ireland) according to manufacturer's instructions,

followed by passage through a Nanosep centrifugal device.

The recovery rate of the sample extraction and clean-up methods was determined by spiking water or control root samples with different phosphite concentrations. The samples were spiked with a range of phosphite concentrations, including 2, 5, 10, 20, 50 and 100 ppm.

Fluorescent enzyme assay

A plasmid containing the phosphite dehydrogenase gene was kindly provided by H. Zhao (University of Illinois at Urnana-Champaign, USA). The plasmid was transformed into an *E. coli* cell line and the enzyme was expressed and purified using standard procedures (Simpson, 2009). The linearity and sensitivity of the assay was evaluated as described by Berkowitz *et al.* (2011).

Orchard trials

Trial design and phosphonate applications

Avocado orchard trials were conducted at two sites. The one orchard (Morgenson) was 2 years old with a 7 x 3.5 m tree spacing (tree size 2 x 3 m, 408 trees/ha) and is situated near Tzaneen. The second orchard (Boschoek) is situated in Mooketsi and is 6 years old with a 10 x 5 m tree spacing (tree size 3 x 5 m, 200 trees/ha). At the Morgenson orchard the foliar spray volume applied was 500 L/ha (1.2 L/tree), whereas that for the Boschoek trial was 1000 L/ha (5 L/tree). The trial design at both orchards was a completely randomized design, with six replicates per treatment. Each replicate contained four trees.

Five of the treatments in the trials were the same in both orchards, whereas the 6th treatment differed. The treatments consisted of:

1) untreated control;
2) 3 foliar sprays at one week intervals at 500 g a.i. phosphorous acid/100 L (Avoguard);
3) 3 foliar sprays at one week intervals at 750 g a.i./100 L (Avoguard);
4) 3 foliar sprays at one week intervals at 1000 g a.i./100 L (Avoguard);
5) summer injection (Avoguard);
6) Morgenson trial: 4 foliar sprays at one week intervals at 750 g a.i./100 L (Avoguard); and
6) Boschoek trial: Spring + summer injections (Avoguard).

All Avoguard foliar spray solutions were adjusted to pH 7.2 using potassium hydroxide to prevent foliar burn. The timing of applications for the different treatments is shown in Table 1.

Sampling of roots for phosphite analyses

Root samples were taken on specific dates after phosphonate applications as indicated in Figure 1. The samples were taken from the centre two trees of each replicate, approximately 20-30 g of roots per tree. Phosphite analyses on the roots were conducted using HPIC analyses as described above.

RESULTS

High performance ion chromatography

Both phosphite and phosphate could be visualised with the system, and separation of all major anions PO_4^{-4}, SO_4^{-2}, Cl^- and PO_3^{-3} was achieved, each having their own distinct peak with different elution times. The standard curves obtained for phosphite and phosphate were linear with high correlation coefficients of > 0.99. The detection limit for phosphite was 1 ppm.

The extraction efficiency and recovery rate of the

Table 1. Application dates of phosphonate treatments in avocado orchard trials at Boschoek and Morgenson.

	Application dates after summer flush hardened off					Application dates after spring flush hardened off		
Treatment	**Trunk injection**	**Foliar spray 1**	**Foliar spray 2**	**Foliar spray 3**	**Foliar spray 4**	**Trunk injection**	**Foliar spray 1**	**Foliar spray 2**
Boschoek								
1 (Control)	-	-	-	-	-	-	-	-
2 (3 x foliar 500 g a.i.)	-	27/05/14	03/06/14	10/06/14	-	-	13/11/14	21/11/14
3 (3 x foliar 750 g a.i.)	-	27/05/14	03/06/14	10/06/14	-	-	13/11/14	21/11/14
4 (3 x foliar 1000 g a.i.)	-	27/05/14	03/06/14	10/06/14	-	-	13/11/14	21/11/14
5 (Summer injection)	19/05/14	-	-	-	-	-	-	-
6 (Spring + summer injection)	19/05/14	-	-	-	-	14/11/13 14/11/14	-	-
Morgenson								
1 (Control)	-	-	-	-	-	-	-	-
2 (3 x foliar 500 g a.i.)	-	20/05/14	27/05/14	03/06/14	-	-	-	-
3 (3 x foliar 750 g a.i.)	-	20/05/14	27/05/14	03/06/14	-	-	-	-
4 (3 x foliar 1000 g a.i.)	-	20/05/14	27/05/14	03/06/14	-	-	-	-
5 Summer injection	20/05/14	-	-	-	-	-	-	-
6 (4 x foliar 750 g a.i.)	-	20/05/14	27/05/14	03/06/14	10/06/14	-	-	-

method was determined with water spiked and roots extract spiked samples. The recovery rate of phosphite from water spiked samples was consistent at 60 ± 5%. However, recovery rates from avocado roots spiked with phosphite varied from 40-70%.

Fluorescent enzyme assay

The fluorescent enzyme assay reaction could be detected in real-time with high sensitivity (Fig. 1A). The standard curve had a linear response with a high correlation efficient (0.99) (Fig. 1B).

Orchard trials

At Morgenson the summer trunk injection yielded relative low phosphite values, which reached ~11 ppm_{dw} one month after application and remained at this level 5 months later when the last measurement was made in October (Fig. 2A). All of the foliar sprays yielded low root phosphite concentrations that were less than 3 ppm_{dw}. There was a slight tendency for the 4 x foliar sprays at 750 g a.i. and 3 x foliar spray at 1000 g a.i. to have higher phosphite concentrations than the other foliar treatments.

The root phosphite concentrations were higher at Boschoek than at Morgenson after the summer flush applications (Fig. 2). At Boschoek, the trunk injections yielded 45 ppm_{dw} one month after application, which decreased to ~ 10 ppm_{dw} five months after application. In contrast, the foliar sprays all yielded more than ten times less phosphite in the roots than the trunk injection and were all below 3 ppm_{dw}. The trunk injections that were applied in spring and summer yielded the highest root phosphite concentrations that were almost 80 ppm_{dw} one month after application, which then declined to 25 ppm_{dw} five months after application.

DISCUSSION

The high performance ion chromatography method had a high sensitivity level (1 ppm) that is comparable to that reported in literature (Ouimette & Coffey, 1988; Roos *et al.*, 1999). The recovery rate for water samples that were spiked with phosphite and cleaned through C_{18} and Nanosep devices, was 60 ± 5%. However, the recovery rate for phosphite spiked root samples was lower and variable (40 – 70%). In literature, phosphite recovery rates for spiked plant samples that have been reported include 70% for avocado roots where C_{18} cartridges were used for sample clean-up (Ouimette & Coffey, 1988). Borza *et al.* (2014) reported a recovery rate of more than 95% from potato leaves and tubers using Amicon centrifugal devices for sample clean-up. The low and variable phosphite recovery rates from roots in the current study might be due to the consistency of the extracted samples that are often viscous in appearance. Future studies will investigate shorter extraction periods from roots, and the dilution of root samples with water prior to clean-up in order to obtain higher and more consistent recovery rates.

The fluorescent enzyme assay published by Berkowitz *et al.* (2011) was successfully validated in the current study. The expressed and purified phosphite dehydrogenase enzyme was successfully used to generate linear standard curves with high correlation coefficients. The sensitivity of the assay was 1 ppm, which is comparable to that reported for HPIC methods and the published fluorescent enzyme assay (0.41 ppm) of Berkowitz *et al.* (2011). Future studies will determine whether the fluorescent enzyme assay can be used to quantify phosphite from avocado roots. This will provide a much higher throughput and cost effective method than HPIC analyses for quantifying phosphite in roots.

In the current study, the amount of phosphite delivered to avocado roots with foliar sprays applied after summer flush hardened off, was much lower (< 3 ppm) than that reported by Duvenhage (2001) and Whiley *et al.* (2001). Ideally one would like to

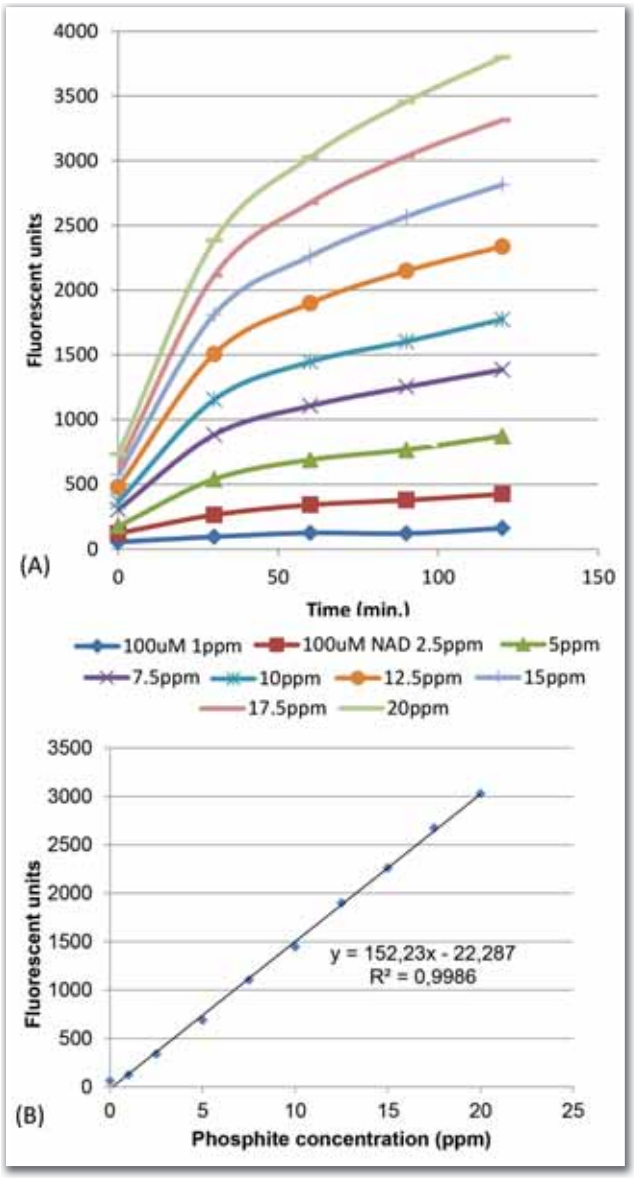

Figure 1. Optimisation of a fluorescent enzyme assay using 1ug of enzyme per reaction. (A) Real-time detection of resorufin production over a 2-hour time period, using 1 to 20 ppm of phosphite. (B) Standard curve obtained from fluorescent assay. The reaction was incubated for 1h.

achieve the same root phosphite concentrations with foliar sprays than with trunk injections. Duvenhage (2001) applied only two sprays at 943 L/ha of a 1% a.i. foliar spray to 8-year old 'Hass/Duke' trees and reported root phosphite levels of ~ 30 ppm almost two months after the last applications. At the Boschoek trial for the trunk injection, our root phosphite concentration detected two months after application was similar than that of the trunk injection achieved by Duvenhage (2001). The poor performance of the trunk injections at the Morgenson trial (10 – 15 ppm) is unclear, but could be due to the young age of the trees, being only two years old at the first injection.

The variability in root phosphite concentrations achieved with phosphonate foliar sprays between different studies is due to several factors that influence the translocation, and ultimately root phosphite concentrations in avocado trees. Crop load can influence root phosphite concentration, in that when crop load is high, there is a tendency for root

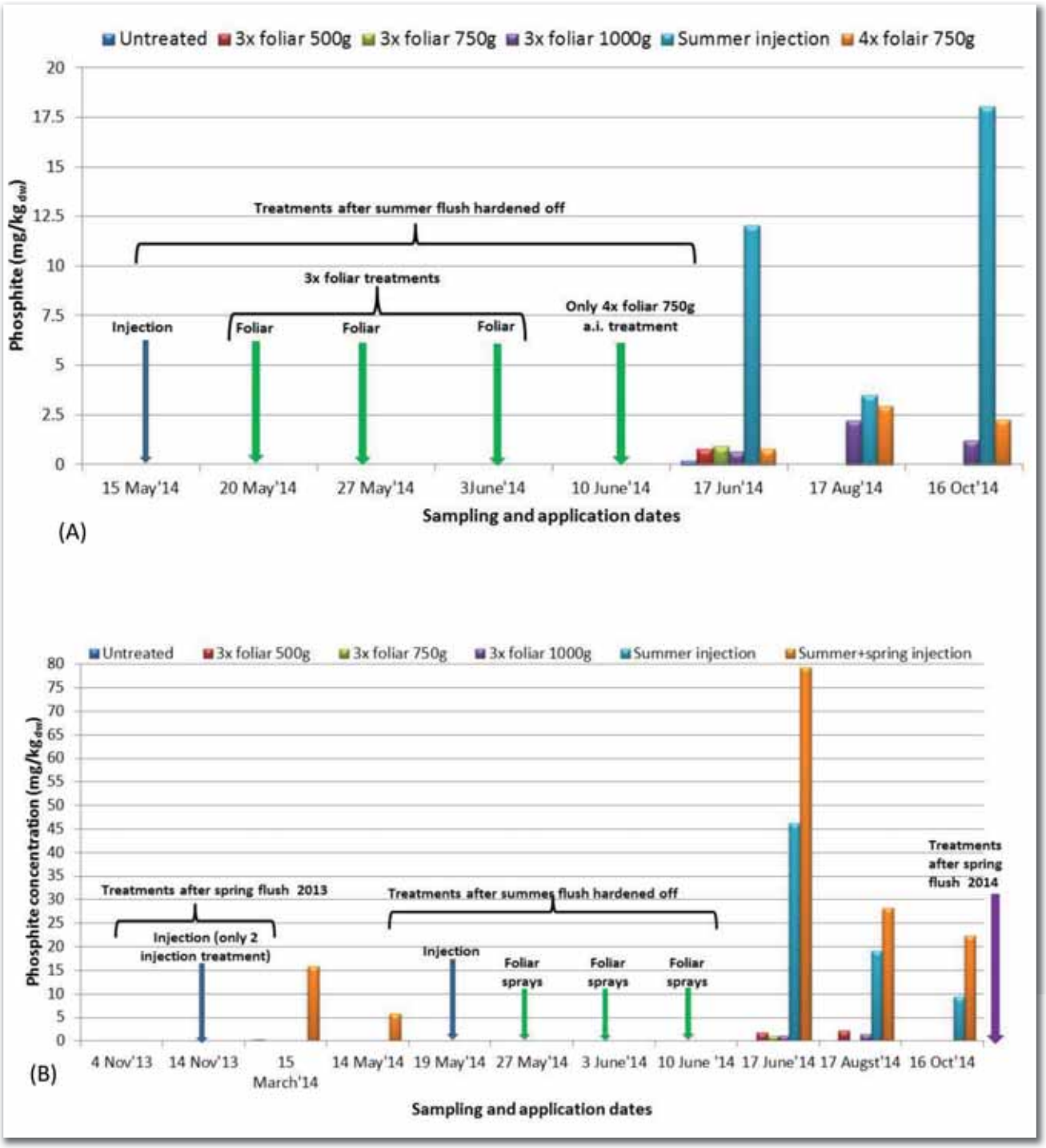

Figure 2. Phosphite concentrations in avocado roots after different phosphonate treatments (foliar or trunk injections) were applied at (A) Morgenson and (B) Boschoek. Phosphonate trunk injections (Avoguard) are indicated by blue arrows and foliar applications (Avoguard) by green arrows. The trial design was a completely randomized block design with six replicates per treatment (each replicate consisted of two trees from which root samples were taken). Roots were analysed for phosphite content using high performance ion chromatography.

phosphite levels to be lower (Whiley *et al*., 2001). Tree phenology is also important, for example, the sink strength of the shoots and fruits at the time of application will determine root phosphite concentrations. There are two important windows for foliar phosphonate applications, which coincide with the strongest sink strength of roots. The best window for building root phosphite concentrations are when applications are made every 7 days to 4 weeks during the late autumn and early winter months when summer leaf and root flush is complete, but before plump flower buds develop on shoots. The second, less optimum window is short (approximately 4 weeks), and occurs once the spring flush has hardened off, but before bud development. Applications in this second window usually results in only a slight increase in root phosphite levels compared to applications made in the first window (Thomas, 2008; personal communication, A.W. Whiley). In the current study, at the Boschoek trial, two foliar applications were also made after spring flush has hardened off. Root samples have been collected following applications, but the roots must still be analysed for phosphite concentration to determine the efficacy of these applications.

Spray volume will also influence root phosphite concentrations. Low volume foliar sprays have been found ineffective in avocado in Australia (personal communication, Whiley; Thomas, 2001). Similarly in native Australian vegetation, only high volume aerial sprays are effective, with low volume sprays having low efficacy (Crane & Shreare, 2014). On avocado it is apparently difficult to define an exact spray volume, but applications in mature orchards under 1000 L/ha are ineffective (Thomas, 2001). According to W.A. Whiley (personal communication), a 0.6% spray is now mostly used in Australia along with a spray volume of 2000 L/ha (trees of approximately 5.5 – 6 m in height) in mature orchards (70% of the orchard floor covered by tree canopies), and 2500 L/ha for trees that are higher. For younger orchards proportionally less spray volume is used, but it is important to thoroughly wet the canopy including the internal parts of the trees, since bark absorption can occur. To prevent resistance problems, applications should not be made so that sprays stream down to the soil (Whiley, personal communication).

The specific season can also influence root phosphite concentrations. Although most growers know the general pattern of expected vegetative- and root flushes, the exact time when the root flush starts and ends will vary with season and location (Thomas, 2001). Translocation of phosphite to roots depends on when during the root flush period (beginning, peak or end) phosphonates are applied. Lastly, the efficacy of foliar sprays may also differ in different locations (Thomas, 2001).

The low phosphite root concentrations in foliar treatments in the current study could be due to the spray volumes being too low at the Morgenson trial, and that perhaps more than three sprays should be applied. At Morgenson, the trees are planted at a high planting density and a spray volume of 500 L/ha only resulted in 1.2 L being applied per tree, whereas at the 6-year old Boschoek trial that has a lower planting density, a spray volume of 1000 L/ha resulted in 5 L being applied per tree. The influence of planting density on the amount of spray applied per tree should be standardised in future by calculating spray volumes per hectare based on tree row volume or foliar leaf wall area. Since the spray volume at Boschoek should have been sufficient based on broad Australian guidelines, the window of application might have been less optimal and contributed to low phosphite root levels for the foliar sprays. Since the foliar sprays were only started in May, the last foliar sprays were applied in June, which is somewhat outside of the optimum application window. Another reason that could affect the root phosphite concentrations measured in our study is the variable phosphite recovery rates obtained with the current HPIC method. Therefore, once the HPIC method is optimised and recovery rates are improved, all the root samples will be re-analysed to ascertain whether the root phosphite concentrations were estimated correctly for all the treatments.

An important aspect of using root phosphite concentrations that must be known is the critical root phosphite concentration, i.e. how much phosphite is required in the roots to suppress the pathogen. Very few studies have attempted to establish a critical (minimum) root phosphite concentration for *P. cinnamomi* in avocado roots. Van der Merwe & Kotze (1994) used glasshouse grown avocado seedlings and a root bioassay to show that when root phosphite levels were less than 9.5 ppm, *P. cinnamomi* was not suppressed. However, at concentrations above 9.5 pp to 53.2 ppm, no significant differences were found in protection of roots against *P. cinnamomi* (Van der Merwe & Kotze, 1994). In Australia, the critical root phosphite concentration for avocados is set at 25 to 40 ppm_{fw}, although the research behind these values was never published, and the phosphite quantification method is protected by commercial laboratories. The Australians obtained their critical root phosphite values from a combination of *in vitro* studies and a large data base of root phosphite analyses of field trees over several years (Giblin *et al.*, 2007; personal communication, A.W. Whiley, Sunshine Horticultural Services Pty Ltd). Obviously, the critical root phosphite value will depend on the extraction efficiency and recovery rates of the method used for phosphite quantification. Since these parameters are unknown for the Australian studies and for the study of Van der Merwe & Kotze (1994), it is difficult to determine the biological significance of the measured root phosphite concentrations obtained in our trials. Therefore, an important aim of the current research project is to determine the critical root phosphite concentrations for our optimised phosphite quantification method.

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